Mechanisms and Modulation of Neuropathic Pain by Neurotrophin-3

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Abstract

Neuropathic pain is a complex clinical syndrome characterized by increased sensitivity to thermal and/or mechanical stimuli that may or may not be accompanied by the phenomenon of spontaneous or aberrant pain sensations.

Over the past decade, the mechanisms underlying the behavioral manifestations of inflammatory neuropathic pain have become more clearly elucidated. These include the involvement of: 1) transient receptor potential vanilloid receptor 1 (TRPV1) in the generation of thermal hyperalgesia; 2) acid sensing ion channel 3 (ASIC3) in some aspects of the development/maintenance of mechanical hypersensitivity; 3) the tetrodotoxin resistant sodium channels $Na_v1.8$ and $Na_v1.9$ in both hyperalgesia and spontaneous pain; and 4) activation of the MAP Kinases p38 and ERK1/2 in the regulation of expression of the aforementioned molecules.

Interestingly, it is the pro-inflammatory neurotrophin nerve growth factor (NGF) that is the common link between all of these mediators of neuropathic pain. Increased availability of NGF under conditions of inflammation has been shown to drive increased expression/upregulation of TRPV1, ASIC3, $Na_v1.8$ and $Na_v1.9$, as well as phospho-p38 and phospho-ERK1/2.

Evidence presented here continues to support a role for neurotrophin-3 (NT-3) in antagonizing the effects of increased NGF on trkA signaling, neuropathic pain behaviors and some of the molecules associated with the generation of such behaviors.

More specifically, the work culminating in this thesis demonstrates a novel role for NT-3 in negative modulation of TRPV1, ASIC3, $Na_v1.8$ and $Na_v1.9$, as well as phospho-p38 expression in response to the chronic constriction injury model of neuropathic pain. Finally, initial insights into how this negative regulation of these nociceptive markers might occur is elucidated in studies demonstrating that NT-3 differentially affects levels of the key signaling molecule phospho-ERK in trkA-positive versus trkC-positive neurons in naïve dorsal root ganglia (DRG).

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Dedication

As it comes time to look back upon the past years, it seems almost impossible to express my gratitude and thanks to those who have helped me so much. I am humbled as I know that this accomplishment would not have been possible without the many helping hands I have received along the way.

It is with a heart overflowing with pride that I dedicate this thesis:

To my son, Spencer: On days that aren't going so well, your smile and your hugs and your kisses can lighten the heaviest of loads. You have made me realize that the challenges of graduate work that seemed so daunting really pale in comparison to the challenges of parenting. Love you, Buddy.

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List of Abbreviations

ASIC3	acid sensing ion channel 3
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CCI	chronic constriction injury
CFA	complete Freund's adjuvant
CGRP	calcitonin gene related peptide
CNS	central nervous system
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
DRASIC	dorsal root acid sensing ion channel
DRG	dorsal root ganglion
DTT	dithiothreitol
ENaC/DEG	epithelial sodium channel/degenerins
ERK	extracellular signal related kinase
G-C	guanine-cytosine
GDNF	glial derived neurotrophic factor
HRP	horseradish peroxidase
IASP	international association for the study of pain
IB-4	isolectin B-4
IL-6	interleukin-6
IP_3	inositol tri-phosphate
JNK	c-jun N-terminal kinase
L4	lumbar segment 4
L5	lumbar segment 5
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
mRNA	messenger ribonucleic acid

NHERF-1	Na^+/H^+ exchanger regulatory factor-1
NGF	nerve growth factor
NPY	neuropeptide tyrosine
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
PACAP	pituitary adenylate cyclase activating polypeptide
PBS	phosphate buffered saline
PGE2	prostaglandin E2
PLC	phospholipase C
^{35}S	sulphur-35
s.e.m.	standard error of the mean
SP	substance P
SSC	sodium chloride/sodium citrate
STZ	streptozotocin
TNF-α	tumor necrosis factor - alpha
trks	tropomyosin related kinase
TRPV1	transient receptor potential vanilloid receptor-1
TTX-R	tetrodotoxin-resistant

1 Introduction

Neuropathic pain is a chronic pain syndrome resulting from injury to a peripheral nerve that is characterized by both an increased sensitivity to thermal stimuli (thermal hyperalgesia), mechanical stimuli (mechanical allodynia) and aberrant pain sensations (spontaneous pain). The chronic condition of this pain state can be both physically and mentally debilitating to those who are afflicted and to date no successful treatment regimens are available. The symptoms and syndromes associated with neuropathic pain are well known, but until the underlying mechanisms that drive the development of these syndromes are identified and understood, effective global treatments are not likely to be available. It is for this reason that the investigation of both behavioral and biochemical aspects of neuropathic pain remain a priority.

The chronic constriction injury (CCI) of the sciatic nerve is a widely used model of neuropathic pain. This model produces neuropathic pain sensations similar to those observed in humans and evokes a series of molecular, biochemical and cytoarchitectural changes in primary sensory neurons – believed to contribute to the ensuing pain state (Bennett and Xie, 1988; Kajander and Bennett, 1992; Nahin et al., 1994; Miki et al., 1998). In addition, some key molecules involved in the generation and maintenance of neuropathic pain states have been identified. Of these, the neurotrophin nerve growth factor (NGF), which has been demonstrated to influence the phenotype of these primary sensory neurons and be a major regulator of inflammatory and homeostatic pain states (Lewin and Mendell, 1993; Woolf et al., 1994; Verge et al., 1995; Woolf, 1996; Herzberg et al., 1997; Ramer et al., 1998; Theodosiou et al., 1999), is the most commonly known. More recently, a novel potential role for another member of the NGF family of neurotrophins – neurotrophin-3 (NT-3) – in acting to antagonize the pro-inflammatory NGF pathway and algesia-associated phenotype has been revealed (Gratto and Verge, 2003; Wilson-Gerwing and Verge, 2006). My thesis will examine this novel

role by elucidating the behavioral and biochemical implications of NT-3's capacity to alter nociceptive pathways using the CCI model of neuropathic pain.

1.1 An overview of primary sensory neurons associated with pain transmission

Biochemical changes that are associated with neuropathic pain can be examined at various levels of the nervous system. These include, but are not limited to, changes at the level of the brain, the level of the spinal cord and at the level of the primary sensory neurons - the first relay point. For the purpose of this thesis, I have chosen to focus in on those changes that occur at the level of the primary sensory neuron.

Primary sensory neurons are pseudo-unipolar (reviewed in Hokfelt et al., 1997; Nolte, 1999), with their cell bodies residing within the dorsal root ganglion (DRG). The axonal processes exit the cell body at a single site with one process projecting to peripheral tissues, and a second process projecting centrally and terminating in either the dorsal horn of the spinal cord or sending a collateral via the dorsal columns to terminate in brainstem nuclei (reviewed in Hokfelt et al., 1997). These neurons are responsible for the gathering of sensory information from the external environment and transmitting it to the central nervous system so that the incoming sensory information can be dealt with accordingly (Woolf, 1996).

Primary sensory neurons that convey nociceptive information can be classified according to their morphology and physiology. With this type of classification, three basic categories are evident for those sensory neurons believed involved in the neuropathic pain response. The first is comprised of large, myelinated axons that respond primarily to innocuous mechanical stimuli (Aβ fibers) (Willis and Coggeshall, 1991; reviewed in Millan, 1999). Aβ fibers have very rapid conduction velocities due to the extensive myelin surrounding the axons (30-100 m/s) (reviewed in Millan, 1999; Nolte, 1999). The second group, Aδ fibers, are of medium size and have thinly myelinated axons (Furst, 1999; reviewed in Millan, 1999; Nolte, 1999). The Aδ fibers rapidly transduce action potentials (reviewed in Stucky et al., 2001) at a rate of 5-30 m/s

(reviewed in Millan, 1999; Nolte, 1999) and are nociceptors that are responsible for the experience of fast, prickling, sharp pain (Furst, 1999; Nolte, 1999; reviewed in Stucky et al., 2001). The final category consists of small neurons whose axons are not myelinated (C fibers) (Willis and Coggeshall, 1991; Nolte, 1999; reviewed in Stucky et al., 2001) and have slow conduction velocities (0.5-2 m/s) (reviewed in Millan, 1999; Nolte, 1999; reviewed in Stucky et al., 2001). C fibers are also known as polymodal nociceptors (Willis and Coggeshall, 1991; reviewed in Millan, 1999) and mediate the experience of slow, dull, aching pain and chemosensation (Furst, 1999; reviewed in Millan, 1999; Nolte, 1999; reviewed in Stucky et al., 2001).

Just as important as the physiological distinctions between these three classes of nociceptive sensory neurons are their specific sites of termination within the spinal cord. The dorsal horn of the spinal cord receives sensory input from the periphery. Under normal physiological conditions, large Aβ fibers project deep into the dorsal horn to terminate in laminas III and V (Shortland et al., 1989; Furst, 1999; reviewed in Millan, 1999; Nolte, 1999). Intermediate Aδ fibers, and small C fibers, terminate primarily in lamina I (marginal zone) and lamina II_{outer} (substantia gelatinosa) (Light and Perl, 1979; Furst, 1999; reviewed in Millan, 1999; Nolte, 1999).

Sensory neurons can also be classified on a histochemical basis into three broad categories. While these categories are largely histochemically distinct, it is important to realize that there is some degree of overlap among them. The first group of DRG cells can be identified by the presence of phosphorylated heavy-chain neurofilament (Lawson et al., 1984) that can be identified using the antibody RT97 (McMahon, 1996). This is the cell group that was initially identified as the "Large Light" neurons (reviewed in Lawson, 1992). Morphologically, the "Large Light" neurons have myelinated axons and it is believed that they transmit information from peripheral mechanoreceptors (Lawson et al., 1984; reviewed in McMahon et al., 1997). The second grouping of DRG cells is made up of populations of cells that express neuropeptides and receptor tyrosine kinases (trks) (reviewed in McMahon, 1996; McMahon et al., 1997). The best single histochemical marker for this population is calcitonin gene-related peptide (CGRP) (McMahon, 1996; reviewed in McMahon et al., 1997; Stucky et al., 2001). Morphologically, this group of cells are primarily small to medium in size and their

axons are unmyelinated (reviewed in McMahon, 1996; McMahon et al., 1997). This is the cell group that corresponds to those cells initially identified as the "Small Dark" neurons (reviewed in Lawson, 1992). These "Small Dark" neurons are believed to transmit information from nociceptors and thermoreceptors (reviewed in Lawson, 1992; reviewed in McMahon, 1996). The final histochemical category of DRG sensory neurons are those that bind the plant lectin *Griffonia Simplifolia* IB4 and are generally non-peptidergic and lack trk receptors (Silverman and Kruger, 1990; Alvarez et al., 1991; reviewed in Stucky et al., 2001). Morphologically, these neurons, like the peptidergic population described above, are small, their axons are not myelinated, and receive sensory information primarily from nociceptors and thermoreceptors (Willis and Coggeshall, 1991).

Of particular interest to this thesis are those sensory neurons that respond to noxious stimuli – the nociceptors. Nociceptors are very diverse in their ability to respond to various types of stimulation. Specifically, they can be activated by thermal, chemical and/or mechanical stimuli (Snider and McMahon, 1998; Furst, 1999; reviewed in Stucky, 2001). The predominant type of nociceptor is capable of responding to all three of these stimuli and is referred to as a polymodal nociceptor (reviewed in Snider and McMahon, 1998; reviewed in Koltzenburg, 1999). A second specific type of nociceptor is the "silent" or "sleeping" nociceptor that becomes activated as a result of inflammation or tissue injury (Snider and McMahon, 1998; reviewed in Millan, 1999). Further properties of these specialized sensory neurons will be discussed in subsequent sections.

Characteristically, the phenotypic profiles and functional properties of all primary sensory neurons vary depending on the physiological state of the neuron (for example, homeostasis or neuropathic pain). The signals regulating these states are trophic molecules and cytokines secreted by target, glial, or immune cell sources. One of the most profound regulators of the differentiated state of sensory neurons is the NGF family of neurotrophic molecules. The elevation of NGF expression in inflammatory states contributes to the associated increase in thermal hypersensitivity. It is the understanding of these changes and their regulation that becomes the basis for

understanding the transition from normal to neuropathic pain states following nerve injury.

1.2 An introduction to neurotrophins and their receptors in sensory neurons

Neurotrophins are secreted proteins (McMahon et al., 1997) having molecular weights of 13-15 kDa (Ross and Riopelle, 2001). The neurotrophin family members are structurally homologous (McMahon et al., 1997) and exist as non-covalently linked homodimers (Ross and Riopelle, 2001). Neurotrophins play important roles with regards to the survival, proliferation, differentiation and phenotypic regulation of developing sensory neurons (Levi-Montalcini, 1987; Barde, 1989; Otten et al., 1994; McMahon and Priestly, 1995; reviewed in McMahon et al., 1997; Ross and Riopelle, 2001; Stucky, 2001). In mature sensory neurons, neurotrophins have been demonstrated to influence the phenotypic maintenance of overlapping subpopulations of these cells (reviewed in Woolf and Mannion, 1999).

Under normal physiological conditions, DRG sensory neurons derive their primary source of neurotrophins from the peripheral target tissues that these neurons innervate (Korsching and Thoenen, 1983a; Heumann et al., 1984; Korsching and Thoenen, 1985; Goedert et al., 1986; Ernfors et al., 1990; Wetmore and Olson, 1995). The neurotrophins bind to receptors on the neurons and are transported retrogradely as signaling endosomes (reviewed in Ginty and Segal, 2002) to the cell body (Korsching and Thoenen, 1983b; DiStefano et al., 1992; reviewed in Stucky, 2001; Miller and Kaplan, 2002).

The first neurotrophic factor to be described was nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951). Subsequently, brain derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990: Maisonpierre et al., 1990; Rosenthal et al., 1990) and neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbook et al., 1991) - all members of the NGF family of neurotrophins - were also isolated.

The actions of neurotrophins are mediated via select high-affinity interactions with the trk family of receptor tyrosine kinases (Chao, 1992; Barbacid, 1994; Bothwell,

1995), although all of the neurotrophins interact with similar affinity with the common neurotrophin receptor – p75 (Johnson et al., 1986; Radeke et al., 1987; Bothwell, 1996; McMahon et al., 1997). To date NGF, BDNF and NT-3 have been shown to have the most profound actions on mature sensory neurons, details of which will be elucidated below.

1.2.1 Nerve growth factor (NGF)

As described above, NGF was the first target-derived neurotrophic factor to be isolated and described (Levi-Montalcini and Hamburger, 1951; reviewed in Lewin and Barde, 1996). As a trophic factor, NGF has been demonstrated to promote survival, growth and differentiation of neural crest-derived sensory neurons and peripheral sensory neurons (Scully and Otten, 1995; Ramer et al., 1998). In support of a role in survival, it has been shown that many sensory neurons do not survive early embryonic life in the absence of NGF (Johnson et al., 1986; Ruit et al., 1992; reviewed in Acheson and Lindsay, 1996). Additionally, removal of the target and consequent removal of target-derived NGF also results in neuronal death (reviewed in Acheson and Lindsay, 1996). Conversely, if the target size is increased – thereby increasing the amount of NGF available - there is a subsequent increase in neuronal survival (reviewed in Acheson and Lindsay, 1996). Further evidence for NGF's role in neuronal survival comes from experiments that use anti-sera to sequester NGF during embryonic development, resulting in dramatic neuronal loss (Cohen, 1960; Levi-Montalcini and Angeletti, 1966).

NGF is also a potent mediator of neuronal phenotype in adult sensory neurons. The model that has dominated this research is that of sciatic nerve injury which ranges from partial nerve injury to complete transection of the nerve. Injury of the sciatic nerve interrupts the normal retrograde flow of NGF from the target tissue (Korsching and Thoenen, 1983b; Raivich et al., 1991) leading to phenotypic changes in DRG neurons (Villar et al., 1989; Gold et al., 1991; reviewed in Verge et al., 2002). The NGF responsive subpopulation of sensory neurons express the neuropeptides substance P (SP)

and calcitonin gene related peptide (CGRP) – both key molecules in the regulation of pain states (Verge et al., 1989a). Administration of exogenous, intrathecal NGF has been shown to both prevent (when given at the time of peripheral nerve injury) and reverse (when given after phenotypic changes due to peripheral nerve injury have been allowed to occur) many phenotypic changes in DRG neurons following peripheral nerve injury including alterations in the neuropeptides SP and CGRP (Yip et al., 1984; Fitzgerald et al., 1985; Rich et al., 1987; Verge et al., 1989b; Verge et al., 1990; Gold et al., 1991; Verge et al., 1995).

More recently, a role for NGF in both neuropathic pain and inflammation has come to light (for reviews see Otten et al., 1994; McMahon, 1996). These aspects of NGF will be discussed in detail in a subsequent section entitled "Neurotrophins and Pain".

1.2.2 Brain derived neurotrophic factor (BDNF)

The second member of the NGF family of neurotrophins to be described was BDNF purified from pig brain (Barde et al., 1982; Hofer and Barde, 1988). Hofer and Barde (1988) and Lindsay et al. (1985) both demonstrated that BDNF is capable of promoting the survival of a population of peripheral sensory neurons that do not require NGF for survival. In vitro studies reveal that placode-derived sensory neurons, neural crest-derived sensory neurons and motor neurons are responsive to BDNF (Lindsay et al., 1985; Davies et al., 1986; Hohn et al., 1990; Wong et al., 1993; reviewed in Cho et al., 1999). With regard to primary sensory neurons, BDNF is a trophic factor for Aβ fibers (reviewed in Millan, 1999) and is essential for proper mechanosensitivity of Merkel cells (reviewed in Koltzenburg, 1999). A significant loss of sensory neurons is observed in BDNF knock-out animals, although it is not as severe as observed in NGF knock-out mice (about 30% of total DRG neurons as opposed to about 80% in NGF knock-out animals) (Klein, 1994; reviewed in Acheson and Lindsay, 1996; McMahon et al., 1997; Mogil and Grisel, 1998).

Although BDNF shares many commonalties with NGF, there are two major differences with respect to sensory neuron biology. First, BDNF mRNA is detectable in normal DRG neurons (Ernfors et al., 1990; Wetmore and Olson, 1995; Tonra, 1999) whereas NGF mRNA is not (Davies et al., 1987; Ernfors et al., 1990; Wetmore and Olson, 1995). Specifically, under normal physiological conditions, it is the nociceptive, NGF responsive, trkA-positive sensory neurons (C fibers) that express BDNF (Michael et al., 1997; Mannion et al., 1999; Zhou et al., 1999). Interestingly, after peripheral nerve injury, these small neurons decrease their synthesis of BDNF and large neurons expressing the cognate receptors for BDNF and NT-3, trkB and trkC respectively, now express BDNF (Zhou et al., 1999). The second dissimilarity is that neuronally synthesized BDNF is transported anterogradely to the spinal cord in secretory vesicles (Zhou and Rush, 1996; Mannion et al., 1999; Tonra, 1999) in addition to the traditional target derived BDNF that is retrogradely transported to the cell body (Tonra, 1999). This supports possible roles for BDNF as both an autocrine and/or paracrine regulator of sensory neurons (Acheson et al., 1995; Acheson and Lindsay, 1996; Tonra, 1999).

1.2.3 Neurotrophin-3 (NT-3)

The third member of the NGF family of neurotrophins to be described was NT-3. As an NGF homologue, it is not surprising that NT-3's trophic effects include neuronal survival, growth and phenotypic modulation of neurons.

Developmentally, NT-3 plays an important role in survival of slowly adapting mechanoreceptors that innervate Merkel cells (Airaksinen et al., 1996; reviewed in Koltzenburg, 1999), muscle sensory afferents (innervating muscle spindle afferents and Golgi tendon organs) (Helgren et al., 1997; reviewed in Koltzenburg, 1999) and sympathetic neurons (Zhou et al., 1999). Additional support for NT-3's role as a survival factor stems from NT-3 knock-out animals that completely lack proprioceptive neurons (lack of muscle spindles) (Ernfors et al., 1994; Farinas et al., 1994; Klein, 1994; Klein et al., 1994; Tessarollo et al., 1994).

Phenotypic modulation of DRG neurons has also been demonstrated for NT-3. Nerve injury-induced phenotypic changes in large DRG neurons can be reversed by providing exogenous NT-3. These changes include gene expression for neurotrophin receptors trkC and p75 (Gratto, 2002), cytoskeletal components, immediate early genes (Verge et al., 1996), neuropeptides (Jongsma Wallin et al., 2001) and the neurotrophin BDNF (Karchewski et al., 2002). In addition to our group, Sterne et al. (1998) report that administration of exogenous NT-3 following peripheral nerve transection was capable of influencing neuropeptide tyrosine (NPY) expression in DRG neurons.

Similar to NGF, NT-3 is produced in peripheral targets (skin, muscle, and viscera) and is retrogradely transported to the cell body (Helgren et al., 1997; Zhou et al., 1999). Like BDNF, NT-3 also undergoes anterograde transport to neurons and target cells potentially functioning as a neuromodulator and trophic factor (Altar and DiStefano, 1998).

1.2.4 The trk family of receptors

The receptor tyrosine kinase family of neurotrophin receptors, characterized by a cytoplasmic tyrosine kinase domain and extracellularly, two cysteine clusters, leucine rich domains, and two cysteine rich domains (Chao and Hempstead, 1995) is composed of three members – tropomyosin-related kinase (trk)A, trkB and trkC – that selectively bind neurotrophins with similar high affinity (reviewed in McMahon et al., 1997). NGF binds to trkA (Kaplan et al., 1991; Klein et al., 1991), BDNF and NT-4/5 both bind to trkB (Klein et al., 1990; Soppet et al., 1991; Squinto et al., 1991) and NT-3 binds primarily to trkC, but can also interact with trkB and an isoform of trkA that contains a 6 amino acid insert in the extracellular binding domain (Lambelle et al., 1991; Ip et al., 1993). Activation of the trks is dependent on ligand-mediated dimerization of the receptor (Hantzopoulous et al., 1994) and results in activation of several signaling pathways (i.e. MAPK, Akt, IP_3) that in turn promote changes in survival, gene expression and neurite outgrowth (reviewed in Miller and Kaplan, 2002).

Extensive work has been undertaken to characterize the prevalence of the trk receptors in adult lumbar sensory neurons. It has been described that approximately 40-45% of DRG sensory neurons express trkA, about 30% express trkB and there are approximately 40% that express trkC (Karchewski et al., 1999). It is easy to see that together these numbers encompass greater than 100% of the sensory neuron population. This is due to the overlap of trk receptors that was previously mentioned. Karchewski et al. (1999) went on to further characterize these DRG neurons and found that about 10% of DRG neurons expressed both trkA and trkB, about 20% expressed both trkA and trkC, about 15% expressed both trkB and trkC and finally, there are about 3% of DRG neurons that express all three trk receptors. There is also a subpopulation of sensory neurons (approximately 20%) that do not express any neurotrophin receptors, bind the lectin IB-4 and are responsive to the growth factor glial derived neurotrophic factor (GDNF) (McMahon et al., 1994; Bennett et al., 1998; Karchewski et al., 1999).

Transgenic studies using knock-out mice have been used to assess the key developmental roles of these trk receptors. Deletion of the trkA receptor (as in deletion of NGF) results in the loss of approximately 70-80% of small diameter sensory neurons (reviewed in McMahon et al., 1997; Mogil and Grisel, 1998). Additionally, these knock-out mice were insensitive to both thermal and mechanical stimulation (Mogil and Grisel, 1998). The selective deletion of the trkB receptor (as seen with deletion of BDNF) displays a loss of sensory neurons (not as profound as with the trkA knock-outs), a subsequent loss of sensitivity to tactile stimulation, and also a loss of motor neurons (Klein, 1994; reviewed in McMahon et al., 1997). Mice deficient in the trkC receptor (as seen with NT-3 knock-out animals) have a loss of sensory neurons responsible for proprioception, the result being abnormal movements (Klein, 1994; Klein et al., 1994).

1.2.5 p75

The p75 neurotrophin receptor is capable of binding all members of the NGF family of neurotrophins with approximately equal specificity, but a lower affinity than the trk receptors and (reviewed in Bothwell, 1996 and McMahon et al., 1997). In the

presence of trk, p75's primary role appears to be modulatory (Bothwell, 1996) creating a higher affinity state for NGF binding (Chao and Hemstead, 1995; Ross et al., 1998; Shamovsky et al., 1999), although in the absence of trk expression, it can actively participate in cell death programs (Rabizadeh et al., 1993; Bothwell, 1996; Frade et al., 1996). Approximately 80% of all DRG neurons express p75 (this equates to virtually every neuron that expresses a trk receptor) (Karchewski et al., 1999). p75 knock-out mice develop a dramatic insensitivity to thermal stimuli compared to wild-type mice (Mogil and Grisel, 1998); however, it is interesting to note that thermal hyperalgesia can be induced by NGF even in the absence of p75 (Bergmann et al., 1998). The function of p75, then, is still not well understood.

1.3 An introduction to neuropathic pain

In order to begin a discussion about neuropathic pain, it is important to understand what is meant by the term 'nociception'. Nociception refers to the process whereby specialized sensory neurons – primarily Aδ and C fibers (nociceptors) - detect the presence of noxious stimuli (Caterina et al., 1997; Furst, 1999; Loeser and Melzak, 1999). Pain, as defined by IASP Task Force on Toxonomy (1994), is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage".

1.3.1 Normal pain transmission

Under normal, physiological conditions, the transmission of nociceptive information from the periphery to the central nervous system (CNS) takes place via a specific pathway. First, nociceptive afferent fibers (Aδ and C fibers) are activated by noxious stimuli that is thermal, mechanical, or chemical in nature (Caterina et al., 1997; Furst, 1999; Loeser and Melzack, 1999). These nociceptive fibers transmit this

information to the substantia gelatinosa of the spinal cord where second order neurons project this information to the thalamus (Furst, 1999; Nolte, 1999). Finally, the cerebral cortex receives the nociceptive information from the thalamus (Furst, 1999; Nolte, 1999). This system is highly organized and precise. However, under pathological conditions, such as neuropathic pain, the mechanisms of pain transduction become highly variable and convoluted.

1.3.2 Neuropathic pain

In its essence, neuropathic pain is the type of pain that occurs as a result of injury to a nerve and becomes a pathological condition. Neuropathic pain is observed in such conditions as causalgia, reflex sympathetic dystrophy and diabetic neuropathy. For the purpose of this thesis, the pathological condition resulting in neuropathic pain will be limited to pain resulting from injury to a peripheral nerve – namely the sciatic nerve.

The study of neuropathic pain is both essential and intriguing. Essential in the respect that the mechanisms of neuropathic pain are not well understood and current treatments for neuropathic pain are not very effective (Woolf and Mannion, 1999). Intriguing in the respect that the etiology of neuropathic pain is diverse and it is not likely that there exists one solitary solution to its treatment.

There are three important differences between neuropathic pain and normal, non-pathological pain. First, neuropathic pain is persistent (as opposed to transient) and appears to serve no biological function (reviewed in Millan, 1999; Schwartzman and Maleki, 1999; Vaillancourt and Langevin, 1999). The principal role of physiological pain is to protect the organism (for example, to remove the foot from a sharp rock or to take the hand away from a hot stove). Second, neuropathic pain results in an increased sensitivity to stimuli (Woolf and Costigan, 1999; Woolf and Mannion, 1999). Specifically, this stimulus-evoked pain can be characterized as either hyperalgesia or allodynia (see below) (Woolf and Mannion, 1999). These two hallmarks of neuropathic pain are believed to result from the sensitization of peripheral nociceptors (Mannion et al., 1999; Urban and Gebhart, 1999) and/or the sprouting of Aβ fibers from lamina V

into lamina II of the spinal cord (where nociceptive afferents terminate) (Woolf and Decosterd, 1999). Finally, it is the phenomenon of stimulus-independent, or spontaneous pain that distinguishes neuropathic pain from physiological pain (Woolf and Mannion, 1999). Spontaneous pain, as its name suggests, takes place in the apparent absence of any stimuli. Possible reasons for the production of stimulus-independent pain are spontaneous activity of the A and C fibers (Mannion et al., 1999; Woolf and Decosterd, 1999; Woolf and Mannion, 1999) and/or the formation of sympathetic 'baskets' around the large sized neurons in the DRG (Ramer et al., 1999; Woolf and Decosterd, 1999; Woolf and Mannion, 1999).

1.3.3 The chronic constriction injury (CCI) model of neuropathic pain

The chronic constriction injury (CCI) model of neuropathic pain involves a partial lesion of the sciatic nerve induced by tying loosely constrictive chromic gut sutures around the sciatic nerve (Bennett and Xie, 1988). This results in the slow, edematous axotomy of predominantly large diameter myelinated axons (90% of Aβ and Aδ fibers and 30% of C fibers) by three days post-ligation (Kajander and Bennett, 1992) while the uninjured, smaller axons remain intact, but exposed to the Wallerian degenerating nerve in which many pro-inflammatory molecules are produced – a state akin to that observed in man (Bennett and Xie, 1988). The CCI model, similar to pain sensations seen in man, generates a reproducible thermal hyperalgesia and mechanical allodynia (Bennett and Xie, 1988).

1.3.4 Inflammation and pain

As previously described, the CCI model exposes the uninjured axons to an inflamed environment resulting, in part, from the presence of inflammatory and immune cells (such as mast cells and macrophages) that are recruited to aid in the Wallerian

degeneration of the injured nerve and to facilitate its regeneration (Perkins and Tracey, 2000). These inflammatory and immune cells release a variety of neuroactive agents including cytokines (interleukin-6 (IL-6), for example) and growth factors (NGF, for example) (Woolf and Costigan, 1999). Given the ability of NGF to directly regulate pain responses and the elevation of endogenous NGF levels in a variety of inflammatory states (reviewed in McMahon, 1996) it is not surprising that NGF appears to play an important role in the development of abnormal sensations in several models of persistent inflammatory/ neuropathic pain.

1.3.5 Thermal hyperalgesia

Hyperalgesia following nerve injury is identified as an increased sensitivity to a normally noxious stimulus (a decreased threshold) and/or suprathreshold stimulation producing an abnormally exaggerated response (reviewed in Millan, 1999; Woolf and Mannion, 1999). Thermal hyperalgesia then, refers to an increased sensitivity to heat stimuli. It is believed that the unmyelinated C fibers (in hairy skin) and the Aδ fibers (in non-hairy skin) contribute to this hyperalgesic state (Millan, 1999).

The development of thermal hyperalgesia has been demonstrated to result from a vast array of biological molecules. These molecules include: pro-inflammatory cytokines (specifically, tumor necrosis factor alpha – TNF-α) (Sommer et al., 1998); growth factors (such as NGF) (Theodosiou et al., 1999); free radicals interacting with nitric oxide (Khalil et al., 1999); inflammatory mediators (such as prostaglandins) (Syriatowicz et al., 1999); and inflammatory cells (such as neutrophils) (Perkins and Tracey, 2000). More recently, the capsaicin receptor - transient receptor potential vanilloid receptor-1 (TRPV1) - which transduces noxious thermal stimuli (Caterina et al., 1997), has become a candidate mechanism responsible for the experience of thermal hyperalgesia (Caterina et al., 2000; Caterina and Julius, 2001).

It is also believed that thermal hyperalgesia is a phenomenon that is mediated entirely by peripheral mechanisms. These mechanisms are reviewed in Millan (1999)

and include the excitation of nociceptive fibers, the subsequent sensitization of these nociceptive fibers, phenotypic alterations in sensory neurons, as well as indirect modulation of sensory neurons resulting from interactions with components of the immune system and the sympathetic nervous system.

1.3.6 Mechanical allodynia

Following nerve injury, painful sensations can also be elicited by a normally non-noxious stimulus – commonly referred to as allodynia (Woolf and Mannion, 1999). Although the term allodynia has been accepted by many, there exists a controversy in the field regarding whether or not the behavior elicited by mechanical stimuli is in fact painful, or simply reflects a heightened awareness of the stimuli. For this reason, throughout the remainder of this thesis, the terms mechanical allodynia and mechanical hypersensitivity will be used interchangeably. It has been proposed that two different pathways can result in allodynic experiences (Millan, 1999; Woolf and Mannion, 1999). First, myelinated mechanosensitive Aβ fibers (that do not normally convey painful information) become responsive to sympathetic stimulation (basket formation around these fibers) (Deng et al., 2000; Sun et al., 2001) and unrelatedly, Aβ fibers are also believed to sprout into lamina II of the dorsal horn (see above) (Woolf and Decosterd, 1999). Further evidence of this comes from Field et al. (1999) who demonstrated that Aδ and Aβ/capsaicin insensitive primary sensory neurons were responsible for the signaling of allodynia in the rat. Second, reduced thresholds or peripheral nociceptors may be able to elicit a painful response from a non-noxious stimulus (reviewed in Millan, 1999; Woolf and Mannion, 1999).

Unlike hyperalgesia, allodynia (mechanical hypersensitivity) is believed to be under the control of central (rather than peripheral) mechanisms. Millan (1999) reviews three central mechanisms: increased excitability and/or sensitization of dorsal horn neurons; alterations in ascending and descending mechanisms of nociception; and adaptive changes in higher centers (thalamus, cortex, etc). Pursuant to this, it has

recently been demonstrated that tactile allodynia requires ascending input to supraspinal sites (Sun et al., 2001).

It is apparent that the mechanisms that underlie allodynia are not yet clearly understood, and only minimal information regarding its development is readily available.

1.4 Neurotrophins and pain

The mediation of nociception by neurotrophins has been best characterized for NGF with its ability to influence peptides, cytokines, ion channels, and signaling pathways involved in the transfer of nociceptive information. In the intact state, these peptides are markers of the trkA population of sensory neurons and include substance P (SP), calcitonin gene related peptide (CGRP) and more recently pituitary adenylate cyclase activating peptide (PACAP) (Verge et al., 1989a, 1989b, 1992, 1995; Jongsma Wallin et al., 2001). Expression of these peptides is elevated in the presence of excess levels of NGF, such as observed with exogenous application of NGF (Verge et al., 1995; Amann et al., 1996; Fernyhough et al., 1998; Schuligoi and Amann, 1998) or with exposure to inflamed tissue that also has elevated levels of NGF (Verge et al., 1995; Zhang et al., 1995; Dickinson et al., 1999; Jongsma Wallin et al., 2001).

The role of NGF in nociception extends beyond the phenotypic regulation of the trkA-positive population of DRG neurons. Both local and systemic administration of NGF induce pain as reflected by the presence of hyperalgesia (Lewin et al., 1993; Woolf et al., 1994; Amann et al., 1996; Malcangio et al., 1997; Schuligoi and Amann, 1998), whereas sequestration of NGF using antibodies to NGF mitigates the development of hyperalgesia following nerve injury (Herzberg et al., 1997; Ro et al., 1999; Theodosiou et al., 1999). Spontaneous pain, another aspect of neuropathic pain, is potentially influenced by sympathetic basket formation around the larger sized neurons in the DRG as previously described. This sympathetic sprouting is encouraged by intrathecal NGF (Jones et al., 1999) and in a separate set of experiments, anti-NGF administration

differentially impeded this sprouting depending on the nerve injury model employed (Ro et al., 1996; Ramer and Bisby, 1999; Deng et al., 2000).

Although there is a plethora of evidence for the involvement of NGF in the development of nociception, Ren et al. (1995) report that NGF infused onto the ligated nerve immediately after ligation served to prevent the development of both thermal and mechanical hypersensitivity. In addition, the sprouting of Aβ fibers from lamina V into lamina II (a possible mechanism for the establishment of hyperalgesia and/or allodynia) is effectively prevented by intrathecal administration of NGF (Bennett et al., 1996). This evidence strengthens many people's hypothesis that NGF plays a key role in nociception, whereas its absence following injury may also influence plasticity events and neuropathic pain associated with this state.

A role for BDNF in neuropathic pain has also been well characterized, and although it is not the direct focus of this thesis, bears mentioning. Under inflammatory conditions, BDNF expression is elevated in an NGF dependent fashion (Cho et al., 1997a, 1997b) and appears to be an important mediator of central sensitization derived inflammatory pain (Kerr et al., 1999; Mannion et al., 1999; reviewed in Obata and Noguchi, 2006). In the uninjured state, BDNF is localized primarily to the NGF – responsive (trkA-positive) population of DRG sensory neurons (Apfel et al., 1996; Kashiba et al., 1997; Michael et al., 1997, 1999; Karchewski et al., 1999), but undergoes a phenotypic switch in response to nerve injury whereby BDNF expression in trkA-positive neurons is decreased and expression in trkB- and trkC-positive neurons is increased (Michael et al., 1999; Zhou et al., 1999; Karchewski et al., 2002). However, under conditions of inflammation or NGF administration, BDNF is upregulated in the small and medium sized (trkA-positive) DRG neurons (Apfel et al., 1996; Verge et al., 1996; Michael et al., 1997). A direct role for BDNF in the generation of neuropathic pain behaviors has also been demonstrated whereby exogenous BDNF was able to induce both thermal hyperalgesia and mechanical allodynia (Shu et al., 1999; Miki et al., 2000; Zhou et al., 2000). Subsequently, hyperalgesia elicited by BDNF sensitization of nociceptive afferents has been shown to be blocked by inhibitors of BDNF (Kerr et al., 1999; Thompson et al., 1999; Pezet et al., 2002). There is also compelling evidence that the process of inflammatory pain involves endogenous BDNF (Kerr et al., 1999;

Mannion et al., 1999; Thompson et al., 1999; Groth and Aanonsen, 2002; Matayoshi et al., 2005). It appears that BDNF signals through the DRG to the dorsal horn of the spinal cord promoting the development of chronic (central) pain (Obata et al., 2006; reviewed in Obata and Noguchi, 2006).

In contrast to NGF and BDNF, the role for NT-3 in nociceptive modulation is not yet clearly understood. NT-3 is able to effect changes in peptide content in both small and large fiber sensory neurons following complete or partial injuries that are believed to contribute to the ensuing pain state. Following injury, large fiber populations that express trkB and trkC begin to express many of the peptides and molecules that, in the intact state, are associated with nociceptive function in the trkA subpopulation (i.e. SP, CGRP, PACAP, BDNF) (Zhou et al., 1999; Jongsma et al., 2000; Malcangio et al., 2000; Jongsma Wallin et al., 2001; Karchewski et al., 2002).

A role for NT-3 in counteracting some of the changes in neuropeptide content in this diverse size range of neurons is strongly supported by its ability to mitigate injury-associated increases in Galanin, NPY, PACAP, BDNF (Gratto et al., 1994, 1995; Sterne et al., 1998; Karchewski et al., 2002; Wilson-Gerwing and Verge, 2006), SP (Gratto et al., 1994, 1995; Gratto 2002) and the pro-inflammatory, pro-nociceptive cytokine IL-6 (Verge and Johnston, 2000) that is also elevated in this population of chronically transected neurons (Murphy et al., 1995; Murphy et al., 1999).

Concerning the impact of NT-3 on the development of thermal and/or mechanical hypersensitivity, reports are not consistent. While I have previously reported that intrathecal infusion of NT-3 at the time of CCI can effectively prevent the development of thermal hyperalgesia and not impact mechanical hypersensitivity (Wilson-Gerwing et al., 2005), Gandhi et al. (2004) have reported that NT-3 can effectively treat mechanical hypersensitivity resulting from intramuscular acid injections. Malcangio et al. (1997) found that exogenous NT-3 induced hypoalgesia to mechanical, but not to thermal stimuli, while White (1998) found that NT-3 significantly reduced mechanical nociceptive thresholds as compared to saline controls. Finally, Shu et al. (1999) reports that NT-3 does not elicit sensitization to thermal stimuli. Clearly, more research must be undertaken to sort out NT-3's role in affecting changes to nociceptive threshold.

1.5 Recent molecules of interest to pain research

Over the past decade, the focus of investigations into the mechanisms and modulators of neuropathic pain has shifted from the examination of peptides and their related receptors to that of a variety of ion channels. Of primary interest to the development and maintenance of thermal hyperalgesia has been the capsaicin receptor transient receptor potential vanilloid receptor-1 (TRPV1) (Caterina et al., 1997, 2000; Davis et al., 2000; Garcia-Martinez et al., 2002). Another channel that has garnered much attention and controversy regarding its function is the acid sensing ion channel 3 (ASIC3) that is known to respond to tissue acidosis as occurs under inflammatory conditions (Steen et al., 1992; Rang et al., 1991; Reeh and Steen, 1996; Helminger et al., 1997; Jacobus et al., 1977). It has been proposed that ASIC3 plays a functional role in both nociception (Krishtal and Pidoplichko, 1981; Benson et al., 1999) and mechanosensation (Garcia-Anoveras et al., 2001; Price et al., 2001; Sluka et al., 2003; Mogil et al., 2005). Lastly, much attention has been paid to the tetrodotoxin resistant (TTX-R) sodium channels $Na_v1.8$ and $Na_v1.9$. One of the hallmarks of neuropathic pain is a change in the sensitivity of nociceptors (reviewed in Devor, 2006). It has long been known that the key modulators of cellular excitability are sodium channels (Hodgkin and Huxley, 1952). More recently, the involvement of sodium channels in the conveyance of increased neuronal sensitivity following nerve injury and inflammation have been the subject of vast amounts of research (for reviews see Lee et al., 2005; Amir et al., 2006; Rogers et al., 2006).

These channels (TRPV1, ASIC3, $Na_v1.8$ and $Na_v1.9$) are of particular interest to this thesis work as they have each been demonstrated to be positively regulated by NGF (Dib-Hajj et al., 1998; Fjell et al., 1999; Ji et al., 2002b; Mamet et al., 2002; Mamet et al., 2003; Fang et al., 2005). For each of TRPV1, ASIC3, and $Na_v1.8$, this NGF-related regulation occurs as a result of activation of various trkA/mitogen activated protein kinase (MAPK) pathways (Ji et al., 2002b; Mamet et al., 2003; Fang et al., 2005). In light of these reports, an examination of the MAPKs phospho-p38 and phospho-extracellular signal related kinase (pERK) were also examined as they have been shown to be activated by inflammatory mediators and are proposed to participate in the

generation and perhaps maintenance of pain syndromes following nerve injury (Ji et al., 2002a; Obata et al., 2004; reviewed in Ma and Quirion, 2005).

2 Hypothesis and Specific Aims

Neuropathic pain is a complex clinical problem occurring in response to peripheral nerve damage and characterized by hyperalgesia, allodynia, and spontaneous pain. The signals regulating these responses have become better elucidated in the past decade, and include the promotion of thermal hyperalgesia, nociceptor phenotype and pain-related ion channels by the neurotrophin nerve growth factor (NGF). Recent evidence in our lab suggests that another member of this family of molecules, neurotrophin-3 (NT-3), acts in an antagonistic fashion to NGF in regulation of nociceptor phenotype.

The chronic constriction injury (CCI) model of neuropathic pain involves a partial lesion of the sciatic nerve induced by loosely tying the sciatic nerve with chromic gut sutures. This injury results in the slow, edematous axotomy of predominantly large diameter axons while leaving the intact smaller diameter axons exposed to a pro-inflammatory environment. The behavioral components of this injury include an increase in sensitivity to both thermal and mechanical stimuli, and the development of spontaneous pain. Thus, employing the CCI in rats, with or without intrathecal infusion of NT-3 via a mini osmotic pump, I have previously demonstrated that NT-3 can prevent some of the changes of sensory neurons that occur in response to CCI injury and are believed to contribute to neuropathic pain states. These findings demonstrated that NT-3 can antagonize the development of thermal hyperalgesia and molecules complicit in this behavior. This was the first direct demonstration of a role for NT-3 in antagonizing aspects of the NGF/trkA interaction associated neuropathic pain state. However, the true extent of NT-3's ability to antagonize nociceptive phenotype in sensory neurons, especially the trkA subpopulation, remained to be elucidated and is the major focus of my PhD thesis.

To achieve this I continued to investigate my original hypothesis that *NT-3 mitigates neuropathic pain and cellular signaling associated with inflamed states by attenuating signaling associating with NGF mediated trkA activation and thus presumably mitigating the normal upregulation of key molecules believed to contribute to this pain state.*

Specific Aims

To test this hypothesis and to reveal the behavioral and biochemical consequences of NT-3 affects on CCI-associated neuropathic changes, I addressed the following aims:

1) To assess the ability of exogenous NT-3 to reverse neuropathic pain behaviors if administered after their establishment.

2) To assess the ability of NT-3 to modulate the transient receptor potential vanilloid receptor-1 (TRPV1) pathway involved in thermal hyperalgesia

3) To assess the ability of exogenous NT-3 to mitigate elevated acid sensing ion channel 3 (ASIC3), phospho-p38 mitogen activated kinase (MAPK), and activated (phosphorylated)-extracellular regulated kinase (phospho-ERK) signaling associated with inflammation.

4) To assess the ability of exogenous NT-3 to mitigate sodium channel expression implicated in neuropathic pain.

3 Neurotrophin-3 suppresses thermal hyperalgesia associated with neuropathic pain and attenuates transient receptor potential vanilloid receptor-1 expression in adult sensory neurons

3.1 Abstract

Neurotrophin-3 (NT-3) negatively modulates nerve growth factor (NGF) receptor expression and associated nociceptive phenotype in intact neurons, suggesting a beneficial role in treating aspects of neuropathic pain mediated by NGF. I report that NT-3 is effective at suppressing thermal hyperalgesia associated with chronic constriction injury (CCI); however, NT-3 does not alter the mechanical hypersensitivity that also develops with CCI. Thermal hyperalgesia is critically linked to expression and activation of the capsaicin receptor, transient receptor potential vanilloid receptor-1 (TRPV1). Thus, its modulation by NT-3 after CCI was examined. CCI results in elevated TRPV1 expression at both the mRNA and protein levels in predominantly small-to-medium neurons, with the percentage of neurons expressing TRPV1 remaining unchanged at ~56%. Attenuation of thermal hyperalgesia mediated by NT-3 correlates with decreased TRPV1 expression such that only ~26% of neurons ipsilateral to CCI expressed detectable TRPV1 mRNA. NT-3 effected a decrease in expression of the activated component of the signaling pathway linked to regulation of TRPV1 expression, phospho-p38 MAPK (Ji et al., 2002b), in neurons ipsilateral to CCI. Exogenous NT-3 could both prevent the onset of thermal hyperalgesia and reverse established thermal hyperalgesia and elevated TRPV1 expression 1 week after CCI. Continuous infusion is required for the suppression of both thermal hyperalgesia and TRPV1 expression, because removal of NT-3 resulted in a prompt reestablishment of the

hyperalgesic state and corresponding CCI-associated TRPV1 phenotype. In conclusion, although NGF drives inflammation-associated thermal hyperalgesia via its regulation of TRPV1 expression, NT-3 is now identified as a potent negative modulator of this state.

3.2 Introduction

Neuropathic pain is a complex clinical problem that occurs in response to peripheral nerve damage and is characterized, in part, by hypersensitivity to thermal and/or mechanical stimuli. The signals regulating these responses are complex and include the promotion of thermal hyperalgesia and nociceptor phenotype by the neurotrophin nerve growth factor (NGF) through activation of its receptor, tropomyosin-related kinase A (trkA) (Verge et al., 1989b, 1995; Lewin and Mendell, 1993; Jongsma Wallin et al., 2001, 2003). Recently, it has been shown that the upregulation of NGF after peripheral inflammation leads to the activation of the mitogen-activated protein kinase (MAPK) p38 (Ji et al., 2002b). Widmann et al. (1999) and Ji and Woolf (2001) characterized p38 MAPK as a protein kinase that is stress-activated and plays a role in both injury responses and cell death. Secondary to p38 MAPK activation by NGF is the upregulation of the transient receptor potential vanilloid receptor-1 (TRPV1) (Ji et al., 2002b). TRPV1, in turn, plays a critical role in inflammation and injury-induced thermal hyperalgesia (Caterina et al., 1997, 2000; Davis et al., 2000; Garcia-Martinez et al., 2002). Thus, NGF is linked to TRPV1-mediated thermal hyperalgesia, having a role in both sensitization and positive regulation of TRPV1 expression (Chuang et al., 2001; Winston et al., 2001; Ji et al., 2002b).

Evidence emerging from our lab indicates that another neurotrophin, neurotrophin-3 (NT-3), acts in a manner that is antagonistic to NGF. Although trkA is the cognate receptor for NGF, NT-3 is also capable of activating trkA and trkB in addition to its own cognate receptor (trkC) (for review, see Lindsay, 1996). Our findings reveal that in intact neurons, NT-3 effects a notable reduction in trkA, high-affinity NGF binding sites, brain-derived neurotrophic factor (BDNF), substance P, calcitonin gene-related peptide, and pituitary cAMP-activated peptide levels (Verge et

al., 1989a,b, 1992, 1995; Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003). This ability of NT-3 to antagonize nociceptive phenotype in sensory neurons led me to speculate that exogenous NT-3 may prove beneficial in the treatment of neuropathic pain. Thus, in this study, I examined whether NT-3 could functionally antagonize neuropathic pain associated with chronic constriction injury (CCI), an injury that elicits a series of changes in primary sensory neurons believed to contribute to the ensuing neuropathic pain state (Bennett and Xie, 1988; Kajander and Bennett, 1992; Nahin et al., 1994; Miki et al., 1998). The evaluation focuses on the ability of NT-3 to alter activation of p38 MAPK and subsequent elevation of TRPV1, as well as the resultant thermal hyperalgesia associated with CCI.

3.3 Material and methods

3.3.1 Animal surgery

All animal procedures were conducted in accordance with the National Institutes of Health policy on the use of animals in research and the University of Saskatchewan animal care committee guidelines (protocol 19920164). A total of 45 young adult male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 250-300 g were used. Animals were anesthetised for surgery with sodium pentobarbital (Somnitol, 65 mg/kg; MTC Pharm, Cambridge, Ontario, Canada). Preoperative and postoperative (for 24 h) subcutaneous injections of buprenorphine (Temgesic, 0.1-0.2 mg/kg) were given to alleviate any postoperative discomfort. To examine the influence of NT-3 on development of neuropathic pain responses, 10 rats were used: 5 underwent 7 d unilateral CCI of the right sciatic nerve as described by Bennett and Xie (1988) during which the sciatic nerve was exposed and loosely ligated with four chromic gut sutures (intact; CCI); and 5 rats received 7 d unilateral CCI with intrathecal infusion of NT-3 for the duration of the injury (intact + NT-3, CCI + NT-3). To examine the ability of NT-3 to reverse neuropathic pain responses and whether continuous infusion is required for

the effect, 21 rats were used: 7 underwent 14 d unilateral CCI; 7 received intrathecal infusion of NT-3 on day 7 after CCI [intact + NT-3 (delayed), CCI + NT-3 (delayed)]; and 7 rats received intrathecal infusion of NT-3 at the time of CCI with pump removal at 7 d after CCI [intact + NT-3 (immediately); CCI + NT-3 (immediately)]. To examine the effect of NT-3 on total protein levels using Western blot analysis, 12 rats were used: 6 rats underwent 7 d unilateral CCI and 6 rats received 7 d unilateral CCI with intrathecal infusion of NT-3 for the duration of the injury (intact + NT-3; CCI + NT-3).

NT-3 was delivered intrathecally for 7 d via mini-osmotic pumps (model 2001; Alza, Cupertino, CA) inserted at the lumbar sacral junction as per Verge et al., (1989a) at a concentration and rate of 600 ng/µl/hr (Karchewski et al., 2002) in a solution of PBS containing rat serum albumin (1 mg/ml), streptomycin (100 U/ml), and penicillin (100 U/ml). This dose of NT-3 was the minimum dose found to selectively reverse injury-associated gene expression in injured trkC-expressing neurons (Verge et al., 1996; Jongsma Wallin et al., 2001; Karchewski et al., 2002). At the conclusion of the experiments, rats were killed, and tissue was dissected and processed for *in situ* hybridization and/or immunohistochemistry as described below.

3.3.2 *In situ* hybridization

Deeply anesthetised animals were perfused via the aorta with 0.1 M PBS, pH 7.4, followed by rapid dissection and freezing of right and left L4 and L5 dorsal root ganglia (DRG) in OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN) in a cryomold (Tissue Tek; Miles Laboratories, Elkhart, IN). Before sectioning, block containing pairs of experimental and control DRG were fused to ensure processing under identical conditions. Sections were cut at 6 µm on a Micron cryostat (Zeiss, Edmonton, Alberta, Canada), thaw mounted onto Probe-On^{+} slides (Fisher Scientific, Edmonton, Alberta, Canada), and stored with desiccant at -20 ^{o}C until hybridization.

Oligonucleotide probes complementary to and selective for TRPV1 mRNA [complementary to bases 509-542 and bases 2601-2634 (Caterina et al., 1997; Michael and Priestly, 1999)] were synthesized (University of Calgary DNA services, Calgary,

Alberta, Canada). All probes were checked against the GenBank database (National Institutes of Health, Bethesda, MD) to ensure that no more than 60% homology was found to sequences other than the cognate transcript. The probes were labelled at the 3'-end with α-[^{35}S]dATP |(New England Nuclear, Boston MA) using terminal deoxynucleotidyl-transferase (Amersham Biosciences, Piscataway, NJ) in a buffer containing 10 mM $CoCl_2$, 1 mM dithiothreitol (DTT), 300 mM Tris base, and 1.4 M potassium cacodylate, pH 7.2, and purified through Bio-Spin Disposable Chromatograph Columns (BioRad Laboratories, Hercules, CA) containing 200 mg of NENSORB PREP Nucleic Acid Purification Resin (DuPont NEN, Boston, MA). Dithiothreitol was added to a final concentration of 10 nM. Specific activities ranged from 4.5 to 5.0 X 10^6 cpm/ng oligonucleotide.

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

The specificity of hybridization signal for the TRPV1 probes used in the study was confirmed using serial sections hybridized with labelled probe, labelled probe with a 1000-fold excess of cold probe (which effectively competed all specific binding of labelled probe), or labelled probe with a 1000-fold excess of another, dissimilar cold probe of the same length and similar "G-C" content (which did not alter the hybridization signal pattern).

3.3.3 Quantification and analysis

All slides were analyzed qualitatively, and relative changes in hybridization signal from one experimental group to another were noted for sections mounted on the same slide to avoid bias attributable to the variance in signal observed from slide to slide. Slides selected for quantitative analysis had similar numbers of neurons in all DRG sections. Relative changes in hybridization signal over individual neurons with a visible nucleus were determined in DRG from different experimental treatments mounted on the same slide using computer-assisted image analysis as described previously (Karchewski et al., 1999). Analysis was performed as follows: for TRPV1 mRNA (7 d injury) on 12 DRG sections or 2823 neuronal profiles (intact: n=3 animals; CCI: n=3 animals; intact + NT-3: n=3 animals; CCI + NT-3: n=3 animals); for TRPV1 mRNA (14 d injury) on 12 DRG sections or 1992 neuronal profiles [intact: n=2 animals; CCI: n=2 animals; intact + NT-3(immediately): n=2 animals; CCI + NT-3(immediately): n=2 animals; intact + NT-3(delayed): n=2 animals; CCI + NT-3(delayed): n=2 animals].

Cells were considered labelled if they had more than five times background levels of silver grains, as determined by averaging grain densities over defined areas of the neuropil devoid of positively labelled cell bodies. This criterion of determining labelled neuronal profiles correlates well with the identification of labelled versus unlabeled neurons as determined manually using a 63X oil immersion objective.

3.3.4 Immunohistochemistry

Deeply anesthetised animals were perfused via the aorta with 0.1M PBS followed by 4% paraformaldehyde. Right and left L4 and L5 DRG were dissected, postfixed for 1-1.5 h, and cryoprotected in 20% sucrose overnight at 4 ^{o}C. Paired experimental and control tissues were frozen in the same cryomold to ensure processing under identical conditions. Transverse 10 µm sections were cut on the cryostat (Zeiss, Edmonton, Alberta, Canada) thaw-mounted onto Probe-On^{+} slides (Fisher Scientific, Edmonton, Alberta, Canada), and processed for immunohistochemistry.

For TRPV1, sections were washed three times for 10 min in 0.1 M PBS, blocked in 1.5% BSA, 4% horse serum, and 0.1% Triton X-100 in 0.1M PBS for 1 h at 4 ^{o}C, incubated overnight with rabbit anti-VR-1 (TRPV1) (10 µg/ml; Alpha Diagnostic International, San Antonio, TX), diluted in 0.5% BSA, 2% horse serum, and 0.1% Triton X-100 in PBS (0.1 M) at 4 ^{o}C, and visualized with Alexa Fluor 488 goat anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR) in PBS (0.1 M) with 0.5% BSA and 2% horse serum for 50 min at room temperature. Slides were washed and coverslipped with 50% glycerol/50% PBS. For phosphorylated p38 MAPK (phospho-p38), sections were washed in 0.1 M PBS, blocked with 10% horse serum and 0.1% Triton X-100 in 0.1M PBS for 1 h at room temperature, incubated overnight with rabbit anti-phospho-p38 MAPK (Cell Signaling Technology, Beverly, MA), diluted 1:50 in 1% BSA and 0.1% Triton X-100 in PBS (0.1 M) at 4 ^{o}C, and visualized using donkey anti-rabbit F(ab')$_2$ Cy3 conjugate (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.1 M PBS for 1 h at room temperature. The slides were washed and coverslipped with Citifluor (Marivac, Halifax, Nova Scotia, Canada). Control sections were processed in the same manner, but without the primary antibody. Results were viewed using a Zeiss Axioscope 50 microscope equipped with incident-light fluorescence optics and a CCD camera.

3.3.5 Western blots

The L4 and L5 DRG were dissected out and immediately placed in ice-chilled RIPA buffer (50 µl per three DRG) containing an anti-protease mixture (Sigma Aldrich, Oakville, Ontario, Canada) and 1 M DTT with 10 mM Na acetate, pH 5.3. The DRG were flash frozen/thawed three times and homogenized (Tissue Tearor; Biospec Products, Bartlesville, OK), and protein was extracted using pulse sonication for 3 s. The samples were ultracentrifuged at 14.5 K rpm at 4 ^{o}C for 30 min. A Bradford assay was conducted on the lysates so an equivalent quantity of protein could be loaded onto each gel. Equivalent loading was verified by Coomassie staining after transfer of the SDS-PAGE.

Protein samples were separated by SDS-PAGE and transferred onto a nitrocellulose paper. The blots were blocked with 7.5% milk powder and 0.5% Tween 20 overnight at 4 ^{o}C. The blots were then incubated with the phospho-p38 MAPK (Thr180/Tyr182) (1:1000; Cell Signaling Technology, Beverly, MA) or VR-1 (TRPV1) (1:1000; Alpha Diagnostic International, San Antonio, TX) primary antibody in PBS containing 5% milk powder and 0.5% Tween 20 overnight at 4 ^{o}C, followed by incubation in the HRP-linked secondary antibody (anti-rabbit IgG, 1:2000; Cell Signaling Technology, Beverly, MA) for 45 min at room temperature. All washes were done using PBS containing 0.1% Tween 20. A Western Lightening Chemiluminescence kit was used to visualize the bound HRP-conjugated secondary (PerkinElmer Life Sciences, Boston, MA). The membrane was exposed to Kodak X-Omat AR film and developed.

3.3.6 Assessment of behavioral responses

Male Wistar rats (250g) (Charles River Laboratories, Wilmington, MA) were acclimatized to the facility before the start of testing. Animals were housed individually in clear plastic cages and maintained in a 12 h light/dark cycle. Food and water were available *ad libitum*. All behavioral testing was performed at the same time of day to avoid hormonal cycling of the male rats.

Thermal hyperalgesia was assessed using the Hargreaves Plantar Test (Hargreaves et al., 1988) (Ugo Basile, Comerio, Italy). The animal was placed in a clear Plexiglas chamber atop a clear glass surface that housed a radiant heat source and acclimatized (~5 min). The heat source was placed directly under the plantar surface of the hindpaw and activated, starting a digital timer. The timer stopped when the paw was withdrawn from the heat source, indicating the paw withdrawal latency. A maximum time of 30 s was in place to prevent tissue damage. Five readings for each paw were taken (allowing 5 min intervals between paws to prevent sensitization). Baseline levels were established for 5 d before CCI and/or pump implantation. Behavioral testing resumed 2 d after surgery and continued on days 2, 4, 6, 9, 11, and 13 after surgery.

Because the thermal sensitivity of the uninjured paw contralateral to CCI (intact) was not altered throughout the experiments (Figure 3-1), the withdrawal latency of the CCI paw was divided by the withdrawal latency of the uninjured contralateral paw for each day of testing. The highest and lowest ratios were excluded. The remaining ratios were transferred into Prism graphing software (GraphPad, Version 4.0; GraphPad Software, San Diego, CA), and graphs were generated using the mean value for each day of testing +/- standard error of the mean (s.e.m.). ANOVA was used to determine significant differences between experimental (NT-3 infused) and control groups (CCI alone) (significance $p<0.05$).

Sensitivity of the hindpaw to mechanical stimulation was measured using calibrated von Frey filaments (Stoelting, Kiel, WI). Animals were placed in a clear Plexiglas chamber atop a modified semi-opaque Plexiglas platform [built according to the specifications of Pitcher et al. (1999) with slight modifications] and acclimatized (~5 min). Briefly, a 3 mm-thick piece of semi-opaque Plexiglas was cut to 30 X 30 cm. Small holes (1.5 mm diameter) were drilled in a grid pattern 5 mm apart to cover the entire surface. This testing surface was then placed on top of a metal frame. Each van Frey filament was applied perpendicular to the plantar surface of the hindpaw five times, and the number of times that the paw was withdrawn from the hair was recorded. Increasing sizes of hairs were applied until the hindpaw was withdrawn four times out of five applications. This was defined as the threshold for withdrawal. The interval between application of increasing size filaments and between paws was 5 min (to prevent sensitization). Baseline levels were established for 5 d before CCI and/or pump implantation. Behavioral testing resumed on days 2, 4, 6, 9, 11, and 13 after surgery.

The data obtained from the assessment of mechanical sensitivity, with withdrawal threshold (in grams), for the CCI paw for each day of testing were normalized such that the mean withdrawal thresholds for baseline were given a value of 1. All data collected were graphed in Prism (GraphPad, Version 4.0; GraphPad Software, San Diego, CA). Graphs were generated using the mean values of all normalized withdrawal thresholds for each day of testing +/- s.e.m. Significant

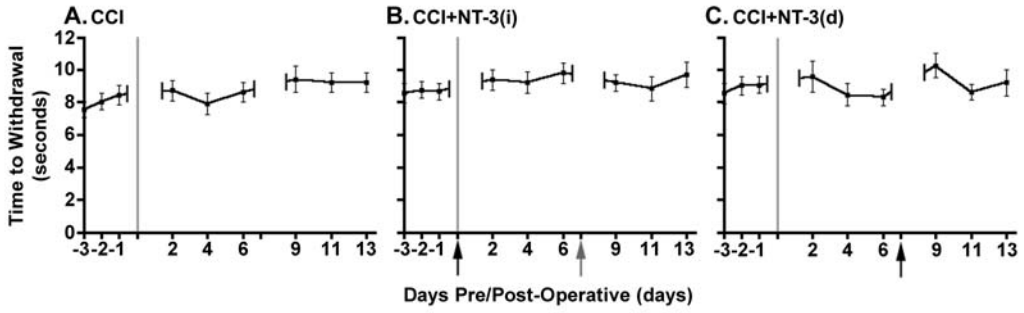

Figure 3- 1: NT-3 infusion does not alter withdrawal latency to thermal stimulation of contralateral intact hindpaw.

Hyperalgesia index plots represent changes in paw withdrawal latency (seconds) to thermal stimulation of the contralateral intact hind paw in response to 14 day CCI (A), 14 day CCI with immediate infusion of NT-3 [followed by removal for the last 7d (B)], and 14 day CCI with delayed infusion of NT-3 [7d post CCI (D)]. A, Paw withdrawal latency on the contralateral intact hind paw is not significantly altered by CCI (n=7). B, Immediate infusion of NT-3 does not significantly alter paw withdrawal latency on the contralateral intact hind paw (n=7). C, Delayed infusion of NT-3 does not significantly alter paw withdrawal latency on the contralateral intact hind paw (n=7). Gray line indicates the time of CCI; black arrow indicates the start of NT-3 infusion; gray arrow indicates the time of pump removal.

differences between experimental and control groups were determined using ANOVA ($p<0.05$).

3.4 Results

3.4.1 The effect of NT-3 on neuropathic pain indices associated with CCI: NT-3 significantly attenuates thermal hyperalgesia

The chronic constriction injury model was used to examine whether NT-3 (in its ability to downregulate trkA and associated phenotypes) alters behavioral indices of neuropathic pain. To determine whether NT-3 might be effective at reversing thermal

hyperalgesia once established and if continuous infusion of NT-3 is required for this effect, animals underwent a 14 d unilateral CCI of the sciatic nerve. These animals developed a significant increase in sensitivity to thermal stimulation in the injured hindpaw ($p<0.0001$) when compared to baseline levels (Figure 3-2A). Delayed infusion of NT-3 (days 7-14 after CCI) resulted in a significant reversal of the thermal hyperalgesia compared to those animals having undergone CCI alone ($p<0.0001$) (Figure 3-2C). In contrast, although immediate infusion of NT-3 significantly attenuated the development of thermal hyperalgesia ($p<0.0001$), removal of the NT-3 pumps at 7 d after CCI resulted in a quick reestablishment of the thermal hyperalgesic state (Figure 3-2B). Thus, the NT-3 mediated attenuation of thermal hyperalgesia associated with CCI requires continuous infusion of NT-3.

3.4.2 NT-3 has no significant influence on mechanical hypersensitivity associated with CCI

To determine whether delayed infusion of NT-3 or removal of exogenous NT-3 might alter mechanical responses, the 14 d unilateral CCI model was used. Animals having undergone unilateral CCI of the sciatic nerve for 14 d developed a significant increase in mechanical sensitivity ($p<0.0001$) (Figure 3-2D). Delayed infusion of NT-3 after CCI did not result in any perceptible changes in mechanical sensitivity compared with those having undergone CCI alone (Figure 3-2F). Furthermore, removal of NT-3 pumps at 7 d after injury did not impact the mechanical hypersensitivity (Figure 3-2E).

3.4.3 NT-3 prevents and counteracts CCI-associated increases in TRPV1 expression

Because of the dramatic influence of NT-3 on neuropathic thermal sensation, the regulation of TRPV1 expression was examined in the DRG of animals used for the behavioral studies. Analysis of sections processed for *in situ* hybridization to detect

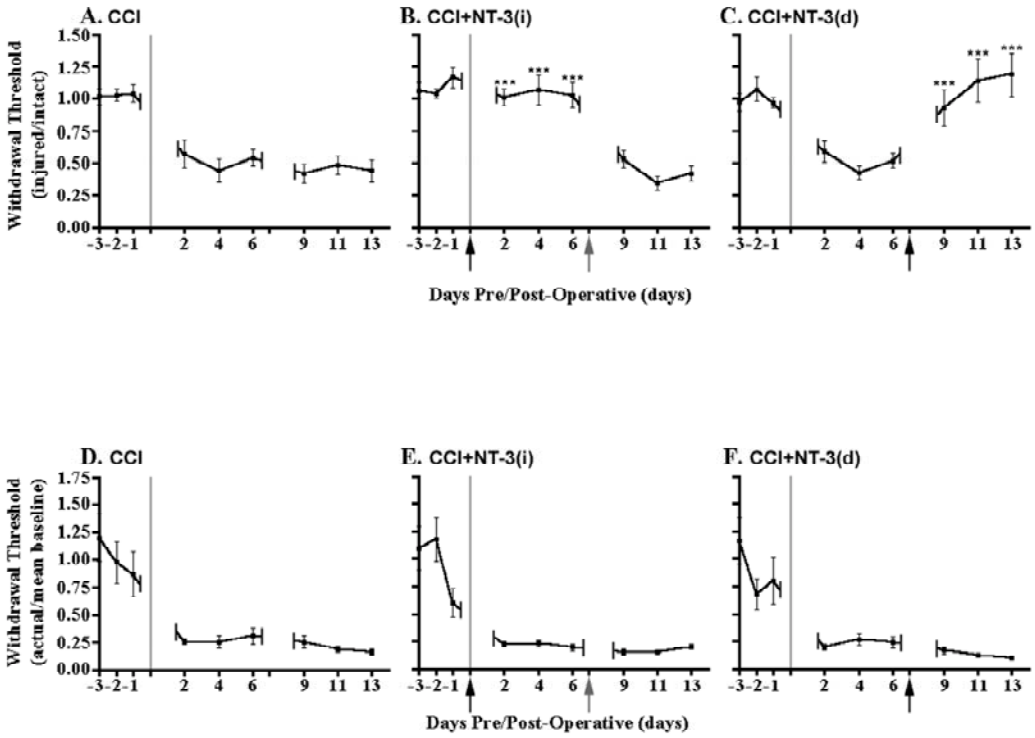

Figure 3- 2: Delayed infusion of NT-3 can reverse established thermal hyperalgesia but not mechanical sensitivity after CCI.

Hyperalgesic index plots represent alterations in thermal (A-C) and mechanical (D-F) sensitivities in 14d CCI animals in response to immediate NT-3 [followed by removal for the last 7d (B, E)] or delayed NT-3 [7d post CCI (C, F)]. Gray line indicates the time of CCI; black arrow indicates the start of NT-3 infusion; gray arrow indicates the time of pump removal. Asterisks indicate significant difference from CCI alone. A, Thermal hyperalgesia is significantly increased compared with baseline levels after unilateral CCI and is maintained for the duration of the experiment (n=7). B, Infusion of NT-3 is required for continuous prevention of thermal hyperalgesia after CCI. Infusion of NT-3 significantly attenuates the development of thermal hyperalgesia after unilateral CCI and is maintained for the duration of the 7d infusion (***$p<0.0001$). After removal of the NT-3 pump, thermal hyperalgesia was reestablished and did not significantly differ from CCI alone (n=7). C, Delayed infusion of NT-3 significantly reverses thermal hyperalgesia compared with CCI alone (***$p<0.0001$) and is maintained for the duration of the infusion (7d) (n=7). D, Mechanical sensitivity is significantly increased compared with baseline levels after unilateral CCI and is maintained for the duration of the experiment (n=7). E, Immediate NT-3 infusion does not alter mechanical sensitivity compared with CCI alone. Stopping NT-3 infusion does not alter mechanical sensitivity compared with CCI alone (n=7). F, Delayed infusion of NT-3 does not alter mechanical sensitivity compared with CCI alone.

neuronal expression of TRPV1 mRNA revealed that in the DRG contralateral to CCI (intact), detectable hybridization signal was localized over small and medium neurons (<40 μm) representing 56.13 +/- 0.99% (s.e.m.) of all neurons measured (Figure 3-3). Neurons expressing moderate to high levels of hybridization signal (>20 X background) accounted for 24.77 +/- 1.88% (s.e.m.) of all neurons measured. Seven days after CCI, relative levels of hybridization signal over small and medium neurons were elevated and detectable over a few large neurons, albeit at relatively low levels (Figure 3-3). Despite these alterations in expression, the overall percentage of neurons expressing TRPV1 mRNA remained similar to that seem in the DRG contralateral to CCI [55.90 +/- 2.95% (s.e.m.)] with no significant change in the percentage of neurons with moderate to high levels of hybridization signal [25.57 +/- 5.19% (s.e.m.)].

NT-3 infusion effected a reduction in the levels of neuronal TRPV1 mRNA expression both ipsilateral (CCI + NT-3) and contralateral (intact + NT-3) to CCI (Figure 3-3). This effect was most profound for neurons ipsilateral to CCI. In DRG contralateral to CCI, levels of TRPV1 mRNA hybridization signal were modestly attenuated after infusion of NT-3 (Figure 3-3), with the overall percentage of neurons expressing detectable levels reduced from 56.13 +/- 0.99% (s.e.m.) to 45.50 +/- 6.98% (s.e.m.). In contrast, a far more dramatic attenuation of TRPV1 expression was observed in 7 d CCI animals that received NT-3 infusion for the duration of the injury [from 55.90 +/- 2.95% (s.e.m.) to 26.13 +/- 0.61% (s.e.m.)] (Figure 3-3). The decrease in TRPV1 mRNA was observed in all size ranges of neurons, with those expressing moderate to high levels of hybridization signal now representing only 5.07 +/- 1.69% (s.e.m.) of the neuronal population compared with 24.77 +/- 1.88% (s.e.m.) in animals with CCI alone. Notably, TRPV1 expression was no longer detectable in large-size neurons after NT-3 infusion (Figure 3-3).

Analysis of sections processed to detect neuronal expression of TRPV1 mRNA from animals having undergone 14 d CCI with either immediate (days 0–7) or delayed (days 7-14) infusion of NT-3 revealed that the patterns of TRPV1 expression from CCI plus immediate NT-3 appeared very similar to those of CCI alone. Quantification of hybridization signal over individual neurons from CCI or CCI plus immediate NT-3 revealed that detectable TRPV1 mRNA hybridization signal was observed

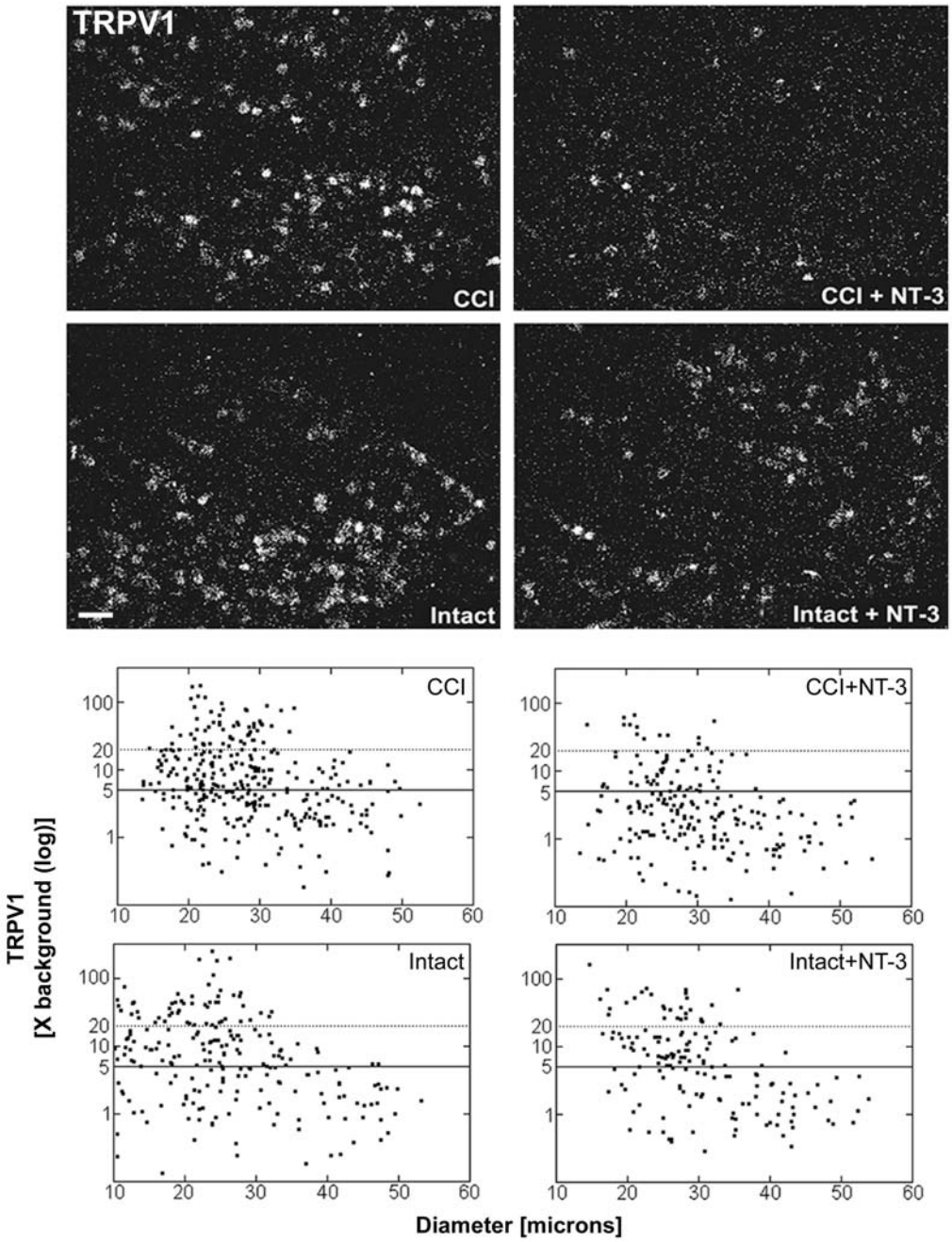

Figure 3- 3: Message levels for TRPV1 are reduced after NT-3 treatment.

Top: Darkfield photomicrographs of 6 µm thick adult rat L5 DRG sections processed for *in situ* hybridization to detect TRPV1 transcripts contralateral (Intact) or ipsilateral to 7d CCI (CCI) and after 7d unilateral CCI plus intrathecal infusion of 600 ng/µl/hr NT-3 (Intact+NT-3; CCI+NT-3). Scale bar, 100 µm. Note: NT-3 infusion results in the reduction in relative levels of hybridization signal for TRPV1 over individual neurons, with the influence most apparent after CCI.

Bottom: Representative scatterplots whereby each point represents the labeling index of an individual neuron identified in 6 μm thick sections of L5 DRG processed to detect TRPV1 mRNA. The relationship between TRPV1 mRNA labeling intensity (y-axis, log scale) and perikaryal diameter (x-axis) is depicted. Experimental states are indicated at the top right of each graph are as described above. Labeling refers to the ratio of silver grain density over the neuronal cytoplasm to grain density over areas of the neuropil devoid of positive hybridization signal. Solid lines divide the plots into labeled and unlabeled populations; dotted lines separate lightly labeled from moderate to heavily labeled populations of TRPV1-expressing neurons. Note: In DRG contralateral to CCI, TRPV1 is expressed predominantly in small to medium neurons. CCI results in elevated TRPV1 expression in small to medium neurons and a novel but low level of expression in large neurons. NT-3 infusion produced a reduction in the levels and percentage of neurons expressing detectable TRPV1 mRNA in DRG both ipsilateral and contralateral to CCI, with a more pronounced effect ipsilateral to injury.

predominantly over small-to-medium neurons, with some large neurons also expressing at 14 d after CCI (Figure 3-4). As with 7 d CCI, neurons expressing moderate to high levels of hybridization signal (> 20X background) were observed almost exclusively over the small-to-medium-sized neurons (Figure 3-4). This suggests that removal of exogenous NT-3 results in a reinstatement of the CCI phenotype. In contrast, animals receiving delayed NT-3 infusion, relative levels of TRPV1 mRNA expression were reduced with the effect most prominent for neurons ipsilateral to CCI [Figure 3-4, CCI + NT-3(d)].

Tissue sections were also processed for immunohistochemistry to ascertain whether modulation of TRPV1 protein expression by CCI and exogenous NT-3 corresponds to that observed at the mRNA level. Immunohistochemistry revealed that that in the DRG contralateral to CCI (intact), staining for TRPV1 protein was intense in a subpopulation of small neurons, whereas a more moderate level of staining was observed in other small and medium neurons. Seven day CCI resulted in a dramatic increase in the relative numbers of neurons that were intensely stained for TRPV1, with predominantly small and medium neurons, but also the occasional large neuron, fall into this category (Figure 3-5). Infusion of NT-3 for the duration of CCI (7 d) resulted in decreased intensity of TRPV1 staining that was most evident in the medium and large neurons from DRG ipsilateral to CCI. In DRG contralateral to CCI, NT-3 infusion did

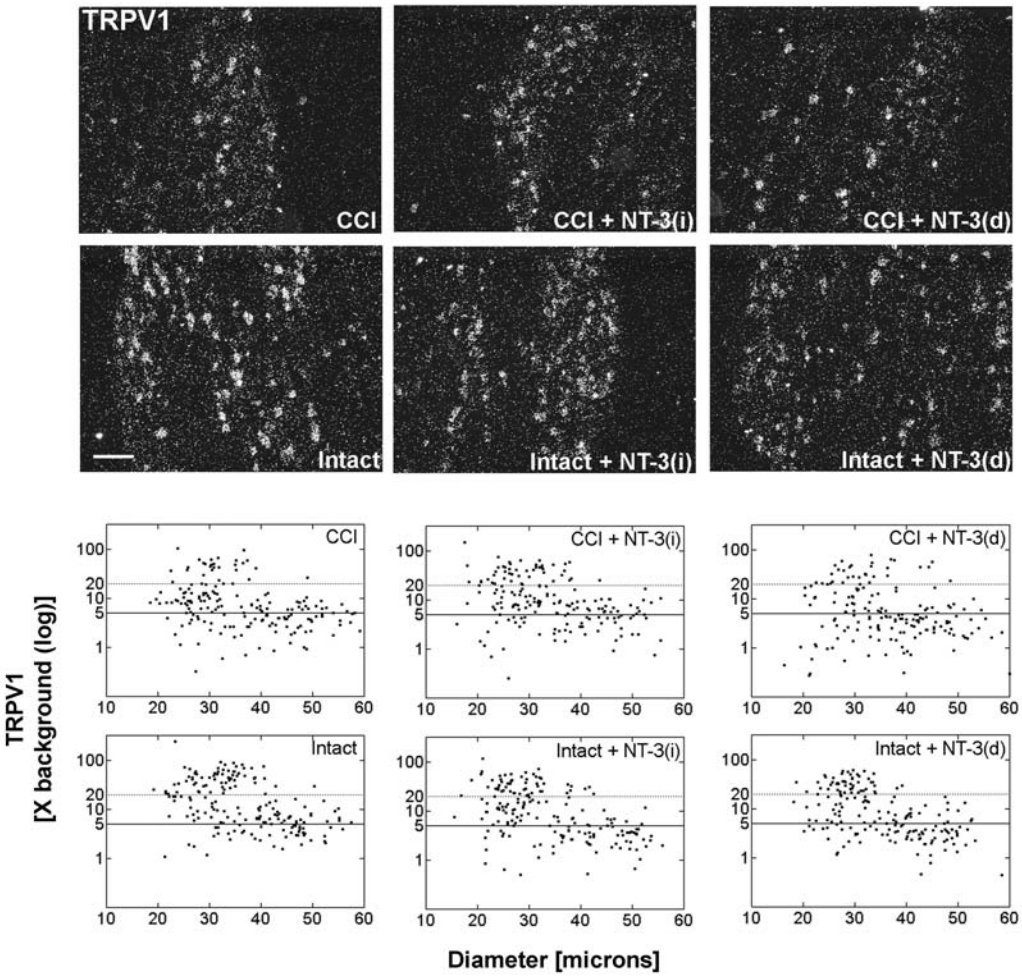

Figure 3- 4: Delayed NT-3 infusion reverses the increase in TRPV1 mRNA detected in DRG neurons subjected to CCI.

Top: Darkfield photomicrographs of 6 µm sections of L5 DRG ipsilateral or contralateral to 14d unilateral CCI, in response to 7d 600 ng/ µl/hr NT-3 infusion for the first 7d of a 14d unilateral CCI [CCI+NT-3(i); Intact+NT-3(i)], or in response to 7d 600 ng/ µl/hr NT-3 infusion for the last 7d of a 14d unilateral CCI [CCI+NT-3(d); Intact+NT-3(d)], as indicated. Scale bar, 60 µm. Note: Delayed infusion of NT-3 [CCI+NT-3(d)] results in a decrease in the relative levels of TRPV1 mRNA detected.

Bottom: Representative scatterplots whereby each point represents the labeling index of an individual neuron identified in 6 µm thick sections of L5 DRG processed to detect TRPV1 mRNA. The relationship between TRPV1 mRNA labeling intensity (y-axis, log scale) and perikaryal diameter (x-axis) is depicted. Experimental states as indicated are described above. Labeling refers to the ratio of silver grain density over the neuronal cytoplasm to grain density over areas of the neuropil devoid of positive hybridization signal. Solid lines divide the plots into labeled and unlabeled populations; dotted lines separate lightly labeled from moderate to heavily labeled populations of TRPV1

expressing neurons. Note: Delayed infusion of NT-3 [CCI+NT-3(d)] results in a decrease in the relative levels of TRPV1 most prominent in medium to large neurons.

not have a dramatic impact on TRPV1 immunostaining; however, more intense staining was observed over some small neurons (intact + NT-3) (Figure 3-5). Consistent with the immunohistochemistry findings, Western blot analysis revealed that TRPV1 protein levels were elevated after 7 d CCI. NT-3 infusion effectively mitigated this increase in DRG ipsilateral to CCI (CCI + NT-3), whereas slightly increased levels were observed in DRG contralateral to CCI (intact + NT-3) (Figure 3-5).

As with 7 d CCI, expression of TRPV1 protein in DRG ipsilateral to 14 d CCI was increased in small, medium, and some large neurons, relative to contralateral DRG (Figure 3-6). Delayed infusion of NT-3 resulted in decreased levels of TRPV1, such that the pattern and level of expression in neurons subjected to CCI [CCI + NT-3(d)] now approximated that observed in the DRG contralateral to CCI (intact) (Figure 3-6). The effect of delayed NT-3 infusion was less evident for neurons from DRG contralateral to CCI [intact + NT-3(d)], with only slight decreased expression observed in some medium neurons. As observed at the mRNA level, the ability of NT-3 to modulate TRPV1 protein expression requires continuous infusion of NT-3, because patterns of protein localization and expression levels resembled those of 14 d CCI animals when NT-3 infusion was halted for the last 7 d of injury (Figure 3-6).

3.4.4 NT-3 reduces the level of activated p38 MAPK after CCI

Tissue sections were processed for immunohistochemistry to ascertain whether CCI and exogenous NT-3 effect a coordinate change in activated p38 MAPK protein expression, a component of the signaling pathway shown previously to directly regulate TRPV1 expression in sensory neurons (Ji et al., 2002b). In DRG contralateral to CCI (intact), staining for phospho-p38 MAPK protein was very intense in a subpopulation of small neurons, whereas a more moderate level of staining was observed in other small

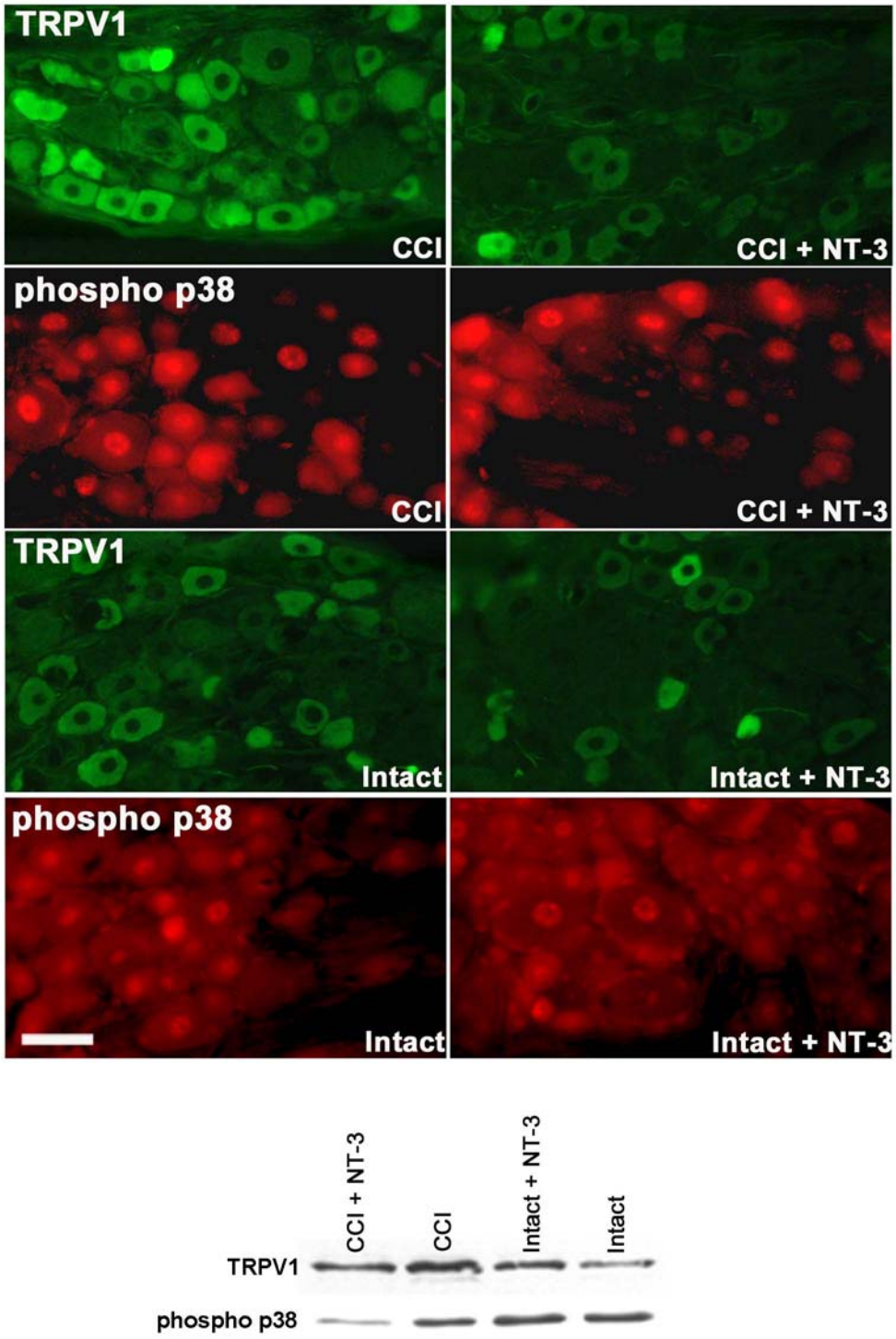

Figure 3- 5: NT-3 infusion results in decreased expression of TRPV1 protein.

Top: Fluorescence photomicrographs demonstrate levels of TRPV1 and phospho-p38MAPK-like immunoreactivity in 10 µm sections of DRG ipsilateral (CCI) and

contralateral to CCI (Intact) L5 DRG with or without immediate intrathecal infusion of NT-3 (CCI+NT-3; Intact+NT-3), as indicated. Scale bar, 60 μm. Note: In the DRG contralateral to CCI (Intact), levels of TRPV1 protein are highest in small neurons, with lower levels of expression observed in several small to medium dorsal root ganglion neurons. Seven days after CCI, levels of expression have increased relative to the intact state, with protein now being detected in a few large neurons. Intrathecal infusion of NT-3 at the time of injury results in reduced levels of TRPV1 protein, most notable in medium to large CCI neurons. Levels of phospho-p38MAPK nuclear expression do not appear to be altered by CCI. Cytoplasmic expression of phospho-p38MAPK does decrease slightly with CCI. Infusion of NT-3 results in a dramatic downregulation of phospho-p38MAPK staining.

Bottom: Representative Western bolts of TRPV1 (n=4) and phospho-p38MAPK (n=5) reflect the staining patterns as described above. Experimental states are as indicated.

and medium neurons. Seven day CCI resulted in a slight decrease in the cytoplasmic staining. This does not appear to be reflected in the pattern of nuclear staining that remained relatively unchanged (CCI) (Figure 3-5). Infusion of NT-3 effected a marked decrease in the intensity of phospho-p38 MAPK across all size ranges of neurons ipsilateral to CCI, with the exception of a subpopulation of small neurons that expressed elevated levels of phospho-p38 MAPK (CCI + NT-3) (Figure 3-5). In DRG contralateral to CCI, NT-3 infusion did not appear to alter neuronal nuclear staining patterns; however, there was a noticeable increase in perineuronal staining (intact + NT-3) (Figure 3-5). Consistent with the immunohistochemistry findings, Western blot analysis revealed that a 7 d CCI resulted in a slight decrease in the levels of phospho-p38 MAPK compared with DRG contralateral to CCI (intact). Infusion of NT-3 dramatically reduced the total phospho-p38 MAPK protein present in the DRG ipsilateral to CCI (CCI + NT-3), whereas it slightly increased levels of phospho-p38 MAPK in the DRG contralateral to CCI (intact + NT-3) (Figure 3-5).

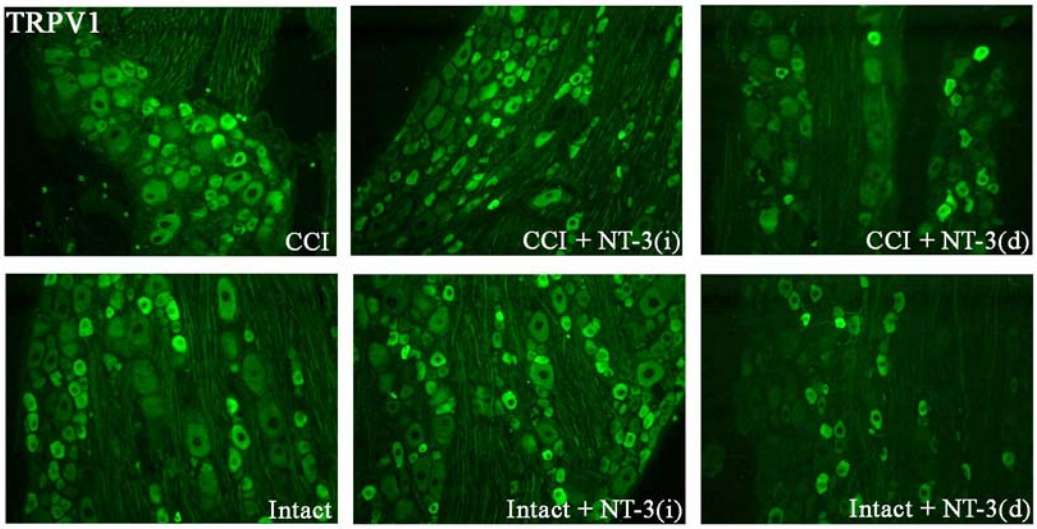

Figure 3- 6: Delayed NT-3 infusion reverses the increase in TRPV1 protein detected in DRG neurons subjected to CCI.

Fluorescence photomicrographs demonstrate levels of TRPV1-like immunoreactivity in 10 μm sections of L5 DRG representing DRG ipsilateral (CCI) and contralateral (Intact) to a 14d CCI, in response to immediate NT-3 infusion for 7d after CCI, followed by removal for the last 7d of the injury [CCI+NT-3(i); Intact+NT-3(i)], or in response to delayed NT-3 infusion for 7d starting 7d after CCI [CCI+NT-3(d); Intact+NT-3(d)], as indicated. Scale bar, 60 μm. Note: Delayed intrathecal infusion of NT-3 results in a dramatic decrease in the levels of TRPV1 protein detected [CCI+NT-3(d)], most notably in medium to large neurons. This affect is less pronounced on the DRG contralateral to CCI [Intact+NT-3(d)] (bottom center). Intrathecal infusion of NT-3 for 7d at the time of CCI, followed by removal for the last 7d of injury [CCI+NT-3(i)] (top right) results in similar levels of TRPV1 protein detected compared with CCI alone, suggesting that chronic infusion is required for mitigation of CCI-associated elevated TRPV1 expression.

3.5 Discussion

Neuropathic pain is a clinical challenge because of the complex mechanisms underlying this pathology. NT-3 can affect a downregulation in trkA expression, NGF high-affinity binding sites, and associated nociceptive phenotype in intact sensory neurons (Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003).

Given that enhanced NGF synthesis contributes to various neuropathic pain syndromes, I hypothesized that NT-3 could attenuate these pain syndromes and neuronal expression of molecules complicit in this state through its antagonistic influence on the trkA pathway. The present study supports this hypothesis, identifying NT-3 as a novel potent modulator of thermal hyperalgesia that influences behavioral responses and neuronal expression of TRPV1 and activated p38 MAPK.

NT-3 influences both trkC- and trkA- expressing neurons. There are two lines of evidence suggesting that its influence is selective for these populations. NT-3 reverses injury-associated changes only in the trkC population of sensory neurons (Verge et al., 1996; Jongsma Wallin et al., 2001; Karchewski et al., 2002). Furthermore, NT-3 does not alter somatostatin levels in either intact or injured sensory neurons that are responsive to GDNF and do not colocalize with trkA (Verge at al., 1995; Bennett et al., 1998; K.A. Gratto and V.M.K. Verge, unpublished observations). Finally, the influence of NT-3 on trkA phenotype is most apparent for that subpopulation of trkA neurons that do not express trkC (Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003).

3.5.1 NT-3 influences thermal hyperalgesia but not mechanical hypersensitivity associated with CCI

The ability of NT-3 to prevent and reverse thermal hyperalgesia, believed signalled by C-fibers (Field et al., 1999), is a novel finding with respect to modulation of neuropathic pain. NT-3 is generally thought to influence only large, myelinated proprioceptive neurons, despite evidence that it modulates expression of molecules associated with nociception (Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003).

Research examining the influence of NT-3 on neuropathic pain-related behaviors is limited. Inflammatory mechanical hyperalgesia was acutely and transiently reversed by local hindpaw injection of NT-3 (Watanabe et al., 2000); however, NT-3 altered neither thermal nor mechanical hypersensitivity resulting from spinal nerve ligation

(Boucher et al., 2000). This suggests that the degree of inflammation and/or type of injury may dictate whether NT-3 mitigates C-fiber pathologies. Neuroinflammation resulting from CCI plays a major role in the ensuing hyperalgesic pain state (Maves et al., 1993) and is associated with elevated NGF and cytokine levels (Maves et al., 1993; Sorkin, 2002). Furthermore, CCI preferentially axotomizes large-diameter neurons (Kajander and Bennett, 1992), leaving the remaining intact neurons exposed to a highly inflamed environment in which there is a strong infiltration of immune cells (TDW-G, unpublished observations).

The ability of NT-3 to prevent the development of thermal hyperalgesia is not likely caused by the elimination of sensory neurons contributing to this state because removal of NT-3 leads to a prompt reestablishment of the hyperalgesic state and CCI-associated TRPV1 phenotype. The reversion to this state indicates that the influence of NT-3 is transient. NT-3 can also reverse established thermal hyperalgesia. Both the prevention and abolishment of thermal hyperalgesia are likely impacted by the ability of NT-3 to suppress p38 MAPK activation and TRPV1 expression.

In contrast, the mechanical hypersensitivity observed after CCI is not altered by NT-3, despite downregulation of injury-associated peptide expression in larger neurons (Wilson and Verge, 2001). Mechanotransduction is mediated by specialized channels (Garcia-Anoveros et al., 2001; Price et al., 2001; Suzuki et al., 2003a, 2003b), but the manner in which expression or sensitization of these channels is altered by this form of injury or NT-3 is unknown; however, a role for NT-3 in the treatment of chronic mechanical hypersensitivity resulting from intramuscular acid injection indicates a potential role for NT-3 in the treatment of muscular pain (Gandhi et al., 2004).

3.5.2 Attenuation of thermal hyperalgesia by NT-3 correlates with a reduction in TRPV1 and activated p38 MAPK expression

Localization of TRPV1 expression to primarily small and medium neurons in contralateral intact DRG is in agreement with past studies (Caterina et al., 1997; Eglen et al., 1999; Ma, 2002). Although axotomy results in a dramatic reduction in TRPV1

expression (Michael and Priestly, 1999), spared neurons express elevated levels of TRPV1 (Fukuoka et al., 2001; Hudson et al., 2001), the latter presumably via NGF-mediated activation of p38 MAPK (Ji et al., 2002b). Although CCI resulted in slightly elevated TRPV1 expression observed in the size range of neurons consistent with C-fibers, it also induced a low level of expression in a novel population of large neurons. It is unknown whether this phenotypic switch factored into the observed thermal hyperalgesia. Alterations in TRPV1 expression effected by NT-3 were most robust for neurons ipsilateral to injury. In accordance with Ji et al. (2002b), the observed modulation of TRPV1 expression was most apparent at the protein versus mRNA level. The alterations in TRPV1 expression effected by CCI and exogenous NT-3 were mirrored by a coordinate modulation of activated p38 MAPK, a known regulator of TRPV1 expression (Ji et al., 2002b), implying a direct link to outcome; however, when infusion was halted, TRPV1 expression reverted back to that observed with CCI alone, as did the thermal hyperalgesia, suggesting a suppressive and not curative role for NT-3 in modulation of this state.

3.5.3 Potential mechanisms underlying NT-3 antagonism of thermal hyperalgesia

Thermal hyperalgesia associated with inflammation is conditioned by NGF and requires trkA and TRPV1 (Michael and Priestly, 1999; Shu et al., 1999; Davis et al., 2000; Galoyan et al., 2003). NGF promotes the sensitization of TRPV1 via release from PtdIns(4, 5)P_2-mediated inhibition (Chuang et al., 2001). Furthermore, trkA signaling can be enhanced by transactivation via G-protein-coupled receptors that are activated by inflammatory mediators and the pituitary adenylate cyclase-activating peptide, the latter being expressed at high levels in an NGF-dependent manner in trkA neurons and downregulated by NT-3 (Jongsma Wallin et al., 2001, 2003). The ability of NT-3 to affect a robust downregulation in TRPV1 expression after CCI and a coordinate decrease in trkA (TDW-G, unpublished observations) and activate p38 MAPK expression would impact on the degree to which an individual neuron responds to noxious heat. The rapid NT-3-mediated abolition or reestablishment of the response

after NT-3 removal implies that it is not solely dependent on alterations in protein expression but may also involve direct influence on signaling pathways that either alter the efficiency of trkA signaling or mediate sensitization of TRPV1. Whether endogenous nerve-derived sources of NT-3 serve to tonically inhibit this pathway is not known; however, constitutive levels of NT-3 in normal skin are higher than that of NGF, and they drop dramatically in the acute phase of the inflammatory response, suggesting a role in tonic inhibition in the uninjured state (Watanabe et al., 2000). Finally, because the NT-3 was delivered intrathecally, one cannot exclude a potential effect on NT-3 on central sensitization events that impact on the development of thermal hyperalgesia (for review, see Melzak et al., 2001; Romanelli and Esposito, 2004). Although not the focus of this study, previous work has shown that this form of NT-3 delivery mitigates the level of BDNF expressed by primary sensory afferents and linked to central sensitization events (Groth and Aanonsen, 2002; Garraway et al., 2003; Lever et al., 2003). The degree to which other molecules or cell types involved in this process are similarly influenced remains to be elucidated.

In conclusion, past studies reveal a major role for NGF in driving inflammation-associated thermal hyperalgesia via its activation of p38 MAPK and subsequent regulation of TRPV1 expression and other mediators of inflammatory responses. NT-3 is now identified as a potent negative modulator of this state. The extent to which it might ameliorate other aspects of inflammatory neuropathic pain responses remains to be elucidated.

4 Neurotrophin-3 attenuates expression of acid-sensing ion channel 3 in adult rat dorsal root ganglion following chronic constriction injury

4.1 Abstract

Chronic constriction injury (CCI) results in a partial nerve injury and marked inflammation in the nerve leading to a robust neuronal cell body response in the corresponding DRG. We have previously demonstrated that exogenous NT-3 can prevent/reverse a number of these changes including the nerve growth factor (NGF) dependent upregulation of transient receptor potential vanilloid receptor-1 (TRPV1) and p38 mitogen-activated protein kinase. ASIC3, a proton-gated sodium channel, localized primarily to nociceptive neurons of the DRG, has been shown to be upregulated through the trkA/JNK/p38MAPK pathway via NGF under inflammatory conditions. As NT-3 is a potent negative modulator of this pathway, I hypothesized that NT-3 would negatively modulate ASIC3 expression in neurons associated with CCI. In response to either 7 or 14 day CCI, ASIC3 hybridization signal was increased over all size ranges of neurons. NT-3 treatment for 7d at the time of CCI significantly prevented the increase in ASIC3 mRNA hybridization signal. Whether NT-3 was infused either immediately or in a delayed fashion, the net influence was decreased expression of ASIC3. Interestingly, continuous infusion of NT-3 was not required for this effect, as removal of the pump did not result in reversal of the effect, contrary to that previously reported for TRPV1 and thermal hyperalgesia (Wilson-Gerwing et al., 2005). In conclusion, NT-3 infusion disables the mechanism by which ASIC3 is upregulated in response to CCI.

4.2 Introduction

Neuroinflammation following injury to a peripheral nerve results in a series of events that contribute to the ensuing neuropathic pain state. These events include the activation of mast cells (Olsson, 1967), the recruitment of neutrophils and macrophages (Perry et al., 1987), and the increased expression of pro-inflammatory cytokines (Cunha et al., 1992; Ferreira et al., 1988). This "inflammatory soup", as it has been referred to, contributes to the sensitization of peripheral nerve terminals and leads to a drop in extracellular pH of the surrounding tissue (for review, see Moalem and Tracey, 2006; Myers et al., 2006).

The chronic constriction injury (CCI) model of neuropathic pain developed by Bennett and Xie (1988) results in a complex physiological response characterized by the preferential axotomy of large-diameter axons and exposure of the uninjured axons to the Wallerian degenerating nerve and denervated skin where many proinflammatory molecules, including NGF, are produced (Kajander and Bennett, 1992). The inflammatory condition also results in tissue acidosis brought about by a drop in extracellular pH (Steen et al., 1992; Rang et al., 1991; Reeh and Steen, 1996; Helminger et al., 1997; Jacobus et al., 1977) which contributes to the ensuing pain states (Steen and Reeh, 1993; Steen et al, 1995; Issberner et al, 1996; Reeh and Steen, 1996).

The molecular basis by which sensory neurons detect these alterations in pH have only recently been elucidated. In 1997, Waldmann et al. cloned a channel that opened in response to a decrease in extracellular pH that was specific to sensory neurons (DRASIC). This channel was subsequently renamed acid-sensing ion channel 3 (ASIC3). ASIC3 is a sodium channel belonging to the epithelial sodium channel/degenerins (ENaC/DEG) superfamily (Waldman and Lazdunski, 1998) whose proposed functions include roles in nociception (Krishtal and Pidoplichko, 1981; Benson et al., 1999) and mechanosenstation (Garcia-Anoveras et al., 2001; Price et al., 2001; Sluka et al., 2003; Mogil et al., 2005). Of the 6 ASIC isoforms that have been identified (Waldmann et al., 1996; Bassilana et al., 1997; Garcia-Anoveros et al., 1997; Lingueglia et al., 1997; Waldmann et al., 1997b; Waldmann et al., 1997a; Chen et al., 1998;

Grunder et al., 2000), ASIC3 is the most sensitive to protons, opening in response to a pH drop of as little at 0.4 (from 7.4 to 7.0) (Sutherland et al, 2001).

Consistent with a potential role in the generation and modulation of peripheral pain, ASIC3 expression is specific to dorsal root ganglion (DRG) neurons (Chen et al., 1998; Waldmann and Lazdunski, 1998; Voilley et al., 2001) and is expressed primarily by small to medium sized neurons (currently recognized as nociceptors) (Chen et al., 1998; Waldmann et al., 1997b; Molliver et al., 2005; Voilley et al., 2001). Most importantly, basal expression of ASIC3 is controlled by a low, endogenous level of the neurotrophin nerve growth factor (NGF) (Mamet et al., 2002). Subsequently, under conditions of inflammation, where NGF levels are increased, ASIC3 expression is upregulated through a trkA/c-jun N-terminal kinase/p38 mitogen activated protein kinase pathway (trkA/JNK/p38MAPK) (Mamet et al., 2003).

Our lab has previously demonstrated that neurotrophin-3 (NT-3) can effectively antagonize a number of the pro-inflammatory effects of NGF (Verge et al., 1989a, b, 1992, 1995; Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003; Wilson-Gerwing et al., 2005). As well, we have previously demonstrated that NT-3 can both prevent and reverse the development of thermal hyperalgesia following CCI, likely through its ability to dampen activation of the p38 MAPK pathway. This effect was also associated with reduced expression of the protein complicit in this behavioral response, transient receptor potential vanilloid receptor 1 (TRPV1) (Wilson-Gerwing et al., 2005). Thus, in this study, we examine whether the ability of NT-3 to regulate phospho-p38MAPK following chronic constriction injury (CCI) translates into an ability to also modulate ASIC3 expression.

4.3 Methods

4.3.1 Animal surgery

All animal procedures were conducted in accordance with the National Institutes of Health policy on the use of animals in research and the University of Saskatchewan animal care committee guidelines (protocol 19920164). A total of 47 young adult male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 250-300 g were used. Animals were anesthetized for surgery with sodium pentobarbital (Somnitol, 65 mg/kg; MTC Pharm, Cambridge, Ontario, Canada). Pre- and post-operative (for 24 h) subcutaneous injections of buprenorphine (Temgesic, 0.1-0.2 mg/kg) were given to alleviate any post-operative discomfort. To examine the effect of NT-3 on the expression of ASIC3, 26 rats were used: 13 underwent 7 d unilateral CCI of the sciatic nerve (Bennett and Xie, 1988) and 13 received 7 d unilateral CCI with intrathecal infusion of NT-3 for the duration of the injury. To examine the ability of NT-3 to reverse the upregulation of ASIC3 after CCI and whether continuous infusion is required for this effect, 21 rats were used: 7 underwent 14 d unilateral CCI; 7 received intrathecal infusion of NT-3 at the time of CCI with pump removal at 7 d after CCI [Intact + NT-3(immediately), CCI + NT-3(immediately)]; 7 received intrathecal infusion of NT-3 on day 7 after CCI [Intact + NT-3(delayed), CCI + NT-3(delayed)].

NT-3 was delivered intrathecally for 7 d via mini-osmotic pumps (model 2001; Alza, Cupertino, CA) inserted at the lumbar sacral junction as per Verge et al. (1989a) at a concentration and rate of 600 ng/μl/hr (Karchewski et al., 2002) in a solution of PBS containing rat serum albumin (1 mg/ml), streptomycin (100 U/ml), and penicillin (100 U/ml). This dose of NT-3 was the minimum dose found to selectively reverse injury-associated gene expression in injured trkC-expressing neurons (Verge et al., 1996; Jongsma Wallin et al., 2001; Karchewski et al., 2002). At the conclusion of the experiments, rats were killed, and tissue was dissected and processed for *in situ* hybridization and/or immunohistochemistry as described below. Previous studies have demonstrated a lack of influence ipsilateral and contralateral to injury when vehicle is

infused intrathecally (Verge et al., 1989a; Verge et al., 1995; Jongsma Wallin et al., 2001; Wilson-Gerwing et al., 2005).

4.3.2 *In situ* hybridization

Deeply anesthetized animals were perfused via the aorta with 0.1 M PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1M PBS. The right and left L4 and L5 DRG were rapidly dissected, postfixed for 1 hour in the same fixative, and cryoprotected in 20% sucrose in 0.1M PBS overnight. Paired experimental and control tissues were mounted in the same cryomold (to ensure processing under identical conditions), covered with OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN, USA) and frozen in cooled isopentane. Transverse sections were cut at 6 μm on a Micron cryostat (Zeiss, Canada), thaw mounted onto Probe-On^{+} slides (Fisher Scientific, Edmonton, AB, Canada) and stored with desiccant at –20 ^{o}C until hybridization.

Prior to hybridization, slides were air dried for 15 minutes, followed by fixation in 4% paraformaldehyde for 20 minutes. Slides were washed 3 X 5 minutes in 1X PBS. Sections were then treated with proteinase K (20 μg/ml) containing 10 ml 1M Tris-HCl (pH 7.6), 2 ml 0.5 M EDTA, 200 μl proteinase K stock (20 mg/ml) and 188 μl dd$H_2$0 for 6 minutes. Slides were then rinsed for 5 minutes in 1 X PBS and post-fixed for 5 minutes in 4% paraformaldehyde. Slides were then rinsed 2 X 5 minutes in 1 X PBS, 1 X 5 minutes in DEPC-H_2O, and dehydrated in ascending alcohols.

An oligonucleotide probe complementary to and selective for ASIC3 mRNA [complementary to bases 851-898 (Waldmann et al., 1997b)] was synthesized (University of Calgary DNA services, Alberta, Canada). The probe was checked against the GenBank database (NIH) to ensure no greater than 60% homology was found to sequences other than the cognate transcript. The probe was labeled at the 3'-end with α-[^{35}S]dATP (New England Nuclear, Boston, MA, USA) using terminal deoxynucleotidyl-transferase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in a buffer containing 10 mM $CoCl_2$, 1 mM dithiothreitol, 300 mM Tris base and 1.4 M-potassium cacodylate (pH 7.2), and purified through Bio-Spin® Disposable Chromatograph Columns (Bio-Rad

laboratories, Hercules, CA, USA) containing 200 mg of NENSORB™ PREP Nucleic Acid Purification Resin (NEN®, Boston, MA, USA). Dithiothreitol was added to a final concentration of 10 nM.

Hybridization was carried out according to published procedures (Dagerlind et al., 1992) on a minimum of 5 slides from each group of experimental and control animals. Briefly, the sections were hybridized at 43 ^{o}C for 14-18 hours in a buffer containing 50% formamide (Sigma Aldrich, Oakville, ON, Canada), 4X SSC (1X SSC – 0.15 M NaCl, 0.015 sodium citrate), 1X Denhart's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl (N-laurylsarcosine), 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 500 µg/ml heat-denatured sheared salmon sperm DNA, 200 mM dithiothreitol and 10^7 cpm/ml of probe. After hybridization, the slides were washed for 4 X 15 mins in 1X SSC at 55^{o}C, dehydrated in ascending alcohols, processed for radioautography as per Karchewski et al., 2002 and exposed for 7 to 10 days before developing in D-19 (Kodak, Rochester, NY, USA).

The specificity of hybridization signal for the ASIC3 probe was confirmed as described in Wilson-Gerwing et al., 2005.

4.3.3 Quantification and analysis

All slides from the 13 groupings of 7 d experimental and control animals and all slides from the 7 groupings of the 14 d experimental and control animals were analyzed qualitatively and relative changes in hybridization signal from one group to another noted for sections mounted on the same slide to avoid bias due to the variance in hybridization signal observed from slide to slide. Representative slides from four (7 d group) and 3 (14 d group) were selected and subjected to further quantitative analysis. These slides had a similar number of neurons in all DRG sections. Photomontages of each section to be analyzed were prepared and individual neurons with a visible nucleus were identified. Using a 40X light objective and a 2X optivar with an interactive computer-assisted image-analysis system (Richardson et al., 1989), cross-sectional areas of individual neurons and the percentages of cytoplasmic areas covered by silver grains

were measured for each DRG. The area per grain was constant for all neurons and a correction for grain overlap was made to obtain a parameter linearly related to density of silver grains (Richardson et al., 1989). Software for the image analysis system was Northern Eclipse, Version 7.0 (Empix Imaging, Mississauga, ON, Canada) and supplemented with Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA) and Prizm 4.0 (Graph Pad Software, San Diego, CA). Cells were considered labeled if they had more than five times background levels of silver grains, as determined by averaging grain densities over defined areas of the neuropil devoid of positively labeled cell bodies. This criterion of determining labeled neuronal profiles correlates well with the identification of labeled versus unlabeled neurons as determined manually using a 63X oil immersion objective. A stereological approach was not employed in this study and therefore the data quantification may represent a biased estimate of (i) the percentage of the population expressing individual markers (based on incidence in the sections examined whereby all neurons with a nucleus present in that section were included in the analysis) and (ii) the actual neuronal size (which was determined based on the assumption that the presence of a nucleus in the section correlated will with the maximal diameter for that cell).

Analysis was performed in each instance on all neurons with a nucleus present in the section being quantified: for 7 d group – on 16 DRG sections or 3087 neuronal profiles (Intact: n = 4 animals, CCI: n = 4 animals, Intact + NT-3: n = 4 animals, CCI+NT-3: n = 4 animals); for 14 d group – on 18 DRG sections or 3855 neuronal profiles (Intact: n = 3 animals, CCI: n = 3 animals, Intact+NT-3(i): n = 3 animals, CCI+NT-3(i): n = 3 animals, Intact+NT-3(d): n = 3 animals, CCI+NT-3(d): n = 3 animals). Previous research from our lab has demonstrated that CCI does not induce bilateral hyperalgesia (Wilson-Gerwing et al., 2005) and we have not discerned any qualitative differences between sham-operated or naïve DRG and contralateral intact DRG with respect to ASIC3. Therefore, the contralateral intact DRG was used as an intact control.

4.3.4 Immunohistochemistry

Transverse 10 μm sections were cut on the cryostat, thaw-mounted onto Probe-ON^{+} slides (Fisher Scientific), and processed for immunohistochemistry. For ASIC3: sections were washed three times for 10 min in 0.1 M PBS, blocked in 2% goat serum and 0.3% Triton X-100 in 0.1 M PBS for 1 h at room temperature, incubated overnight with guinea pig anti-ASIC3 (1:100; Neuromics Antibodies, Bloomington, MN), diluted in 2% goat serum and 0.3% Triton X-100 in 0.1 M PBS at 4 ^{o}C, and visualized with goat anti-guinea pig F(ab')$_2$ Cy3 conjugate (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) in 2% goat serum in 0.1 M PBS for 1 h at room temperature. For phospho-p38MAPK: sections were washed three times for 10 min in 0.1 M PBS, blocked in 10% horse serum and 0.1% Triton X-100 in 0.1 M PBS for 1 h at room temperature, incubated overnight with rabbit anti-phospho-p38 MAPK (1:50; Cell Signaling Technology, Beverly, MA) diluted in 1.0% BSA and 0.1% Triton X-100 in 0.1 M PBS at 4 ^{o}C, and visualized with donkey anti-rabbit F(ab')$_2$ Cy3 conjugate (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.1 M PBS for 1 h at room temperature. Slides were washed and coverslipped with 50% glycerol/50% PBS. Control sections were processed in the same manner, but without the primary antibody. Results were visualized using a Zeiss Axioscope 50 microscope equipped with incident-light fluorescence optics and a digital camera.

4.4 Results

4.4.1 NT-3 prevents CCI-associated increases in ASIC3 mRNA

Analysis of sections processed for ISH to detect neuronal expression of ASIC3 mRNA revealed that in the DRG contralateral to CCI (Intact), detectable hybridization signal was localized over all size ranges of neurons. Seven days after CCI, relative levels or hybridization signal were elevated, predominantly in the small to medium sized

neuronal population but also in a small population of large neurons (Figure 4-1). This overall increased incidence of expression resulted in 25% more neurons expressing detectable ASIC3 mRNA [from 39.38% +/- 4.200 (s.e.m) to 49.18% +/- 3.766 (s.e.m.)]. Those neurons expressing moderate to high levels of ASIC3 mRNA were increased from 5.575% +/- 3.978 (s.e.m.) to 13.9% +/- 5.156 (s.e.m.) (Figure 4-1). This increase in expression was also reflected in the mean labeling index for these neurons [from 5.970 +/- 1.150 (s.e.m.) to 9.396 +/- 2.093 (s.e.m.)] (Figure 4-2).

NT-3's ability to effect reduced ASIC3 mRNA expression was predominantly evident on those neurons ipsilateral to CCI (CCI + NT-3) (Figure 4-1). In DRG contralateral to CCI (Intact + NT-3) the mean labeling index was not significantly altered [from 5.970 +/- 1.150 (s.e.m.) to 5.491 +/- 1.241 (s.e.m.)] (Figure 4-2) nor was the percentage of neurons expressing detectable levels of ASIC3 [from 39.20% +/- 4.126 to 33.58% +/- 5.381] (Figure 4-1). In contrast, a dramatic attenuation of ASIC3 expression was observed in the NT-3 treated DRG ipsilateral to CCI (CCI + NT-3). Mean neuronal labeling index was significantly decreased with NT-3 treatment [from 9.396 +/- 2.093 (s.e.m.) to 5.523 +/- 1.131 (s.e.m.)] (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 4-2). The percentages of neurons expressing detectable levels of ASIC3 mRNA were also reduced [from 42.79% +/- 4.989 (s.e.m.) to 33.63% +/- 4.163 (s.e.m.)]. This decrease in the relative level of ASIC3 expression was most noticeable in those neurons expressing moderate to high levels of ASIC3 [from 13.90% +/- 5.156 (s.e.m.) to 5.225 +/- 3.041 (s.e.m.)] (Figure 4-1). The infusion of NT-3 decreased levels of ASIC3 expression in all sizes of neurons, with no discernible difference between the decrease observed in small to medium versus medium to large sized neurons.

4.4.2 NT-3 suppresses and attenuates CCI-associated increases in ASIC3 mRNA

Analysis of sections processed to detect neuronal expression of ASIC3 mRNA from animals having undergone 14d CCI with either immediate (days 0–7) or delayed

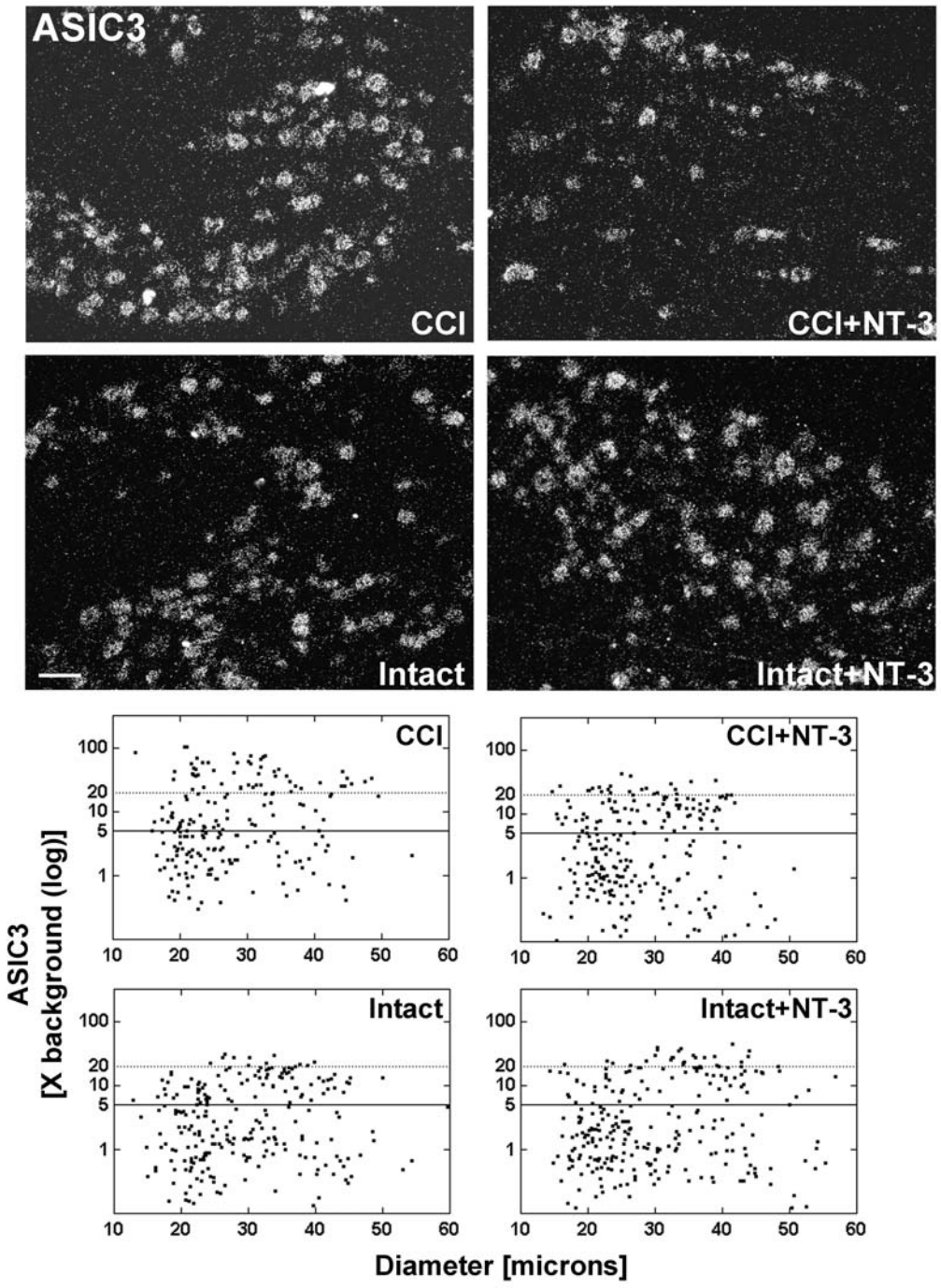

Figure 4- 1: ASIC3 mRNA levels are reduced in response to NT-3 treatment.

Top: Darkfield photomicrographs of 6 μm thick adult rat L5 DRG sections processed for *in situ* hybridization to detect ASIC3 transcripts contralateral (Intact) or ipsilateral to 7d CCI (CCI) and after 7d unilateral CCI plus intrathecal infusion of 600 ng/μl/hr NT-3 (Intact+NT-3; CCI+NT-3). Scale bar, 100 μm. Note: NT-3 infusion results in the reduction in relative levels of hybridization signal for ASIC3 over individual neurons after CCI.

Bottom: Representative scatterplots whereby each point represents the labeling index of an individual neuron identified in 6 µm thick sections of L5 DRG processed to detect ASIC3 mRNA. The relationship between ASIC3 mRNA labeling intensity (y-axis, log scale) and perikaryal diameter (x-axis) is depicted. Experimental states are indicated at the top right of each graph are as described above. Labeling refers to the ratio of silver grain density over the neuronal cytoplasm to grain density over areas of the neuropil devoid of positive hybridization signal. Solid lines divide the plots into labeled and unlabeled populations; dotted lines separate lightly labeled from moderate to heavily labeled populations of ASIC3-expressing neurons. Note: In DRG contralateral to CCI, ASIC3 is expressed in all size ranges of neurons. CCI results in elevated ASIC3 expression primarily in small to medium sized neurons and a small population of large neurons. NT-3 infusion resulted in a reduction in the levels and percentage of neurons expressing detectable ASIC3 mRNA ipsilateral to CCI.

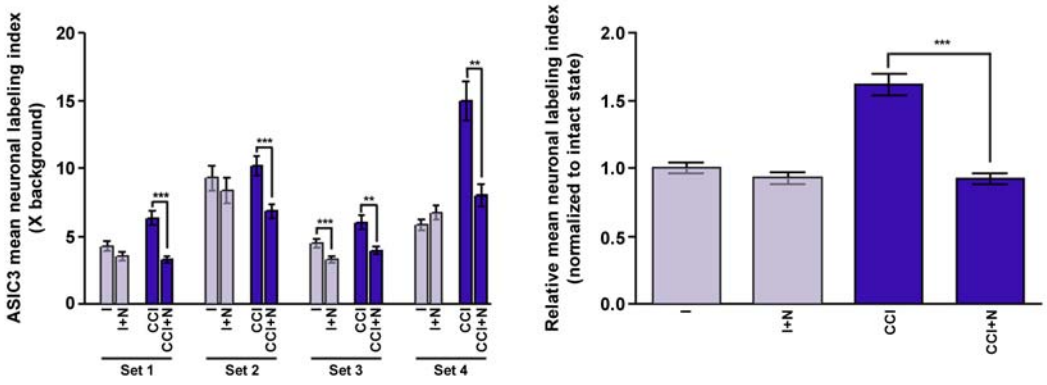

Figure 4- 2: Mean neuronal ASIC3 mRNA labeling indices following CCI are significantly decreased with NT-3 treatment.

Left: Graph depicts quantification of relative differences between experimental groups (as indicated) in ASIC3 mRNA mean labeling indices for all DRG neurons measured following *in situ* hybridization for 4 separate experiments [representing 4 animals having undergone 7 day unilateral CCI [Intact (I) and CCI] and 4 animals having undergone 7 day unilateral CCI with intrathecal infusion of NT-3 [Intact+NT-3 (I+N) and CCI+NT-3 (CCI+N)]. Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$, ** $p<0.01$). NT-3 infusion results in a significant decrease in mean ASIC3 mRNA expression in L5 neurons ipsilateral to CCI.

Right: Pooled data depicting the relative differences in mean labeling indices in ASIC3 mRNA normalized to the intact state in response to unilateral chronic constriction injury with or without NT-3 infusion (n=4). Experimental conditions are as described above.

Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). NT-3 infusion results in a significant decrease in mean ASIC3 mRNA expression in L5 neurons ipsilateral to CCI.

(days 7–14) infusion of NT-3 revealed that NT-3 effectively reduced levels of ASIC3 expression regardless of the timing of infusion. Quantification of hybridization signal over individual neurons from 14 d CCI revealed that detectable ASIC3 mRNA hybridization signal was observed over all size ranges of neurons (Figure 4-3). Those neurons expressing moderate to high levels of ASIC3 mRNA are significantly increased 2 weeks following CCI from 9.460% +/- 1.196 (s.e.m.) to 29.83% +/- 1.302 (s.e.m.) (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.05$). The mean neuronal labeling index is also increased [from 8.060 +/- 0.08729 (s.e.m.) to 15.55 +/- 1.110 (s.e.m.)]. Immediate infusion of NT-3 [CCI + NT-3(i)] significantly decreased the ASIC3 mean labeling index to a level equivalent to that of neurons contralateral to CCI from 15.55 +/- 1.110 (s.e.m.) to 7.844 +/- 0.8070 (s.e.m.) (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 4-4) despite the fact that the NT-3 pump was removed for the last 7 days of injury. The incidence of expression was also decreased from 51.57% +/- 0.6333 (s.e.m.) to 41.43% +/- 5.460 (s.e.m.). This decrease was most prevalent in those neurons expressing moderate to high levels of ASIC3 [from 29.83% +/- 1.302 (s.e.m.) to 12.43% +/- 1.466 (s.e.m.)] (Figure 4-3). Delayed infusion of NT-3 also significantly reduced the ASIC3 mean labeling index [from 15.55 +/- 1.110 (s.e.m.) to 5.017 +/- 0.08471 (s.e.m.)] (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.0001$) (Figure 4-4). Again, the percentage of neurons expressing ASIC3 mRNA was decreased [from 51.57% +/- 0.6333 (s.e.m.) to 33.27% +/- 1.545 (s.e.m.)], most apparent in those neurons expressing moderate to high levels of ASIC3 [from 29.83% +/- 1.302 (s.e.m.) to 4.267% +/- 0.4410 (s.e.m.)] (Figure 4-3). These decreases in expression were observed over all size ranges of neurons. This suggests that a single treatment of exogenous NT-3 is effective at both preventing and reversing

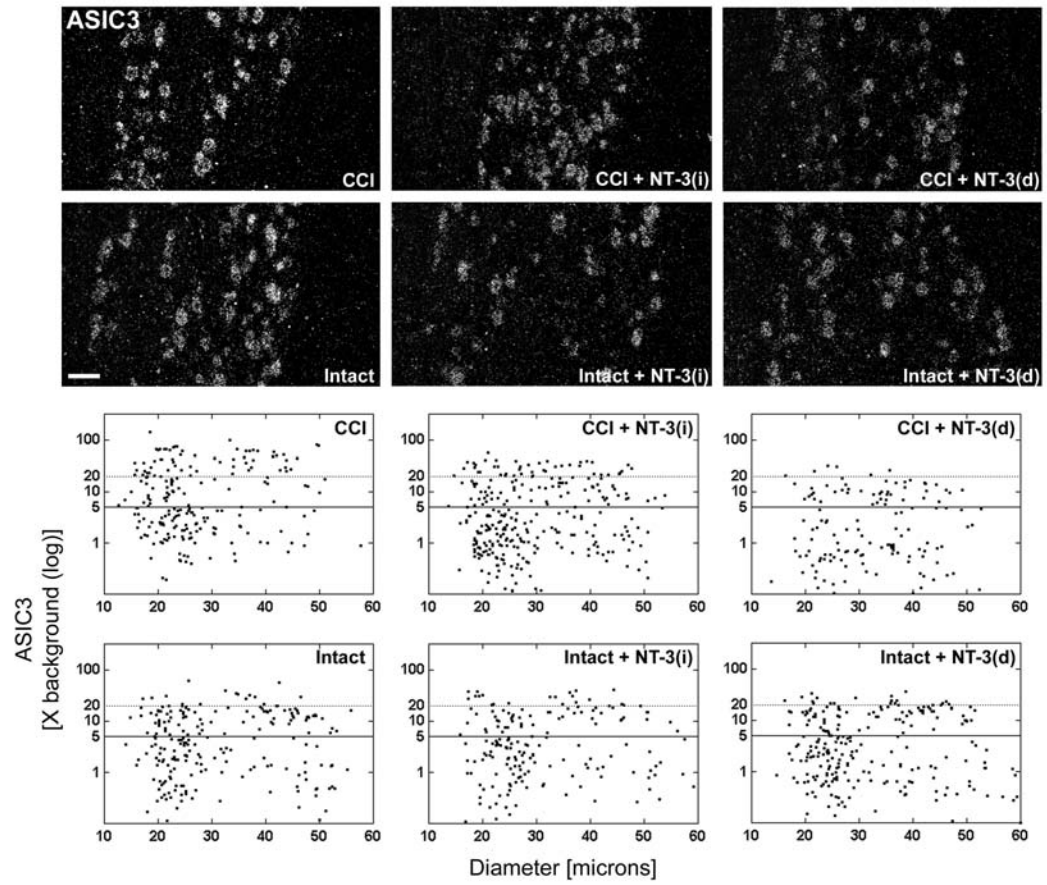

Figure 4- 3: Continuous NT-3 infusion is not required to attenuate CCI-associated ASIC3 mRNA expression in DRG neurons subjected to CCI.

Top: Darkfield photomicrographs of 6μm sections of L5 DRG ipsilateral and contralateral to 14d unilateral CCI [CCI and Intact] in response to 600 ng/μl/hr NT-3 infusion for the first 7 days of a 14d unilateral CCI [CCI+NT-3(i) and Intact+NT-3(i)], or in response to 600 ng/μl/hr NT-3 infusion for the last 7 days of a 14d unilateral CCI [CCI+NT-3(d) and Intact+NT-3(d)], as indicated. Note: Both immediate and delayed infusion result in a decrease in the relative levels of ASIC3 mRNA detected, with the decrease being more pronounced following delayed NT-3 treatment. Scale bar = 100 μm

Bottom: Representative scatterplots whereby each point represents the labeling index of an individual neuron identified in 6 μm thick sections of L5 DRG processed to detect ASIC3 mRNA. The relationship between ASIC3 mRNA labeling intensity (y-axis, log scale) and perikaryal diameter (x-axis) is depicted. Experimental states, as indicated, are described above. Labeling refers to the ratio of silver grain density over the neuronal cytoplasm to grain density over areas of the neuropil devoid of positive hybridization signal. Solid lines divide the plots into labeled and unlabeled populations; dotted lines separate lightly labeled from moderate to heavily labeled populations of ASIC3-expressing neurons. Note: 2 week CCI results in elevated ASIC3 expression in a

subpopulation of small, medium and large size sensory neurons. Both immediate and delayed infusion of NT-3 result in a decrease in the relative levels of ASIC3 mRNA expression ipsilateral to CCI, with the decrease being more pronounced with delayed NT-3 treatment.

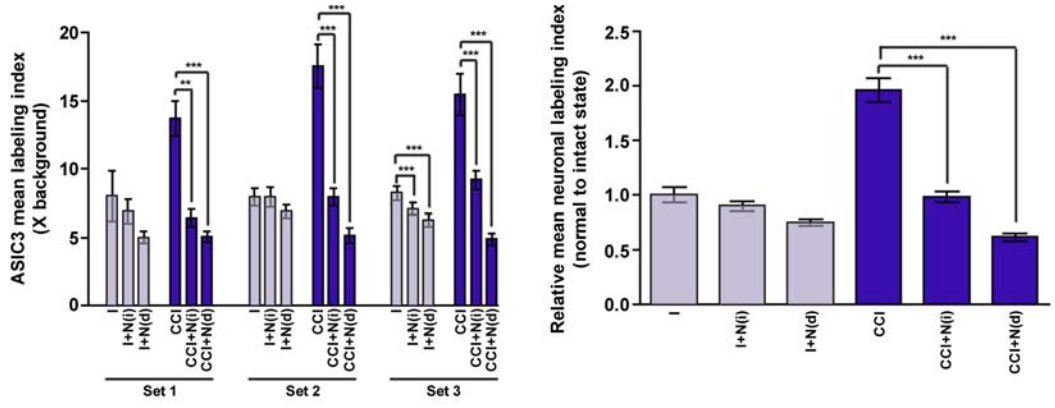

Figure 4- 4: Both immediate and delayed NT-3 treatment significantly decrease mean ASIC3 labeling indices following CCI.

Left: Graph depicts quantification of relative differences between experimental groups (as indicated) in ASIC3 mRNA mean labeling indices for all DRG neurons measured following *in situ* hybridization for 3 separate experiments [representing 3 animals having undergone 14 day unilateral CCI (Intact and CCI), 3 animals having undergone 14 day unilateral CCI with intrathecal infusion of NT-3 for the first 7 days of the injury [I+N(i) and C+N(i)], and 3 animals having undergone 14 day unilateral CCI with intrathecal infusion of NT-3 for the last 7 days of the injury [I+N(d) and C+N(d)]. Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$, ** $p<0.01$). NT-3 infusion results in a significant decrease in mean ASIC3 mRNA expression in L5 neurons ipsilateral to CCI when given for either the first or the last 7 days of a 14 day injury, the former demonstrating that continuous infusion of NT-3 is not required for this effect.

Right: Pooled data depicting the relative differences in mean labeling indices in ASIC3 mRNA normalized to the intact state in response to unilateral chronic constriction injury without NT-3 infusion or with immediate or delayed NT-3 infusion (n=3). Experimental conditions are as described above. Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). NT-3 infusion results in a significant decrease in mean ASIC3 mRNA expression in L5 neurons ipsilateral to CCI when given for either the first or the last 7 days of a 14 day injury. Continuous infusion of NT-3 is not required for this effect.

the ASIC3 CCI-associated phenotype and that continuous infusion of NT-3 is not required for the former effect.

4.4.3 Levels of ASIC3 protein are decreased following NT-3 treatment

Immunohistochemistry revealed that cytoplasmic ASIC3 protein levels were elevated both 7 and 14 days after CCI. This elevated expression was observed across all size ranges of neurons. However, while increases in message expression were more readily apparent in the small and medium sized neurons, alterations in protein expression were most apparent in the large neurons. CCI appeared to induce an increase in membrane localized expression of ASIC3. Infusion of NT-3 resulted in reduced levels of cytoplasmic and membrane localized ASIC3 protein expression most prominent in the DRG ipsilateral to CCI and regardless of the timing of the infusion. Again, it appears that continuous infusion of NT-3 is not required for this effect as levels did not recover to those of control injuries as reported for animals where the pump was removed for the last 7 days of the injury period (Figure 4-5 and Figure 4-6).

4.4.4 Levels of activated p38 MAPK are decreased in response to NT-3 treatment following CCI

We next examined whether CCI and/or NT-3 infusion had an impact on the level of activation of a transcription factor implicated in the regulation of ASIC3 expression. Immunohistochemistry to detect levels of phospho-p38MAPK in DRG sections from animals having undergone 14d unilateral CCI with or without immediate or delayed infusion of NT-3 revealed that following CCI, levels of phospho-p38MAPK were increased in all size ranges of neurons. Both immediate and delayed infusion of NT-3 result in a decrease in the relative levels of nuclear localized phospho-p38MAPK-like immunoreactivity detected. This decrease is more pronounced with delayed NT-3 treatment [CCI + NT-3(d)] (Figure 4-7).

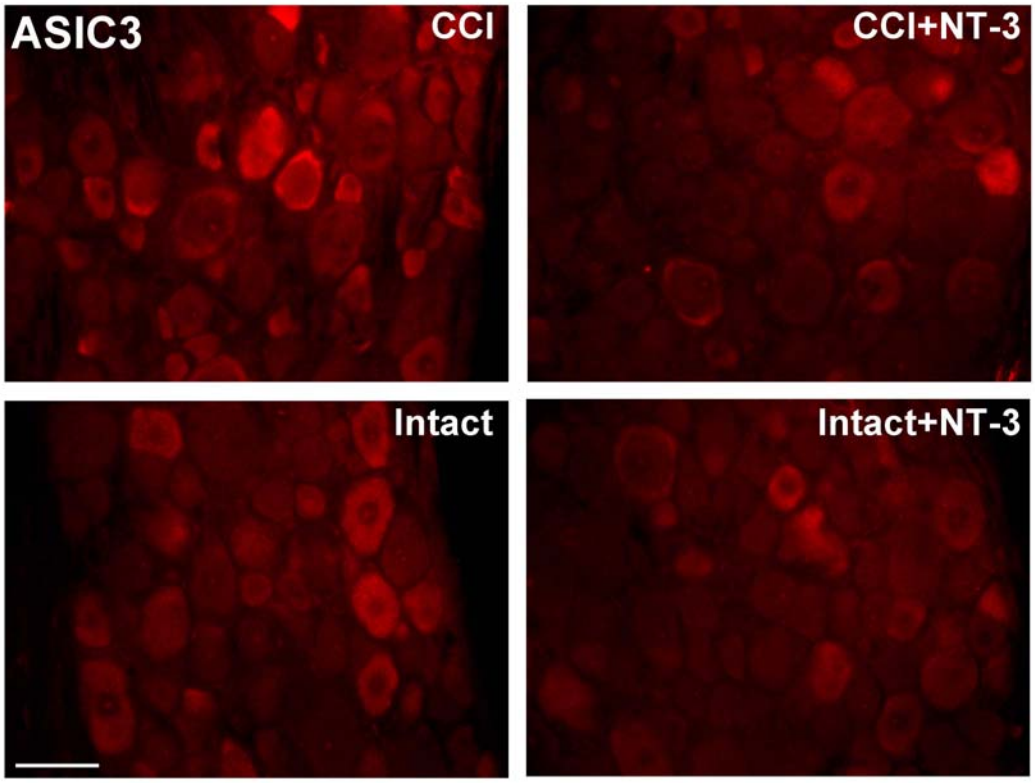

Figure 4- 5: NT-3 infusion results in decreased expression of ASIC3 protein.

Fluorescence photomicrographs demonstrate levels of ASIC3-like immunoreactivity in 10 μm sections of DRG ipsilateral (CCI) and contralateral to CCI (Intact) L5 DRG with or without immediate intrathecal infusion of NT-3 (CCI+NT-3; Intact+NT-3), as indicated. Scale bar, 60 μm. Note: In the DRG contralateral to CCI (Intact), levels of ASIC3 protein are highest in medium sized dorsal root ganglion neurons. Seven days after CCI, levels of expression have increased relative to the intact state, with the highest levels of protein being detected in primarily small and medium sized neurons. Intrathecal infusion of NT-3 at the time of injury attenuates levels of ASIC3 protein expression ipsilateral to injury.

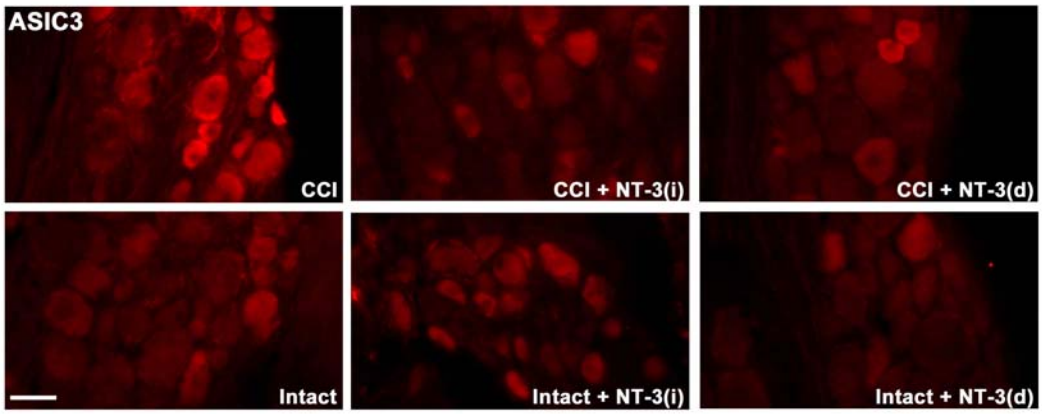

Figure 4- 6: Continuous NT-3 infusion is not required to mitigate CCI-induced changes in ASIC3-like immunoreactivity in DRG neurons.

Fluorescence photomicrographs depict levels of ASIC3-like immunoreactivity in 10 μm sections of L5 DRG representing DRG ipsilateral and contralateral to 14d unilateral CCI [CCI and Intact] in response to 600 ng/μl/hr NT-3 infusion for the first 7 days of a 14d unilateral CCI [CCI+NT-3(i) and Intact+NT-3(i)], or in response to 600 ng/μl/hr NT-3 infusion for the last 7 days of a 14d unilateral CCI [CCI+NT-3(d) and Intact+NT-3(d)], as indicated. Note: 2 week CCI results in elevated ASIC3 protein expression. Both immediate and delayed infusion decrease the relative levels of ASIC3-like immunoreactivity detected ipsilateral to CCI, with the decrease being more pronounced with delayed NT-3 treatment. Scale bar = 40 μm

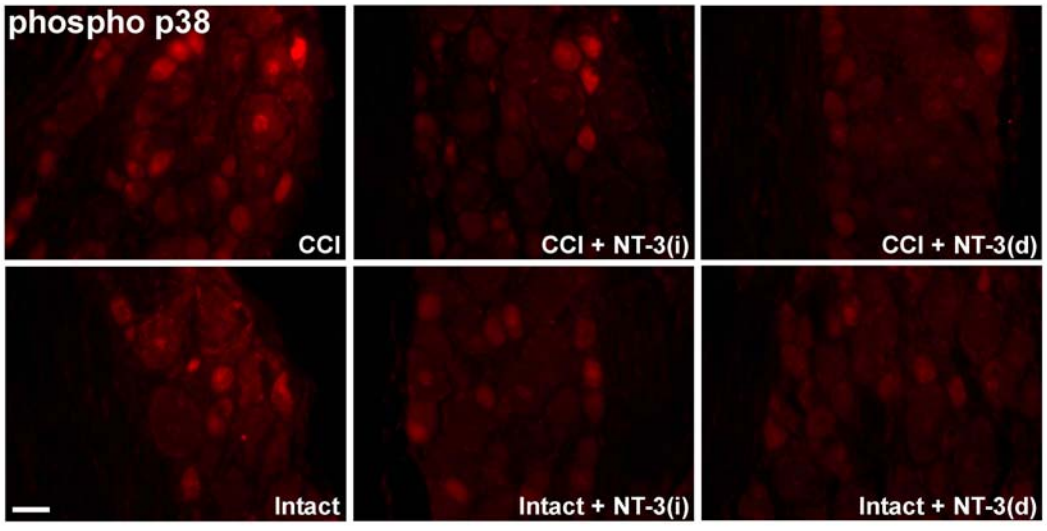

Figure 4- 7: Continuous NT-3 infusion is not required to reverse the increase in phospho-p38 MAP Kinase label detected in DRG neurons subjected to CCI.

Fluorescence photomicrographs demonstrate levels of phospho-p38 MAP Kinase -like immunoreactivity in 10 μm sections of L5 DRG representing DRG ipsilateral and contralateral to 14d unilateral CCI [CCI and Intact] in response to 600 ng/μl/hr NT-3 infusion for the first 7 days of a 14d unilateral CCI [CCI+NT-3(i) and Intact+NT-3(i)], or in response to 600 ng/μl/hr NT-3 infusion for the last 7 days of a 14d unilateral CCI [CCI+NT-3(d) and Intact+NT-3(d)], as indicated. Note: CCI results in increased phospho-p38 MAPK-like nuclear immunoreactivity. Both immediate and delayed infusion result in a decrease in the relative levels of phospho-p38 MAPK-like immunoreactivity detected, with the decrease being more pronounced with delayed NT-3 treatment. Scale bar = 50 μm

4.5 Discussion

Damage to a peripheral nerve and the subsequent invasion of multiple inflammatory mediators leads to a condition known as neuroinflammation which has been demonstrated to contribute to the sensitization of peripheral nerve terminals leading to exaggerated pain responses. The main contributor to these neuroinflammation induced pain states is believed to be alterations in pH sensing channels such as ASIC3. Given that enhanced NGF synthesis contributes to various neuropathic pain syndromes and has been linked to the upregulation of ASIC3 under conditions of inflammation (Mamet et al., 2003), and that NT-3 is able to antagonize many pathways involving NGF, I hypothesized that NT-3 could attenuate changes in expression of ASIC3 in response to CCI. The present study supports this hypothesis, identifying NT-3 as a novel potent modulator of the pH sensing channel, ASIC3.

This study describes for the first time that: 1) following CCI, levels of ASIC3 are increased in the cytoplasm and possibly membrane presentation seen at both the message and protein levels; 2) NT-3 significantly attenuates levels of both mRNA and protein ASIC3 independent of the timing of infusion – continuous infusion does not appear to be required for this effect as ASIC3 levels remain low and appear slow to re-establish following pump removal.

4.5.1 CCI induces alterations in level and localization of ASIC3 expression

Consistent with previous reports, in the intact state, expression of ASIC3 was observed predominantly in small to medium sized DRG neurons, with some large neurons also expressing detectable levels of ASIC3 (Waldmann et al., 1997b; Chen et al., 1998; Voilley et al., 2001; Molliver et al., 2005) in approximately 35 to 40% of DRG neurons (Ugawa et al., 2005; Molliver et al., 2005). We describe for the first time that following CCI, levels of ASIC3 expression were significantly upregulated, primarily in the small to medium sized (nociceptive) DRG neurons, as well as a small population of large sized neurons. This supports insights by Mamet et al. (2002, 2003) that the increased levels of NGF available under conditions of inflammation drive the increased expression/overexpression of ASIC3.

Following CCI, there appears to be an increased localization of ASIC3 at the cell membrane. This shift in localization is most likely the result of an interaction between ASIC3 and the adaptor protein Na^+/H^+ exchanger regulatory factor-1 (NHERF-1). It has been suggested by Deval et al. (2006) that the interaction between ASIC3 and NHERF-1 plays a role in the membrane surface expression of ASIC3. It is thus probable that under conditions of inflammation, the mobilization of ASIC3 towards the cell membrane would be enhanced in an attempt to respond to the changing pH of the extracellular environment.

4.5.2 Continuous delivery of NT-3 is not required for suppression of ASIC3 expression

The current work also demonstrates that, following CCI, exogenous NT-3 can effectively counter the upregulation of ASIC3 expression, reducing levels to approximately that of basal expression. Surprisingly, examination of 14 day CCI with infusion of NT-3 for the first 7 days of the injury reveals that upon removal of exogenous NT-3, ASIC3 levels remain depressed. Interestingly, examination of 14 day CCI with infusion of NT-3 for the last 7 days of the injury reveals that even if ASIC3

levels have increased for the first 7 days of the injury, exogenous NT-3 is capable of reversing this response. These findings are contrary to those reported by Gandhi et al. (2004) in which NT-3 was unable to reverse the muscle acid-induced hyperalgesia once it had developed. These conflicting results can likely be explained as a result of the differences between the two models. In the CCI model, there is not only a drop in the pH of the extracellular environment, but also a massive production of inflammatory molecules that can impact the situation. The intramuscular acid injection model, however, produces a focal, peripheral drop in pH. Furthermore, while in our previous study utilizing the same chronic injury model, continuous infusion of NT-3 was required in order to attenuate expression of TRPV1 and thermal hyperalgesia (Wilson-Gerwing et al., 2005). This did not appear to be the case for ASIC3 expression which remained at a significantly lower level of expression relative to control even 1 week following NT-3 removal. This demonstrates, for the first time, that continuous NT-3 is not required to reverse the increase in ASIC3 label detected in DRG neurons subjected to CCI, as we have previously observed with TRPV1 (Wilson-Gerwing et al., 2005).

4.5.3 Attenuation of ASIC3 upregulation by NT-3 does not correlate with the regulation of thermal hyperalgesia by NT-3 following CCI

We have reported previously that exogenous NT-3 could effectively prevent and reverse thermal hyperalgesia but had no net effect on mechanical hypersensitivity (von Frey stimulation) following CCI and that removal of NT-3 resulted in the rapid reestablishment of hyperalgesia (Wilson-Gerwing et al., 2005). Here, we report that exogenous NT-3 applied intrathecally is capable of disabling the mechanism by which ASIC3 is upregulated in the DRG in response to CCI. Thus, we find that downregulation of ASIC3 does not appear to influence mechanical hypersensitivity or the re-establishment of the thermal hyperalgesic state following NT-3 pump removal.

4.5.4 Modulation of ASIC3 by NT-3 is likely due to its impact on the trkA/JNK/p38MAPK pathway

Activation of the trkA/JNK/p38MAPK pathway by inflammation–associated increases in NGF has been reported to be responsible for the upregulation of ASIC3 under inflammatory conditions (Mamet et al., 2003). Previously, we have reported that following CCI, NT-3 is able to downregulate the neurotrophin receptor, tropomyosin-related kinase A (trkA) (Wilson-Gerwing and Verge, 2006) as well as levels of phospho-p38MAPK (Wilson-Gerwing et al., 2005). As observed with the regulation of ASIC3 following CCI, phospho-p38 MAPK expression remains depressed upon removal of exogenous NT-3. Therefore, if the downstream transcriptional action of phospho-p38MAPK is inhibited, it is likely to have an impact on ASIC3 transcription (Mamet et al., 2003). Interestingly, although levels of phoshpo-p38 MAPK remain depressed, this does not inhibit the prompt reestablishment of thermal hyperalgesia following removal of exogenous NT-3 (Wilson-Gerwing et al., 2005). This is likely due to the previously reported findings that levels of TRPV1 mRNA are not significantly altered in the 2 week model of CCI +/- NT-3, therefore allowing for reactivation of thermal hyperalgesia without the immediate need to engage the p38 MAPK pathway.

4.5.5 A role for ASIC3 in neuropathic pain?

Exploring the potential roles for ASIC3 as a generator or modulator of pain sensations demonstrates that the use of ASIC3 transgenic mice have been widely employed with varied results. These range from ASIC3 knock outs 1) being more responsive to light touch, but less responsive to noxious pinch and noxious heat (Price et al., 2001); 2) being more sensitive to moderate to high noxious stimuli (Chen et al., 2002); and 3) exhibiting the absence of mechanical hyperalgesia and central sensitization (Sluka et al., 2003). More recently, it has been reported that ASIC3 transgenics are more sensitive to both mechanical and chemical/inflammatory pain (Mogil et al., 2005) and conversely, that ASIC3 does not mediate responses to

mechanical stimuli (Drew et al., 2004). In concert with those results reported by Drew et al. (2004), the findings reported here are indicative of the lack of a critical role for ASIC3 in mechanical hypersensitivity following CCI and also the lack of a critical role for ASIC3 in the reestablishment of thermal hyperalgesia following CCI. While one might predict from all of the research performed on ASIC3 transgenic mice that there is a critical role for ASIC3 in the neuropathic pain state, it should be noted that there may be species driven differences in the role of ASIC3 in the generation and maintenance of neuropathic pain behaviors as described in Leffler et al. (2006).

Although we observed changes within the cell body at both the message and protein level, we are assuming that ASIC3 did not make a major contribution in the rapid reestablishment of the thermal hyperalgesia that was observed. The possibility that there was a redistribution of ASIC3 to the terminal regions of the small fibers that still remained following NT-3 treatment cannot be excluded. However, it is unlikely that elevated terminal expression would still be present following NT-3 infusion and be actively contributing to the thermal hyperalgesia state still observed. It is also important to note that unlike other inflammatory models where local inflammation is the main driver of the local response, the nerve inflammation observed with CCI is far removed from the hindpaw where the behavioral testing is taking place, thus involving a more complex strategy for the treatment of this type of inflammation.

While ASIC3 activation is brought about by a drop in extracellular pH (Waldmann et al., 1997b), it is not the only channel sensitive to such a shift in pH (i.e. TRPV1) (Caterina et al., 1997), providing insight that perhaps ASIC3 must work in conjunction with additional channels and/or molecules to effect changes in nociception at the level of the DRG. In conclusion, ASIC3 is undoubtedly involved in some aspect of the generation and/or maintenance of neuropathic pain, but it is not a critical channel in the CCI model of neuropathic pain.

5 Exogenous neurotrophin-3 treatment significantly reduces sodium channel expression linked to neuropathic pain states

5.1 Abstract

The development of neuropathic pain syndromes as a result of a chronic constriction injury (CCI) is critically linked to the sensitization of peripheral nociceptors. Changes in the electrophysiological properties of these neurons are controlled by the presence of voltage gated sodium channels. These channels are major contributors to this sensitized state and their expression can be upregulated by the presence of nerve growth factor (NGF). We have previously demonstrated that another neurotrophin, neurotrophin-3 (NT-3) can effectively antagonize a number of changes brought about by increased NGF, including its high-affinity receptor, tropomyosin related kinase A (trkA), and thermal hyperalgesia. Two tetrodotoxin resistant (TTX-R) sodium channels, $Na_v1.8$ and $Na_v1.9$, are upregulated in the presence of proinflammatory mediators, including NGF, and play a role in the development of neuronal hypersensitivity in a number of models of neuropathic pain. As NT-3 is a potent negative modulator of this pathway, I hypothesized that increases in available NT-3 would effectively reduce expression of $Na_v1.8$ and $Na_v1.9$ in DRG neurons ipsilateral to CCI. Neuronal levels of $Na_v1.8$ and $Na_v1.9$ mRNA and protein were decreased following 7 day CCI. Intrathecal infusion of NT-3 for 7 days at the time of injury significantly reduced levels of both $Na_v1.8$ and $Na_v1.9$ contralateral and most notably ipsilateral to CCI. This ability of NT-3 to dramatically reduce levels of the TTX-R sodium channels $Na_v1.8$ and $Na_v1.9$ is potentially linked to the ability of NT-3 to interfere with the sensitization of sensory neurons and the ensuing development of thermal hyperalgesia following CCI.

5.2 Introduction

Neuropathic pain has become well characterized over the last decade, including the behaviors associated with this chronic pain syndrome. Among these behaviors is the increased sensitivity to noxious stimuli (defined as hyperalgesia) as well as the generation of spontaneous pain (related to ectopic discharge of neurons) (reviewed in Woolf and Mannion, 1999). Complicit in the development of hyperalgesia is the sensitization of peripheral nerve terminals (nociceptors) whereby the threshold required for activation of the nociceptor is decreased. Ectopic discharges resulting from the spontaneous firing of the nociceptive neuron in the absence of a stimulus is also a contributing factor to this pain state. It has been well established that both the activation threshold of a neuron and the potential for spontaneous firing is regulated by sodium channels (Hodgkin and Huxley, 1952; Catterall, 1995). This cumulative hyperexcitability of sensory neurons following nerve injury has been associated with altered expression and the redistribution of voltage gated sodium channels to the tips of the injured axons and/or the neuromas (Devor et al., 1989; England et al., 1994, 1996; Amir et al., 1999).

Sodium channels are characterized on the basis of their ability to respond to the puffer fish toxin, tetrodotoxin (TTX). This results in two diverse groups of sodium channels – those that are TTX-sensitive (TTX-S) and those that are TTX-resistant (TTX-R). It is the TTX-R sodium channels that have garnered much attention with respect to the generation of neuropathic pain syndromes, specifically $Na_v1.8$ and $Na_v1.9$. In general, the TTX-R currents have been detected primarily in small diameter, but also in medium and large diameter DRG neurons (Akopian et al., 1996; Black et al., 1996; Cummins et al., 1999; Dib-Hajj et al., 1999; Rush et al., 1998; Renganathan et al., 2000; Hong and Wiley, 2006) are associated with nociceptive neurons (Djourhi et al., 2003; Fang et al., 2002).

Expression of the $Na_v1.8$ isoform is linked to the development of nociceptor hyperexcitability. More specifically, the development of thermal hyperalgesia and mechanical allodynia in nerve ligation models of neuropathic pain is reduced with $Na_v1.8$ antisense treatment (Porreca et al., 1999; Lai et al., 2002; Joshi et al., 2006),

while ectopic firing of neurons is correlated with $Na_v1.8$ protein expression (Novakovic et al., 1998; Gold et al., 2003). In contrast to $Na_v1.8$, early evidence of a direct role of $Na_v1.9$ in the development of thermal and mechanical hyperalgesia was lacking in rats with neuropathic pain states induced by spinal nerve ligation (Porecca et al., 1999) and partial ligation of the sciatic nerve (Priest et al., 2005). However, $Na_v1.9$ may play a significant role in the increased excitability and sensitization of nociceptive axons during inflammation (Herzog et al., 2001; Baker et al., 2003; Rush and Waxman, 2004; Priest et al., 2005) although this is still a matter of debate (Hillsley et al., 2006).

Alterations in the levels of expression of both $Na_v1.8$ and $Na_v1.9$ in response to chronic constriction injury (CCI) are conflicting. Following CCI, neuronal $Na_v1.8$ has been found to decrease in small diameter neurons (Dib-Hajj et al., 1999), decrease only in axotomized cells (Decosterd et al., 2002), or undergo no change at all (Novakovic et al., 1998). Similarly, neuronal levels of $Na_v1.9$ have been found to decrease in small diameter neurons (Dib-Hajj et al., 1999) or to decrease only in axotomized cells (Decosterd et al., 2002). It is of interest that these two channels respond differently to inflammation, with $Na_v1.8$ being upregulated primarily in small neurons (Tanaka et al., 1998; Black et al., 2004; Gould et al., 2004) while there appears to be no change in the expression of $Na_v1.9$ (Black at al., 2004). The variations in these reported findings may be due, in part, to the complexity of the CCI model itself producing preferential axotomy of large sized DRG neurons and exposure of the remaining intact axons to an inflammatory environment (Bennett and Xie, 1988; Kajander and Bennett, 1992) and increased levels of nerve growth factor (NGF) that has been shown to increase injury-induced expression of these two isoforms (Dib-Hajj et al., 1998; Fjell et al., 1999). As such, it appears that the levels of $Na_v1.8$ as regulated by NGF are dependent on the levels of trkA expression, but apparently not $Na_v1.9$ (Fang et al., 2005).

I have previously demonstrated that another neurotrophin, neurotrophin-3 (NT-3), is able to negatively modulate levels of the high-affinity NGF receptor, tropomyosin related kinase A (trkA) (Wilson-Gerwing and Verge, 2006), to regulate the development and maintenance of thermal hyperalgesia, and in general, to act in a manner that is antagonistic to NGF in the CCI model of neuropathic pain (Wilson-Gerwing et al., 2005). In accordance with these findings, I have thus investigated whether NT-3 is

capable of regulating levels of expression of the TTX-R sodium channel isoforms $Na_v1.8$ and $Na_v1.9$ following CCI.

5.3 Methods

5.3.1 Animal surgery

All animal procedures were conducted in accordance with the National Institutes of Health policy on the use of animals in research and the University of Saskatchewan animal care committee guidelines (protocol 19920164). A total of 32 young adult male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 250-300 g were used. Animals were anesthetized for surgery with sodium pentobarbital (Somnitol, 65 mg/kg; MTC Pharm, Cambridge, Ontario, Canada). Pre- and post-operative (for 24 h) subcutaneous injections of buprenorphine (Temgesic, 0.1-0.2 mg/kg) were given to alleviate any post-operative discomfort. To examine the effect of NT-3 on the expression of $Na_v1.8$ and $Na_v1.9$, 32 rats were used: 13 underwent 7 d unilateral CCI of the sciatic nerve (Bennett and Xie, 1988), 3 received sham CCI surgeries whereby the sciatic nerve was exposed but not ligated, 13 received 7 d unilateral CCI with intrathecal infusion of NT-3 for the duration of the injury, and 3 received 7 d unilateral CCI with sham pump implantation whereby the dorsal roots were exposed, the dura opened as with the CCI + NT-3 procedure, but no pump was implanted.

NT-3 (generously supplied by Regeneron Pharmaceuticals, Tarrytown, NY) was delivered intrathecally for 7 d via mini-osmotic pumps (model 2001; Alza, Cupertino, CA) inserted at the lumbar sacral junction as per Verge et al. (1989a) at a concentration and rate of 600 ng/µl/hr (Karchewski et al., 2002) in a solution of PBS containing rat serum albumin (1 mg/ml), streptomycin (100 U/ml), and penicillin (100 U/ml). This dose of NT-3 was the minimum dose found to selectively reverse injury-associated gene expression in injured trkC-expressing neurons (Verge et al., 1996; Jongsma Wallin et al., 2001; Karchewski et al., 2002). At the conclusion of the experiments, rats were killed, and tissue was dissected and processed for *in situ* hybridization and/or

immunohistochemistry as described below. Previous studies have demonstrated a lack of influence ipsilateral and contralateral to injury when vehicle is infused intrathecally (Verge et al., 1989a; Verge et al., 1995; Jongsma Wallin et al., 2001; Wilson-Gerwing et al., 2005).

5.3.2 *In situ* hybridization

Deeply anesthetized animals were perfused via the aorta with 0.1 M PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1M PBS. The right and left L4 and L5 DRG were rapidly dissected, postfixed for 1 hour in the same fixative, and cryoprotected in 20% sucrose in 0.1M PBS overnight. Paired experimental and control tissues were mounted in the same cryomold (to ensure processing under identical conditions), covered with OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN, USA) and frozen in cooled isopentane. Transverse sections were cut at 6 μm on a Micron cryostat (Zeiss, Canada), thaw mounted onto Probe-On^{+} slides (Fisher Scientific, Edmonton, AB, Canada) and stored with desiccant at –20 ^{o}C until hybridization.

Prior to hybridization, slides were air dried for 15 minutes, followed by fixation in 4% paraformaldehyde for 20 minutes. Slides were washed 3 X 5 minutes in 1X PBS. Sections were then treated with proteinase K (20 μg/ml) containing 10 ml 1M Tris-HCl (pH 7.6), 2 ml 0.5 M EDTA, 200 μl proteinase K stock (20 mg/ml) and 188 μl ddH_20 for 6 minutes. Slides were then rinsed for 5 minutes in 1 X PBS and post-fixed for 5 minutes in 4% paraformaldehyde. Slides were then rinsed 2 X 5 minutes in 1 X PBS, 1 X 5 minutes in DEPC-H_2O, and dehydrated in ascending alcohols.

Oligonucleotide probes complementary to and selective for $Na_v1.8$ mRNA [complementary to bases 640-687 (Akopian et al., 1996)] and $Na_v1.9$ mRNA [complementary to bases 2811-2858 (Dib-Hajj et al., 1998)] were synthesized (University of Calgary DNA services, Alberta, Canada). The probes were checked against the GenBank database (NIH) to ensure no greater than 60% homology was found to sequences other than the cognate transcript. The probes were labeled at the 3'-end with α-[^{35}S]dATP (New England Nuclear, Boston, MA, USA) using terminal deoxynucleotidyl-transferase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in a

buffer containing 10 mM $CoCl_2$, 1 mM dithiothreitol, 300 mM Tris base and 1.4 M-potassium cacodylate (pH 7.2), and purified through Bio-Spin® Disposable Chromatograph Columns (Bio-Rad laboratories, Hercules, CA, USA) containing 200 mg of NENSORB™ PREP Nucleic Acid Purification Resin (NEN®, Boston, MA, USA). Dithiothreitol was added to a final concentration of 10 nM.

Hybridization was carried out according to published procedures (Dagerlind et al., 1992) on a minimum of 5 slides/probe from each of the experimental and control groupings. Briefly, the sections were hybridized at 43 ^oC for 14-18 hours in a buffer containing 50% formamide (Sigma Aldrich, Oakville, ON, Canada), 4X SSC (1X SSC – 0.15 M NaCl, 0.015 sodium citrate), 1X Denhart's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl (N-laurylsarcosine), 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 500 µg/ml heat-denatured sheared salmon sperm DNA, 200 mM dithiothreitol and 10^7 cpm/ml of probe. After hybridization, the slides were washed for 4 X 15 mins in 1X SSC at 55^oC, dehydrated in ascending alcohols, processed for radioautography as per Karchewski et al., 2002 and exposed for 7 to 10 days before developing in D-19 (Kodak, Rochester, NY, USA).

The specificity of hybridization signal for the Na_v 1.8 and Na_v 1.9 probes were confirmed as described in Wilson-Gerwing et al., 2005.

5.3.3 Quantification and analysis

All slides from the 13 groupings of 7 d experimental and control animals were analyzed qualitatively and relative changes in hybridization signal from one group to another noted for sections mounted on the same slide to avoid bias due to the variance in hybridization signal observed from slide to slide. Representative slides were selected for further quantitative analysis. These slides had a similar number of neurons in all DRG sections. Photomontages of each section to be analyzed were prepared and individual neurons with a visible nucleus were identified. Using a 40X light objective and a 2X optivar with an interactive computer-assisted image-analysis system (Richardson et al., 1989), cross-sectional areas of individual neurons and the percentages of cytoplasmic areas covered by silver grains were measured for each DRG. The area per grain was

constant for all neurons and a correction for grain overlap was made to obtain a parameter linearly related to density of silver grains (Richardson et al., 1989). Software for the image analysis system was Northern Eclipse, Version 7.0 (Empix Imaging, Mississauga, ON, Canada) and supplemented with Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA) and Prizm 4.0 (Graph Pad Software, San Diego, CA). Cells were considered labeled if they had more than five times background levels of silver grains, as determined by averaging grain densities over defined areas of the neuropil devoid of positively labeled cell bodies. This criterion for determining labeled neuronal profiles correlates well with the identification of labeled versus unlabeled neurons as determined manually using a 63X oil immersion objective. A stereological approach was not employed in this study and therefore the data quantification may represent a biased estimate of (i) the percentage of the population expressing individual markers (based on incidence in the sections examined whereby all neurons with a nucleus present in that section were included in the analysis) and (ii) the actual neuronal size (which was determined based on the assumption that the presence of a nucleus in the section correlates with the maximal diameter for that cell).

Analysis was performed in each instance on all neurons with a nucleus present in the section being quantified: for $Na_v1.8$ this represents 12 DRG sections or 2236 neuronal profiles (Intact: n = 3 animals, CCI: n = 3 animals, Intact + NT-3: n = 3 animals, CCI+NT-3: n = 3 animals); while for $Na_v1.9$ this represents 12 DRG sections or 2025 neuronal profiles (Intact: n = 3 animals, CCI: n = 3 animals, Intact + NT-3: n = 3 animals, CCI+NT-3: n = 3 animals). Previous research from our lab has demonstrated that CCI does not induce bilateral hyperalgesia (Wilson-Gerwing et al., 2005) and we have not discerned any qualitative differences between sham-operated or naïve DRG and contralateral intact DRG for $Na_v1.8$ or $Na_v1.9$. Therefore, the contralateral intact DRG was used as an intact control.

5.3.4 Immunohistochemistry

Deeply anesthetized animals were perfused via the aorta with 0.1M PBS followed by 4% paraformaldehyde. Right and left L4 and L5 DRG were dissected, post-fixed for 1 – 1.5 h, and cryoprotected in 20% sucrose overnight at 4 ^{o}C. Paired experimental and control tissues were frozen in the same cryomold to ensure processing under identical conditions. Transverse 10 μm sections were cut on the cryostat, thaw-mounted onto Probe-ON^{+} slides (Fisher Scientific), and processed for immunohistochemistry.

For $Na_v1.8$, sections were washed three times for 5 minutes in 0.1 M PBS, then permeabilized with 0.3% Triton X-100 in 0.1 M PBS for 45 minutes at room temperature. Sections were blocked overnight at 4 ^{o}C in 10% goat serum and 0.3% Triton X-100 in 0.1 M PBS, then incubated overnight at 4 ^{o}C with rabbit anti-Na_v 1.8 Affinity Purified Polyclonal Antibody (1:200; Chemicon International, Temecula, CA, USA) diluted with 10% goat serum and 0.3% Triton X-100 in 0.1 M PBS. Sections were visualized with Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-rabbit IgG (H+L) (1:250; Molecular Probes, Eugene, OR, USA) in 2% goat serum in 0.1 M PBS for 2 hours at room temperature. Slides were washed and coverslipped with 50% glycerol/50% PBS. For $Na_v1.9$, sections were washed three times for 5 minutes in 0.1 M PBS, then permeabilized with 0.3% Triton X-100 in 0.1 M PBS for 45 minutes at room temperature. Sections were blocked overnight at 4 ^{o}C in 10% goat serum, 3% BSA, and 0.3% Triton X-100 in 0.1 M PBS, then incubated overnight at 4 ^{o}C with rabbit anti-$Na_v1.9$ Affinity Purified Polyclonal Antibody (1:100; Chemicon International, Temecula, CA, USA) diluted with 10% goat serum, 3% BSA, and 0.3% Triton X-100 in 0.1 M PBS. Sections were visualized with Alexa Fluor® 546 goat anti-rabbit IgG (H+L), F(ab')$_2$ fragment conjugate (1:250; Molecular Probes, Eugene, OR, USA) in 2% goat serum for 2 hours at room temperature. Slides were washed and coverslipped with 50% glycerol/50% PBS. Control sections were processed in the same manner, but without the primary antibody. Results were visualized using a Zeiss Axioscope 50 microscope equipped with incident-light fluorescence optics and a digital camera.

5.4 Results

5.4.1 NT-3 significantly reduces levels of $Na_v1.8$ mRNA

Analysis of sections processed for *in situ* hybridization to detect neuronal expression of $Na_v1.8$ mRNA revealed that in the DRG contralateral to CCI (Intact), detectable hybridization signal was observed in ~72% of neurons analyzed (Table 5-1). This was localized primarily over small to medium sized (<35 μm in diameter) neurons with ~88% of the population of small to medium size neurons expressing this message and only ~53% of the population of larger sized (>35 μm in diameter) neurons doing so at detectable levels (Table 5-1). Seven days after CCI, there is a reduction in the relative levels of hybridization signal (Figure 5-1). This reduction is also reflected in the percentage of neurons expressing detectable levels of $Na_v1.8$ (Table 5-1). The mean labeling index for these neurons is significantly reduced from a normalized value of 1 in the intact state to 0.94 following CCI. This reduction is most apparent in those small to medium sized neurons (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 5-2; Table 5-2).

NT-3 infusion effected a marked reduction in the levels of $Na_v1.8$ mRNA expression observed in neurons both contralateral and ipsilateral to CCI (Intact + NT-3 and CCI + NT-3) (Figure 5-1). In NT-3 infused DRG contralateral to CCI (Intact + NT-3) the mean labeling index was significantly decreased from a normalized value of 1 in the intact state to 0.77 in the NT-3 treated group (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 5-2; Table 5-2) as was the percentage of neurons expressing detectable levels of $Na_v1.8$ (Figure 5-1; Table 5-1). A more dramatic attenuation of $Na_v1.8$ expression was observed in the NT-3 treated DRG ipsilateral to CCI (CCI + NT-3). Mean labeling index was significantly decreased with NT-3 treatment from 0.94 to 0.41 (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 5-2; Table 5-2). The percentages of neurons expressing detectable levels of $Na_v1.8$ mRNA were also reduced (Table 5-1). This decrease in the relative level of $Na_v1.8$ expression is most noticeable in those small to medium sized neurons (Figure 5-1; Table 5-1).

Nav 1.8	Population of total neurons examined (%)		% of small to medium neuronal population (neurons < 35µm)		% of large neuronal population (neurons > 35µm)	
	> 5X bkgd	> 20X bkgd	> 5X bkgd	> 20X bkgd	> 5X bkgd	> 20X bkgd
Intact	72.27 +/- 6.750%	40.60 +/- 3.156%	87.67 +/- 2.685%	52.80 +/- 8.235%	36.27 +/- 9.275%	11.63 +/- 3.115%
Intact + NT-3	55.47 +/- 4.226%	31.13 +/- 3.593%	73.67 +/- 3.494%	46.17 +/- 4.529%	30.33 +/- 2.945%	11.67 +/- 4.796%
CCI	52.23 +/- 10.98%	34.17 +/- 1.994%	70.3 +/- 3.012%	43.77 +/- 5.940%	23.13 +/- 6.020%	11.53 +/- 1.810%
CCI + NT-3	36.67 +/- 2.955%	15.30 +/- 4.105%	43.47 +/- 4.311%	17.30 +/- 3.851%	16.10 +/- 8.504%	8.533 +/- 6.416%

Table 5- 1: Effects of CCI +/- NT-3 infusion on numbers of neurons expressing $Na_v1.8$ mRNA.

The above table summarizes the alterations in the incidence (percentage) of $Na_v1.8$ mRNA positive neurons in L5 DRG neurons under various experimental conditions as indicated in the left column. Those neurons < 35 µm are characterized as small to medium in size and those neurons > 35 µm are characterized as medium to large in size. Detectable levels of hybridization signal are all those > 5X background where as moderate to high levels of hybridization signal are those > 20X background. CCI produces a decrease in the percentages of neurons positively labeled for $Na_v1.8$. Infusion of NT-3 further decreases the percentage of $Na_v1.8$ mRNA positive neurons detected most evident ipsilateral to CCI.

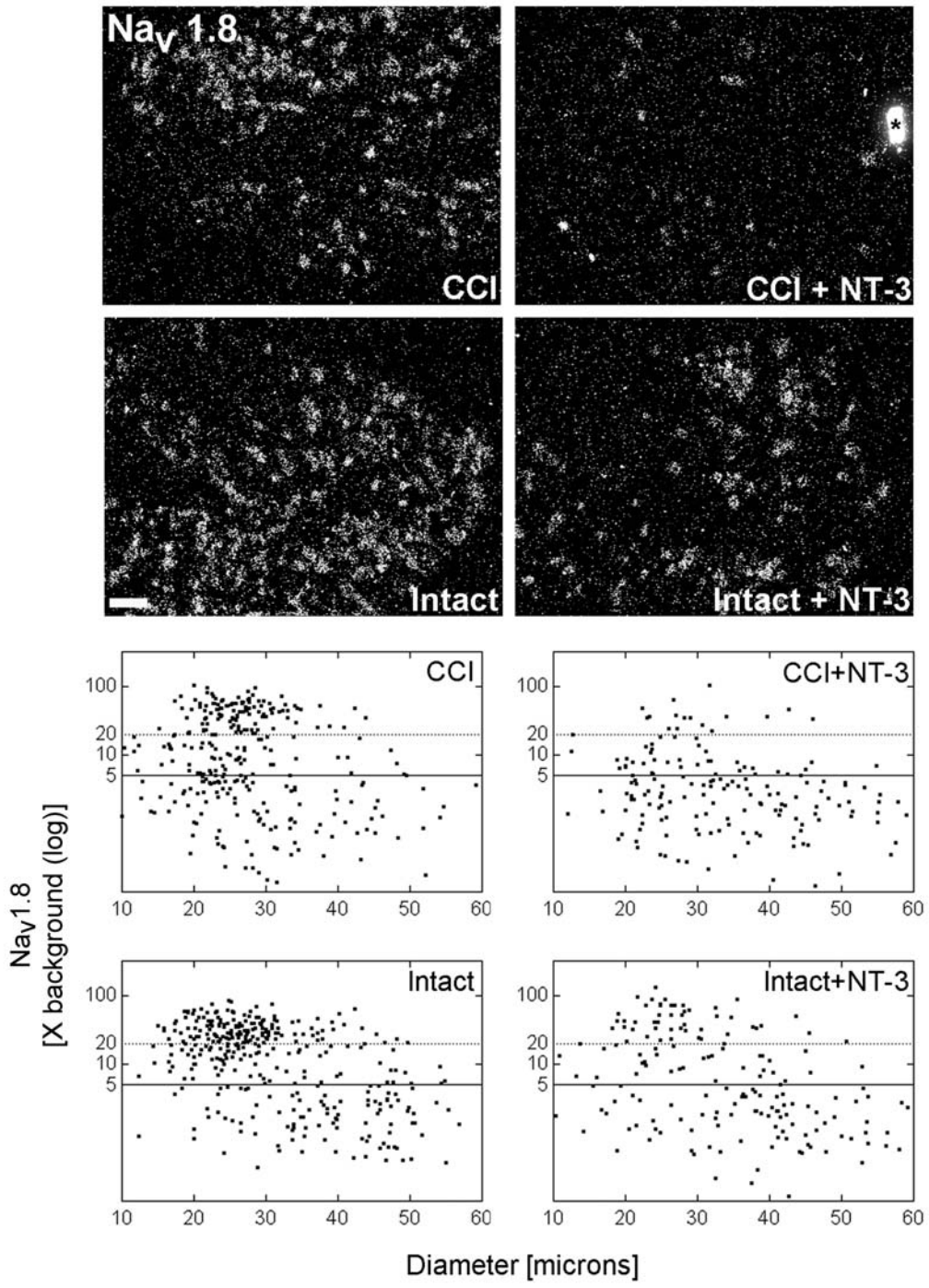

Figure 5- 1: Message levels for the tetrodotoxin resistant sodium channel $Na_v1.8$ are reduced in response to NT-3 treatment.

Top: Dark-field photomicrographs of 6 μm thick adult rat L5 DRG sections processed for *in situ* hybridization to detect $Na_v1.8$ transcripts contralateral (Intact) or ipsilateral to 7d CCI (CCI) and after a 7d unilateral CCI plus intrathecal infusion of 600 ng/μl/hr NT-

3 (Intact+NT-3; CCI+NT-3). Scale bar = 100 µm. Note: Treatment with NT-3 dramatically reduces levels of $Na_v1.8$ mRNA following CCI.

Bottom: Representative scatterplots whereby each point represents the labeling index of an individual neuron identified in 6 µm thick sections of L5 DRG processed to detect $Na_v1.8$ mRNA. The relationship between $Na_v1.8$ mRNA labeling intensity (y-axis, log scale) and perikaryal diameter (x-axis) is depicted. Experimental states are indicated at the top right of each graph and are as described above. Labeling refers to the ratio of silver grain density over the neuronal cytoplasm to grain density over areas of the neuropil devoid of positive hybridization signal. Solid lines divide the plots into labeled and unlabeled populations; dotted lines separate lightly labeled from moderate to heavily labeled populations of $Na_v1.8$ –expressing neurons. Note: In DRG contralateral to CCI, $Na_v1.8$ is expressed predominantly in small to medium neurons with some large neurons also expressing detectable levels. CCI results in a slight decrease in $Na_v1.8$ expression. NT-3 infusion results in a decrease in the levels of detectable $Na_v1.8$ mRNA in DRG both ipsilateral and contralateral to CCI, with a more pronounced effect ipsilateral to injury.

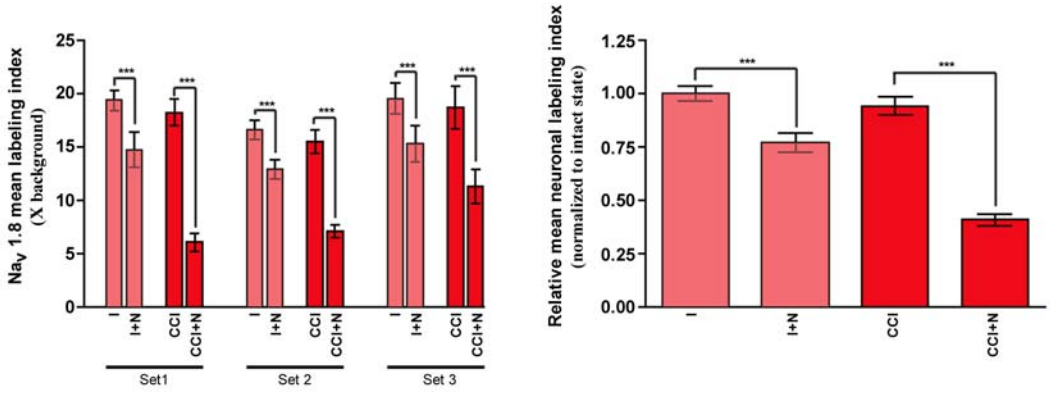

Figure 5- 2: NT-3 treatment significantly reduces mean $Na_v1.8$ mRNA labeling indices.

Left: Graph depicts quantification of relative differences between experimental groups (as indicated) in $Na_v1.8$ mRNA mean labeling indices for all DRG neurons measured following *in situ* hybridization for 3 separate experiments [representing 3 animals having undergone 7 day unilateral CCI [Intact (I) and CCI] and 3 animals having undergone 7 day unilateral CCI with intrathecal infusion of NT-3 [Intact+NT-3 (I+N) and CCI+NT-3 (CCI+N)]. Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). NT-3 infusion results in a significant decrease in mean $Na_v1.8$ mRNA expression in L5 neurons both ipsilateral and contralateral to CCI.

Right: Pooled data depicting the relative differences in mean labeling indices in $Na_v1.8$ mRNA normalized to the intact state in response to unilateral chronic constriction injury with or without NT-3 infusion (n=3). Experimental conditions are as described above. Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). NT-3 infusion results in a significant decrease in mean $Na_v1.8$ mRNA expression in L5 neurons both ipsilateral and contralateral to CCI.

Nav 1.8	Total population of neurons examined (mean labeling index)	Small to medium neuronal population (neurons < 35μm) (mean labeling index)	Large neuronal population (neurons > 35μm) (mean labeling index)
Intact	1.0000 +/- 0.03496	1.0000 +/- 0.03140	1.0000 +/- 0.1170
Intact + NT-3	0.7717 +/- 0.04497	0.8847 +/- 0.05304	0.9569 +/- 0.1271
CCI	0.9428 +/- 0.04333	0.8978 +/- 0.04004	0.9946 +/- 0.1980
CCI + NT-3	0.4096 +/- 0.02767	0.3776 +/- 0.02719	0.8743 +/- 0.1741

Table 5- 2: Effects of CCI +/- NT-3 infusion on the $Na_v1.8$ mRNA mean labeling index.

The above table summarizes the alterations in $Na_v1.8$ mRNA mean labeling index of pooled data normalized to intact mean labeling index within each of the 3 experimental groupings of animals analyzed in L5 DRG neurons under various experimental conditions as indicated in the left column. Those neurons < 35 μm are characterized as small to medium in size and those neurons > 35 μm are characterized as medium to large in size. CCI produces a decrease in the $Na_v1.8$ mean labeling index in the total neurons most apparent in those small to medium neurons. Infusion of NT-3 decreases levels of $Na_v1.8$ mRNA both ipsilateral and contralateral to CCI in all size ranges of neurons.

5.4.2 Levels of $Na_v1.8$ protein are decreased following NT-3 treatment

Immunohistochemistry revealed that in the intact state, $Na_v1.8$ protein was observed most prominently in the smaller DRG neurons, with some medium to large sized neurons also expressing low to moderate levels of $Na_v1.8$ protein. Levels of $Na_v1.8$ protein were reduced 7 days following CCI (Figure 5-3). This reduction was most obvious in the medium to large sized neurons that appeared devoid of any $Na_v1.8$ protein. Infusion of NT-3 effected a decrease in the levels of $Na_v1.8$ protein in the DRG both contralateral and ipsilateral to CCI (Intact + NT-3; CCI + NT-3) (Figure 5-3).

5.4.3 NT-3 significantly reduces levels of Nav1.9 mRNA

Analysis of sections processed for *in situ* hybridization to detect neuronal expression of $Na_v1.9$ mRNA revealed that in the DRG contralateral to CCI (Intact), detectable hybridization signal was observed in ~73% of neurons. This was localized primarily over small to medium sized neurons representing ~90% of the population of small-medium size neurons with ~37% of the population of large sized neurons also expressing detectable levels (Figure 5-4; Table 5-3). Seven days after CCI, there is a reduction in the percentage of neurons expressing detectable levels of $Na_v1.9$ (Figure 5-4: Table 5-3) and the mean labeling index for these neurons was significantly decreased from normalized value of 1 in the intact state to 0.77 following CCI (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 5-5; Table 5-4).

NT-3 infusion effected a marked reduction in the levels of $Na_v1.9$ mRNA expression observed in neurons both contralateral and ipsilateral to CCI (Intact + NT-3 and CCI + NT-3) (Figure 5-4). In DRG contralateral to CCI (Intact + NT-3) the mean labeling index was significantly decreased from a normalized value of 1 in the intact state to 0.53 in the NT-3 treated group (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 5-5; Table 5-4) as was the percentage of neurons expressing detectable levels of $Na_v1.9$ (Figure 5-4; Table 5-3). A more dramatic attenuation of $Na_v1.9$ expression was observed in the

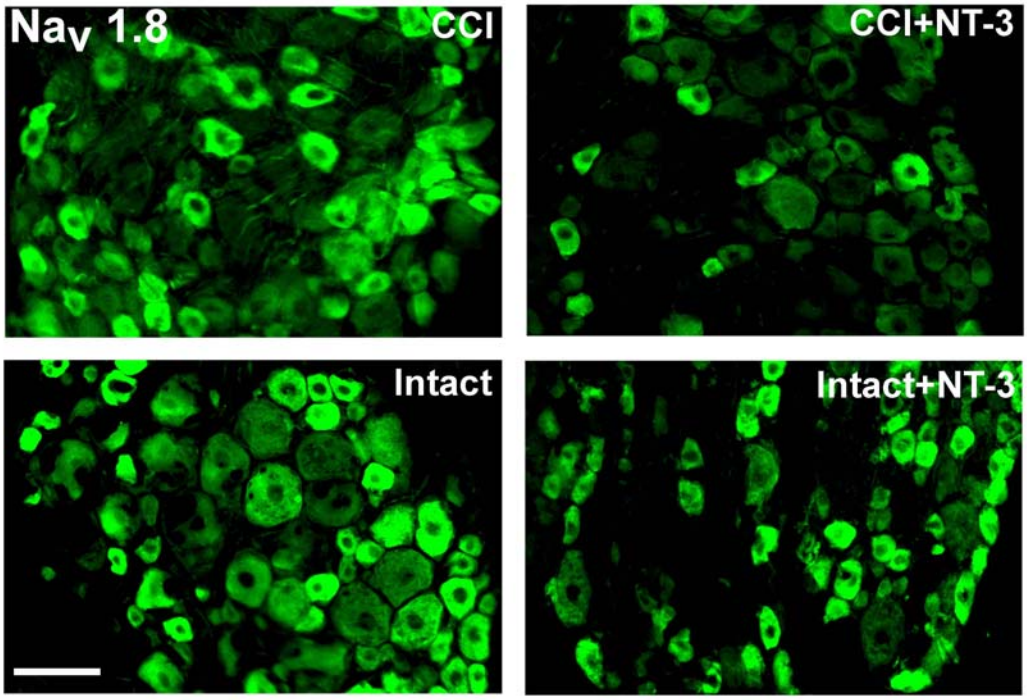

Figure 5- 3: NT-3 infusion attenuates $Na_v1.8$ protein expression.

Fluorescence photomicrographs depict levels of $Na_v1.8$-like immunoreactivity in 10 μm sections of DRG ipsilateral (CCI) and contralateral to CCI (Intact) L5 DRG with or without immediate intrathecal infusion of NT-3 (CCI+NT-3; Intact+NT-3), as indicated. Scale bar, 60 μm. Note: In the DRG contralateral to CCI (Intact), levels of $Na_v1.8$ protein are highest in small to medium sized dorsal root ganglion neurons. Seven days after CCI, levels of expression have decreased slightly relative to the intact state most evident in the medium sized neurons. Intrathecal infusion of NT-3 at the time of injury also results in markedly reduced levels of $Na_v1.8$ protein expression ipsilateral to CCI.

NT-3 treated DRG ipsilateral to CCI (CCI + NT-3). The mean labeling index was significantly decreased with NT-3 treatment from 0.77 to 0.26 (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 5-5; Table 5-4). The percentages of neurons expressing detectable levels of $Na_v1.9$ mRNA were also reduced (Table 5-3). This decrease in the relative level of $Na_v1.9$ expression (Figure 5-5; Table 5-3) as well as the significant decrease in the mean labeling index (Kruskal Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Table 5-4) is most apparent in those small to medium sized neurons.

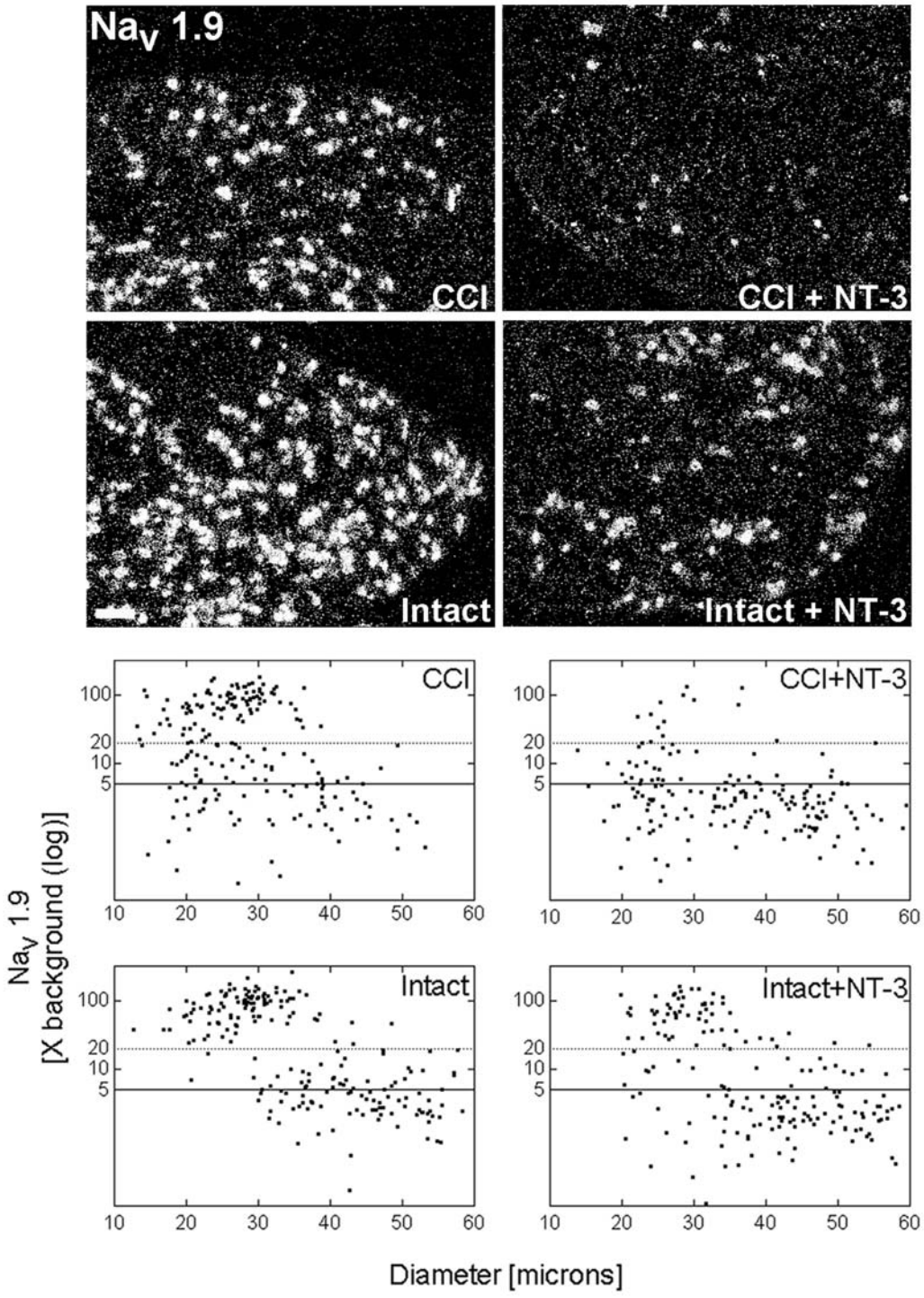

Figure 5- 4: Message levels for the tetrodotoxin resistant sodium channel $Na_v1.9$ are reduced in response to NT-3 treatment.

Top: Dark-field photomicrographs of 6 μm thick adult rat L5 DRG sections processed for *in situ* hybridization to detect $Na_v1.9$ mRNA transcripts contralateral (Intact) or ipsilateral to 7d CCI (CCI) and after a 7d unilateral CCI plus intrathecal infusion of 600

ng/µl/hr NT-3 (Intact+NT-3; CCI+NT-3). Scale bar = 100 µm. Treatment with NT-3 dramatically reduces levels of $Na_v1.9$ mRNA following CCI.

Bottom: Representative scatterplots whereby each point represents the labeling index of an individual neuron identified in 6 µm thick sections of L5 DRG processed to detect $Na_v1.9$ mRNA. The relationship between $Na_v1.9$ mRNA labeling intensity (y-axis, log scale) and perikaryal diameter (x-axis) is depicted. Experimental states are indicated at the top right of each graph and are as described above. Labeling refers to the ratio of silver grain density over the neuronal cytoplasm to grain density over areas of the neuropil devoid of positive hybridization signal. Solid lines divide the plots into labeled and unlabeled populations; dotted lines separate lightly labeled from moderate to heavily labeled populations of $Na_v1.9$–expressing neurons. Note: In DRG contralateral to CCI, $Na_v1.9$ is expressed predominantly in small to medium neurons with a low level of expression detectable in some large neurons. CCI results in a slight decrease in $Na_v1.9$ expression. NT-3 infusion results in a decrease in the levels of detectable $Na_v1.9$ mRNA in DRG both ipsilateral and contralateral to CCI, with a more pronounced effect ipsilateral to injury.

Nav 1.9	Population of total neurons examined (%)		% of small to medium neuronal population (neurons < 35µm)		% of large neuronal population (neurons > 35µm)	
	> 5X bkgd	> 20X bkgd	> 5X bkgd	> 20X bkgd	> 5X bkgd	> 20X bkgd
Intact	73.07 +/- 3.259%	50.50 +/- 0.4933%	89.97 +/- 1.433%	73.80 +/- 7.976%	37.07 +/- 4.651%	6.467 +/- 3.234%
Intact + NT-3	50.67 +/- 2.017%	28.90 +/- 3.816%	76.13 +/- 1.648%	50.67 +/- 8.161%	21.87 +/- 1.638%	4.700 +/- 2.658%
CCI	61.43 +/- 4.653%	40.43 +/- 3.931%	74.63 +/- 3.320%	53.37 +/- 5.393%	25.70 +/- 4.200%	6.333 +/- 3.483%
CCI + NT-3	33.27 +/- 6.000%	11.50+/- 2.458%	44.07 +/- 5.380%	16.67 +/- 3.048%	13.90 +/- 5.284%	1.133 +/- 1.133%

Table 5- 3: Effects of CCI +/- NT-3 infusion on numbers of neurons expressing $Na_v1.9$ mRNA.

The above table summarizes the alterations in the incidence (percentage) of $Na_v1.9$ mRNA positive neurons in L5 DRG under various experimental conditions as indicated in the left column. Those neurons < 35 µm are characterized as small to medium in size

and those neurons > 35 μm are characterized as medium to large in size. Detectable levels of hybridization signal are all those > 5X background where as moderate to high levels of hybridization signal are those > 20X background. CCI produces a decrease in the percentages of neurons positively labeled for $Na_v1.9$. Infusion of NT-3 further decreases the percentage of $Na_v1.9$ mRNA positive neurons detected most evident ipsilateral to CCI.

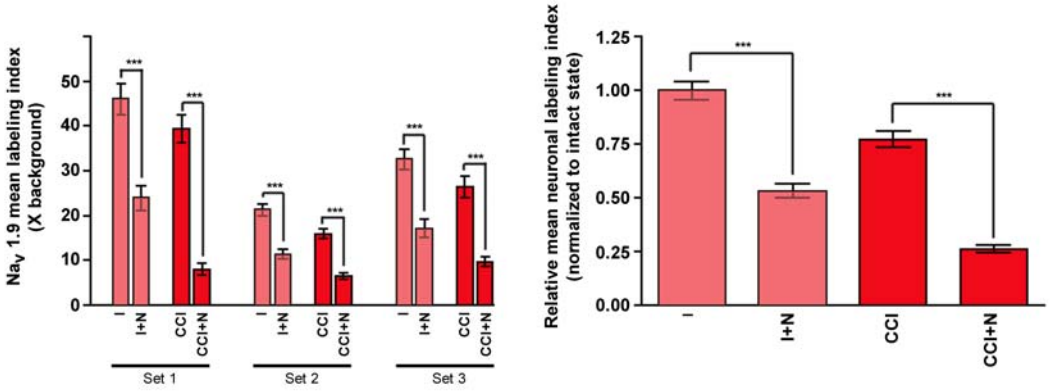

Figure 5- 5: Mean $Na_v1.9$ mRNA labeling indices are significantly decreased following NT-3 treatment.

Left: Graph depicts quantification of relative differences between experimental groups (as indicated) in $Na_v1.9$ mRNA mean labeling indices for all DRG neurons measured following *in situ* hybridization for 3 separate experiments [representing 3 animals having undergone 7 day unilateral CCI [Intact (I) and CCI] and 3 animals having undergone 7 day unilateral CCI with intrathecal infusion of NT-3 [Intact+NT-3 (I+N) and CCI+NT-3 (CCI+N)]. Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). NT-3 infusion results in a significant decrease in mean $Na_v1.9$ mRNA expression in L5 neurons both ipsilateral and contralateral to CCI.

Right: Pooled data depicting the relative differences in mean labeling indices in $Na_v1.9$ mRNA normalized to the intact state in response to unilateral chronic constriction injury with or without NT-3 infusion (n=3). Experimental conditions are as described above. Bars represent the standard error of the mean (s.e.m.). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). NT-3 infusion results in a significant decrease in mean $Na_v1.9$ mRNA expression in L5 neurons both ipsilateral and contralateral to CCI.

Nav 1.9	Total population of neurons examined (mean labeling index)	Small to medium neuronal population (neurons < 35μm) (mean labeling index)	Large neuronal population (neurons > 35μm) (mean labeling index)
Intact	1.0000 +/- 0.04309	1.0000 +/- 0.03327	1.0000 +/- 0.1192
Intact + NT-3	0.5324 +/- 0.03385	0.6460 +/- 0.03636	0.6069 +/- 0.06689
CCI	0.7724 +/- 0.03891	0.7381 +/- 0.03229	0.8273 +/- 0.1116
CCI + NT-3	0.2631 +/- 0.01864	0.2946 +/- 0.02172	0.4271 +/- 0.06281

Table 5- 4: Effects of CCI +/- NT-3 infusion on the $Na_v1.9$ mRNA mean labeling index.

The above table summarizes the alterations in $Na_v1.9$ mRNA mean labeling index of pooled data normalized to intact mean labeling index within each of the 3 experimental groupings of animals analyzed in L5 DRG neurons under various experimental conditions as indicated in left column. Those neurons < 35 μm are characterized as small to medium in size and those neurons > 35 μm are characterized as medium to large in size. CCI produces a decrease in the $Na_v1.9$ mean labeling index in the total neurons that is reflected in both those small to medium and medium to large size neurons. Infusion of NT-3 decreases levels of $Na_v1.9$ mRNA both ipsilateral and contralateral to CCI in all size ranges of neurons.

5.4.4 Levels of $Na_v1.9$ protein are decreased following NT-3 treatment

Immunohistochemistry revealed that in the intact state, $Na_v1.9$ protein was observed predominantly in the small to medium sized DRG neurons. Levels of $Na_v1.9$ protein were reduced 7 days following CCI (Figure 5-6) with fewer small neurons expressing $Na_v1.9$ and at lower levels. Infusion of NT-3 effected a decrease in both the levels of $Na_v1.9$ protein and the numbers of neurons expressing in the DRG both contralateral and ipsilateral to CCI (Intact + NT-3; CCI + NT-3) (Figure 5-6).

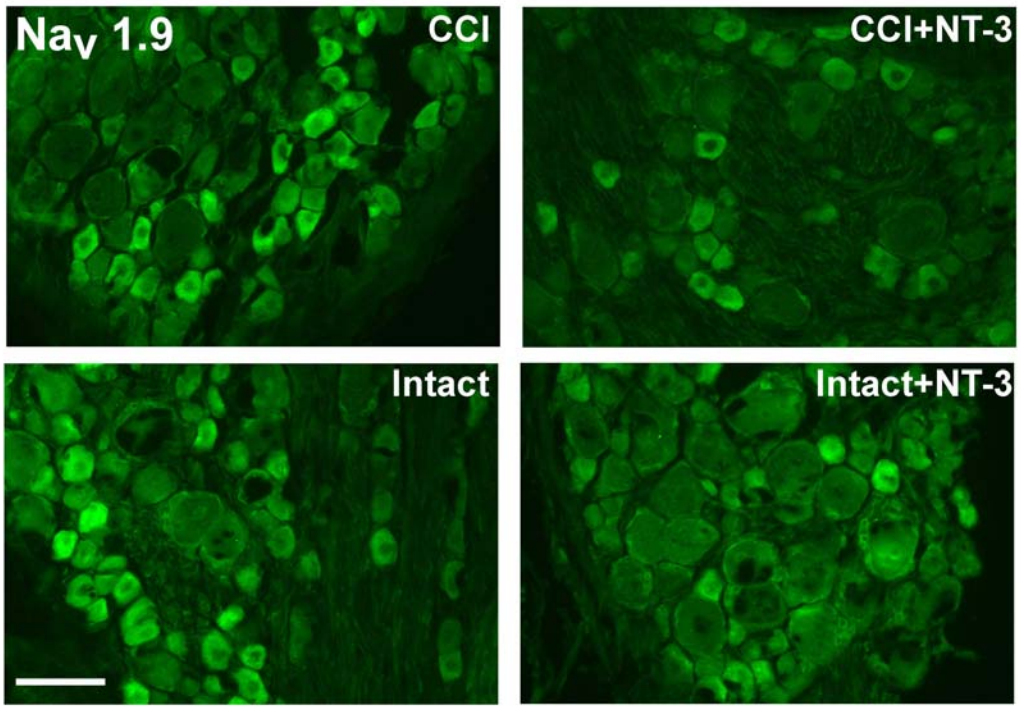

Figure 5- 6: NT-3 infusion results in decreased expression of $Na_v1.9$ protein.

Fluorescence photomicrographs demonstrate levels of $Na_v1.9$-like immunoreactivity in 10 μm sections of DRG ipsilateral (CCI) and contralateral to CCI (Intact) L5 DRG with or without immediate intrathecal infusion of NT-3 (CCI+NT-3; Intact+NT-3), as indicated. Scale bar, 60 μm. Note: In the DRG contralateral to CCI (Intact), levels of $Na_v1.9$ protein are highest in small sized dorsal root ganglion neurons. Seven days after CCI, levels of expression have decreased relative to the intact state. Intrathecal infusion of NT-3 at the time of injury results in markedly reduced levels of $Na_v1.9$ protein ipsilateral to CCI.

5.5 Discussion

5.5.1 NT-3 significantly reduces levels of $Na_v1.8$ and $Na_v1.9$ following CCI

Consistent with previous reports, I have found that in the intact state, both $Na_v1.8$ and $Na_v1.9$ mRNA and protein are localized primarily over small diameter DRG neurons, with some medium and large sized neurons also expressing (Akopian et al.,

1996; Black et al., 1996; Cummins et al., 1999; Dib-Hajj et al., 1999). Following 7d unilateral CCI, the $Na_v1.8$ mRNA mean labeling index was significantly reduced, primarily in those small to medium diameter neurons (consistent with those findings of Dib-Hajj et al., 1999) (Table 5-4). The smaller decrease in the larger cells (presumably those that are axotomized with CCI) is consistent with that observed by Decosterd et al. (2002). These results are paralleled in the $Na_v1.8$ protein levels.

The $Na_v1.9$ mRNA mean labeling is also significantly decreased following CCI. This decrease is reflected in both the small to medium and the larger sized neurons (Table 5-4). This decrease in the small sized neurons was observed by Dib-Hajj et al. (1999), while the decrease in the larger sized neurons (presumably those axotomized by CCI) was observed by Decosterd et al. (2002). These observations are also observed for $Na_v1.9$ protein.

The current work demonstrates the novel finding that NT-3 can dramatically effect a further significant decrease in the mRNA levels and this is also reflected in changes observed protein levels of $Na_v1.8$ and $Na_v1.9$ following 7d unilateral CCI in the DRG neurons both ipsilateral and contralateral to CCI. For $Na_v1.8$, this decreased level of expression is obvious across all size ranges of sensory neurons in both ipsilateral and contralateral DRG. Alternatively, for $Na_v1.9$, this decrease is most apparent in those small to medium sized neurons both ipsilateral and contralateral DRG.

5.5.2 Attenuation of $Na_v1.8$ and $Na_v1.9$ expression correlates with the regulation of thermal hyperalgesia by NT-3 following CCI

Previously, I have reported that administration of exogenous NT-3 at the time of CCI successfully prevents the development of thermal hyperalgesia, while having no effect on the ensuing mechanical hypersensitivity (Wilson-Gerwing et al., 2005). In concert with these findings, I have now shown that exogenous NT-3 at the time of injury is also capable of significantly decreasing levels of the two TTX-R sodium channels $Na_v1.8$ and $Na_v1.9$.

In support of a role for $Na_v1.8$ in the development of thermal hyperalgesia, it has been shown that if $Na_v1.8$ is blocked by antisense oligonucleotides, the development of

thermal hyperalgesia is reduced in both the spinal nerve ligation and CCI models (Porreca et al., 1999; Lai et al., 2002; Joshi et al., 2006). However, if antisense oligonucleotides to $Na_v1.9$ or genetic ablation of $Na_v1.9$ are employed, there appears to be no effect on thermal or mechanical hypersensitivity in the neuropathic rat (Porreca et al., 1999; Priest et al., 2005; Amaya et al., 2006). Interestingly, it appears that $Na_v1.9$ does play a role in hypersensitivity produced by the application of inflammatory mediators to the peripheral terminals of the nociceptors (Amaya et al., 2006). It has been proposed that $Na_v1.9$ plays a crucial role in setting the resting membrane potential of a neuron and that an increased density of this channel, such as is seen with the accumulation of voltage gated sodium channels at the tips of the injured neurons (Devor et al., 1989; England et al., 1994, 1996), may hyperpolarize the neuron (Herzog et al., 2001). It thus appears that the decreased expression of $Na_v1.8$ by exogenous NT-3 likely plays an important role in preventing the development of thermal hyperalgesia, while the decreased expression of $Na_v1.9$ may prevent hyperexcitablity and/or repetitive firing of the neuron by increasing the resting membrane potential of these neurons.

5.5.3 Potential mechanisms of modulation of sodium channel expression by NT-3

It has been well characterized that inflammatory mediators (including NGF) are capable of increasing the expression voltage gated sodium channels (Dib-Hajj et al., 1998; Fjell et al., 1999; Gould et al., 2000; Fang et al., 2005; Amaya et al., 2006). It has also been postulated that the NGF regulation of $Na_v1.8$ is limited by the levels of trkA available (Fang et al., 2005). It therefore seems plausible that the ability of NT-3 to downregulate expression of trkA (Wilson-Gerwing and Verge, 2006) and to act in an antagonistic fashion to the pro-inflammatory effects of NGF (Verge et al., 1989a, 1989b, 1992, 1995; Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003; Wilson-Gerwing et al., 2005) may also underlie its ability to effect downregulation of the TTX-R sodium channel $Na_v1.8$ and possibly $Na_v1.9$. Another growth factor, glial derived neurotrophic factor (GDNF), has also been shown to upregulate $Na_v1.8$ and $Na_v1.9$ following sciatic nerve transection (Cummins et al., 2000) and to reduce ectopic neuronal discharges (spontaneous pain) after nerve injury

(Boucher et al., 2000) – a property of neuropathic pain attributed to $Na_v1.9$. There is some evidence that perhaps, NT-3 may be able to act through the GDNF receptor c-Ret (Kobayashi and Masuoka, 2000) and thus may exert its effects on $Na_v1.9$ in this manner.

Following nerve injury, it has been described that voltage gated sodium channels are redistributed to the tips of the injured axons and/or neuromas (Devor et al., 1989; England et al., 1994, 1996; Amir et al., 1999). It then becomes a plausible question whether the reduced levels of sodium channels at the DRG are simply the result of this translocation. While this was not directly examined, I would propose that initially there is a redistribution of sodium channels, but the extremely low transcript levels following NT-3 treatment would support that it is less likely that protein is still abundant at the nerve sites at the week post injury time examined as NT-3 was infused for this duration. It is also unknown whether or not treatment with exogenous NT-3 dampens/influences the inflammatory response to CCI, and may therefore alter the signals that effect this translocation.

5.5.4 Clinical relevance of reduced $Na_v1.8$ and $Na_v1.9$ expression

The use of sodium channel blockers to treat both neuropathic and inflammatory pain in a clinical setting is well known to result in analgesia (Clayton et al., 1997; Evans et al., 1997; Galer, 1995; McQuay et al., 1995; Trezise and Xie, 1997). These include such treatments as topical creams (for example, lidocaine), anticonvulsants, and tricyclic antidepressants (reviewed in Rogers et al., 2006).

It therefore becomes important to ask: Does the decreased expression of $Na_v1.8$ and $Na_v1.9$ also result in a physiological blockade of these channels? It has been demonstrated that a significant downregulation of these two channels also resulted in the significant reduction of the TTX-R sodium current (Dib-Hajj et al., 1999). Preliminary evidence from electrophysiological studies of DRG neurons subjected to both CCI and exogenous NT-3 treatment suggests that there is an overall reduction in sodium current and a complete lack of a TTX-R current in these neurons (TDW-G and Cheryl Stucky, unpublished observations).

In conclusion, the investigation into the modulation by exogenous NT-3 of two TTX-R sodium channels, $Na_v1.8$ and $Na_v1.9$, implicated in the generation of neuropathic pain has once again shown that NT-3 can effectively antagonize yet another pro-inflammatory aspect of this pain state and in doing so presumably alter the electrophysiological properties of these neurons.

6 Neurotrophin-3 differentially modulates levels of activated extracellular signal-related kinase nuclear localization in trkA versus trkC subpopulations of adult rat dorsal root ganglia neurons

6.1 Abstract

NT-3 is a negative modulator of trkA expression and associated phenotype in intact sensory neurons, while positively regulating trkC and associated phenotype. However, how NT-3 effects this response is less clear. NGF mediates its positive influence on trkA neurons by activating members of the mitogen-activated protein kinase family (i.e. ERKs). This is an important form of signaling associated with NGF's role in regulation of neuronal phenotype in nociceptive neurons. The present study addresses whether NT-3 in its capacity to differentially modulate gene expression in distinct subpopulations of sensory neurons in the intact state, exerts a differential influence on levels of activated ERK1/2 in the DRG of adult rats in trk-A versus trkC-mRNA positive subpopulations of neurons. Analysis focused on defining the level of activated phospho-ERK1/2 immunofluorescence signal detected in the nucleus of sensory neurons expressing trkA alone, trkC alone, or both trkA and trkC transcripts.

NT-3 intrathecal infusion decreased the overall percentage of neurons expressing detectable trkA only, while increasing the overall percentage of neurons expressing detectable trkC only. The incidence of neurons coexpressing trkA and trkC was not altered by infusion of NT-3. NT-3 also had a differential influence on nuclear phospho-ERK1/2 levels detected in neurons expressing trkA alone or trkC alone. Phospho-ERK1/2 labeling intensity was significantly decreased over nuclei of those neurons expressing trkA alone and significantly increased over the nuclei of those neurons expressing trkC alone. Neurons expressing either both trkA and trkC or neurons

expressing neither trkA nor trkC showed no significant alteration in levels of phospho-ERK signaling. In conclusion, it appears that exogenous NT-3 can effect a differential regulation of ERK signaling in trkA- versus trkC-only subpopulations of sensory neurons that may contribute to the differential influence of NT-3 on downstream gene expression in these two subpopulations.

6.2 Introduction

Sensory neurons of the dorsal root ganglion (DRG) are able to recognize and respond to a variety of extracellular stimuli (reviewed in Roux and Blennis, 2004). Stimuli such as growth factors and other mediators of cellular stress lead to the activation of signaling pathways within the neuron - primarily the mitogen-activated protein kinase (MAPK) pathway – leading, in part, to the modification of gene expression (Keyse, 2000; Camps et al., 2000; Sun et al., 1993; Keyse and Emslie, 1992). While multiple MAPK pathways exist within any given cell, it is the extracellular signal-regulated kinases 1 and 2 (ERK1/2) MAPK family that is of interest from a neurotrophin perspective as they are preferentially activated in response to growth factors (reviewed in Pearson et al., 2001). Set in motion by dimerization of a receptor tyrosine kinase binding to a growth factor, the Raf/MEK/ERK signaling cascade (English et al., 1999) leads to activation and translocation of phospho-ERK to the nucleus where it regulates transcriptional events (Lewis et al., 1998).

Members of the MAPK family, such as ERK1/2, p38 MAPK and ERK 5 are activated by inflammatory mediators and are proposed to participate in the generation and perhaps maintenance of pain syndromes following nerve injury (Ji et al., 2002a; Obata et al., 2004; Mizushima et al., 2007; reviewed in Ma and Quirion, 2005).

Nerve growth factor (NGF) has been well characterized as influencing the phenotype of nociceptive primary sensory neurons (trkA neurons) and regulating both inflammatory and homeostatic pain states (Lewin and Mendell, 1993; Woolf et al., 1994; Verge et al., 1995; Woolf, 1996; Herzberg et al., 1997; Ramer et al., 1998; Theodosiou et al., 1999). The primary manner in which NGF mediates its positive influence on trkA

sensory neurons is by activating members of the MAPK family, such as ERK1/2 (Averill et al., 2001; Zhuang et al., 2004; Donnerer et al., 2005). Neurotrophin-3 (NT-3), another member of the NGF family, has its primary influence on the growth, development, and maintenance of large, myelinated proprioceptive sensory neurons that are trkC-positive (Ernfors et al., 1994; Farinas et al., 1994). More recently, it has been demonstrated that NT-3 can modulate expression of peptides, molecules and behaviors implicated in nociception in an antagonistic fashion to NGF (Jongsma Wallin et al., 2001; Gratto and Verge, 2003; Wilson-Gerwing et al., 2005; Wilson-Gerwing and Verge, 2006).

Thus, NT-3 is a negative modulator of trkA expression and associated phenotype in sensory neurons, while positively regulating trkC and associated phenotype. However, how NT-3 effects this response is less clear. NGF mediates its positive influence on trkA neurons by activating members of the MAPK family (i.e. ERKs). This is an important form of signaling associated with NGF's role in inflammatory pain. The present study addresses whether the ability of NT-3 to differentially modulate gene expression in trkA versus trkC subpopulations of adult sensory neurons, in the intact state, is associated with differential influence on levels of activated (phospho) ERK1/2 in the DRG of adult rats in these subpopulations of neurons.

6.3 Methods

6.3.1 Animal surgery

All animal procedures were conducted in accordance with the National Institutes of Health policy on the use of animals in research and the University of Saskatchewan animal care committee guidelines (protocol 19920164). A total of 17 young adult male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 250-300 g were used. Animals were anesthetized for surgery with sodium pentobarbital (Somnitol, 65 mg/kg; MTC Pharm, Cambridge, Ontario, Canada). Pre- and post-operative (for 24 h)

subcutaneous injections of buprenorphine (Temgesic, 0.1-0.2 mg/kg) were given to alleviate any post-operative discomfort. To examine the effect of NT-3 on the expression of phospho-ERK1/2, 17 rats were used: 6 underwent intrathecal infusion of NT-3 for 7 days, 6 underwent intrathecal infusion of 1X Vehicle for 7 days, and 5 remained as naïve controls.

NT-3 was delivered intrathecally for 7 d via mini-osmotic pumps (model 2001; Alza, Cupertino, CA) inserted at the lumbar sacral junction as per Verge et al. (1989a) at a concentration and rate of 600 ng/µl/hr (Karchewski et al., 2002) in a solution of PBS containing rat serum albumin (1 mg/ml), streptomycin (100 U/ml), and penicillin (100 U/ml). This dose of NT-3 was the minimum dose found to selectively reverse injury-associated gene expression in injured trkC-expressing neurons (Verge et al., 1996; Jongsma Wallin et al., 2001; Karchewski et al., 2002). 1X Vehicle consisting of PBS containing rat serum albumin (1 mg/ml), streptomycin (100 U/ml), and penicillin (100 U/ml) was delivered intrathecally for 7 d as described above. At the conclusion of the experiments, rats were killed, and tissue was dissected and processed for *in situ* hybridization and/or immunohistochemistry as described below.

6.3.2 *In situ* hybridization

Deeply anesthetized animals were perfused via the aorta with 0.1 M PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1M PBS. The right and left L4 and L5 DRG were rapidly dissected, postfixed for 1 hour in the same fixative, and cryoprotected in 20% sucrose in 0.1M PBS overnight. Paired experimental and control tissues were mounted in the same cryomold (to ensure processing under identical conditions), covered with OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN, USA) and frozen in cooled isopentane. Transverse serial sections were cut at 6 µm on a Micron cryostat (Zeiss, Canada), thaw mounted onto Probe-On^{+} slides (Fisher Scientific, Edmonton, AB, Canada) and prepared for hybridization.

Prior to hybridization, slides were air dried for 15 minutes, followed by fixation in 4% paraformaldehyde for 20 minutes. Slides were washed 3 X 5 minutes in 1X PBS. Sections were then treated with proteinase K (20 µg/ml) containing 10 ml 1M Tris-HCl

(pH 7.6), 2 ml 0.5 M EDTA, 200 µl proteinase K stock (20 mg/ml) and 188 µl ddH_20 for 6 minutes. Slides were then rinsed for 5 minutes in 1 X PBS and post-fixed for 5 minutes in 4% paraformaldehyde. Slides were then rinsed 2 X 5 minutes in 1 X PBS, 1 X 5 minutes in DEPC-H_2O, and dehydrated in ascending alcohols.

An oligonucleotide probes complementary to and selective for trkA [complementary to bases 1198-1245 (Barker et al., 1993)] and trkC [complementary to bases 1189-1236 (Merlio et al., 1992)] messenger RNA (mRNA) were synthesized (University of Calgary DNA services, Alberta, Canada). The probes were checked against the GenBank database (NIH) to ensure no greater than 60% homology was found to sequences other than the cognate transcript. The probes were labeled at the 3'-end with α-[^{35}S]dATP (New England Nuclear, Boston, MA, USA) using terminal deoxynucleotidyl-transferase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in a buffer containing 10 mM $CoCl_2$, 1 mM dithiothreitol, 300 mM Tris base and 1.4 M-potassium cacodylate (pH 7.2), and purified through Bio-Spin® Disposable Chromatograph Columns (Bio-Rad laboratories, Hercules, CA, USA) containing 200 mg of NENSORB™ PREP Nucleic Acid Purification Resin (NEN®, Boston, MA, USA). Dithiothreitol was added to a final concentration of 10 nM.

Hybridization was carried out according to published procedures (Dagerlind et al., 1992) on a minimum of 5 slides/probe from each group of experimental and control animals. Briefly, the sections were hybridized at 43 ^oC for 14-18 hours in a buffer containing 50% formamide (Sigma Aldrich, Oakville, ON, Canada), 4X SSC (1X SSC – 0.15 M NaCl, 0.015 sodium citrate), 1X Denhart's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sacrosyl (N-laurylsarcosine), 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 500 µg/ml heat-denatured sheared salmon sperm DNA, 200 mM dithiothreitol and 10^7 cpm/ml of probe. After hybridization, the slides were washed for 4 X 15 mins in 1X SSC at 55^oC, dehydrated in ascending alcohols, processed for radioautography as per Karchewski et al., 2002 and exposed for 7 to 10 days before developing in D-19 (Kodak, Rochester, NY, USA).

The specificity of hybridization signal for the trkA and trkC probes were confirmed as described in Wilson-Gerwing et al., 2005.

6.3.3 Immunohistochemistry

Transverse 6 μm sections were cut on the cryostat, thaw-mounted onto Probe-ON^{+} slides (Fisher Scientific), and processed for immunohistochemistry. Sections were washed three times for 10 min in 0.1 M PBS, blocked in 2% goat serum and 0.3% Triton X-100 in 0.1 M PBS for 1 h at room temperature, incubated overnight with rabbit anti-phospho-p44/42 MAPK (ERK1/2) (1:3000; Cell Signaling Technology, Beverly, MA), diluted in 2% goat serum and 0.3% Triton X-100 in 0.1 M PBS at 4 ^{o}C, and visualized with a tyramide signal amplification kit (Molecular Probes, Inc., Eugene, OR). Briefly, slides were incubated for 45 min with HRP-conjugated secondary antibody (1:100) diluted in 2% goat serum and 0.3% Triton S-100 in 0.1 M PBS at room temperature, washed, and incubated for 5 minutes with tyramide stock solution (1:100) in amplification buffer. Slides were washed and coverslipped with 50% glycerol/50% PBS. Control sections were processed in the same manner, but without the primary antibody. Results were visualized using a Zeiss Axioscope 50 microscope equipped with incident-light fluorescence optics and a digital camera. Antibody specificity was confirmed by Western blot analysis (McDaid et al., 2005).

6.3.4 Quantification and analysis

Prior to *in situ* hybridization and immunohistochemistry, serial sections were arranged such that those slides processed to detect phospho-ERK1/2 were flanked by slides processed to detect trkA mRNA on one side and trkC mRNA on the other side (trkA mRNA/ anti-phospho-ERK1/2/ trkC mRNA) such that each DRG neuron identified in the phospho-ERK1/2 sections could be trilocalized with those neurons in the trkA and trkC sections.

Rapid qualitative analysis was performed on all slides from the 12 groupings of NT-3, 1X Vehicle, and naive animals (to avoid fading of the phospho-ERK1/2 immunofluoresence) and relative changes in the intensity of phospho-ERK1/2 fluorescence from one group to another noted for sections mounted on the same slide to avoid bias due to the variance in signal observed from slide to slide. Representative

slides from 3 different experimental and control groupings were selected based on having similar numbers of neurons in each DRG section on the slide and subjected to further quantitative analysis. Photomontages of each phospho-ERK1/2 section to be analyzed (and the corresponding trkA and trkC sections) were prepared and individual neurons with a visible nucleus in phospho-ERK sections were identified in the flanking serial sections. Using a 63X oil fluorescent objective, images of all neurons identified on the phospho-ERK1/2 montages were rapidly captured under identical conditions. Using the Northern Eclipse software package, Version 7.0 (Empix Imaging, Mississauga, ON, Canada), the area of the neuron was measured followed by measuring the intensity of phospho-ERK1/2 immunofluoresence in the nucleus. Data analysis was supplemented with Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA) and Prizm 4.0 (Graph Pad Software, San Diego, CA).

Analysis was performed in each instance on all neurons from 9 DRG sections or 1704 nuclear profiles (Naive: n = 3 animals, Naïve + Vehicle: n = 3 animals, Naïve + NT-3: n = 3 animals).

Following preliminary analysis of each experimental and control grouping, the presence of phospho-ERK1/2 was detected in both the cytoplasm and the nucleus of the DRG neurons examined with the most notable influence detected in the nuclei. As phospho-ERK1/2 levels in the nucleus are correlated with the impact this pathway on gene expression, only nuclear phospho-ERK1/2 labeling intensity was quantified.

6.4 Results

6.4.1 Experimental Controls

In the sections from naïve DRG (n=3), phospho-ERK1/2 signal was detected over the nuclei of all size ranges of neurons with a higher level of signal intensity in those neurons that are medium to large in diameter (Figure 6-1 and Figure 6-2).

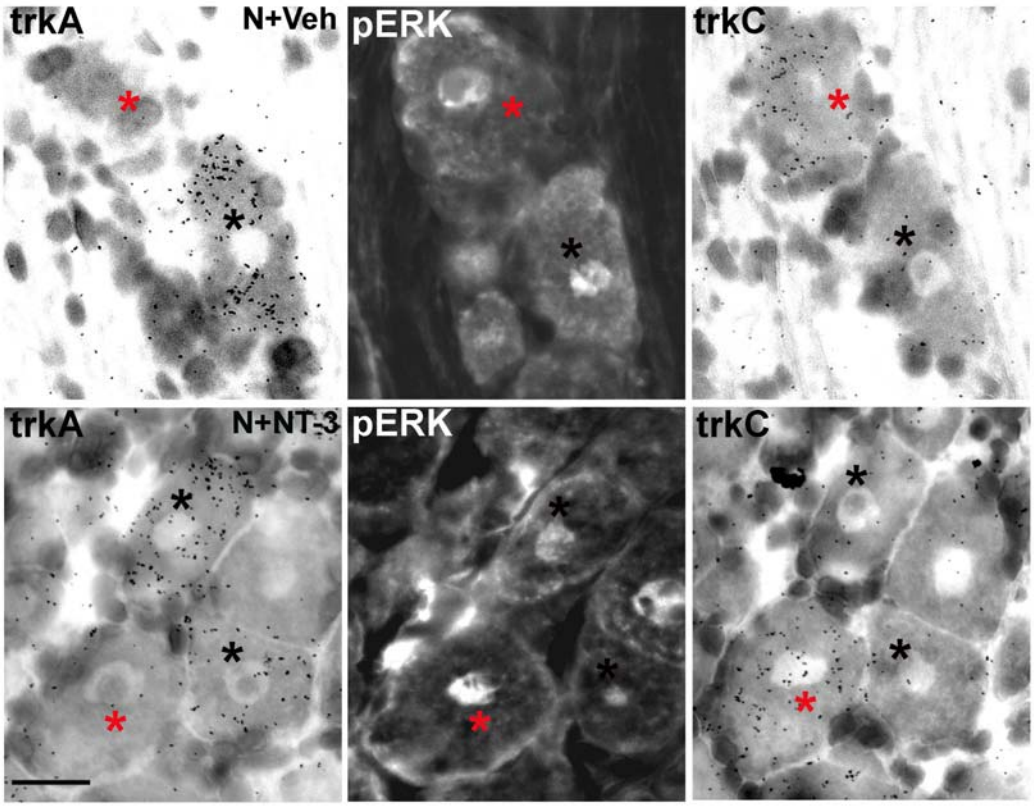

Figure 6- 1: Exogenous NT-3 differentially modulates the intensity of immunofluorescence signal depicting degree of nuclear localized activated ERK1/2 in trkC versus trkA subpopulations of sensory neurons.

Brightfield and fluorescence images representing DRG neurons in adjacent sections processed to detect trkA mRNA (left), activated (phospho) ERK 1/2 (center), and trkC mRNA (right) in naïve animals receiving either 1X Vehicle [N+Veh (top)] or 600 ng/µl/hr NT-3 infusions [N+NT-3 (bottom)]. Note: Following NT-3 treatment (N+NT-3), levels of nuclear phospho-ERK1/2 immunofluorescence are increased in trkC positive neurons (red asterisks) and decreased in trkA positive neurons (black asterisks). Scale bar = 100 µm

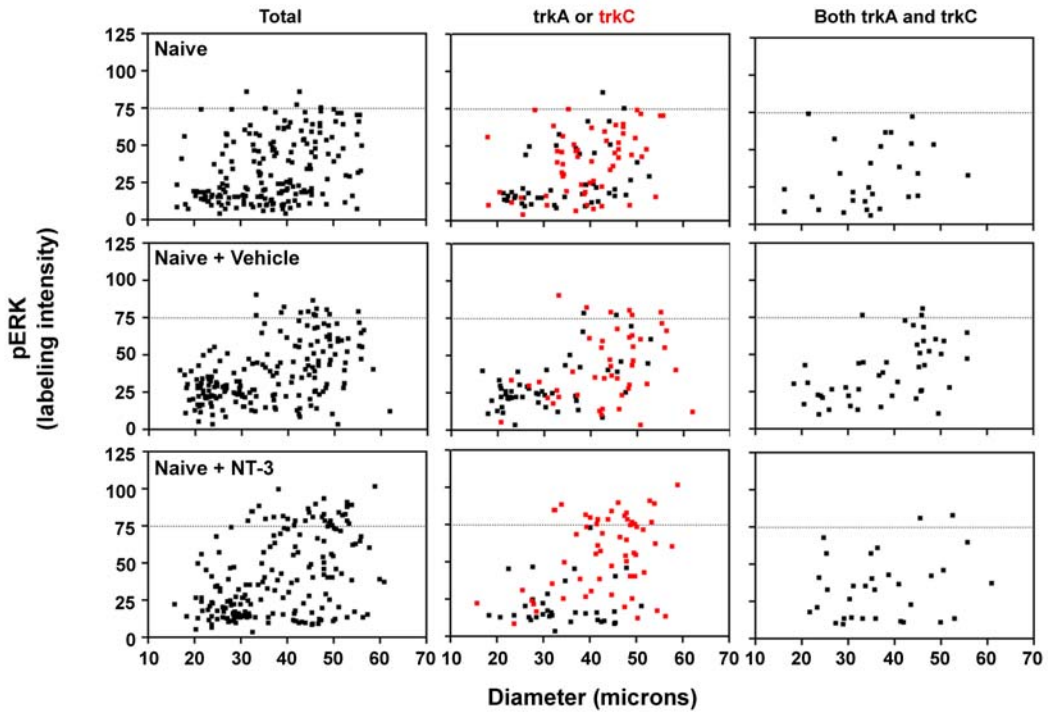

Figure 6- 2: NT-3 differentially modulates the levels of activated ERK1/2 immunofluorescence detected in the nucleus of sensory neurons expressing only trkA mRNA versus those expressing only trkC mRNA.

Representative scatterplots whereby each point represents the intensity of immunofluorescence signal over the nucleus of individual neurons identified as expressing trkA, trkC or both trkA and trkC mRNA in flanking adjacent sections processed for trkA or trkC *in* situ hybridization. The relationship between phospho-ERK1/2-like labeling intensity (y-axis) and perikaryal diameter (x-axis) is depicted for all neurons with a visible nucleus present in the DRG section sampled (left), those neurons that are trkA-positive only (black; middle) or, those neurons that are trkC-positive only (red; middle), and those neurons that are positively labeled for both trkA and trkC (right) for each of three conditions: Naïve animals (top row), Naïve animals receiving 1X Vehicle (middle row), and Naïve animals receiving 600 ng/µl/hr NT-3 (bottom row). Dotted lines separate graphs into light to moderate levels of immunofluorescence signal intensity and high levels of immunofluorescence signal intensity. Note: Following NT-3 treatment (N+NT-3), levels of phospho-ERK1/2 are increased in trkC-positive neurons and decreased in trkA-positive neurons.

Infusion of 1X Vehicle (n=3) did not significantly alter the frequency, distribution (Figure 6-2), or the mean labeling intensity (Figure 6-3) of phospho-ERK1/2. As a result, data obtained from each of the three experimental groups were pooled after first

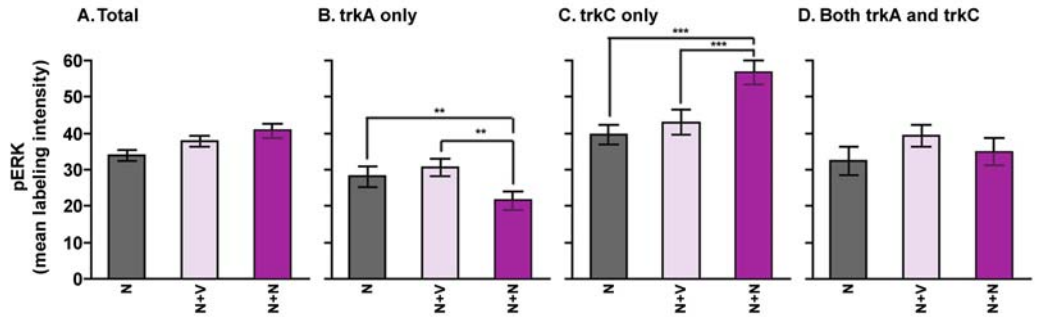

Figure 6- 3: NT-3 differentially affects the overall intensity of activated ERK1/2 immunofluorescence signal over the nucleus of DRG neurons expressing only trkA or only and trkC mRNA.

Bar graphs of relative changes in the average phospho-ERK1/2 immunofluorescence signal intensity in the nucleus of individual neurons in DRG sections for each of: A, All neurons with a visible nucleus present in the DRG section sampled (total); B, Neurons positive for trkA only; C, Neurons positive for trkC only; D, Neurons positive for both trkA and trkC. Experimental states as indicated on the graphs are: Naive (N); Naive + Vehicle (N + Veh); Naive + NT-3 (N + NT-3). Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$; ** $p<0.01$). Note: Treatment with NT-3 significantly decreases mean phospho-ERK1/2 nuclear immunofluorescence signal intensity in neurons expressing trkA only and significantly increases mean phospho-ERK1/2 nuclear labeling intensity in neurons expressing trkC only. There are no significant differences between Naïve versus Naïve + Vehicle treated animals.

being normalized to total nuclear phospho-ERK mean labeling intensity of Naïve + Vehicle values for each individual grouping of animals (Figure 6-4 and Figure 6-5).

6.4.2 Effect of NT-3 infusion on individual and mean nuclear phospho-ERK1/2 localization for entire neuronal population

When the impact of NT-3 infusion on nuclear level of phospho-ERK1/2 signal was examined for the entire population of individual neurons in the DRG sections, there appeared to be an increase in the incidence of medium to large sized DRG neurons displaying a high intensity of phospho-ERK1/2 signal as shown in the scatterplot

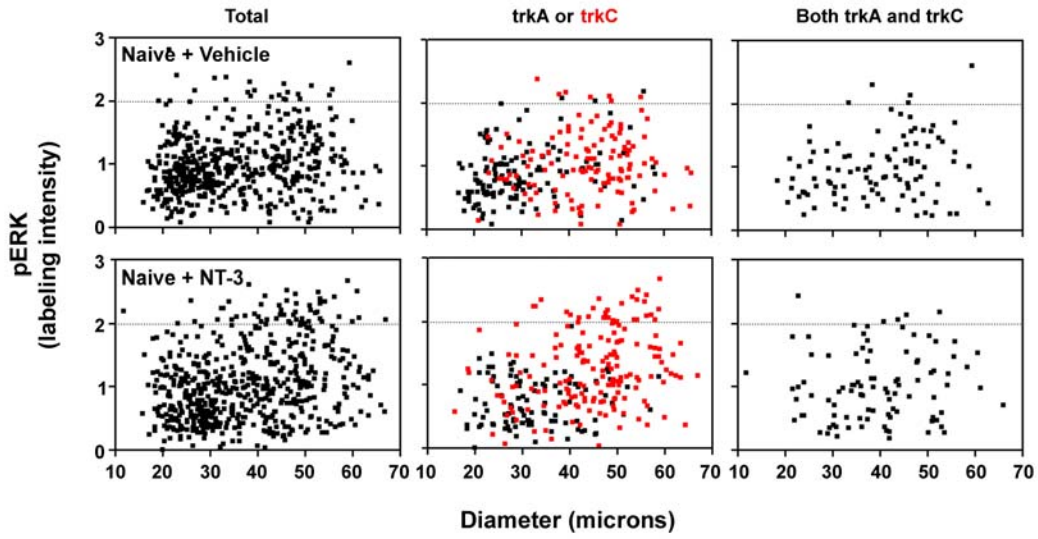

Figure 6- 4: NT-3 differentially affects the degree and incidence of nuclear localized activated ERK1/2 in DRG neurons positive for trkA only and trkC only.

Scatterplots of the total population of all neurons measured (n = 3 animals) whereby each point represents the labeling intensity of immunofluorescence signal over nuclei of individual neurons identified in 5 μm thick sections of L5 DRG and normalized to the mean signal intensity neurons from the naïve animal in its grouping. The relationship between phospho-ERK1/2-like immunofluorescence signal intensity (y-axis) and perikaryal diameter (x-axis) is depicted for all neurons with a visible nucleus present in the DRG sections sampled (left), those neurons that are trkA-positive only (black; middle), those neurons that are trkC-positive only (red; middle), or those neurons that are positively labeled for both trkA and trkC (right). Changes in mean phospho-ERK1/2 immunofluorescence signal intensity by NT-3 [Naïve + NT-3 (bottom row)] are depicted relative to Naïve animals receiving 1X Vehicle [Naïve + Vehicle (top row)]. Dotted lines separate graphs into light to moderate levels of labeling intensity and high levels of labeling intensity. Note: Following NT-3 treatment (N+NT-3), levels of phospho-ERK1/2 are increased in neurons expressing only trkC (red; middle) and decreased in neurons expressing only trkA (black; middle).

analysis (i.e. those having greater than signal >2; Figure 6-2). However, when one determined the impact of NT-3 infusion on the mean labeling index of phospho-ERK1/2 signal for the entire neuronal population (n=3) no significant difference was observed for each of the individual sets of experimental versus control animals analyzed (Figure 6-3). Similarly, when data from the NT-3 infused animals was normalized to the total nuclear phospho-ERK mean labeling intensity of Naïve + Vehicle values for each

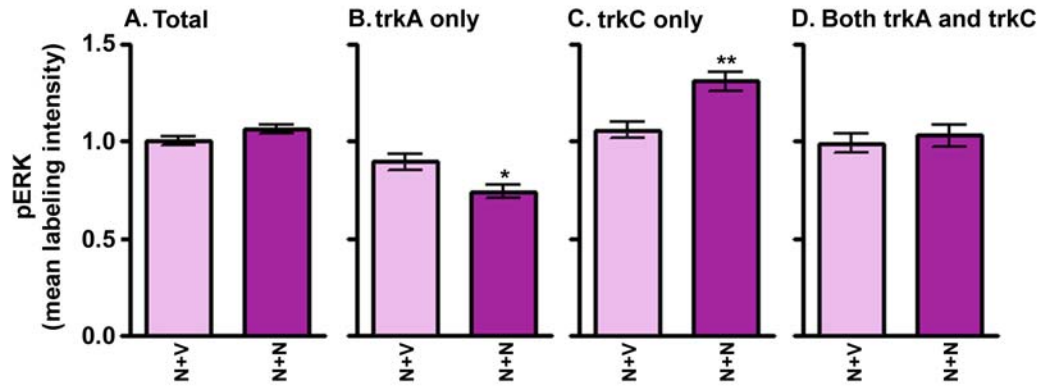

Figure 6- 5: NT-3 significantly affects the overall intensity of activated ERK1/2 signal in the pooled population of DRG neurons positive for trkA only or trkC only.

Summary bar graphs of data presented in Figure 6-4 showing relative changes in the average phospho-ERK1/2 immunofluorescence signal intensity in the nucleus of individual neurons in DRG sections for each of: A, All neurons with a nucleus present in the DRG sections sampled (total); B, Neurons positive for trkA only; C, Neurons positive for trkC only; D, Neurons positive for both trkA and trkC. Changes in mean phospho-ERK1/2 immunofluorescence signal intensity by NT-3 [Naïve + NT-3 (bottom row)] are depicted relative to Naïve animals receiving 1X Vehicle [Naïve + Vehicle (top row)]. Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; ** $p<0.01$; * $p<0.05$). Note: Treatment with NT-3 significantly decreases mean phospho-ERK1/2 immunofluorescence signal intensity in those neurons expressing only trkA and significantly increases mean phopsho-ERK1/2 immunofluorescence signal intensity in those neurons expressing only trkC.

individual grouping of animals and then pooled (Figure 6-4 and Figure 6-5), the incidence of neurons expressing high labeling intensities (>2) were increased by NT-3 infusion (from 4.31% to 10.20%) (Figure 6-4). However, there was no significant difference in mean labeling intensity as compared to controls [1.0 +/- 0.0206 (s.e.m.) to 1.061 +/- 0.02551 (s.e.m.) (Figure 6-5)]. Thus, it became apparent that in order to ascertain whether there might be selective alterations in the level of nuclear phospho-ERK1/2 signal observed in the trkA versus trkC subpopulations of sensory neurons the subpopulations would have to be identified and analyzed individually. Further, as we have previously demonstrated that there is extensive overalp in the trkA and trkC subpopulations (Karchewski et al., 1999), trilocalization of all three parameters would

be necessary in order to draw appropriate conclusions for those neurons expressing only trkA or only trkC versus those co-expressing the two receptor mRNAs.

6.4.3 Effect of NT-3 treatment on the subpopulation of sensory neurons expressing only trkA

Those DRG neurons that were identified as expressing trkA mRNA only where primarily, although not exclusively, small to medium in size (Figure 6-2 and Figure 6-4). Infusion of NT-3 decreased the incidence of trkA only positive neurons being detected (from 24.53% to 17.86%). Those neurons expressing high labeling intensities of phospho-ERK1/2 (>2) were also reduced (from 2.29% to 0%) (Figure 6-4). In addition, exogenous NT-3 effected a significant decrease in the mean labeling intensity of phospho-ERK1/2 in this population of neurons [from 0.8927 +/- 0.03807 (s.e.m.) to 0.7416 +/- 0.03684(s.e.m.) (Figure 6-5)].

6.4.4 Effect of NT-3 treatment on the subpopulation of sensory neurons expressing only trkC only

Those DRG neurons that were identified as expressing trkC mRNA only were primarily medium to large in size, with some small to medium sized neurons also observed (Figure 6-2 and Figure 6-4). Infusion of NT-3 slightly increased the incidence of detectable trkC only positive neurons (from 23.78% to 29.76%) while markedly increasing the percentage of those neurons expressing high labeling intensities of phospho-ERK1/2 (>2) from 5.51% to 18.29% (Figure 6-4). NT-3 infusion also had a significant impact on the mean labeling intensity of phospho-ERK1/2 in this population of neurons increasing it from 1.057 +/- 0.0438 (s.e.m.) to 1.307 +/- 0.04723 (s.e.m.) (Figure 6-5).

6.4.5 Effect of NT-3 treatment on the subpopulation of sensory neurons expressing both trkA and trkC

The population of DRG neurons identified as expressing both trkA and trkC mRNA was distributed over all size ranges of neurons (Figure 6-2 and Figure 6-4). Infusion of NT-3 did not markedly alter the incidence of neurons expressing both trkA and trkC (18.91% versus16.33%), nor the incidence of neurons expressing high labeling intensities of phospho-ERK1/2 (4.95% versus 5.21%). Further, it did not significantly influence the mean labeling signal intensity of phospho-ERK1/2 in this population of neurons [0.988 +/- 0.0499 (s.e.m.) versus 1.031 +/- 0.0562 (s.e.m.) (Figure 6-5)].

6.5 Discussion

In this study we have shown for the first time a differential regulation by NT-3 of nuclear localized phospho-ERK1/2 in intact lumbar sensory neurons expressing only trkA versus those expressing only trkC *in vivo*. These findings are consistent with our previous studies demonstrating that NT-3 modulates trkA-associated neuronal phenotype in an opposite manner to that of NGF in intact sensory neurons (Jongsma Wallin et al., 2001; Karchewski et al., 2002; Gratto and Verge, 2003). Subsequently we have also shown that NT-3 can effectively prevent and reverse the development of thermal hyperalgesia, associated phenotype and signaling molecules complicit in this response in the chronic constriction injury (CCI) model of neuropathic pain, a behavioral response/phenotype attributable largely to the trkA subpopulation (Wilson-Gerwing et al., 2005). All these lines of evidence support a role for NT-3 acting antagonistically to NGF on the trkA subpopulation of sensory neurons.

6.5.1 phospho-ERK1/2 is present in the nucleus of naïve DRG neurons.

In the naïve DRG, it was determined that there was a detectable level of phospho-ERK1/2 in the nuclei of the neurons examined. This is in contrast to other published reports whereby phospho-ERK staining was weak or absent in the majority of DRG cells (Averill et al., 2001; Dai et al., 2002; Takahashi et al., 2006). It has been described that phospho-ERK1/2 is present throughout unstimulated cells, but a significant amount is only translocated to the nucleus in response to stimulation which may underlie the technical difficulties in visualizing it under homeostatic conditions (Chen et al., 1992; Gonzalez et al. 1993; Lenormand et al., 1993). The findings of this study demonstrate that phospho-ERK1/2 is present in both the cytoplasm and the nucleus of unstimulated cells. One possible explanation for these contradictory findings is the sensitivity of the phospho-ERK1/2 antibody and protocol employed. The use of a tyramide amplification system likely allowed for the enhanced detection of extremely low levels of phospho-ERK1/2 within the nucleus. Further, similar results were attainable with the protocol in the absence of the tyramide amplification step when classic avidin biotin approaches were employed although at a reduced signal to noise ratio (TDW-G, personal observation). Intuitively, one would expect there to be a low level of continuous activation as constant retrograde signaling by target-derived NGF or NT-3 to these fibers must occur if homeostatic phenotype is to be maintained.

This is also the first analysis of the presence and distribution of phospho-ERK1/2 in the nucleus of those cells contained within both the trkA and the trkC subpopulations of DRG neurons. The finding that those neurons containing only trkA are primarily small to medium in diameter, but also contain some large sized neurons and that those neurons containing only trkC are primarily medium to large in size but also contain some small neurons support previous findings on the heterogeneity of trk-containing neurons (Karchewski et al., 1999; Jongsma Wallin et al., 2001; Karchewski et al., 2002). This is further supported by the population of DRG neurons shown here containing both trkA and trkC and consisting of all size ranges. Interestingly, the mean level of nuclear localized phospho-ERK1/2 was higher in the trkC only expressing neurons versus the trkA only. This is consistent with the potential higher level of activation that one would

expect to see in the former population which consists largely of proprioceptive fibers which should receive continual input/stimulation as the animal locomotes around his environment.

6.5.2 NT-3 differentially modulates phospho-ERK1/2 levels in those neurons positive for either trkA or trkC.

In the naïve DRG, NT-3 effectively decreased both the incidence of neurons expressing trkA alone as well as the labeling intensity of phospho-ERK1/2 within this population. Conversely, but not unexpectedly, NT-3 infusion effectively increased both the percentage of neurons expressing detectable trkC alone as well as the labeling intensity of phospho-ERK1/2 within this population. There appeared to be no net effect of NT-3 infusion on those neurons expressing both trkA and trkC presumably due to the differential influence on each receptor. These findings are consistent with a role for exogenous NT-3 in negatively modulating gene expression in the trkA population of neurons while positively regulating that in the trkC population that we have previously observed in regards to phenotype modulation (Verge et al., 1989a, 1989b; 1992; 1995; 1996 Jongsma Wallin; 2001; Karchewski et al., 2002; Gratto and Verge, 2003; Wilson-Gerwing et al., 2005, 2006).

6.5.3 Possible roles for phospho-ERK1/2 modulation by NT-3 in nociception.

Numerous lines of research have demonstrated an important role for phospho-ERK in the promotion of neuropathic pain syndromes in rats (Mizushima et al., 2007; reviewed in Ma and Quirion, 2005; Ji et al., 2002a; Dai et al., 2002; Obata et al., 2004; Aley et al., 2001; Averill et al., 2001, Obata et al., 2003; Dina et al., 2003; Song et al., 2005; reviewed in Obata and Noguchi, 2004). It has been shown that NGF also promotes neuropathic pain syndromes (Lewin and Mendell, 1993; Woolf et al., 1994;

Herzberg et al., 1997; Ramer et al., 1998; Theodosiou et al., 1999), associated neuronal phenotype (Verge et al., 1995; Woolf, 1996) and an increase in the expression of phospho-ERK (Hundle et al., 1995; Averill et al., 2001). This increase in phospho-ERK, in turn correlates with increases in the expression of the pain-related neuropeptide SP (Kawasaki et al., 2004), and the neurotrophin brain derived neurotrophic factor (BDNF) (Yajima et al., 2002; Kawasaki et al., 2004). Finally, NT-3 has been demonstrated to antagonize thermal hyperalgesia (Wilson-Gerwing et al., 2005), the expression of certain pain-related neuropeptides (Jongsma Wallin et al., 2001; Gratto and Verge, 2003; Wilson-Gerwing and Verge, 2006) and BDNF (Karchewski et al., 2002; TDW-G, unpublished observations).

Much of the examination into the role of phospho-ERK in nociceptive processing, has focused on the upregulation BDNF by NGF in models of peripheral inflammation from the perspective that activation of the phospho-ERK signaling cascade results in the transcription of BDNF (Woolf and Costigan, 1999; Ji and Woolf, 2001). From this standpoint, it is reasonable to assume that NT-3 is capable of inhibiting phospho-ERK's role in nociception as 1) NT-3 has been demonstrated to act antagonistically to NGF in numerous incidences; 2) NT-3 is now shown to differentially regulate levels of phospho-ERK in the nuclei of trkA or trkC expressing DRG neurons; and 3) NT-3 has been observed to mitigate increased expression of BDNF protein and mRNA normally observed following CCI (TDW-G, unpublished observations). How NT-3 is mediating this effect on the trkA associated signaling pathways is still unknown. It remains to be determined whether NT-3 through its ability to bind to an isoform of trkA (Barker et al., 1993; Clary and Reichardt, 1994; Barbacid, 1994) that we have shown is expressed by all neurons in this subpopulation of trkA-positive sensory neurons (Karchewski et al., 1999) is having a direct effect on levels of phospho-ERK. The recent description of hotspots for NT-3 binding on the trkA receptor when expressed in HEK293 cells support a complex role for NT-3 in serving as both an agonist or antagonist to NGF-mediated responses (Ivanisevic et al., 2007). This also raises the possibility that some of yet unexamined roles for NT-3 in the nociceptive subpopulation may actually be additive to the NGF-mediated response. One can also not exclude that the ability of NT-3 to dampen the MAPK signaling pathway may be

linked to interactions with the p75 common neurotrophin receptor that is also expressed by the trkA population (Verge et al., 1992). This has recently been shown to be the case for BDNF where it is believed that BDNF binding to p75 may interfere with the ability of NGF to bind with high-affinity to trkA (Obata et al., 2006). While these are intriguing possibilities it is unlikely that in the present model where we infuse NT-3 intrathecally thereby giving it access to receptors on the dorsal roots that there is a major interference by NT-3 with NGF binding to p75 and trkA as the major source of NGF is derived from the peripheral target of these fibers and not centrally. Thus it is likely that the net influence of NT-3 on the MAPK pathway observed is due to the convergent alterations by NT-3 on the NGF/trkA signaling pathway.

In conclusion, the findings of this study once again support a biologically relevant role for NT-3 in the regulation of nociceptive pathways.

7 Discussion

7.1 CCI as a neuropathic pain model

Understanding neuropathic pain associated with pathologies of the sciatic nerve remains somewhat clouded even in spite of the large bodies of research that have taken place over the years. The difficulty in obtaining a clear understanding of this condition results from the inherent complexities of the associated pain syndromes whereby multiple symptoms may be elicited by a single mechanism or, conversely, various single mechanisms may all produce the same symptom (Woolf and Decosterd, 1999).

For a number of years, the complete sciatic nerve transection model was employed to examine cellular and molecular changes that occurred at various levels of the peripheral and central nervous systems without the ability to easily correlate these changes and the behavior of the animal due to the target no longer being innervated. With the development of the CCI model of neuropathic pain (Bennett and Xie, 1988), whereby there is only a partial injury coupled with an inflammatory response, it became possible to determine whether or not phenotypic changes in the dorsal root ganglia (DRG) as a result of injury (or treatment) correlated with behavioral changes. For these reasons, the CCI model was chosen to examine the behavioral and biochemical changes as a result of peripheral nerve injury and the ability of exogenous neurotrophin-3 (NT-3) to alter these changes.

The body of knowledge that has been obtained from this thesis work provides novel insights into the ability of NT-3 to modulate certain aspects of the homeostatic state of sensory neurons and neuropathic pain syndromes. I also confirm portions of the existing knowledge regarding the phenotype of intact neurons and select biochemical changes in the DRG as a result of CCI.

7.2 Review of chronic constriction injury model (CCI), inflammation, and neuropathic pain behaviors

The chronic constriction injury (CCI) model of neuropathic pain involves a partial lesion of the sciatic nerve induced by tying loosely constrictive chromic gut sutures around the sciatic nerve (Bennett and Xie, 1988). This results in the slow, edematous axotomy of predominantly large diameter myelinated axons (90% of Aβ and Aδ fibers and 30% of C fibers) by three days post-ligation (Kajander and Bennett, 1992) while the uninjured, smaller axons remain intact, but exposed to the Wallerian degenerating nerve in which many pro-inflammatory molecules are produced – a state akin to that observed in man (Bennett and Xie, 1988). These inflammatory and immune cells release a variety of neuroactive agents including cytokines (IL-6, for example) and growth factors (NGF, for example) (Woolf and Costigan, 1999).

Neuropathic pain that develops following CCI is characterized by the presence of abnormal sensory behaviors. These include thermal hyperalgesia, mechanical allodynia/hypersensitivity, and spontaneous pain. Hyperalgesia following nerve injury is identified as an increased sensitivity to a normally noxious stimulus (a decreased threshold) and/or suprathreshold stimulation producing an abnormally exaggerated response (reviewed in Millan, 1999; Woolf and Mannion, 1999). Following nerve injury, painful sensations can also be elicited by a normally non-noxious stimulus – commonly referred to as allodynia (Woolf and Mannion, 1999). Finally, spontaneous pain is the result of aberrant pain sensations in the apparent absence of any stimuli (Mannion et al., 1999; Woolf and Decosterd, 1999; Woolf and Mannion, 1999; Han et al., 2000; Liu et al., 2000)

7.3 Summary of major findings

7.3.1 Behavioral analysis

The ability of NT-3 to prevent and reverse thermal hyperalgesia following CCI is a novel finding with respect to modulation of neuropathic pain. Included in this finding is that continuous infusion of NT-3 is required for this effect, as removal of exogenous NT-3 results in the rapid reestablishment of the thermal hyperalgesic state. In contrast, the mechanical hypersensitivity observed after CCI is not altered by NT-3, despite downregulation of injury-associated peptide expression in larger neurons.

7.3.2 TRPV1 analysis

Localization of TRPV1 expression to primarily small and medium neurons in contralateral intact DRG is in agreement with past studies (Caterina et al., 1997; Eglen et al., 1999; Ma, 2002). Although CCI resulted in slightly elevated TRPV1 expression observed in the size range of neurons consistent with C-fibers, it also induced a low level of expression in a novel population of large neurons. Alterations in TRPV1 expression effected by NT-3 were most robust for neurons ipsilateral to injury. In accordance with Ji et al. (2002b), the observed modulation of TRPV1 expression was most apparent at the protein versus mRNA level.

7.3.3 ASIC3 analysis

Consistent with previous reports, in the intact state, expression of ASIC3 was observed predominantly in small to medium sized DRG neurons, with some large neurons also expressing detectable levels of ASIC3 (Waldmann et al., 1997b; Chen et al., 1998; Voilley et al., 2001; Molliver et al., 2005) in approximately 35 to 40% of DRG

neurons (Ugawa et al., 2005; Molliver et al., 2005). We describe for the first time that following CCI, levels of ASIC3 expression were significantly upregulated, primarily in the small to medium sized (nociceptive) DRG neurons, and also in a small population of large sized neurons. The current work also demonstrates that, following CCI, exogenous NT-3 can effectively counter the upregulation of ASIC3 expression, reducing levels to approximately that of basal expression. Surprisingly, examination of 14 day CCI with infusion of NT-3 for the first 7 days of the injury reveals that upon removal of exogenous NT-3, ASIC3 levels remain depressed, suggesting that continuous infusion of NT-3 is not required for this effect.

7.3.4 $Na_v1.8$ and $Na_v1.9$ analysis

Consistent with previous reports, I have found that in the intact state, both $Na_v1.8$ and $Na_v1.9$ mRNA and protein are localized primarily over small diameter DRG neurons, with some medium and large sized neurons also expressing these channels (Akopian et al., 1996; Black et al., 1996; Cummins et al., 1999; Dib-Hajj et al., 1999). Following 7d unilateral CCI, the $Na_v1.8$ mRNA was significantly reduced, primarily in those small to medium diameter neurons (similar to those findings of Dib-Hajj et al., 1999). Neuronal expression of $Na_v1.9$ mRNA is also significantly decreased following CCI. The current work demonstrates the novel finding that NT-3 can significantly decrease both the mRNA and protein levels of $Na_v1.8$ and $Na_v1.9$ following 7d unilateral CCI in the DRG neurons both ipsilateral and contralateral to CCI.

7.3.5 Phospho-p38 MAPK analysis

While the levels of phospho-p38 MAPK expression were found to be unaltered by 7 d CCI, exogenous NT-3 effected a dramatic downregulation of phospho-p38

MAPK. Further experiments demonstrate that exogenous NT-3 can effectively prevent and reverse the CCI-associated increases in phospho-p38 MAPK expression. Upon removal of exogenous NT-3, levels of phospho-p38 MAK remain low, again suggesting that continuous infusion is not required for this effect.

7.3.6 Phospho-ERK analysis

In the naïve DRG, it was determined that phospho-ERK1/2 can be detected in the nuclei of the neurons examined. This is in contrast to other published reports whereby phospho-ERK staining was weak or absent in the majority of DRG cells (Averill et al., 2001; Dai et al., 2002; Takahashi et al., 2006). In the naïve DRG, NT-3 effectively decreased both the incidence of neurons expressing trkA alone as well as the phospho-ERK1/2 immunofluorescence signal over the nuclei of neurons within this population. Conversely, but not unexpectedly, NT-3 effectively increased both the percentage of neurons expressing trkC alone as well as the phospho-ERK1/2 immunofluorescence signal over the nuclei of neurons within this population. There appeared to be no net effect of those neurons expressing both trkA and trkC.

7.4 Thermal hyperalgesia with respect to TRPV1, ASIC3, and sodium channels

The demonstration that intrathecal NT-3 infused at the time of CCI is able to prevent the development of thermal hyperalgesia, while intrathecal NT-3 infused 7 d post-CCI is able to reverse established thermal hyperalgesia is a novel finding with respect to the modulation of neuropathic pain. NT-3 is generally thought of as being a neurotrophin that influences large, myelinated sensory neurons despite evidence that it is capable of modulating peptides and molecules that are associated with nociceptive function. Research exploring the ability of NT-3 to influence pain-related behaviors in an injury model is limited, but expanding. In one such study, inflammatory mechanical hyperalgesia – induced by intraplantar injection of complete Freund's adjuvant (CFA) –

was transiently reversed by local hindpaw injection of NT-3 (Watanabe et al., 2000). This study, in accordance with my findings on thermal hyperalgesia and associated phenotype, suggests that NT-3 (in the CCI model) might exert its effect by altering the inflammatory response of uninjured nociceptors.

Many of the studies exploring the role of NT-3 in modulation of pain states are from naïve animals and examine whether NT-3 might alter baseline nociceptive thresholds. Conflicting results have been obtained. Shu et al. (1999) report that intradermal injection of NT-3 (200ng) into an uninjured hindpaw had no influence over thermal sensitivity. This is supported by my previous finding that NT-3 infused into normal animals did not alter thermal sensitivity (Wilson-Gerwing et al., 2005). In contrast, Theodosiou et al. (1999) found that significant thermal sensitivity was induced by a subcutaneous injection of NT-3 (500ng) into an uninjured rat hindpaw. The higher dose of injected NT-3 – or the place of injection – may result in local responses not observed at the lower dose (for example, mast cell activation) although this possibility was not ascertained in the study. It remains unknown whether intrathecal infusion of NT-3 can effect changes in the environment surrounding the peripheral nerve terminal. This may be feasible as anterograde transport of NT-3 has been demonstrated in the optic nerve (von Bartheld and Butowt, 2000).

The vanilloid receptor, TRPV1, has been described as being expressed primarily on small, nociceptive neurons in the intact state (Caterina et al., 1997; Eglen et al., 1999; Ma, 2002). My results are in agreement with this. Following CCI, my results demonstrate that there is a dramatic increase in the numbers of neurons that express TRPV1 including small, medium and large sized DRG neurons. Hudson et al. (2001) and Fukuoka et al. (2002) have previously reported similar findings. Not surprisingly, Winston et al. (2001) demonstrated that, in culture, DRG neurons treated with NGF increased TRPV1 expression and that these effects were dependent on the trkA receptor. One of the novel findings that have emerged from this body of thesis work is that exogenous NT-3, given at the time of injury, results in a reduction in TRPV1 expression. While this may be a direct effect of NT-3 on the TRPV1 receptor expression, it is also plausible that the further downregulation of trkA by NT-3 after injury may also play a role. It has been shown that TRPV1 knock-out mice are deficient in their responses to

heat, vanilloid compounds and protons (all of which activate the TRPV1 receptor) (Caterina et al., 2000). More recently, it has also been shown that by blocking the TRPV1 receptor, sensitivity to thermal stimulation is significantly attenuated (Garcia-Martinez et al., 2002). Jin and Gereau (2006) have also demonstrated a role for TRPV1 in inflammation-induced thermal hyperalgesia. Collectively, these demonstrate that TRPV1 is necessary for the development of sensitization to thermal stimuli following nerve injury. These findings, taken together with the ability of NT-3 to prevent an increase in TRPV1 expression following CCI, suggest that the relationship between reduced TRPV1 expression and reduced thermal hyperalgesia as a result of NT-3 treatment is likely a causal one.

The proton sensitive ion channel, ASIC3, plays no apparent role in thermal hyperalgesia. My finding that ASIC3 levels remain depressed following discontinuation of NT-3 treatment provide novel insight into the lack of a necessary role for ASIC3 in the reestablishment of thermal hyperalgesia following CCI.

Previous reports have described that, in the intact state, both $Na_v1.8$ and $Na_v1.9$ mRNA and protein as being localized primarily over small diameter DRG neurons, with some medium and large sized neurons also expressing (Akopian et al., 1996; Black et al., 1996; Cummins et al., 1999; Dib-Hajj et al., 1999). My findings are in accordance with these studies. Following CCI, the levels of both $Na_v.8$ and $Na_v1.9$ mRNA and protein were significantly decreased similar to the findings of Dib-Hajj et al. (1999). The current work demonstrates the novel finding that NT-3 can significantly decrease both the mRNA and protein levels of $Na_v1.8$ and $Na_v1.9$ following 7d unilateral CCI in the DRG neurons both ipsilateral and contralateral to CCI. It has not yet been determined whether the downregulation of $Na_v1.8$ and $Na_v1.9$ mRNA/ protein corresponds to a negative modulation of the electrophysiological properties of these channels. In support of a role for $Na_v1.8$ in the development of thermal hyperalgesia, it has been shown that if $Na_v1.8$ is blocked by antisense oligonucleotides, the development of thermal hyperalgesia is reduced in both the spinal nerve ligation and CCI models (Porreca et al., 1999; Lai et al., 2002; Joshi et al., 2006). Alternatively, Nassar et al. (2005) found that $Na_v1.8$ played no role in the development of neuropathic pain. However, if antisense oligonucleotides to $Na_v1.9$ or genetic ablation of $Na_v1.9$ are

employed, there appears to be no effect on thermal or mechanical hypersensitivity in the neuropathic rat (Porreca et al., 1999; Priest et al., 2005; Amaya et al., 2006). In contrast, Amaya et al. (2006) found that under conditions of CFA inflammation, $Na_v1.9$ (-/-) mice display remarkably reduced thermal, but not mechanical, hypersensitivity. While from the conflicting reports, it is not possible to draw any sound conclusions regarding the role(s), or lack of role(s), of these two sodium channels in the generation of thermal hyperalgesia, this thesis work establishes a correlation between the decreased expression of these channels and the prevention of thermal hyperalgesia following CCI. The use of sodium channel blockers to treat both neuropathic and inflammatory pain in a clinical setting is well known to result in analgesia (Clayton et al., 1997; Evans et al., 1997; Galer, 1995; McQuay et al., 1995; Trezise and Xie, 1997). This in itself provides further evidence that it is likely that at least one of the isoforms studied plays a crucial role in the development of neuropathic pain.

Although, at first glance, it appears that the regulation of TRPV1 following CCI can account for the development of thermal hyperalgesia, it is important to remember that more than one mechanism may be responsible for the generation of a single symptom (Woolf and Decosterd, 1999). After chronic constriction of the sciatic nerve, there are a number of additional changes that take place outside of the DRG, all of which may also be responsible for the development of neuropathic pain behaviors, including alterations in axonal firing which are attributable to the decreased activation threshold of TTX-R sodium channels. For these reasons, a potential role for $Na_v1.8$ and/or $Na_v1.9$ in thermal hyperalgesia cannot be dismissed. As CCI results in both peripheral nerve injury and inflammation, there is a large inflammatory response that takes place - the components of which have been demonstrated to influence neuropathic pain behaviors (Bennett et al., 1998; Sommer and Schafers, 1998; Khalil et al., 1999; Syriatowicz et al., 1999; Cui et al., 2000; Perkins and Tracey, 2000; Zhao et al., 2000). In addition, it has been hypothesized that the formation of sympathetic baskets around large size DRG neurons may play a role in the development and maintenance of neuropathic pain (Ramer et al., 1999; Woolf and Mannion, 1999; Zhou et al., 1999).

7.5 Mechanical hypersensitivity with respect to TRPV1, ASIC3, and sodium channels

In my experiments, it was determined that following CCI, intrathecal NT-3 did neither prevent, nor reverse, the development of mechanical hypersensitivity. This directly contradicts the findings of Gandhi et al. (2004) in which they report that NT-3 can effectively reverse mechanical hyperalgesia produced by intramuscular acid injection in mice. Currently, much of the literature that examines the effect of exogenous NT-3 on mechanical sensitivity is based on the injured state. It has been reported, however, that both antibodies to NT-3 (delivered directly to injured DRG) and antisera to NT-3 (injected intraperitoneally) attenuates nerve lesion-induced increases in von Frey hair-evoked foot withdrawals during later stages of nerve injury (Deng et al., 2000; Zhou et al., 2000). This suggests that although intrathecal infusion of NT-3 in concert with CCI does not alter the ensuing mechanical hypersensitivity, it may still influence it when delivered in alternate manners or for different durations. In support of this, Zhou et al. (2000) also found that intrathecal infusion of NT-3 did not significantly alter mechanical allodynia. In another experiment during which NT-3 was infused intrathecally for 14 days, White (1998) determined that this treatment significantly decreased mechanical thresholds. Given the controversy surrounding the effect of NT-3 on mechanical thresholds, it appears that the dosage, duration and route of administration may critically alter the outcomes of these experiments.

While the primary role for TRPV1 appears to be that of thermal hyperalgesia modulation, there is some emerging evidence that it may also play a role in mechanical hypersensitivity. Kanai et al. (2005) report that upregulation of spinal TRPV1 in the rat CCI model shows involvement in the development and/or maintenance of mechanical allodynia. Liedtke (2007) demonstrates that transduction of mechanical stimuli involves TRPV1, and Christoph et al. (2007) used a TRPV1 antagonist applied intravenously following rat spinal nerve ligation to reduce mechanical hypersensitivity. My findings do not support a critical role for TRPV1 in mechanical hypersensitivity.

Consistent with previous reports, in the intact state, expression of ASIC3 was observed predominantly in small to medium sized DRG neurons, with some large

neurons also expressing detectable levels of ASIC3 (Waldmann et al., 1997b; Chen et al., 1998; Voilley et al., 2001; Molliver et al., 2005). We describe for the first time that following CCI, levels of ASIC3 expression were significantly upregulated, primarily in the small to medium sized (nociceptive) DRG neurons, as well as a small population of large sized neurons. This supports insights by Mamet et al., (2002, 2003) that the increased levels of NGF available under conditions of inflammation drive the increased expression/overexpression of ASIC3. The current work also demonstrates the novel finding that, following CCI, exogenous NT-3 can effectively counter the upregulation of ASIC3 expression, reducing levels to approximately that of basal expression. Surprisingly, examination of 14 day CCI with infusion of NT-3 for the first 7 days of the injury reveals that upon removal of exogenous NT-3, ASIC3 levels remain depressed. There have been numerous studies undertaken to establish a critical role for ASIC3 in the development of mechanical hypersensitivity. Sluka et al. (2003, 2007) have described a critical role for ASIC3 on muscle afferents in the development of mechanical hyperalgesia resulting from intramuscular acid injection in. It was determined by Mogil et al. (2005) that while ASICs are involved in the increased sensitivity to mechanical stimuli, they are not likely directly involved in the heightened response to nociceptive stimuli, and Drew et al. (2004) reports that ASIC3 is not important in mechanotransduction in the DRG. Additional evidence of a role for ASIC3 in mechanical allodynia comes from Ohtori et al. (2006), who found that the level of mechanical allodynia was significantly decreased following lidocaine application to spinal nerve roots affected by disc herniation and a concurrent decrease in ASIC3 immunoreactivity in DRG neurons. A critical evaluation of the findings of Ohtori et al. (2006) leads me to the conclusion that the downregulation of ASIC3 observed was coincident, similar to my findings. In addition, the ability of lidocaine to decrease mechanical allodynia would lead me to surmise an important role of sodium channels in this pathology.

There is little evidence to either support of refute a role for $Na_v1.8$ and/or $Na_v1.9$ in mechanical hypersensitivity. It has been proposed that $Na_v1.8$ does play a role in the development and maintenance of persistent mechanical hypernociception following intraplantar treatment with prostaglandin E2 (PGE2) (Villarreal et al., 2005). In

contrast, the deletion or inactivation of $Na_v1.9$ reportedly had little or no effect on mechanical hypersensitivity in the neuropathic rat (Porreca et al., 1999; Priest et al., 2005). Again, my findings support the lack of a role for these two sodium channels in mechanical hypersensitivity.

Taken together, it appears that of the channels examined throughout this thesis, ASIC3 presents itself as the most likely candidate to be involved in the generation and/or maintenance of mechanical hypersensitivity. This role would most likely be based on the activation of ASIC3 resulting from a drop in extracellular pH (Waldmann et al., 1997b). However, ASIC3 is not the only channel examined that is sensitive to such a shift in pH (i.e. TRPV1) (Caterina et al., 1997). Both the patterns of expression of TRPV1 and ASIC3 and their functional properties suggest that they are the primary channels involved in sensing changes in pH (Leffler et al., 2006). Together, this provides insight that perhaps ASIC3 must work in conjunction with additional channels and/or molecules to effect changes in nociception at the level of the DRG. In conclusion, ASIC3 is undoubtedly involved in some aspect of the generation and/or maintenance of neuropathic pain, but it is not a critical channel in behavioral responses associated with the CCI model.

7.6 Spontaneous pain

While the behavioral assessment of spontaneous pain was not included in the scope of this thesis, it is of interest to the study of neuropathic pain. As the term suggests, spontaneous pain observed after nerve injury is the result of non-elicited activity of the sensory neuron. This activity occurs as a consequence of an increase in oscillations in membrane potential and a decreased neuronal firing threshold, both under the influence of sodium channels (Amir et al., 1999). There is much debate regarding which fibers are actually involved in spontaneous firing, with some labs claiming it is the uninjured C fibers that contribute to this pain state (Wu et al., 2001; Wu et al., 2002; Djouhri et al., 2006). Others find that this ectopic discharge does not occur in C fibers (where expression of the sodium channel $Na_v1.8$ is reduced) (Cummins et al., 1997; Dib-

Hajj et al., 1996; Dib-Hajj et al., 1998; Novakovic et al., 1998; Sleeper et al., 2000; Lai et al., 2003), but rather in the medium and large size fibers where the alterations in $Na_v1.8$ are minimal (Dib-Hajj et al., 1996; Novakovic et al., 1998; Lai et al., 2003). Conversely, spontaneous firing has been demonstrate in injured DRG neurons by Gallego et al. (1987) and Gurtu and Smith (1988) and has been attributed to the translocation of sodium channel to the tips of the injured axons and/or neuromas (Devor et al., 1989; England et al., 1994, 1996). In separate experiments, $Na_v1.8$ has been deemed essential to (Roza et al., 2003) and $Na_v1.9$ has been described as contributing to (Priest et al., 2005) the development of spontaneous pain following nerve injury. As the study of neuropathic pain behaviors in animals seldom includes the assessment of neuropathic pain, it remains elusive as to which voltage-gated sodium channel(s) are intrinsically involved in its generation. What is clear is that both $Na_v1.8$ and $Na_v1.9$ are downregulated by treatment with exogenous NT-3. It is easy to hypothesize then, that exogenous NT-3 may dampen the generation of ectopic discharges leading to spontaneous pain, however, comprehensive studies would need to be undertaken to support this hypothesis.

7.7 The neurotrophin receptors, trk signaling, and MAPK in neuropathic pain

One of the biggest hurdles when presenting these findings to those not intimately associated with neurotrophins and their receptors is the fact that NT-3 can be a ligand for each of these receptors. The receptor tyrosine kinase (trk) family of neurotrophin receptors is composed of three members – trkA, trkB and trkC – that selectively bind neurotrophins with similar high affinity (reviewed in Huang and Reichardt, 2003). NGF binds to trkA (Kaplan et al., 1991; Klein et al., 1991), BDNF and NT-4/5 both bind to trkB (Klein et al., 1990; Soppet et al., 1991; Squinto et al., 1991) and NT-3 binds primarily to trkC, but can also interact with trkB and an isoform of trkA that contains a 6 amino acid insert in the extracellular domain (reviewed in Huang and Reichardt, 2003). There also exists the p75 neurotrophin receptor that is capable of binding all members of the NGF family of neurotrophins with approximately equal specificity, but a lower

affinity than the trk receptors (reviewed in Bothwell, 1996 and McMahon et al., 1997) (Figure 7-1).

As NT-3 is considered to be a "promiscuous" neurotrophin based on its ability to interact with number of receptors, there are two characteristics of NT-3 that are of particular importance when contemplating its role in antagonizing NGF to modulate neuropathic pain. First, the isoform of trkA that does not have the 6 amino acid insert is only activated by NGF, while the trkA isoform with the insert allows for increased activation by NT-3, but does not affect the ability of NGF to activate it (Clary and Reichardt, 1994). Second, high-affinity binding of NGF to trkA to occur, p75 must also be present (Hempstead et al., 1991; Benedetti et al., 1993; Davies et al., 1993; Clary and Reichardt, 1994; Bibel et al., 1999). Finally, since the specificity for trkA to bind its primary ligand (i.e. NGF) is increased by p75, this makes NT-3 less effective at activating trkA when p75 is present (Clary and Reichardt, 1994; Lee et al., 1994; Bibel et al., 1999; Benedetti et al., 1993; Mischel et al., 2001; Brennan et al., 1999) although the latter has not been specifically tested in sensory neurons.

Upon the binding of ligand to the trk receptors, there are three primary signaling pathways that may be activated. These are: 1) the Ras-MAPK signaling pathway; 2) the PLC-γ1 signaling pathway; and 3) the PI3 Kinase signaling pathway (reviewed in Huang and Reichardt, 2003) (Figure 7-2). Of particular interest to this thesis is the Ras-MAPK pathway, as it was two of the MAPK pathways that were examined with respect to NT-3's ability to modulate neuropathic pain syndromes. However, a preliminary understanding of the other two pathways becomes important for understanding potential alternative modes of modulation by this neurotrophin.

Briefly, phosphorylation of the trk receptor at key tyrosine residues results in the binding to the signaling protein Shc which in turn activates the Ras-MAPK signaling pathway whereby the MAPKKK Raf leads to activation of the MAPKK MEK which activates the MAPK ERK (English et al., 1999; reviewed in Huang and Reichardt, 2003). Phosphorylated ERK then phosphorylates target proteins and is also translocated to the nucleus where it phosphorylates and activates transcription factors involved in the transcription of immediate early genes (reviewed in Segal, 2003). Alternatively, the p38 MAPK pathway may be activated by numerous extracellular stimuli in response to

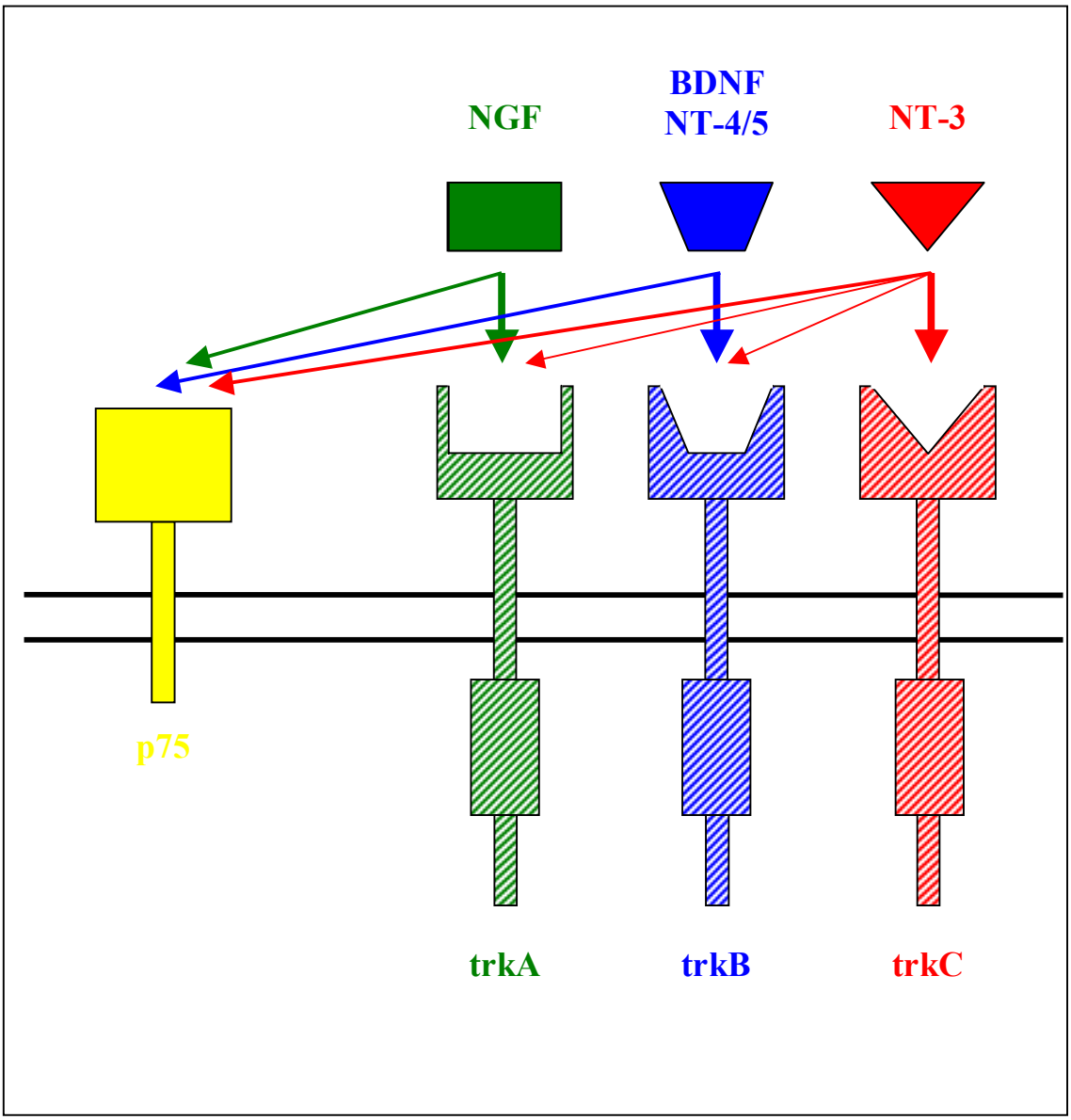

Figure 7- 1: The nerve growth factor (NGF) family of neurotrophins and their receptors.

The receptor tyrosine kinase (trk) family binds their cognate neurotrophin ligand with similar high-affinity (as identified by thick lines). NGF preferentially binds to trkA, BDNF and NT-4/5 both preferentially bind to trkB and NT-3 binds preferentially to trkC, but can also interact with trkB and an isoform of trkA that contains a 6 amino acid insert in the extracellular domain with a lower affinity (thin red lines). The p75 neurotrophin receptor that is capable of binding all members of the NGF family of neurotrophins with approximately equal specificity, but a lower affinity than the trk receptors.

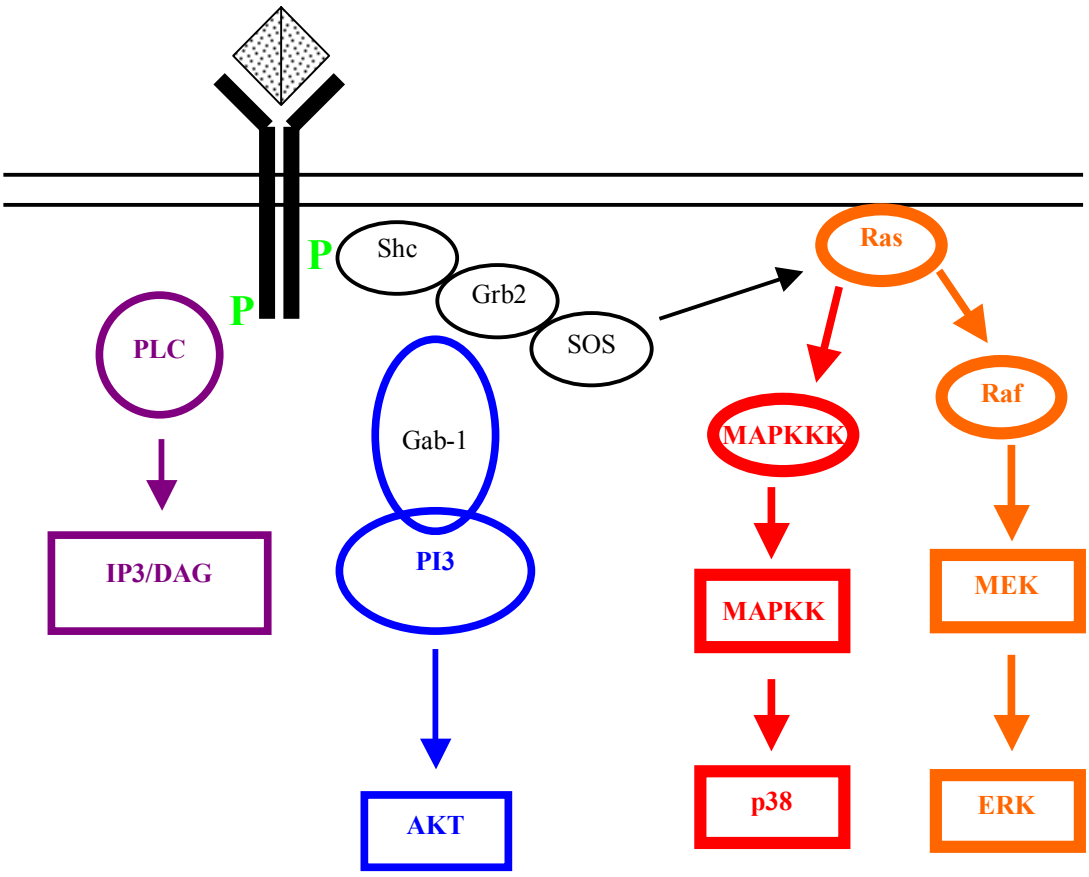

Figure 7- 2: An overview of the basic signaling pathways engaged upon binding of neurotrophin to receptor.

Upon binding of ligand to membrane-associated trk receptors, dimerization of the receptor results in cross activation of the catalytic domains and phosphorylation of the receptors at key tyrosine residues (P) flanking the catalytic domain. These phosphotyrosines bind to intermediate signaling molecules (Shc, PLC) and can activate three primary signaling pathways. These are: 1) the Ras-MAPK signaling pathway [phospho-ERK1/2 (orange), phospho-p38 (red)]; 2) the PLC-γ1 signaling pathway (purple); and 3) the PI3 Kinase signaling pathway (blue).

cellular stresses and inflammation, (reviewed in Huang and Reichardt, 2003; Segal, 2003). As the p38 MAPK pathway can be activated by a number of stimuli, there is a diverse range of MAPKKK (including TAK1, ASK1, DLK, MEKK4) that lead to the activation of the MAPKK (MKK6, MKK3) that ultimately result in the phosphorylation of p38 MAPK (reviewed in Keren et al., 2006). The PLC-γ1 pathway results in the

generation of inositol tri-phosphate (IP3) and diacylglycerol (DAG) upon hydrolization of PtdsIns(4,5)P_2 and the promotion of Ca^{2+} release from internal stores (reviewed in Huang and Reichardt, 2003; Segal, 2003). The final pathway of interest is the PI3-Kinase pathway results in the activation of Akt whose actions include the promotion of cell survival and translation of mRNAs (Kimaball et al., 2002; reviewed in Huang and Reichardt, 2003).

7.8 Potential implications of regulation of phospho-ERK and phospho-p38 MAPK in pain treatment with respect to TRPV1, ASIC3, and sodium channels

7.8.1 Thermal and mechanical

There is evidence from experiments directly linking both phospho-ERK and phospho-p38 MAPK to the development of thermal and/or mechanical hypersensitivity following injury and/or inflammation. By inhibiting MEK (the MAPKK needed for ERK activation), behavioral assessments have shown the attenuation of thermal hyperalgesia after capsaicin injection (Dai et al., 2002). Similarly, CCI-induced thermal and mechanical hypersensitivity induced by CCI can be attenuated by either MEK inhibition or attenuation of ERK expression with antisense oligonucleotides (Song et al., 2005). In a similar study using the spinal nerve ligation model of neuropathic pain, mechanical allodynia was reversed by inhibitors of both ERK and p38 MAPK, while thermal hyperalgesia was only attenuated by the p38 MAPK inhibitor (Obata et al., 2004). The differential regulation of mechanical versus thermal sensitivity by ERK and p38 MAPK suggests that the various models of neuropathic pain and inflammation likely employ different mechanisms in their generation of neuropathic pain behaviors.

7.8.2 TRPV1

A vast amount of research has been undertaken to characterize the signaling cascades involved in regulation of TRPV1. It was originally described by Ji et al. (2002b) that NGF-induced activation of p38 MAPK during inflammation was required for increases in TRPV1 to occur. It was subsequently demonstrated that ERK could positively regulate levels of TRPV1 (Bron et al., 2003) in at least one case in conjunction with the PI3 signaling pathway (Zhuang et al., 2004). It was also described that the PI3 pathway is involved in the generation of inflammatory pain through TRPV1 independent of ERK (Zhuang et al., 2004; Zhang et al., 2005; Stein et al., 2006). While I have previously proposed that the various models of neuropathic pain and inflammation likely employ different mechanisms in their generation of neuropathic pain behaviors, it is also becoming clear that these signaling pathways do not necessarily act in isolation from one another and that more than one pathway may be responsible for the generation of a single pain syndrome and/or molecular change resulting in a single pain behavior.

7.8.3 ASIC3

Far less research has been undertaken to describe the precise signaling pathways that lead to the regulation of ASIC3. It has been shown that ASIC3 expression and activity and are decreased by activation of the ERK1/2 cascade (Zentner et al., 1998; Booth and Stockland, 2003; Frank et al., 2003), but upregulated via the p38 MAPK pathway (Mamet et al., 2003). It is intriguing that these pathways are involved in the differential modulation of this channel.

7.8.4 Sodium channels

Similar to ASIC3, the tetrodotoxin-resistant sodium channels have been demonstrated to be positively regulated by p38 (Jin and Gereau, 2006) but downregulated by activated ERK (Yanagita et al., 2002).

The findings of this thesis are based on the assumption that NT-3 is acting through one or more of the trk receptors (and possibly p75, although this is not addressed) to exert its effects on the CCI-induced changes examined. There is, however, the very real possibility that NT-3 may also be acting through another type of receptor – namely c-RET, the glial-derived neurotrophic factor (GDNF) receptor. Evidence for this hypothesis is based on the findings of Amaya et al. (2004) and Malin et al. (2006). Amaya et al. (2004) found that inflammation rapidly increased NGF and thus TRPV1 while GDNF increased more gradually, but also increased TRPV1 expression. Furthermore, Malin et al. (2006) have described an ability of GDNF to potentiate TRPV1 function at doses up to 100 times lower than NGF and also to increase thermal sensitivity. It is therefore possible that the effects of NT-3 on TRPV1 expression and thermal hyperalgesia – at least in the 14 d CCI model – may be the result of NT-3 counteractions of both NGF and GDNF.

7.9 Potential clinical relevance of NT-3 as a therapeutic treatment for neuropathic pain

Examination of the usefulness of NT-3 in treating neuropathies has occurred primarily in two models of artificial neuropathy. One model, using streptozotocin (STZ)-induced diabetes, is a model of diabetic neuropathy characterized by demyelination (Pradat et al., 2001), altered calcium homeostasis (Huang et al., 2002), slowing of motor and sensory conduction velocities (Mizisin et al., 1999; Pradat et al., 2001; Zochodne et al., 2001), abnormal axonal transport of activated stress activated protein kinases c-jun N-terminal kinase (JNK) and p38 MAPK (Middlemas et al., 2003) and neuronal atrophy (Schmidt et al., 2001; Zochodne et al., 2001). It has been shown

that the cell body response of the neurons is not akin to a nerve injury response, but rather a degenerative one (Zochodne et al., 2001). A second model, employing acrylamide experimental neuropathy, results in diffuse sensorimotor neuropathy (Pradat et al., 2001). Promising studies in STZ-induced diabetic rats have shown that NT-3 treatment (1 month) was able to normalize established sensory and motor neuron conduction velocities (Mizisin et al., 1999; Huang et al., 2005). In a separate study, NT-3 administration was able to prevent the slowing of neuron conduction velocities in these rats (Pradat et al., 2001). Altered calcium homeostasis observed with STZ-induced diabetes can also be corrected with NT-3 therapy (Huang et al., 2002). Findings by Middlemas et al. (2003) also demonstrate that treatment with NT-3 can effectively reduce the abnormal axonal transport of p38 MAPK in diabetic rats. Finally, it has been demonstrated that NT-3 – either alone or in combination with NGF – can significantly increase myelinated innervation compared to control diabetic mice (Christianson et al., 2007). Additionally, muscle innervation was demonstrated to increase in acrylamide experimental neuropathy treated with NT-3 (Pradat et al., 2001).

As a possible role for activated microglia in promoting the inflammatory response is emerging, there are two studies that support a role for NT-3 in preventing this response. In a model of lipopolysaccharide-induced inflammation, the production of the proinflammatory mediators nitric oxide, tumor necrosis factor-α and interleukin-1β have been shown to be inhibited by NT-3 (Tzeng and Huang, 2003; Tzeng et al., 2005). In addition, it was also shown that the ability of NT-3 to attenuate production of inducible nitric oxide synthetase correlates with a similar effect on MAP Kinase and PI3 kinase signaling pathways (Tzeng et al., 2005).

Taken together, NT-3 may prove to be therapeutically useful for treating the symptoms of large fiber neuropathy - including atrophy – as this is the endogenous trophic factor for large sized neurons. Caution must be exercised, however, when any trophic factor is proposed as a therapy for neuronal alterations, as detrimental side effects may result, simply due to the inhibition of a non-target signaling pathway. This was in fact the case in the phase II clinical trials using NGF to treat diabetic polyneuropathy and HIV-related neuropathy. It was proven that painful side effects limited the useful dosage of NGF (Apfel, 2002).

7.10 Concluding comments and summary

Neuropathic pain remains an elusive clinical problem due to its complex etiology and the variety of behavioral syndromes that accompany it. By definition, neuropathic pain occurs as a result of damage to or dysfunction of the nervous system. These can include, but are not limited to, a lesion or disease of the nervous system. The behavioral syndromes associated with this type of pain are just as varied. They include the development of hyperalgesia (a decrease in the threshold of a normally noxious stimuli), allodynia (a non-noxious stimulus becomes noxious), and spontaneous pain (pain in the apparent absence stimuli). To further complicate any attempts at the treatment of neuropathic pain, these pain syndromes seldom occur in isolation and are brought about by a variety of mechanisms.

Over the past decade or so, numerous advances have been made in the area of pain research. These have included the identification, isolation and cloning of certain ion channels that now appear to play an intrinsic role in the development and maintenance of neuropathic pain. They are: the transient receptor potential vanilloid receptor-1 (TRPV1) (Caterina et al., 1997); the acid sensing ion channel 3 (ASIC3) (Waldmann et al., 1997b); and the tetrodotoxin-resistant sodium channels $Na_v1.8$ (Akopian et al., 1996) and $Na_v1.9$ (Dib-Hajj et al., 1998). Numerous avenues of research employing the use of transgenic knock-outs, specific channel blockers, and the inhibition of signaling pathways have been undertaken in an attempt to fully understand the individual contributions of these channels to neuropathic pain. Interestingly, each of these channels appears to be positively regulated by increasing levels of nerve growth factor (NGF) (Dib-Hajj et al., 1998; Fjell et al., 1999; Ji et al., 2002b; Mamet et al., 2003).

Based upon my hypothesis that NT-3 acts antagonistically to NGF to modulate certain aspects of the CCI-associated neuropathic pain phenotype, the findings that NT-3 effected a downregulation of TRPV1, ASIC3, $Na_v1.8$, $Na_v1.9$, and phospho-p38 MAPK following chronic constriction injury (CCI) are in support of this hypothesis. With an understanding of these findings firmly in hand, the differential regulation of phospho-ERK in the trkA versus trkC populations of naive neurons by NT-3 provides a

reasonable mechanism of how NT-3 serves to primarily downregulate CCI-induced changes in phenotype in the nociceptive (trkA) population of dorsal root ganglion (DRG) neurons. From these experiments, it seems likely that NT-3 may effectively act through the isoform of trkA that can bind NT-3. What is not clear, however, is whether or not NT-3 affects the affinity with which NGF is able to bind with its cognate trkA receptor or alternatively is activating aspects of the p75 pathway that lead to this result and have yet to be elucidated. Further studies into this area are therefore required.

Given the apparent "global" ability of NT-3 to antagonize injury/NGF - associated changes in the DRG following CCI, it leads one to question what other possible effects it may be having. It has previously been demonstrated that there is an infiltration of numerous inflammatory mediators following CCI (Olsson, 1967; Perry et al., 1987; Ferreira et al., 1988; Cunha et al., 1992). Perhaps, then, NT-3 may also be effective in reducing the impact of this "inflammatory soup" thereby mitigating the drop in pH that accompanies tissue inflammation (for review, see Moalem and Tracey, 2006; Myers et al., 2006). Another possibility is that exposure to exogenous NT-3 is inhibiting any number of the six signaling pathways through which trkA acts including a MAPK pathway involving ERK1/2, a PI-3 kinase pathway involving serine/threonine kinase Akt, and a PLCγ pathway (reviewed in Friedman and Greene, 1999; Kaplan and Miller, 2000), thus inhibiting gene expressing in this population of nociceptive neurons.

Effective treatments for neuropathic pain will remain elusive until such time as the numerous channels, peptides, trophic factors, receptors, and signaling pathways governing the development and maintenance of hyperalgesia, allodynia, and spontaneous pain are identified and characterized. In addition, it is becoming clear that it is not the involvement of individual molecules that require investigation, but rather the interaction of these molecules collectively on the response. It is only once the complex biochemical interactions of all the players in the neuropathic pain game are understood that hopefully curative, rather than simply palliative, treatments can be developed.

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