The Role of Neurotrophin-3 in Primary Sensory Neurons

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ABSTRACT

Neurotrophin-3 (NT-3) is a member of the nerve growth factor family of neurotrophins. These molecules regulate aspects of sensory neuron survival, proliferation, phenotype, regeneration, and nociception. NT-3 presumably acts upon binding to its preferred receptors, trkC and p75, but much of the literature concerning its role involves embryological studies; little is known regarding its role in the adult, aside from its part in proprioception. Here, the effect of a 7 day intrathecal infusion of NT-3 on the phenotype of intact or axotomized adult rat dorsal root ganglion (DRG) neurons was examined. Serial sections were processed for *in situ* hybridization and computer-assisted image analysis was undertaken to characterize the NT-3 responsive subpopulation and to compare relative levels of mRNA for neurotrophin receptors, neuropeptides, cytoskeletal elements, injury- and regeneration-associated molecules, and other markers in individual neurons. ^{125}I-NGF was utilized to determine the influence of NT-3 on high-affinity NGF binding site densities. Data show that ~ 40% of neurons coexpress trkC and p75 mRNAs; some of these cells also exhibit transcripts for trkA, NFM, Tα1 α-tubulin, α-CGRP, SP, galanin, NPY, GAP-43, cjun, and SNAP-25 — establishing the presence of potentially functionally significant micropopulations within the trkC-positive subset. Following injury, levels of many biochemical markers are altered in a positive or negative fashion. In all cases described here, if the marker colocalizes with trkC, post-trauma treatment with NT-3 allows for a return towards normal message levels, suggesting a role for NT-3 in the maintenance of normal adult phenotype in these cells. In the intact state, NT-3 effects a reduction in trkA, high-affinity NGF binding sites, and SP levels, within non-trkC neurons. The importance of these markers in nociception suggests a role for NT-3 in analgesia. But, in addition, NT-3 also reduces SNAP-25 mRNA levels in otherwise normal trkC-expressing cells, which might negatively alter their functioning. Together, these data indicate that multiple subsets of mature DRG neurons are responsive to NT-3, not all of which express trkC, and not all of which respond to the neurotrophin in the same manner: these factors must be taken into account when considering therapeutic applications for NT-3.

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a.a.	amino acid
aFGF	acidic fibroblast growth factor
AP-1	activator protein-1
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic phosphate
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cyclic adenosine monophosphate response element-binding protein
DAG	diacylglycerol
DEPC	diethyl-pyrocarbonate
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DRG	dorsal root ganglion
DTT	dithiothreitol
E"x"	embryonic day "x"
EGF	epidermal growth factor
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GAP	guanosine triphosphatase-activating protein
GAP-43	43 kDa growth-associated protein (B-50, F1, pp46, pp57, neuromodulin)
GDP	guanosine diphosphate
GDNF	glial cell line-derived neurotrophic factor
GNRP	guanine nucleotide releasing protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HDNF	hippocampus-derived neurotrophic factor (NT-3)
IEG	immediate early gene
IGF	insulin-like growth factor
IL-6	interleukin-6
IP-3	inositol-triphosphate
-ir	-like immunoreactivity, -like immunoreactive
JNK	cJUN NH_2-terminal kinase
L	lumbar
LIF	leukemia inhibitory factor
MAP kinase	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
mRNA	messenger ribonucleic acid

NF-κB	nuclear factor kappa B
NF	neurofilament
NFH	neurofilament high/heavy subunit
NFL	neurofilament low/light subunit
NFM	neurofilament medium/middle subunit
NGF	nerve growth factor
NPY	neuropeptide tyrosine
NSF	N-ethylmoleimide-sensitive fusion protein
NT-3	neurotrophin-3 (HDNF)
NT-4/5	neurotrophin-4/5
NT-6	neurotrophin-6
NTN	neurturin
p75	low-affinity neurotrophin receptor
P"x"	postnatal day "x"
PACAP	pituitary adenylate cyclase-activating polypeptide
PB	phosphate buffer
PBS	phosphate-buffered saline
PC12	pheochromocytoma cell line
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI-3-K	phosphatidylinosital-3-kinase
PKC	protein kinase C
PLCγ	phospholipase-C-γ
PNS	peripheral nervous system
RNase	ribonuclease
RT-PCR	reverse transcriptase- polymerase chain reaction
SH	sarcoma homology
SNAP-25	synaptosomal-associated protein of 25 kDa
SNARE	soluble NSF attachment protein receptor
SOM	somatostatin
SP	substance P
SSC	sodium chloride/sodium citrate
SNT	*suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target
TCA	trichloroacetic acid
TGF	transforming growth factor
TNF	tumour necrosis factor
TRAF6	tumour necrosis factor receptor-associated factor-6
trk	tropomyosin receptor kinase
$trk_{ei"x"}$	isoform of trk containing a "x" a.a. insertion in the extracellular domain
$trk_{ki"x"}$	isoform of trk containing a "x" a.a. insertion in the tyrosine kinase domain
TSA	tyramide signal amplification
Tyr	tyrosine
TX-100	Triton X-100
VAMP	vesicle-associated membrane protein
VIP	vasoactive intestinal polypeptide

"x"$^{+/-}$	heterozygous gene targeted mutant for "x"
"x"$^{-/-}$	homozygous gene targeted mutant for "x"

1. INTRODUCTION

1.1 General Introduction to the Neurotrophins and their Receptors

1.1.1 The Neurotrophins

The neurotrophins are a family of structurally related small proteins, ~ 120 amino acids long, that share ~ 50% amino acid homology (reviewed in: Ebendal, 1992) and are represented by five known molecules: nerve growth factor (NGF) (reviewed in: Levi-Montalcini, 1987a, 1987b), brain-derived neurotrophic factor (BDNF) (Barde *et al.*, 1982; Leibrock *et al.*, 1989), neurotrophin-3 (NT-3; hippocampus-derived neurotrophic factor, HDNF) (Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990a; Rosenthal *et al.*, 1990), neurotrophin-4/5 (NT-4/5) (Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991; Ip *et al.*, 1992a), and neurotrophin-6 (NT-6) (Götz *et al.*, 1994). Of these, NGF was the initial molecule to be identified, a phenomenon that facilitated the discovery of the other family members.

The basis for the discovery of NGF (reviewed in: Levi-Montalcini, 1987a, 1987b) was a series of experiments conducted by E. Bueker during the late 1940s. Portions of mouse sarcoma were implanted along one side of the spinal cord of chicken embryos; 3-5 days later, the dorsal root ganglia (DRG) at the level of, and ipsilateral to, the tumor were enlarged. Furthering these observations, V. Hamburger and R. Levi-Montalcini (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1952) conducted more thorough experimentation and found that not only did ipsilateral DRG increase in size, but so did the juxtapositioned spinal and sympathetic chain ganglia. They then placed tumor-containing tissue on the chorioallantoic membrane, such that the only contact between the tumor and embryo was through the circulatory system of the

embryo. This work yielded comparable results and led to the hypothesis that the observed phenomena were caused by a soluble, diffusible molecule. In later tissue culture experiments Levi-Montalcini observed similar, though far less dramatic effects when normal mouse tissue was used instead of sarcoma. Although at the time Levi-Montalcini and coworkers failed to identify this soluble factor as a potentially beneficial molecule, they continued to study its chemical nature. In the mid to late 1950s, S. Cohen used snake venom to degrade the growth-inducing factor, but instead of resulting in a negative control these tissue culture experiments showed neurite outgrowth even greater than that effected by the tumor alone. Further, the addition of snake venom to chicken ganglia *in vitro* in the absence of tumor tissue, mirrored the tumor-engendered effect (Cohen and Levi-Montalcini, 1956). These findings prompted the examination of the nerve-growth promoting abilities of an extract from mouse submandibular gland, a homologue of the gland producing snake venom; the resulting neurite extension was greater than that observed for any of the previous culture conditions. The growth-inducing molecule was subsequently named NGF; the protein was purified, and its biological activity was, and continues to be, screened (reviewed in: Greene and Shooter, 1980; Thoenen and Barde, 1980; Johnson *et al.*, 1986; Levi-Montalcini, 1987a, 1987b; Levi-Montalcini *et al.*, 1996). It was not until approximately 20 years later that BDNF was identified (Barde *et al.*, 1982). The cloning of this molecule, the identification of a shared partial amino acid sequence homology with NGF (Leibrock *et al.*, 1989), and the use of this sequence region as a probe under low stringency conditions soon led to the discovery of NT-3 (Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990a; Rosenthal *et al.*, 1990). PCR (polymerase chain reaction) analyses, exploiting the sequence similarities between these three neurotrophins, rapidly allowed for the identification of NT-4/5 (Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991; Ip *et al.*, 1992a) and NT-6 (Götz *et al.*, 1994). Although NGF is still the best characterized neurotrophin, the roles of the other family members are being rapidly elucidated.

The neurotrophins have been shown to effect both pre- and postsynaptic modulation (Berninger *et al.*, 1993; Lohof *et al.*, 1993; Kang and Schuman, 1995; Lesser

and Lo, 1995; Levine *et al.*, 1995; Kang *et al.*, 1996; Blochl and Sirrenberg, 1996; D'Arcangelo *et al.*, 1993), through influencing synaptic density (Causing *et al.*, 1997), neurotransmitter production and/or release (Kang and Schuman, 1995; Takei *et al.*, 1997; Fernandes *et al.*, 1998; Sala *et al.*, 1998; Blochl and Sirrenberg, 1996; Chang and Popov, 1999; Rylett *et al.*, 1993; Helke and Verdier-Pinard, 2000), intracellular calcium levels (Berninger *et al.*, 1993; Jiang *et al.*, 1997), neurotransmitter receptor efficacy (Mandelzys *et al.*, 1990; Jarvis *et al.*, 1997; Oyelese *et al.*, 1997; Suen *et al.*, 1997; Levine *et al.*, 1998), and neuronal electrophysiology (Fjell *et al.*, 1999). Further, neurotrophins can affect gene regulation through the alteration of mRNA stability (Nishizawa, 1994) and *via* transcription factors (reviewed in: Bonni and Greenberg, 1997; Segal and Greenberg, 1996; Impey *et al.*, 1999). Through such actions they modulate the seemingly diverse phenomena of neuronal survival and proliferation (Cordon-Cardo *et al.*, 1991; Hory-Lee *et al.*, 1993; Memberg and Hall, 1995; Yao and Cooper, 1995; ElShamy and Ernfors, 1996; Bradbury *et al.*, 1998; reviewed in: Henderson, 1996; Snider and Silos-Santiago, 1996), neurite outgrowth and differentiation (Gundersen and Barret, 1979; Connolly *et al.*, 1981; Richardson and Ebendal, 1982; Wright *et al.*, 1992; Morfini *et al.*, 1994; Ghosh and Greenberg, 1995b; Edström *et al.*, 1996; Gallo *et al.*, 1997; Grabham and Goldberg, 1997; Tuttle and O'Leary, 1998; Bradbury *et al.*, 1999; reviewed in: Campenot, 1994; Clark and Brugge, 1995), embryonic and adult neuronal phenotype (Lindsay and Harmar, 1989; Ma *et al.*, 1992; Verge *et al.*, 1995; Friedel *et al.*, 1997; Miller *et al.*, 1991; reviewed in: Lewin, 1996), neuronal regeneration (Yip and Grafstein, 1982; Zhang *et al.*, 1998; Oudega and Hagg; 1999; reviewed in: Richardson, 1991), pain perception (reviewed in: Lewin and Mendell, 1993; McMahon, 1996; Woolf, 1996; McMahon *et al.*, 1997; Millan, 1999; Stucky, 2001) and antinociceptive effects (Siuciak *et al.*, 1994), free radical scavenging (Olanow, 1993; Goss *et al.*, 1997; Sampath and Perez-Polo, 1997), food uptake (Lapchak and Hefti, 1992), and memory and learning (Koh *et al.*, 1989; Patterson *et al.*, 1992; Kang and Schuman, 1995; Korte *et al.*, 1995; Markowska *et al.*, 1994). Moreover, it appears that neurotrophins are also important in nonneuronal cells, with the potential to act as bridging molecules between the nervous and immune systems (Besser and

Wank, 1999; Reinshagen *et al.*, 2000; reviewed in: Scully and Otten, 1995). In addition, there is evidence that NT-3 plays a critical role in normal cardiac system development (Donovan *et al.*, 1996; Tessarollo *et al.*, 1997; Story *et al.*, 2000).

It was assumed that multiple, but mutually independent, neurotrophic factors were synthesized in nonoverlapping regions, cell types, and/or developmental stages and that following release, potentially from vesicles (Blochl, 1998), these molecules were retrogradely transported to their target cells (reviewed in: Ebendal, 1992; Korsching, 1993). Currently, considerable evidence points towards more complex interactions; ultimately, the downstream effects elicited through the binding of neurotrophins to their receptors (reviewed in:. Ebendal, 1992; Raffioni and Bradshaw, 1993; Davies, 1994; Lindsay *et al.*, 1994; Frade and Barde, 1998a) may be determined through both temporal and spatial specificity (reviewed in: Korsching, 1993; Berninger and Poo, 1996). Spatial specificity occurs through restricted sites of neurotrophin production/release or of neurotrophin receptor localization; temporal specificity is effected by way of the activity-dependent release of neurotrophins, the rate of neurotrophin degeneration or its uptake by nonneuronal cells, or through receptor desensitization.

The neurotrophins and their receptors show both distinct and overlapping patterns of distribution. Developmentally, in the rat central nervous system (CNS) NT-3 is at its highest levels at the earliest stages and BDNF is at its lowest; NT-3 levels decrease during development, with BDNF levels increasing concurrently; CNS levels of NGF mRNA vary as the embryo matures (Maisonpierre *et al.*, 1990b). The levels of expression of the neurotrophin receptors, trks [trkA (Trk), trkB, trkC], also differ during development (Martin-Zanca *et al.*, 1990; Ernfors *et al.*, 1993; Tessarollo *et al.*, 1993). In adult animals NGF, NT-3, and BDNF mRNA are all present in the hippocampus, and while NGF and BDNF may colocalize in a subset of these neurons, NT-3 does not (Ernfors *et al.*, 1990b; Kokaia *et al.*, 1993). Additionally, the neurotrophins may be found in unique structures. For example: unlike NGF and NT-3, BDNF is rarely detected in peripheral tissues but is strongly expressed in DRG (Ernfors *et al.*, 1990b; Wetmore and Olson, 1995; Yamamoto *et al.*, 1996), while transcripts of all three of these neurotrophins are present in heart, spleen, and muscle (Yamamoto *et al.*, 1996). The

expression of trk mRNAs tend to be more restricted to neuronal tissues (Klein *et al.*, 1990a; Yamamoto *et al.*, 1996); the brain and spinal cord show high mRNA levels of trkB and trkC (Yamamoto *et al.*, 1996) and DRG exhibit transcripts of all three trk species. Also of interest is the potential for colocalization or trilocalization of the trks (McMahon *et al.*, 1994; Williams and Ebendal, 1995; Wright and Snider, 1995; Fariñas *et al.*, 1998; Karchewski *et al.*, 1999) or of the neurotrophins themselves (Miranda *et al.*, 1993).

Further, the action of one neurotrophin may depend upon the concomitant presence or interaction with another neurotrophin or trophic factor (reviewed in: Korsching, 1993; Berninger and Poo, 1996; Sieber-Blum, 1998). For example, in pheochromocytoma cell line (PC12) cells both exogenous NGF and BDNF induce the secretion of NT-3 (Kruttgen *et al.*, 1998). In addition, NGF has been shown to increase BDNF mRNA and protein levels (Apfel *et al.*, 1996; Robinson *et al.*, 1996; Michael *et al.*, 1997), while BDNF can induce NT-3 expression in cerebellar granule neurons (Leingärtner *et al.*, 1994) and act synergistically with NT-3 to influence the survival of spiral ganglion neurons (Mou *et al.*, 1997). Also, even though NT-3 supports the survival of a subset of developing sensory neurons *in vitro* and transforming growth factor-beta (TGF-β) does not, in combination NT-3 and TGF-β result in significantly greater numbers of surviving neurons compared to cultures exposed only to NT-3 (Krieglstein and Unsicker, 1996). Moreover, NGF can collaborate with ciliary neurotrophic factor (CNTF) and fibroblast growth factor (FGF) to promote cellular differentiation (Ip *et al.*, 1994); both acidic FGF (aFGF) and basic FGF (bFGF) can induce NGF expression (Figueiredo *et al.*, 1995; Ferhat *et al.*, 1997); CNTF and BDNF can act synergistically to arrest the development of motor neuron disease (Mitsumoto *et al.*, 1994); and NT-3 can collaborate with platelet-derived growth factor (PDGF) to stimulate oligodendrocyte precursor cell proliferation (Barres *et al.*, 1994).

1.1.2 The Neurotrophin Receptors

1.1.2.1 The trks

1.1.2.1.1 trk Structure

Tropomyosin receptor kinases (trks) are members of a class of transmembrane receptors categorized as receptor tyrosine kinases — a group that also includes the receptors for epidermal growth factor (EGF), FGF, insulin, and PDGF. All receptor tyrosine kinases are composed of an extracellular domain connected by a single membrane-spanning region to a cytoplasmic domain (reviewed in: Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992; Segal and Greenberg, 1996). The extracellular region functions in ligand binding and the intracellular portion possesses a tyrosine kinase domain that, upon ligand-induced activation, results in the initiation of signal transduction pathways through which the biological effects of the trophic factors are mediated. In addition, a highly conserved region of secondary protein structure has been identified in the cytoplasmic domain of many of these receptors which purportedly facilitates interactions with downstream signaling proteins (Feinstein and Larhammar, 1990).

All of the trks identified to date [trkA (Martin-Zanca *et al.*, 1989), trkB (Klein *et al.*, 1989), and trkC (Lamballe *et al.*, 1991)] exhibit ligand-disassociation constants of ~ 10^{-11} M (Sutter *et al.*, 1979; Sonnenfeld and Ishii, 1985; Rodriguez-Tébar and Barde, 1988; Rodriguez-Tébar *et al.*, 1990, 1992; reviewed in: Meakin and Shooter, 1992). The kinase domains of the trks share ~ 85% sequence homology, while there is roughly 50% homology among the extracellular regions (Lamballe *et al.*, 1991) and a comparable degree of likeness exists between their ligands (reviewed in: Chao, 1992). However, in spite of these similarities, members of the NGF family show specificity of binding to these receptors at picomolar concentrations of ligand; NGF binds to trkA, BDNF and NT-4/5 to trkB, and NT-3 binds preferentially to trkC (Cordon-Cardo *et al.*, 1991; Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Ip *et al.*, 1993a). NT-3 has also been reported to bind to trkA and trkB (Cordon-Cardo *et al.*, 1991; Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991), but was generally believed to do so at above physiologically relevant concentrations of ligand (Lamballe *et al.*, 1991; Ip *et al.*, 1993a; reviewed in: Davies, 1994), since neurotrophins are not found in excess quantities *in vivo* (reviewed in: Thoenen, 1991). Recently there have been a number of observations that contradict this belief (Davies *et al.*, 1995; Belliveau *et al.*, 1997; Fundin *et al.*, 1997b; Maroney *et*

al., 1997; Fariñas *et al.*, 1998; Nakatani *et al.*, 1998; Huang *et al.*, 1999): The ability of biologically relevant levels of NT-3 to support the *in vitro* survival of trkA- and trkB-positive embryonic neurons from transgenic mice that lack trkC upholds the view that NT-3 can activate these receptors, although the ability of NT-3 to influence these neurons appears to be restricted to certain developmental stages (Davies *et al.*, 1995). Further, reduced numbers of trkB-positive sensory neurons in embryos of NT-3 deficient mice as compared to wild-type, implies that these neurons may have been dependent on NT-3/trkB interactions for survival (Fariñas *et al.*, 1998). In addition, since transgenic mice lacking NT-3 show deficits in the numbers of trkA- and trkB-positive prenatal trigeminal ganglion neurons, it has been proposed that this neurotrophin must promote their *in vivo* survival through the activation of these receptors (Huang *et al.*, 1999). More direct evidence comes from experiments involving neonatal sympathetic neurons, where NT-3 has been shown to bind to trkA, and even though its capacity to influence neuronal survival is poor when compared to that of NGF, its ability to promote neurite outgrowth and the expression of growth-associated genes are as effective (Belliveau *et al.*, 1997). These results may reflect a general ability of NT-3 to bind to trkA, or may be because the trkA present on sympathetic neurons is an isoform with an extracellular insert that confers preferential binding of NT-3 (Barker *et al.*, 1993; Clary and Reichardt, 1994).

Isoforms of the various trk genes have been reported; trkA (Barker *et al.*, 1993; Clary and Reichardt, 1994), trkB (Klein *et al.*, 1990b; Merlio *et al.*, 1992; Ernfors *et al.*, 1993; Eide *et al.*, 1996; Strohmaier *et al.*, 1996), and trkC (Lamballe *et al.*, 1993; Tsoulfas *et al.*, 1993, 1996; Valenzuela *et al.*, 1993; Garner and Large, 1994; Shelton *et al.*, 1995) are known to exist not only as full-length receptor proteins, but also as truncated receptors, lacking at least a portion of the intracellular catalytic domain, or with extracellular or cytoplasmic structural insertions. Numerous forms of the truncated receptors have been identified (Klein *et al.*, 1990b; Merlio *et al.*, 1992; Valenzuela *et al.*, 1993). They show patterns of distribution both similar and dissimilar to that of the corresponding full-length receptors (Klein *et al.*, 1990a, 1990b; Beck *et al.*, 1993; Deckner *et al.*, 1993; Valenzuela *et al.*, 1993; Karchewski *et al.*, 1999), although the truncated forms appear to be primarily (Klein *et al.*, 1990b; Merlio *et al.*, 1992, 1993;

Ernfors *et al.*, 1993; Funacoshi *et al.*, 1993; Valenzuela *et al.*, 1993; Wetmore and Olson, 1995), but not always (Zirrgiebel *et al.*, 1995; Karchewski *et al.*, 1999), localized to glia. The functional significance of these isoforms is unclear, but because they lack portions of the tyrosine kinase catalytic domain they are presumed to play a specialized role, distinct from that of full-length trks (Klein *et al.*, 1990b; Chao, 1992; Valenzuela *et al.*, 1993; reviewed in: Davies, 1994). Truncated receptors may serve to buffer the concentration of ligand by maintaining or prolonging higher local levels of bioavailable neurotrophin, or to reduce the concentration of neurotrophin through direct competition for ligand with full-length trks (Klein *et al.*, 1990b; Beck *et al.*, 1993; Kokaia *et al.*, 1993; Merlio *et al.*, 1993; Valenzuela *et al.*, 1993; Knüsel *et al.*, 1994; Palko *et al.*, 1999). Alternatively, the activity of the full-length receptor may be modulated through the formation of inactive heterodimers with truncated receptors (Valenzuela *et al.*, 1993; Knüsel *et al.*, 1994; Eide *et al.*, 1996). Further, differential expression of full-length and truncated isoforms of trkC may serve to fine-tune neurite outgrowth during development because overexpression of the former results in increased, and the latter results in decreased, axonal process formation (Ichinose and Snider, 2000). Another proposed role for truncated trks may be to transport neurotrophins across the blood-brain barrier, since trkB transcripts have been localized to the choroid plexus and ependymal cells (Klein *et al.*, 1990b). Isoforms with extracellular or cytoplasmic domain inserts may show differing effects. Most importantly, *in vitro* experiments involving cell lines transfected to express mammalian and avian trkC isoforms containing amino acid insertions within their tyrosine kinase or extracellular domains show that these receptors retain their ability to autophosphorylate in response to exogenous NT-3 (Tsoulfas *et al.*, 1993, 1996; Valenzuela *et al.*, 1993; Garner and Large, 1994) and thus, unlike truncated receptors, have the potential to participate in signal transduction. In spite of these findings, receptors with intracellular domain inclusions may differ from the non-insert form in their capacity to function, which suggests that alternative splicing may play an important role in regulating trk signaling (Tsoulfas *et al.*, 1993, 1996; Garner and Large, 1994; Guiton *et al.*, 1995; Meakin *et al.*, 1997; Fernandes *et al.*, 1998). In a fibroblast cell line that does not normally exhibit trk, cells transfected to express trkC or either of the

known trkC kinase insert isoforms show that the isoforms have a reduced ability to participate in at least one aspect of NT-3 induced signal transduction (Guiton *et al.*, 1995). Moreover, NT-3 effects neurite outgrowth in PC12 cells *via* trkC, but not through a mammalian isoform of trkC that contains a 14 amino acid insertion in the tyrosine kinase domain ($trkC_{ki14}$) (Tsoulfas *et al.*, 1993, 1996; Valenzuela *et al.*, 1993). Further, although the kinase insertion in an avian trkC isoform (Garner and Large, 1994) occurs at the same position in the kinase domain as that of the mammalian insert (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993), the amino acid sequence of the avian insert is markedly different. This difference in molecular structure may be reflected in the ability of the receptors to function, since unlike the mammalian form (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993) the avian isoform has been shown to mediate process outgrowth in transfected PC12 cells (Garner and Large, 1994). In addition, trk species with extracellular domain additions or deletions may show altered ligand/receptor interactions: Isoforms of trkB with a deletion in the extracellular domain show contrasting responses to the ligands, BDNF and NT-4/5 (Strohmaier *et al.*, 1996). Similarly, a 6 amino acid insertion in the extracellular domain of trkA ($trkA_{ei6}$) endows the receptor with a heightened responsiveness to NT-3, without apparent alteration in the ability of $trkA_{ei6}$ to bind or respond to NGF (Barker *et al.*, 1993; Clary and Reichardt, 1994), although the biological responses elicited by NT-3 through this isoform differ from those effected by NGF (Belliveau *et al.*, 1997). Interestingly, it appears that in adult rat lumbar DRG neurons the insert-containing isoforms of trkA and trkC colocalize with their respective non-insert-containing receptor subtypes (Karchewski *et al.*, 1999).

1.1.2.1.2 trk Signal Transduction

Tyrosine autophosphorylation is necessary for the catalytic and signaling actions of trk (Cordon-Cardo *et al.*, 1991; Soltoff *et al.*, 1992; Obermeier *et al.*, 1993, 1994; Knüsel *et al.*, 1994; Stephens *et al.*, 1994), and although the phosphorylation of tyrosine represents less than 1% of all cellular protein phosphorylation events, it is crucial to many pathways involving cell growth and differentiation (reviewed in: Russell, 1995). That the tyrosine kinase domain is the most

highly conserved portion of all receptor tyrosine molecules underscores the importance of this region to the functioning of these receptors (Ullrich and Schlessinger, 1990). The onset of neurotrophin-induced trk tyrosine phosphorylation is rapid (Kaplan *et al.*, 1991a, 1991b; Ip *et al.*, 1993b; Volonté *et al.*, 1993b; Knüsel *et al.*, 1996), although the duration of the influence of neurotrophins on trk tyrosine phosphorylation levels appears to be variable. In PC12 cells, *in vitro* trkA tyrosine phosphorylation occurs within 1 minute of treatment with physiologically relevant concentrations of NGF, reaching maximal levels after 5 minutes, and decreasing thereafter (Kaplan *et al.*, 1991a, 1991b). *In vivo* injection of NGF into rat brain also results in immediate trk tyrosine phosphorylation, but phosphorylation levels remain elevated for 1-3 days after NGF injection, with levels lessening but still detectable 5 days after NGF treatment (Knüsel *et al.*, 1996).

It has been documented that both the neurotrophins (Hendry *et al.*, 1974; Rohrer *et al.*, 1982; Richardson and Riopelle, 1984; DiStefano *et al.*, 1992; Fang and Luo, 1996; Grimes *et al.*, 1997; Matheson *et al.*, 1997; Curtis *et al.*, 1998) and trks (Loy *et al.*, 1994; Ehlers *et al.*, 1995; von Bartheld *et al.*, 1996b; Bhattacharyya *et al.*, 1997; Grimes *et al.*, 1997) are retrogradely transported to the perikarya, potentially within vesicles (Rohrer *et al.*, 1982; Bhattacharyya *et al.*, 1997; Grimes *et al.*, 1997). Although the transport of neurotrophins alone does not appear to be adequate for trophic signaling at the cell body (von Bartheld *et al.*, 1996b; Riccio *et al.*, 1997), it seems critical to signal propagation that the NGF/trkA complexes are internalized and retrogradely transported from distal processes and that trkA tyrosine phosphorylation is maintained during transit (Riccio *et al.*, 1997). There is also evidence that distal application of neurotrophin rapidly results in increased internal levels of phosphorylated trk proximal to the site of ligand/receptor interactions (Bhattacharyya *et al.*, 1997; Senger and Campenot, 1997). This appears to occur at a more rapid rate than that reported for the retrograde transport of neurotrophins (Richardson and Riopelle, 1984). Because of this, these findings suggest that neurotrophin-induced signal transduction also involves the propagation of signal that is dependent on the initial ligand-induced activation of trk, but independent of the internalization and transport of neurotrophin/trk complexes (Bhattacharyya *et al.*, 1997;

Senger and Campenot, 1997; MacInnis and Campenot, 2002). In addition, neurotrophins may be transported anterogradely (von Bartheld *et al.*, 1996a; Zhou and Rush, 1996b; reviewed in: Altar and DiStefano, 1998). For example, it has been shown that ocular injection of radioiodinated NT-3 results in the apparent anterograde accumulation of the neurotrophin in presynaptic vesicles of the optic tectum and in postsynaptic dendrites and perikarya (von Bartheld *et al.*, 1996a). Neurotrophins thus conveyed may then be released at synapses, potentially affecting postsynaptic targets (Levine *et al.*, 1995; von Bartheld *et al.*, 1996a) or subserving autocrine functions (Acheson *et al.*, 1995; reviewed in: Davis and Wright, 1995; Acheson and Lindsay, 1996).

Neurotrophin homodimers, the bioactive form of these molecules (McDonald *et al.*, 1991; Radziejewski *et al.*, 1992; reviewed in: Thoenen and Barde, 1980; Ebendal, 1992), bind to trk through interactions between specific residues of individual neurotrophin species and the corresponding receptor (Kullander *et al.*, 1997a; 1997b). Although neurotrophin heterodimers have not been identified under physiologically relevant conditions, they may be synthesized (Radziejewski and Robinson, 1993; Jungbluth *et al.*, 1994; Robinson *et al.*, 1995) and even though they can bind to trk they show reduced biological activity (Jungbluth *et al.*, 1994; Robinson *et al.*, 1995). Ligand binding activates the receptor, inducing or stabilizing receptor homodimerization (Jing *et al.*, 1992; reviewed in: Kaplan and Stephens, 1994; Segal and Greenberg, 1996). This permits a conformational change in the receptors that allows for the juxtaposition of the respective tyrosine kinases, which then rapidly results in the activation of the tyrosine kinase domains and subsequent trans-autophosphorylation of a number of tyrosine residues on each receptor (Ip *et al.*, 1993a; reviewed in: Kaplan and Stephens, 1994; Greene and Kaplan, 1995; Bonni and Greenberg, 1997; Kaplan and Miller, 1997). Three tyrosines have been identified as being phosphorylated within the trkA catalytic domain: Tyr670, Tyr674, and Tyr675 (Stephens *et al.*, 1994; Segal *et al.*, 1996). Additional tyrosine phosphorylation sites are found outside this region: Tyr490, located in the juxtamembrane region of the cytoplasmic domain of trkA, plus Tyr785 and Tyr794, situated in the carboxyl-terminal intracellular tail of trkA (Loeb *et al.*, 1994; Stephens *et al.*, 1994; Segal *et al.*, 1996; MacDonald *et al.*, 2000). Individual tyrosines are not

coordinately phosphorylated, rather the phosphorylation of these tyrosine residues is sequential; Tyr674 is completely phosphorylated *in vivo* before other sites are fully phosphorylated (Segal *et al.*, 1996). In addition, catalytic domain sites undergo rapid dephosphorylation, while phosphorylation at the other receptor sites endures for longer periods (Segal *et al.*, 1996). These phosphotyrosine residues can serve as docking sites for downstream effector substrates through specific binding with a non-catalytic region of 100-110 amino acid residues of some intracellular signaling proteins, termed the *src* (sarcoma) homology 2 (SH2) domain (reviewed in: Schlessinger and Ullrich, 1992), although these are not the only molecules to interact with activated trkA (MacDonald *et al.*, 1999). The majority of SH2 domain-containing proteins are expressed in a wide range of cell types, while others are presumed to have a restricted distribution; the tissue-specific SH2 domain proteins are believed to serve a more specialized role in receptor tyrosine kinase-mediated signal transmission pathways (reviewed in: Schlessinger and Ullrich, 1992). A number of intracellular SH2 domain-containing proteins are known to bind to activated trkA — including Shc, phospholipase-C-γ (PLCγ), and phosphatidylinositol-3-kinase (PI-3-K) (Ohmichi *et al.*, 1991a, 1991b; Obermeier *et al.*, 1993; Loeb *et al.*, 1994; Stephens *et al.*, 1994; Baxter *et al.*, 1995; Zirrgiebel *et al.*, 1995; Segal *et al.*, 1996; Bhattacharyya *et al.*, 1997; Iwasaki *et al.*, 1998). The binding of these substrates to trk enhances their specific activity, either through receptor-induced tyrosine phosphorylation or alterations in protein conformation, therein launching signal transduction cascades (Vetter *et al.*, 1991; Soltoff *et al.*, 1992; Loeb *et al.*, 1992). This allows for signal amplification, a phenomenon important when a signal threshold must be reached in order to effect the appropriate cellular response, especially since there may be degradation of the signal as it travels to the nucleus (Segal *et al.*, 1996). The effects of neurotrophin/trk interactions have been shown to be local, in the immediate vicinity of the receptors *in situ*, and to involve the induction of signal cascades that may ultimately result in the transmission of an intracellular signal along the length of the axon. They can also effect rapid and/or long-term cellular reactions. To be considered a "fast action" the response should occur within one to tens of minutes, before new gene expression and protein synthesis are possible (Berninger and Poo, 1996). Such actions may affect the

physiology and morphology of neurons, serving roles in both pre- and postsynaptic plasticity and neuronal growth (Berninger *et al.*, 1993; Bhattacharyya *et al.*, 1997; Roche *et al.*, 1994; Siegelbaum, 1994; Kang and Schuman, 1995; Suen *et al.*, 1997; Blochl and Sirrenberg, 1996; reviewed in: Berninger and Poo, 1996), and may be necessary before more long-term potentiation is possible (Berninger and Poo, 1996). Neurotrophins have been shown to rapidly potentiate synaptic transmission and neurotransmitter release (Kang and Schuman, 1995; Blochl and Sirrenberg, 1996; reviewed in: Berninger and Poo, 1996), which may result from a neurotrophin-induced elevation in calcium levels at the presynaptic terminal (Berninger *et al.*, 1993). Neurotrophins may also modulate synaptic efficacy through changing postsynaptic responses to the released neurotransmitter (Levine *et al.*, 1995; reviewed in: Berninger and Poo, 1996). The mechanism underlying both pre- and postsynaptic alterations may be through neurotrophin-induced serine/threonine and tyrosine phosphorylation modulations of transmitter receptors and ion channels (Roche *et al.*, 1994; Siegelbaum, 1994). Rapid alterations in neurite outgrowth have been attributed to neurotrophins: Growth cones turn toward a source of NGF within 10-20 minutes (Gundersen and Barret, 1979; Gallo *et al.*, 1997); removal of NGF causes the loss of filopodial and lamellar extensions, while the re-addition of NGF induces growth cone spreading and the formation of new extensions within several minutes (Connolly *et al.*, 1981). Such changes may be due, at least in part, to the neurotrophin-induced phosphorylation of growth-associated protein (GAP-43) (Aigner and Caroni, 1995). Additional changes in outgrowth patterns may be attributed to NGF, since β1 integrins, receptors of extracellular matrix proteins and important directors of neuronal migration, form aggregates at the tips of neuronal filopodia within 10 minutes of exposure to the trophic factor (Grabham and Goldberg, 1997). Neurotrophin-influenced transcriptional regulation takes more time: *In vitro*, exogenous NGF upregulates p75, Tα1 α-tubulin, and tyrosine hydroxylase mRNAs in sympathetic neurons within 12 hours (Ma *et al.*, 1992).

Shc, an adaptor signaling protein containing an SH2 domain, has been shown to bind to Tyr490 (Stephens *et al.*, 1994; Segal *et al.*, 1996; Bhattacharyya *et al.*, 1997). Tyrosine phosphorylated Shc binds to Grb2, an adaptor protein that bridges Shc to Sos

(László and Downward, 1993), although there is recent evidence that Grb2 can also bind to activated trkA independently of Shc (MacDonald *et al.*, 2000). Sos is a guanine nucleotide releasing protein that activates Ras, enhancing the rate of GDP-GTP (guanosine diphosphate-guanosine triphosphate) exchange on members of this small family of GTP-binding proteins (Boriack-Sjodin *et al.*, 1998). In the resting state Ras proteins bind GDP; upon stimulation they release GDP and bind GTP (Carter *et al.* 1995; reviewed in: Segal and Greenberg, 1996; Wittinghofer, 1998). Following trkA activation, the complexes composed of tyrosine phosphorylated Shc-Grb2-Sos do not appear to stably associate with the receptor; the complex is free from trkA during Ras activation (Basu *et al.*, 1994). Activated Ras binds to Raf, a serine/threonine protein kinase, that then phosphorylates, and consequently activates, mitogen-activated protein kinase kinases (MAPKKs) (Lange-Carter and Johnson, 1994; Vaillancourt *et al.*, 1994). Activated MAPKK phosphorylates mitogen-activated protein kinases (MAPKs) on tyrosine and threonine residues, initiating their activation; MAPK phosphorylates and activates the serine/threonine protein kinase, Rsk, and both enzymes translocate to the nucleus (Chen *et al.*, 1992; Qiu and Green, 1992). The resulting activation of transcription factors, including CREB (cyclic adenosine monophosphate response element-binding protein), regulate the expression of NGF-inducible genes (reviewed in: Bonni and Greenberg, 1997; Segal and Greenberg, 1996; Impey *et al.*, 1999). The MAPKK-MAPK phosphorylation cascade serves to amplify the signal from the relatively scarce trks to the plentiful MAPKs, with further augmentation possible due to the presence of isoforms of both MAPKKs and MAPKs (reviewed in: Russell, 1995; Impey *et al.*, 1999). Activation of the Ras pathway is required for NGF-mediated outgrowth, in at least PC12 cells and chicken sensory neurons (reviewed in: Greene and Kaplan, 1995), and is purported to play a role in learning and memory (Impey *et al.*, 1999), but its activation appears insufficient to support the survival of hippocampal neurons (Marsh and Palfrey, 1996).

PI-3-K, another SH2 domain-containing protein, binds to NGF-activated trkA at or near the Shc binding site (Baxter *et al.*, 1995). The binding of the regulatory subunit of PI-3-K to the autophosphorylated receptor activates the catalytic subunit and,

although the exact pathway of this signaling cascade is unclear, data suggest PI-3-K may initiate a Ras-dependent pathway separate from that of Shc (reviewed in: Russell, 1995; Greene and Kaplan, 1995; Segal and Greenberg, 1996). The protein has also been shown to be activated by both NT-3 and BDNF (Widmer *et al.*, 1992; Righi *et al.*, 2000). Neurotrophin-induced PI-3-K activation has been implicated in neuronal survival (Yao and Cooper, 1995; Dudek *et al.*, 1997), neurite outgrowth (Kimura *et al.*, 1994), and neuronal differentiation (Soltoff *et al.*, 1992).

The SH2 domain-containing enzyme, PLCγ has been shown to become tyrosine phosphorylated upon binding to Tyr785 of ligand-activated trkA (Ohmichi *et al.*, 1991a, 1991b; Obermeier *et al.*, 1993; Loeb *et al.*, 1994) and to activated trkB (Zirrgiebel *et al.*, 1995), although the role of this phosphorylation is not clear (Vetter *et al.*, 1991). PLCγ cleaves membrane phosphatidylinositol-bis-phosphate into diacylglycerol (DAG) and inositol-triphosphate (IP-3); DAG activates protein kinase C (PKC), while IP-3 induces the release of stored calcium. Calcium is required for the activation of many PKCs and also activates calcium-calmodulin-dependent protein kinases. Both of these types of kinases have been implicated in promoting neuronal survival (Zirrgiebel *et al.*, 1995) and as mediators of neuronal plasticity (Loeb *et al.*, 1994; reviewed in: Russell, 1995; Segal and Greenberg, 1996).

A fourth SH2 domain-containing protein, Fyn tyrosine kinase, has been reported to bind to the intracellular region of trkB upon BDNF activation and, because of this, is presumed to function in neurotrophin signal transduction pathways (Iwasaki *et al.*, 1998). In addition, rAPS and SH2-B, two molecules closely related to SH2 domain-containing effectors, have been implicated in trk signaling in developing neurons (Qian *et al.*, 1998).

Another neurotrophin-mediated signal transduction pathway involves the protein SNT (*suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target) (Rabin *et al.*, 1993; Meakin *et al.*, 1999). SNT binds to trkA at the same place as Shc (Meakin *et al.*, 1999), undergoes rapid NGF-induced tyrosine phosphorylation (Rabin *et al.*, 1993), and appears to function independently of any other known signaling cascades initiated by trk (Rabin *et al.*, 1993). Interestingly, in embryonic tissues BDNF and NT-3, but not

NGF, induce the tyrosine phosphorylation of SNT (Knüsel *et al.*, 1994). Activation of the SNT signaling pathway appears to result in neurite outgrowth and differentiation, but not neuronal proliferation (Rabin *et al.*, 1993; Peng *et al.*, 1995; Meakin *et al.*, 1999; reviewed in: Bonni and Greenberg, 1997).

Although aspects of specific neurotrophin/trk signal transduction cascades may be unique, redundancy and/or cooperation may also exist within these pathways. Both NGF and BDNF induce neuritogenesis in PC12 cells that coexpress trkA and trkB, but the phenotypic changes appear to be neurotrophin-specific, with exogenous BDNF inducing the formation of thicker, longer neurites and larger, more flattened perikarya (Iwasaki *et al.*, 1997). These results suggest that although each of these neurotrophins effect neurite outgrowth, the specific downstream signal transduction molecules that are activated differ. Contrasting with these observations, MAPK 1 can be activated *via* trkA through either Shc- or PLCγ-dependent signaling pathways (Stephens *et al.*, 1994) and both the Shc/trkA and PLCγ/trkA cascades have been shown to induce PC12 cell differentiation (Obermeier *et al.*, 1994). Further, PI-3-K may initiate a Ras-dependent pathway that is independent of the Shc/Ras cascade (reviewed in: Russell, 1995; Greene and Kaplan, 1995; Segal and Greenberg, 1996). In addition, there is potential for redundancy or overlap in signaling pathway activation through different, although specific, ligand/receptor interactions. The activation of the Ras pathway through the interaction of NGF/trkA, and also by BDNF/trkB (Zirrgiebel *et al.*, 1995), provides evidence for this theory. Moreover, because NGF, BDNF, and NT-3 can all initiate the PI-3-K signaling pathway (Widmer *et al.*, 1992; Righi *et al.*, 2000), and since neurons can coexpress trks (Karchewski *et al.*, 1999), there is the potential for multiple neurotrophins to initiate parallel signal transduction cascades within an individual cell. There is also the possibility that two neurotrophins can interact with a single receptor type, but with each exerting different effects on an individual cell. For instance, mice with a mutation in the Shc binding site show differing effects in response to BDNF and NT-4/5; few NT-4/5 dependent sensory neurons survive, while the BDNF-dependent subpopulation is little affected (Minichiello *et al.*, 1998). Further, the trophic factors EGF and insulin can also activate Ras and MAPKs, although it appears that the specific

ligand/receptor interactions may result in variable downstream effects: NGF induces an extended activation of Ras and MAPKs, while responses to EGF are less strong and of shorter duration (Qiu and Green, 1992; Traverse *et al.*, 1992). Moreover, because NGF, but not EGF or insulin, has been shown to effect neurite outgrowth through the activation of Ras (reviewed in: Greene and Kaplan, 1995), it has been proposed that the distinction between strong/prolonged and weak/transient Ras pathway activation may ultimately be evidenced in the difference between neuronal proliferation and differentiation (Greene and Kaplan, 1995; Marshall, 1995). Another signal transduction cascade that shows overlap between trophic factors is that involving SNT. Both NGF and FGF, but not EGF, induce the phosphorylation of this protein, although potential similarities and differences in their activation of this pathway have not yet been identified (Rabin *et al.*, 1993).

1.1.2.2 p75

All members of the NGF family bind to an additional receptor known as p75, or the low-affinity NGF receptor (Chao *et al.*, 1986; Radeke *et al.*, 1987; Squinto *et al.*, 1991; reviewed in: Chao, 1994). Unrelated to the trks, p75 is a transmembrane glycoprotein composed of an extracellular domain with a short cytoplasmic region. The extracellular portion contains four cysteine-rich repeats (reviewed in: Chao and Hempstead, 1995) and is involved in neurotrophin binding (Welcher *et al.*, 1991; Yan and Chao, 1991). Individual neurotrophins exhibit similar binding affinities with this receptor, but each shows variation in the rate of dissociation, with NGF dissociating from p75 more rapidly than NT-3, and both dissociating more quickly than BDNF (Rodriguez-Tébar *et al.*, 1990, 1992). Further, upon binding, all of the known neurotrophins effect similar, but not identical conformational changes to the binding site of p75 (Shamovsky *et al.*, 1999). Although the intracellular region lacks inherent kinase activity and was initially believed incapable of signal transduction (reviewed in: Meakin and Shooter, 1992; Davis, 1994; Greene and Kaplan, 1995), there is growing evidence that a number of signaling molecules are associated with p75 (Ohmichi *et al.*, 1991c; Volonté *et al.*, 1993a, 1993c; Dobrowsky *et al.*, 1994; Canossa

et al., 1996; Yamashita *et al.*, 1999). Like the trks, p75 is internalized and retrogradely conveyed, and while this may have consequences in neurotrophin/p75 signal transduction, the significance of p75 transport is unclear (Johnson *et al.*, 1987; Raivich *et al.*, 1991; Curtis *et al.*, 1995; von Bartheld *et al.*, 1996b).

Due to similarities in molecular structure, especially in the highly conserved cytoplasmic domain, p75 is classified as a member of the tumour necrosis factor (TNF) receptor superfamily (Smith *et al.*, 1990, 1994; Liepinsh *et al.*, 1997; reviewed in: Bothwell, 1996). TNF receptor family members are known to be involved in programmed cell death (apoptosis) (reviewed in: Nagata and Goldstein, 1995; Fraser *et al.*, 1996; Martinou and Sadoul, 1996) and, because of this, it is not surprising that p75 has been shown to promote this cellular phenomenon (Rabizadeh *et al.*, 1993; Bamji *et al.*, 1998; Frade and Barde, 1998b; reviewed in: Carter and Lewin, 1997; Chao *et al.*, 1998) through its cytoplasmic domain (Majdan *et al.*, 1997). p75 is not necessary for viability since mice with nonfunctional receptors survive (Lee *et al.*, 1992), and although the phenotype of these animals generally resembles that of the wild-type, p75 must function in roles besides apoptosis because these mice exhibit sensory (Lee *et al.*, 1994b; Curtis *et al.*, 1998) and sympathetic (Lee *et al.*, 1994a, 1994b) deficits. In fact, p75 has also been shown to be involved in neuronal sprouting (Kuchel *et al.*, 1992), neurite outgrowth (Dostaler *et al.*, 1996; Yamashita *et al.*, 1999), and Schwann cell migration during development and after injury (Anton *et al.*, 1994; You *et al.*, 1997). There is additional evidence that p75 directly, or indirectly, influences trks and may be a necessary component in trk/neurotrophin signal transduction (reviewed in: Meakin and Shooter, 1992; Chao, 1992; Barker and Shooter, 1994; Hantzopoulos *et al.*, 1994; Chao and Hempstead, 1995).

1.1.2.2.1 Role of p75 in Apoptosis

In the nervous system, apoptosis occurs during development (reviewed in: Raff *et al.*, 1993; Raff, 1998) and following ischemic injury (reviewed in: Thompson, 1995; Choi, 1996), traumatic spinal cord injury (Liu *et al.*, 1997), epilepsy (Roux *et al.*, 1999), and neurodegenerative disorders such as

Alzheimer's disease (reviewed in: Thompson *et al.*, 1995). There is evidence that p75 plays a role in programmed cell death (reviewed in: Bothwell, 1996; Bredesen and Rabizadeh, 1997; Carter and Lewin, 1997; Chao *et al.*, 1998), although current results seem to conflict, with the neurotrophin receptor potentially functioning in both an apoptotic and an antiapoptotic capacity. Application of p75 antisense oligonucleotides to the proximal end of transected sciatic nerve shows that these molecules are retrogradely transported and result in decreased p75 protein levels and a reduction in the axotomy-induced loss of sensory neurons (Cheema *et al.*, 1996). In neuronal-based cell lines death appears to be invoked through p75 in the absence of ligand binding, while the binding of NGF or NT-3 to p75 seems to inhibit apoptosis (Rabizadeh *et al.*, 1993; Cortazzo *et al.*, 1996; Longo *et al.*, 1997; ElShamy *et al.*, 1998). Further, although p75 has been shown to mediate both survival and apoptosis in developing sensory neurons, depending on the developmental stage, the former response has been attributed to the interaction of p75 with trkA, while apoptosis was deemed to involve p75 alone (Barrett and Bartlett, 1994). Conversely, in glia it appears that the binding of NGF to p75 induces apoptosis (Frade *et al.*, 1996; Soilu-Hänninen *et al.*, 1999).

In two glial cell lines, all of the neurotrophins, through binding to p75, have been shown to activate the sphingomyelin cycle and to subsequently generate the second messenger ceramide (Dobrowsky *et al.*, 1994, 1995), an inducer of apoptosis (Obeid *et al.*, 1993; Verheij *et al.*, 1996). Similar results have been reported for postnatal rat oligodendrocytes, where NGF, but not BDNF or NT-3, invokes apoptosis *via* p75 and a subsequent increase in intracellular ceramide (Casaccia-Bonnefil *et al.*, 1996). Interestingly, the ability of NGF/ceramide to induce apoptosis may be dependent on the developmental stage of the cell since NGF exposure does not result in the death of adult human oligodendrocytes (Ladiwala *et al.*, 1998). Ceramide initiates apoptosis through a stress-activated protein kinase (Verheij *et al.*, 1996) which phosphorylates Raf, potentially effecting a link between ceramide activation and the MAPK pathway that is also activated by the trks (Yao and Cooper, 1995).

The role of ceramide is complex, as there is evidence for a second NGF/p75/ceramide signaling pathway. The effect of ceramide appears to be

concentration dependent, with low levels mediating neurite outgrowth and higher ceramide levels inducing apoptosis (Schwarz and Futerman, 1997; Brann *et al.*, 1999). Further, coexpression of trkA in p75-positive cells blocks ligand-mediated ceramide production (Dobrowsky *et al.*, 1995), supporting the hypothesis that p75 alone is responsible for apoptosis induction and that the presence of trkA impedes p75-induced cell death (Barrett and Bartlett, 1994). In postnatal rat oligodendrocyte cultures, activation of introduced trkA negates p75-induced apoptosis through MAPK activation, and this phenomenon appears to involve both the induction of survival signals and the concomitant repression of p75-induced death signal (Yoon *et al.*, 1998). This may involve the production of sphingosine-1-phosphate, a second messenger and downstream product in the ceramide pathway, which has mitogenic properties (Edsall *et al.*, 1997).

Ceramide may also induce the activation of the transcription factor, nuclear factor kappa B (NF-κB) (Quillet-Mary *et al.*, 1997). This protein connects early trophic factor signaling events and downstream changes in gene expression primarily through the regulation of genes involved in immunity and inflammation, although it may also induce genes regulating neuronal development, plasticity, and neurodegeneration (reviewed in: O'Neill and Kaltschmidt, 1997). In rat Schwann cells NGF, but neither BDNF nor NT-3, activates NF-κB upon binding to p75 (Carter *et al.*, 1996), but this apparently occurs only under stress conditions (Bhakar *et al.*, 1999). The interaction of ligand-bound p75 with TRAF6 (tumour necrosis factor receptor-associated factor-6) mediates the nuclear translocation of NF-κB (Khursigara *et al.*, 1999), which allows the transcription factor to modulate gene expression. The inhibition of NF-κB results in apoptosis of PC12 cells and does not permit NGF-mediated rescue (Taglialatela *et al.*, 1997), supporting an additional role for the ceramide pathway in survival.

1.1.2.2.2 Interactions between p75 and trk

Although glial p75 may serve to present neurotrophins to trks on adjacent neurons (Johnson *et al.*, 1988), there is evidence that p75 can act more directly to influence trks and trk/neurotrophin signal transduction; p75 may interact with trkA to form high-affinity binding sites and, seemingly conversely, may also reduce the

ability of trkA to interact with NGF (reviewed in: Meakin and Shooter, 1992; Chao, 1992; Barker and Shooter, 1994; Hantzopoulos *et al.*, 1994; Chao and Hempstead, 1995; Greene and Kaplan, 1995). There is additional evidence that p75 alone, without interaction with trk, may bind NT-3 with high affinity on sympathetic neurons (Dechant *et al.*, 1993, 1997).

Concomitant interaction between p75 and trkA appears necessary for optimal trkA signal transduction, since suboptimal signals are produced when trkA, in the absence of p75, is activated (Battleman *et al.*, 1993; Mahadeo *et al.*, 1994; Maliartchouk and Saragovi, 1997). And while the coexpression of these receptors increases ligand-induced trkA tyrosine phosphorylation (Verdi *et al.*, 1994), there is conflicting evidence as to whether the cytoplasmic domain of p75 is necessary (Battleman *et al.*, 1993; Wolf *et al.*, 1995; Wang *et al.*, 1998) or not (Hantzopoulos *et al.*, 1994) for effective trkA interaction. A specific ratio of p75 to trkA seems to be required to create high-affinity NGF binding sites (Benedetti *et al.*, 1993; Mahedeo *et al.*, 1994), although this may only occur when there are high levels of both receptor species (Huber and Chao, 1995). In p75-deficient mice, primary sensory neurons exhibit a two to three fold decrease in sensitivity to NGF (Lee *et al.*, 1994b), and although sensitivity to the other neurotrophins remains unchanged in these animals, other data indicate that p75 may influence the activation of trkB and trkC (Hantzopoulos *et al.*, 1994).

The ability of trkA to bind and respond to NGF may be controlled by p75, depending on NGF concentration and/or duration of ligand exposure (Barker and Shooter, 1994; Kahle *et al.*, 1994; Twiss *et al.*, 1998; Wang *et al.*, 1998). When high levels of the neurotrophin are present, p75 may decrease NGF-induced trkA signal by sequestering NGF from trkA (Kahle *et al.*, 1994). Conversely, at low ligand levels p75 appears to pool NGF, elevating levels of the neurotrophin available to interact with trkA (Barker and Shooter, 1994) and amplifying ligand-induced trkA phosphorylation (Wang *et al.*, 1998). Further, under short-term NGF exposure p75 appears to enhance ligand-induced trkA activation, while long-term NGF exposure lessens the signal response (Twiss *et al.*, 1998). Interestingly, other data indicate that in the absence of bound ligand p75 confers high-affinity binding properties on trkA, while NGF-activated p75 appears

to reduce the ability of trkA to bind NGF with high affinity (Ross *et al.*, 1998). In addition, in PC12 cells, BDNF effects a notable reduction in trkA tyrosine phosphorylation with a concomitant increase in serine phosphorylation in the cytoplasmic domain of the receptor (MacPhee and Barker, 1997). These findings appear to result from an interaction between that neurotrophin and p75, and can be mimicked by exposure to a ceramide analogue in lieu of BDNF (MacPhee and Barker, 1997).

Affinity-crosslinking and immunoprecipitation experiments show that trkA and p75 may physically interact (Huber and Chao, 1995). This interaction may be facilitated through the juxtapositioning of the two receptor types, and although direct contact appears nonessential for interplay between the two (Kalhe *et al.*, 1994), there is evidence for direct interaction through the possible formation of p75 and trkA heterodimers, resulting from NGF-induced conformational changes in the receptors (Ross *et al.*, 1998). Further, supporting data indicate that a physical interaction between p75 and trkA results in p75 immobilization; the effect of trkB on p75 is more modest (Wolf *et al.*, 1995).

1.2 Primary Sensory Neurons

1.2.1 Introduction to Primary Sensory Neurons

DRG neurons are sensory in function — innervating skin, muscle, joints, or viscera. Information detected by the sensory receptor is transmitted along the afferent fiber of the peripheral nerve and, bypassing the cell body, moves along the central fiber of the dorsal root to synapse in the spinal cord grey matter or the brain stem. The cell bodies, perikarya, of these pseudo-unipolar neurons function primarily in cell maintenance, are located within the DRG, and are surrounded by glial satellite cells. Historically, DRG neurons have been described as a homogenous population because they are all derived from the neural crest, their processes and perikarya are organized in a similar fashion, and they have a common sensory function (reviewed in: Perl, 1992). In spite of this, these neurons represent a diverse group — mirrored through differences in size, cytology, physiology, biochemical markers, and responsiveness to neurotrophic factors (reviewed in: Hunt *et al.*, 1992; Lawson, 1992).

These neurons may be classified according to afferent fiber diameter, sensory receptor type, and cell body morphology. Large, medium, and small diameter myelinated afferent fibers are termed Aα, Aβ, and Aδ, respectively; small unmyelinated fibers are designated as C fibers. A second classification system, with the same defining characteristics but specific for muscle afferents, has labeled these fibers Type I, II, III, and IV, respectively. Conduction velocity correlates with afferent axon diameter; Aα, Aβ, Aδ, and C fibers conduct impulses at 80-120, 35-75, 5-30, and 0.5-2 m/s, respectively (reviewed in: Kandel *et al.*, 1991). Large diameter fibers that innervate muscle project to the spinal cord ventral horn, while in general those that innervate skin project to laminae III and IV of the dorsal horn (Shortland *et al.*, 1989; reviewed in: Fyffe, 1992; Hunt *et al.*, 1992). Of the small diameter afferents, Aδ fibers project to spinal cord laminae I and V (Light and Perl, 1979; reviewed in: Fyffe, 1992; Hunt *et al.*, 1992) with C fibers predominately projecting to dorsal horn laminae I and II (Sugiura *et al.*, 1986; reviewed in: Fyffe, 1992; Hunt *et al.*, 1992).

The peripheral termini of primary afferent fibers are specialized sensory receptors that are individually sensitive to specific stimuli. These receptors have been variously classified based on the type of sensation to which they respond and may be further subdivided according to histological characteristics (reviewed in: Perl, 1992). Nociceptors discriminate the sensation of pain in response to mechanical, thermal, or chemical stimuli, and are typically free nerve endings. Most mechanoreceptors are comprised of specialized end organs that surround the axon terminals, and mediate the sensations of touch (i.e. Merkel cells and Meissner's, Ruffini's, and Pacinian corpuscles) and proprioception, the sense of limb positioning and movement (i.e. muscle spindles and Golgi tendon organs). Virtually all mechanoreceptor subtypes, including proprioceptors, are classed as Aα or Aβ fibers, while nociceptors belong to the Aδ and C fiber groups. Somatosensory information is relayed to the thalamus *via* two primary ascending routes, the posterior column-medial lemniscus pathway and the spinothalamic tract. The former system is important in carrying proprioceptive and tactile information and the latter transmits principally pain and temperature information.

Based on their appearance under light microscopy, DRG cell bodies may be broadly divided into two categories: "large light" and "small dark" neurons and, with few exceptions, DRG cell body size correlates with the corresponding afferent fiber diameter (reviewed in: Lawson, 1992; Perl, 1992). Large light-type neurons are neurofilament (NF)-rich and have large to small sized cell bodies with myelinated fibers, most are presumed to be Aα and Aβ mechanoreceptor neurons; small dark-type neurons have small sized, NF-poor perikarya with Aδ and unmyelinated C fibers (reviewed in: Lawson, 1992). This classification does not reflect the phenotypic complexities of the subpopulations DRG neurons. Through the use of techniques such as immunohistochemistry and *in situ* hybridization, researchers are currently characterizing numerous distinct subgroups of sensory neurons based on their expression of one or more biochemical markers — including trophic factors and their receptors, neuropeptides, cytoskeletal elements, enzymes, and other proteins (reviewed in: Hunt *et al.*, 1992; Lawson, 1992).

The neuronal diversity seen in DRG populations and the relative accessibility of these cells makes them ideal models for studying sensory neurons and for determining potential roles of trophic factors within specific subpopulations. Once unique subgroups of DRG neurons have been identified based on their responsiveness to neurotrophic factors, including the neurotrophins, they may be manipulated; such studies will yield valuable information that could lead to advances in the treatment of neurological diseases or trauma (Maeda *et al.*, 1996; reviewed in: Eddington, 1993; Barinaga, 1994; McMahon and Priestly, 1995; Schätzl, 1995; Anand, 1996; Tomlinson *et al.*, 1996, 1997; Mufson *et al.*, 1999).

1.2.2 Characterization of DRG Neurons with Respect to Neurotrophin Responsiveness

Three distinct, but potentially overlapping, DRG neuronal subpopulations have been identified through micro-injections of iodinated NGF, BDNF, and NT-3 into sciatic nerve (DiStefano *et al.*, 1992). One of these subsets, representing 40-45% of the total (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1989b, 1990a, 1990b, 1992a; Mu *et*

al., 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Bennett *et al.*, 1996a; Kashiba *et al.*, 1996, 1997a; Karchewski *et al.*, 1999), has been characterized based on its ability to respond to the neurotrophin, NGF. These neurons display high-affinity NGF binding sites and express p75 and trkA mRNA (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1989b, 1990b, 1992a). The group includes virtually all substance P- (SP) and most calcitonin gene-related peptide- (CGRP), but not somatostatin- (SOM), expressing DRG neurons (Verge *et al.*, 1989a; Kashiba *et al.*, 1996); all members express GAP-43 mRNA (Verge *et al.*, 1990a). They are mainly small to medium in size (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1989b, 1990a, 1990b, 1992a, 1992b; Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Kashiba *et al.*, 1996, 1997a; Karchewski *et al.*, 1999) and are presumed to be nociceptive in function (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). The subsets of DRG neurons expressing other trks are poorly characterized, even though subpopulations of predominantly medium to large sized cells expressing trkB and trkC have been reported (McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Kashiba *et al.*, 1996, 1997a; Karchewski *et al.*, 1999; reviewed in: Phillips and Armanini, 1996; Lindsay, 1996; Snider and Silos-Santiago, 1996). Several research groups indicate that for adult rat DRG 27%[1], 26%[2], or 5%[3] of neurons express trkB mRNA, while 17%[1], 21%[2], or 15-20%[3] are trkC-positive ([1]McMahon *et al.*, 1994; [2]Wetmore and Olson, 1995; [3]Kashiba *et al.*, 1996, 1997a). In this laboratory, we find that ~ 33% of DRG neurons exhibit detectable levels of trkB transcripts, with 30-35% of the total population being strongly trkC-positive and an additional 10% weakly expressing the signal (Karchewski *et al.*, 1999). Although the subpopulations of neurons expressing trkA, trkB, or trkC are for the most part unique, these receptors may also colocalize, although reported percentages vary (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wright and Snider, 1995; Karchewski *et al.*, 1999). In addition, between 20-34% of DRG neurons do not appear to exhibit detectable levels for any of the trks (McMahon *et al.*, 1994; Wright and Snider, 1995; Karchewski *et al.*, 1999).

1.2.3 Effects of Peripheral Injury on Primary Sensory Neurons

1.2.3.1 Response of Primary Sensory Neuron Perikarya to Injury

Following peripheral nerve injury, the surviving DRG neurons undergo structural, physiological, and biochemical changes; they may lose their ability to respond to various types of stimuli or may gain new reactivity (reviewed in: Lieberman, 1971, 1974; Grafstein and McQuarrie, 1978; Aldskogius *et al.*, 1985, 1992; Titmus and Faber, 1990). A detailed analysis of these changes is necessary to understand disturbances in sensory function after peripheral nerve damage and to understand how these conditions may be prevented, compensated for, or treated. Much of the information concerning the effect of peripheral trauma on these neurons has come from studies involving changes observed within their cell bodies.

Studies involving peripheral nerve injury conclude that there is a significant loss (15-30%) of sensory ganglion neurons (Tessler *et al.*, 1985; Arvidsson *et al.* 1986), although it is unclear if there is a selective loss of any specific subpopulation (reviewed in: Aldskogius *et al.*, 1992). There is a more extensive loss of DRG neurons after a nerve transection as compared to a crush lesion, after proximal compared to distal nerve transection, and in injured immature animals compared to adults (Aldskogius *et al.*, 1985; Redshaw and Bisby, 1985; Navarro *et al.*, 1994). DRG neurons that survive the axonal injury undergo characteristic alterations in morphology (Greenberg and Lasek, 1988; Moskowitz *et al.*, 1993; reviewed in: Lieberman, 1971). Within 1 day, and for at least several weeks following the insult, there is a disintegration and redistribution of the basophilic Nissl substance (chromatolysis), a migration of the nucleus to the periphery of the soma, and changes in the sizes of the nucleolus, nucleus, and perikaryon (reviewed in: Lieberman, 1971, 1974; Grafstein and McQuarrie, 1978; Wells and Vaidya, 1989). These morphological changes reflect significant parallel alterations in the metabolism and cytoplasmic makeup of the neuron, alterations that may play a key role in determining successful axonal regeneration (Wells, 1984, 1987; Wells and Vaidya, 1989; reviewed in: Lieberman, 1971, 1974; Grafstein and McQuarrie, 1978).

Chromatolysis has been revealed by electron microscopy studies to be due to a disorganization of the structure of rough endoplasmic reticulum, with a subsequent increase in the proportion of free polyribosomes (reviewed in: Lieberman, 1971). Free

polyribosomes are generally thought to be responsible for the production of cytoplasmic proteins, whereas membrane-bound ribosomes are responsible for the production of cellular membranes and secretion products and may reflect a shift from normal functioning to a survival response (Palade, 1975). Other indications of changes in RNA metabolism are the observed enlargement of the nucleus and nucleolus, which reflect increased capacity for RNA, and subsequent protein, synthesis (reviewed in: Lieberman, 1971, 1974; Grafstein and McQuarrie, 1978; Hall, 1982). RNA synthesis in injured DRG neurons is biphasic, consisting of two major increases about 7 days apart (Wells, 1984, 1987). The significance of this two-phased increase in RNA synthesis after axonal injury is unclear, but the first phase may be due to an "injury program" that is separated by a period of reorganization from the second, potentially regenerative, phase (Wells and Vaidya, 1989). Although an increase of ~ 30% in total protein synthesis has been noted in DRG neurons following sciatic nerve axotomy (Hall, 1982), there is a reduction in the synthesis of proteins associated with synaptic transmitter metabolism (reviewed in: Grafstein and McQuarrie, 1978), reflecting the finding that transmitting and receiving functions of the neuron may be partially, or wholly, interrupted after axotomy (reviewed in: Lieberman, 1971).

NF protein and mRNA levels in DRG neurons decrease following axotomy (Greenberg and Lasek, 1988; Oblinger and Lasek, 1988; Verge *et al.*, 1990b; Wong and Oblinger, 1987, 1990, 1991; Ernfors *et al.*, 1993; Hoffman *et al.*, 1993), and since neurofilaments function mainly as architectural elements, the observed decrease in somal size may be due to a reduction in NF synthesis (Verge *et al.*, 1990b; Hoffman *et al.*, 1993). Other cytoskeletal components, the microtubule-associated proteins, actins and Classes II and III β-tubulins, increase in proportion to neurofilament levels; these increases reflect an upregulation in intracellular transport levels and axonal regeneration (Oblinger and Lasek, 1988; Skene, 1989; Hoffman *et al.*, 1993; Moskowitz *et al.*, 1993).

Additional axotomy-induced biochemical changes within DRG neurons include the regulation of neuropeptides (reviewed in: Hökfelt *et al.*, 1994a, 1994b). Sciatic nerve transection causes a significant decrease in the levels of α- and β-CGRP (Shehab and Atkinson, 1986; Noguchi *et al.*, 1990b, 1993; Klein *et al.*, 1991; Verge *et al.*, 1995), SP

(Jessell *et al.*, 1979; Tessler *et al.*, 1985; Villar *et al.*, 1989; Klein *et al.*, 1991; Hökfelt *et al.*, 1993; Noguchi *et al.*, 1994, 1995; Verge *et al.*, 1995) and SOM (Noguchi *et al.*, 1993; Verge *et al.*, 1995). Under similar conditions a dramatic upregulation of the following peptides occurs: neuropeptide tyrosine (NPY) (Wakisaka *et al.*, 1991, 1992; Frisén *et al.*, 1992; Noguchi *et al.*, 1993; Zhang *et al.*, 1994c; Ohara *et al.*, 1994, 1995; Verge *et al.*, 1995; Corness *et al.*, 1996), galanin (Hökfelt *et al.*, 1987, 1993; Villar *et al.*, 1989; Klein *et al.*, 1991; Noguchi *et al.*, 1993; Verge *et al.*, 1993b, 1995; Corness *et al.*, 1996), vasoactive intestinal peptide (VIP) (Villar *et al.*, 1989; Klein *et al.*, 1991; Noguchi *et al.*, 1993; Verge *et al.*, 1995), and cholecystokinin (CCK) (Klein *et al.*, 1991; Verge *et al.*, 1993a, 1995; Zhang *et al.*, 1993b). Although the exact physiological import of these neuropeptides are not known, some putative roles have been described (reviewed in: Hunt *et al.*, 1992; Lawson, 1992; Hökfelt *et al.*, 1993; Iversen, 1995). Of the peptides that decrease with injury CGRP and SP may function as an excitatory molecule, having algesic effects, and may also function as a vasodilator, while SOM may be involved in thermoception and also act to reduce the intensity of the immune response. Of the neuropeptides whose expression is upregulated after axotomy VIP is algesic and has been implicated in the modulation of the immune response, NPY may play a role in pain pathways through the inhibition of neurotransmitter release, galanin appears to be important in suppressing states of hyperexcitation after injury; and CCK may be involved in antagonizing analgesic effects.

In adult rat DRG neurons, upregulation of GAP-43 mRNA and protein levels have been shown to increase in some neurons 1-2 days after injury (Skene, 1989; Schreyer and Skene, 1991, 1993; Wiese *et al.*, 1992), and within 1 week almost all of the neurons express GAP-43 transcripts (Skene, 1989; Verge *et al.*, 1990b; Wiese *et al.*, 1992). Putative roles for GAP-43 are in axonal elongation and in the growth and remodeling of synaptic connections (reviewed in: De Graan *et al.*, 1986; Skene, 1989). Another protein that is strongly upregulated in sensory neurons following axotomy is the transcription factor cJUN, encoded by an immediate early gene, cjun, which is rapidly induced in many tissues in response to a variety of stimuli (Gold *et al.*, 1993a, 1994). It has been proposed that cJUN may serve to transcriptionally regulate galanin expression

due to the parallel post-traumatic increase in levels of these two substances in the same neurons (Herdegen *et al.*, 1993).

In normal rat DRG there is a heterogeneous distribution of neurons with high-affinity NGF binding sites, representing ~ 40% of the total lumbar DRG population, but following peripheral nerve injury ^{125}I-NGF binding decreases (Raivich and Kreutzberg, 1987; Verge *et al.*, 1989b), with the number of NGF binding sites falling by more than 80% 2 weeks post-injury (Verge *et al.*, 1989b). Moreover, neurotrophin receptor mRNA expression in DRG neurons for both p75 and the trks decreases post-axotomy (Johnson *et al.*, 1987; Verge *et al.*, 1989b, 1992a; Ernfors *et al.*, 1993; Funakoshi *et al.*, 1993; Sebert and Shooter, 1993; Zhou *et al.*, 1996; Kashiba *et al.*, 1998).

1.2.3.2 Reversal of Injury-Induced Changes

It is not known what factor(s) causes the alterations in neurons following peripheral axotomy, but it has been demonstrated that if regeneration is successful (i.e. neurons reinnervate their targets), many of these changes reverse (Lieberman, 1971, 1974; Hoffman *et al.*, 1993). Several lines of evidence support the current view that retrograde transport of a trophic factor(s) from the peripheral target and/or cells along the distal nerve tract maintain the neuron in its normal biological state; disruption of this retrograde flow triggers the injury-induced state (Lieberman, 1971, 1974; Grafstein and McQuarrie, 1978; Redshaw and Bisby, 1985; Verge *et al.*, 1992a, 1992b, 1995; Mudge, 1993). This theory is supported by the finding that the appearance of chromatolytic changes is correlated with the time period necessary for retrograde axonal transport from the injury site to the soma (Kristensson and Olsson, 1975). Also, damage to the central axon of the dorsal root does not appear to cause cell death, chromatolysis, or changes in protein levels (Lieberman, 1971; Oblinger and Lasek, 1988); such a lesion would not affect retrograde flow from the periphery to the cell body. Additionally, many of the peripheral nerve injury-induced changes can be mimicked by the application of colchicine or vinblastine, inhibitors of microtubule formation, and, therefore, of retrograde transport (Woolf *et al.*, 1990; Kashiba *et al.*, 1992a).

Convincing evidence to support the retrograde-transport theory comes from experiments where exogenous NGF is infused at a point in time after the neuron has responded to peripheral axotomy. Since NGF is produced in limited quantities by target tissue and is moved by retrograde transport to the cell body, where it can exert trophic effects (reviewed in: Korsching and Thoenen, 1985; Ebendal, 1992; Korsching, 1993), and because peripheral nerve injury reduces levels of receptor-mediated NGF uptake and its retrograde transport (Raivich *et al.*, 1991), the axotomy-induced changes may be attributed to the injury-reduced availability of NGF to this neuronal subpopulation responsive to this neurotrophin (Verge *et al.*, 1989b, 1992a). Trauma-induced decreases in neuronal size, NF, α- and β-CGRP, SP, p75, and trkA are counteracted, at least in part, with NGF infusion (Fitzgerald *et al.*, 1985; Rich *et al.*, 1987; Verge *et al.*, 1989a, 1989b, 1990a, 1990b, 1992a, 1992b, 1995; Gold *et al.*, 1991; Wong and Oblinger, 1991; Tetzlaff *et al.*, 1992), while the trauma-induced increases in GAP-43, cjun, VIP, CCK, NPY, and galanin are, at least partially, mitigated (Verge *et al.*, 1990b, 1995; Tetzlaff *et al.*, 1992; Gold *et al.*, 1993a). Further, that exogenous NGF does not appear to influence the injury-induced decrease in SOM expression (Verge *et al.*, 1992b; Verge *et al.*, 1995), is most likely due to a lack of detectable trkA mRNA in the SOM-positive population (Verge *et al.*, 1989b). In addition, intraperitoneal injection of NGF antiserum mimics the axotomy-mediated alterations in neuropeptide expression, resulting in elevated levels of galanin and VIP, and reduced levels of SP and CGRP, mRNA and protein (Shadiack *et al.*, 2001)

Those trk-positive DRG neurons that are not NGF-responsive (McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Kashiba *et al.*, 1996, 1997a; Karchewski *et al.*, 1999) presumably respond to other molecules (Verge *et al.*, 1990a, 1995) and, because an individual neuron may possess more than one trk species (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wright and Snider, 1995; Karchewski *et al.*, 1999), one can expect to encounter both unique and overlapping forms of the regulation of cellular phenotype by the various neurotrophins.

1.3 Objectives

I hypothesize that other members of the NGF family of neurotrophic factors, specifically NT-3, also play a major role in modulating or maintaining the differentiated phenotype of adult primary sensory neurons, and that since NT-3 is retrogradely transported in sciatic nerve (Nitta *et al.*, 1999), that it is part of the signal that allows at least a subpopulation of these axotomized neurons to return to normal following axonal regeneration. Therefore, my objective is to obtain a better understanding, at the cellular level, of the role of NT-3 in the maintenance of adult primary sensory neuron phenotype. This will be accomplished through isotopic *in situ* hybridization processing of intact and peripherally axotomized adult rat DRG neurons from control and NT-3 infused animals. Computer-assisted image analysis of serial DRG sections will be used to identify and characterize the NT-3 responsive subpopulation and to determine and compare relative levels of mRNA expression for selected neurotrophin receptors, neuropeptides, cytoskeletal elements, injury- and regeneration-associated molecules, and other markers in individual neurons under the conditions stated above. This information will be important in furthering the understanding of the biological role of NT-3 and in the planning of clinical studies involving neuronal degeneration, regeneration, and the restoration of normal neuronal function following nerve injury.

2. LITERATURE REVIEW

2.1 Neurotrophin-3 Receptors

2.1.1 trkC

2.1.1.1 trkC in Developing Primary Sensory Neurons

During embryonic development, the neuroepithelium gives rise to a specialized group of migratory, multipotent cells termed the neural crest. These progenitors disperse dorso- and ventrolaterally from their dorsal positions along the neural tube to follow distinct developmental pathways, ultimately establishing a wide range of differentiated cell types including glia, melanocytes, and sensory and autonomic neurons (reviewed in: Kalcheim, 1996). *In vivo* and *in vitro* experiments have shown that trophic factors, including NT-3, are important in supporting the survival and proliferation of neural crest progenitor cells (reviewed in: Kalcheim, 1996), and although during migration they have been reported to express trkC mRNA (Tessarollo *et al.*, 1993), trkC protein is not detectable in these cells (Fariñas *et al.*, 1998). This apparent discrepancy may be because trkC protein has not yet reached detectable levels, or may be due to a repression of the translation of trkC transcripts during this developmental stage (Fariñas *et al.*, 1998). Later in development, the nascent DRG are composed of a mixed population of committed and multipotent precursor/progenitor cells and postmitotic neurons (reviewed in: Kalcheim, 1996). In E11/11.5 mouse lumbar DRG, where greater than 60% of cells are precursors (Fariñas *et al.*, 1996); virtually all postmitotic, differentiated neurons exhibit high levels of trkC transcripts (Lamballe *et al.*, 1994; Phillips and Armanini, 1996; White *et al.*, 1996) and ~ 70% display trkC-like immunoreactivity (-ir) (Fariñas *et al.*, 1996). At later developmental stages, the number of DRG neurons expressing this receptor rapidly decreases (Lamballe *et al.*, 1994;

Fariñas *et al.*, 1996; Phillips and Armanini, 1996; White *et al.*, 1996); by E13 less than 10% of neurons exhibit trkC-ir (Fariñas *et al.*, 1996) and although between E15 and E17.5 trkC mRNA levels remain high, receptor expression continues to be limited to a subset of DRG neurons (Lamballe *et al.*, 1994; Phillips and Armanini, 1996). Data from studies on rat (Ernfors *et al.*, 1993; Mu *et al.*, 1993; Elkabes *et al.*, 1994) and chicken (Williams and Ebendal, 1995) indicate that trkC expression follows a similar pattern in DRG neurons of these species. Further, percentages of trkC-positive neurons, as well as size frequency histograms, indicate that thoracic and lumbar DRG populations are comparable in rat (Mu *et al.*, 1993). Data regarding the developmental expression of isoforms of the receptor are limited, but in chicken it has been reported that $trkC_{full\text{-}length}$ transcripts are detectable prior to those of the truncated form (Escandon *et al.*, 1994). At later stages in development, trkC is primarily localized to neurons with large diameter perikarya (Mu *et al.*, 1993; Oakley *et al.*, 1995; Williams and Ebendal, 1995; Lefcort *et al.*, 1996), although an additional subset of neurons with small soma and low levels of trkC mRNA has also been identified (Lefcort *et al.*, 1996). Together these data indicate that trkC is differentially expressed in sensory neuron precursors and differentiated DRG neurons during development, with the number of trkC-positive cells restricted to a subset of the population in maturing embryos.

2.1.1.2 trkC in Adult Primary Sensory Neurons

A subpopulation of adult murine DRG neurons express trkC mRNA (Ernfors *et al.*, 1993; Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Kashiba *et al.*, 1996, 1997a; Helgren *et al.*, 1997; Karchewski *et al.*, 1999), although reports differ as to the percentages of neurons that exhibit the receptor. As few as 10%(Mu *et al.*, 1993), and from 17%[2], 21%[3], or 15-20% (McMahon *et al.*, 1994; Wetmore and Olson, 1995; Kashiba *et al.*, 1996, 1997a) of adult rat lumbar DRG neurons have been identified as being trkC-positive. In this laboratory, we find that 30-35% of the total L_5 DRG population exhibit high levels of trkC hybridization signal, with up to 10% of additional neurons displaying lower signal levels (Karchewski *et al.*, 1999). These numbers correspond to the ~ 40% of lumbar DRG

neurons labeled with antiserum to NT-3 (Zhou and Rush, 1995), ~ 95% of which also display trkC-ir (Chen *et al.*, 1996). The apparent discrepancies in the number of trkC-positive neurons reported by the different research groups may be attributed to several factors. Even though all of these results were based on *in situ* hybridization studies, the protocols followed vary, with some processing methods more sensitive in the detection of mRNA than others (Tecott *et al.*, 1987). In addition, the duration of exposure of radioautographs prior to the developing of the emulsion is also important in distinguishing between neurons with low levels of trkC transcripts and nonspecific, background labeling. That a number of these researchers have indicated that trkC-positive neurons display relatively uniform labeling intensities (Wetmore and Olson, 1995; Wright and Snider, 1995) implies that the subset with lower levels of labeling (Karchewski *et al.*, 1999) may not have been identified in these studies. Despite the disparity in reported percentages of trkC-positive DRG neurons, all groups concur that these cells are primarily large in size (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Helgren *et al.*, 1997; Karchewski *et al.*, 1999), with ~ 85% of trkC-expressing neurons possessing medium to large diameter perikarya (Karchewski *et al.*, 1999). Hybridization signal for trkC mRNA tends to be of higher density over medium and large soma; small neurons typically display lower signal levels (Karchewski *et al.*, 1999). The percentages of rat L_5 DRG neurons expressing two kinase insert isoforms of trkC ($trkC_{ki14}$ and $trkC_{ki39}$) have also been determined, with 42%, 35%, and 27% of neurons displaying detectable levels of $trkC_{no\ insert}$, $trkC_{ki14}$, and $trkC_{ki39}$ mRNAs, respectively (Karchewski *et al.*, 1999). Transcripts of all three isoforms are primarily detected in medium and large diameter cell bodies, and expression of $trkC_{ki14}$ or $trkC_{ki39}$ almost always colocalizes with the noninsert isoform (Karchewski *et al.*, 1999). In rat, all neural tissues appear to possess mRNAs of both the full-length and truncated trkC isoforms with the potential exception of sciatic nerve, in which only the latter type has been detected (Valenzuela *et al.*, 1993). In human DRG, trkC mRNA is expressed at moderate levels, with a relatively high ratio of $trkC_{full\text{-}length}$ to $trkC_{truncated}$ transcripts (Yamamoto *et al.*, 1996).

2.1.1.3 trkC in the Spinal Cord

Northern blot analyses reveal that trkC mRNA is present in rat spinal cord at differing levels during embryonic development: At E13 and E14 trkC is expressed at low levels, message levels increase over time, peaking at P1 where levels are 12 fold higher than at E14 (Ernfors *et al.*, 1993). In adult rat, trkC transcripts are detectable in spinal cord (Ernfors *et al.*, 1993; Funakoshi *et al.*, 1993), but levels are comparable to those of E13/E14 (Ernfors *et al.*, 1993). In the dorsal horn of adult monkey trkC-ir is detectable in nerve terminals (Arvidsson *et al.*, 1994), while in human spinal cord trkC message is reported to be strongly expressed, with the majority of transcripts believed to encode the truncated isoform of the receptor (Yamamoto *et al.*, 1996).

2.1.2 Coexpression of trks in Adult Primary Sensory Neurons

Although micro-injection of radioiodinated neurotrophins into adult rat sciatic nerve retrogradely labels three distinct subpopulations of DRG neurons in a receptor-mediated fashion — with ^{125}I-NT-3 and ^{125}I-NGF primarily localized to large- and small-sized soma, respectively, and ^{125}I-BDNF labeling perikarya of varying diameters (DiStefano *et al.*, 1992) — the somal size distributions of the labeled populations allow for potential overlap between the expression of the corresponding neurotrophin receptors.

Numerical analyses by McMahon and colleagues (1994) of radioautographs of DRG sections processed for *in situ* hybridization indicate that 44%, 27%, and 17% of L_4/L_5 neurons exhibit transcripts for trkA, trkB, and trkC, respectively. Repeating the experiment, but with a cocktail containing probes for all three trks, they show that 34% of these neurons do not express mRNA for any of the receptors. From these findings they conclude that because 88% of neurons express trks (trkA + trkB + trkC), and since 34% do not exhibit detectable trk transcripts, some neurons must express more than one trk species. They further suggest that trkB would be most likely to colocalize with trkA or trkC, since trkB transcripts are present in cells of varying somal diameters, while the other trks show more restricted size distributions.

Direct evidence for the coexpression of trks is provided through the work of two research groups (Wright and Snider, 1995; Karchewski *et al.*, 1999). Using isotopic and nonisotopic *in situ* hybridization protocols to detect two different trk species mRNAs in individual thoracic DRG neurons, Wright and Snider (1995) show that while the majority of trkA-positive cells lack detectable levels of trkC transcripts, coexpression of the receptors occurs in a subset of larger diameter neurons that represents ~ 6% of the trkA-positive, and ~ 15% of the trkC-positive, subpopulations. Surprisingly, given the somal size frequency distributions of DRG neurons that express each of the trks, they did not identify any neurons in which trkB colocalized with either trkA or trkC. More recently, this laboratory (Karchewski *et al.*, 1999) used 5 μm thick serial sections processed for radioisotopic *in situ* hybridization to indicate the subgroups of L_5 DRG neurons expressing trks. Individual neurons were identified in each series of sections and the relative levels of hybridization signal for the trks expressed by these neurons were quantified. Results show that ~ 40%, 30%, and 40% of neurons exhibit transcripts for trkA, trkB, and trkC, respectively, with size distributions corresponding to previous reports (DiStefano *et al.*, 1992; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Kashiba *et al.*, 1996, 1997a, 1998). In addition, coexpression of trkA and trkB, trkA and trkC, and trkB and trkC occurs in ~ 10%, 20%, and 20% of neurons respectively, with transcripts for all three of the trks detectable in 3-4% of the total DRG population. These data indicate that the colocalization of trk mRNAs is more common than the expression of a single trk species in adult rat DRG neurons.

While there is general agreement that 40-45% of DRG neurons display high-affinity NGF binding sites and trkA mRNA (Verge *et al.*, 1990a, 1990b, 1992a; Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Bennett *et al.*, 1996a; Kashiba *et al.*, 1996, 1997a, 1998; Karchewski *et al.*, 1999), there are considerable discrepancies in the reported percentages of neurons expressing, or coexpressing, the other trks. These differences may be attributed to factors that limit the sensitivity of the *in situ* hybridization protocol; low levels of transcripts are more apt to be detected when tissue is fresh-frozen rather than fixed and if radioisotopic, instead of nonisotopic, probes are used. Moreover, disparities may be magnified because unlike

trkA, where message levels tend to be homogeneously expressed, greater numbers of trkB- and trkC-positive DRG neurons exhibit variable levels of hybridization signal for the respective trks (Karchewski *et al.*, 1999) and, therefore, in some cells transcript levels may be below the level of detection of some protocols.

2.1.3 p75 in Primary Sensory Neurons

The neurotrophin receptor, p75 is detectable in migrating mouse neural crest cells (Fariñas *et al.*, 1998), and hybridization signal levels are high in DRG between E11.5 and P1 (Schecterson and Bothwell, 1992). Transcripts are also present in adult rat DRG (Verge *et al.*, 1992a; Sebert and Shooter, 1993; Wetmore and Olson, 1995; Wright and Snider, 1995; Zhou *et al.*, 1996; Karchewski *et al.*, 1999), and p75 mRNA is widely distributed in human PNS (Yamamoto *et al.*, 1996). Message and protein are expressed heterogenously, both in the level of labeling intensities and in the size range of neurons exhibiting the marker (Verge *et al.*, 1992a; Zhou *et al.*, 1993, 1996; Wetmore and Olson, 1995; Wright and Snider, 1995; Karchewski *et al.*, 1999), with reported percentages of p75-positive cells for L_4/L_5 adult rat DRG neurons ranging from 60[2]-80%[1,3] ([1]Wetmore and Olson, 1995; [2]Zhou *et al.*, 1993, 1996; [3]Karchewski *et al.*, 1999). Further, the distribution of p75 appears to closely correlate with trk expression; few neurons express one type of neurotrophin receptor and lack the other (Wright and Snider, 1995; Karchewski *et al.*, 1999).

2.2 Neurotrophin-3

2.2.1 Sources of Neurotrophin-3 for Primary Sensory Neurons

2.2.1.1 Neurotrophin-3 in Developing Primary Sensory Neurons

That NT-3 is expressed in early developing CNS (Maisonpierre *et al.*, 1990a) is consistent with studies illustrating the presence of trophic factors in the neural tube, necessary to the survival of migrating neural crest cells (reviewed in: Kalcheim, 1996). Following migration and early in DRG formation, NT-3 has been identified in target tissues (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992; Copray and Brouwer, 1994; White *et al.*, 1996), but at this developmental time point

trkC-positive DRG cells (Ernfors *et al.*, 1993; Mu *et al.*, 1993; Elkabes *et al.*, 1994; Lamballe *et al.*, 1994; Williams and Ebendal, 1995; Fariñas *et al.*, 1996) do not have contact with target tissues, and therefore, cannot access NT-3 from these sources. Although reports vary as to the initial developmental stage at which NT-3 mRNA is detectable in mouse (Schecterson and Bothwell, 1992; Ernfors *et al.*, 1993; ElShamy and Ernfors, 1996; Tojo *et al.*, 1996), the neurotrophin has been shown to be present at low levels as early as E11 using an RNase protection assay (ElShamy and Ernfors, 1996). By E15.5, 10-15% of mouse primary sensory neurons display low levels of hybridization signal for NT-3 (Schecterson and Bothwell, 1992), but by E17.5 the marker is no longer detectable in DRG using *in situ* hybridization (Schecterson and Bothwell, 1992). Further, by P15, levels of NT-3 mRNA expression are below the detection limit of RNase protection assays (ElShamy and Ernfors, 1996). Similar developmental patterns of expression have been reported in embryonic rat DRG (Ernfors and Persson, 1991; Elkabes *et al.*, 1994). These data suggest that during development NT-3 is produced within DRG and consequently may act locally on DRG neurons, although such interactions may be more important in early DRG since levels of the neurotrophin decrease postnatally.

2.2.1.2 Neurotrophin-3 in Adult Primary Sensory Neurons

Even though NT-3 mRNA has been identified in regions of adult CNS (Ernfors *et al.*, 1990b; Maisonpierre *et al.*, 1990a; Kokaia *et al.*, 1993), there are conflicting reports as to the presence of the neurotrophin in adult DRG. Using *in situ* hybridization, three research groups were unable to detect NT-3 mRNA in adult murine lumbar DRG (Ernfors *et al.*, 1990b; Wetmore and Olson, 1995; Mearow, 1998), nor was message for the neurotrophin detected in similar tissue in either RNase or ribonuclease protection assay studies (ElShamy and Ernfors, 1996; Shen *et al.*, 1999). In contrast, one laboratory has shown the presence of both NT-3 transcripts and protein in adult rat DRG, although the mRNA and protein appear to label two different subsets of neurons (Zhou and Rush, 1995; Chen *et al.*, 1996; Zhou *et al.*, 1999a). In lumbar DRG many large, and some small, neurons exhibit low levels of *in situ* hybridization signal for NT-3 (Zhou *et*

al., 1999a); whereas NT-3-ir is localized to ~ 40% of DRG neurons, primarily those with large perikarya (Zhou and Rush, 1995; Chen *et al.*, 1996). There is a positive correlation between NT-3-ir and the trkC-positive subpopulation, with trkC-ir colocalizing in 94% of NT-3-ir neurons, implying that the presence of NT-3-ir is due to internalization of the neurotrophin after trkC activation (Chen *et al.*, 1996). Further, this group has shown that even though satellite cells do not normally express NT-3 mRNA, 48 hours after sciatic nerve transection, and for at least 2 weeks after, NT-3 message levels are upregulated within cells surrounding a subset of large, and some small, neuronal soma in ipsilateral DRG (Zhou *et al.*, 1999a). Moreover, a second group has used reverse transcriptase-polymerase chain reaction (RT-PCR) to demonstrate the presence of low levels of NT-3 mRNA in human DRG (Yamamoto *et al.*, 1996). The localization of NT-3 transcripts to mature DRG neurons and satellite cells suggests potential autocrine and/or paracrine roles for the neurotrophin.

2.2.1.3 Neurotrophin-3 in Target Tissues

Although transcripts for NT-3 have been identified in mature DRG (Yamamoto *et al.*, 1996; Zhou *et al.*, 1999a), the primary source of NT-3 for primary sensory neurons may be target-derived. In adult rats, within 1 day of sciatic nerve transection NT-3 protein accumulates inside axons of the distal stump; levels return to normal values by 3 days post-surgery (Nitta *et al.*, 1999). For both the proximal and distal nerve segments, axotomy does not appear to alter NT-3 message levels, nor is there an injury-induced change in the preexisting low NT-3 protein levels in Schwann cells (Nitta *et al.*, 1999). These findings indicate that in the intact state, although NT-3 mRNA is present in sciatic nerve, significant levels of NT-3 protein appear to be retrogradely transported in many peripheral nerve axons, and thus may function in sensory neuron maintenance.

An *in situ* hybridization study involving developing mouse embryos shows that NT-3 mRNA is present in skin dermal mesenchyme at moderate to high levels between E11.5 and E15.5; by E17.5 signal levels are weak and fall to below detectable values by P1 (Schecterson and Bothwell, 1992). NT-3 is not detectable in hair follicles between

E11.5 and E12.5, but by E14.5 levels are strong; transcripts decline to moderate levels by E15.5, decrease further by E17.5, and fall below the limits of detection by P1 (Schecterson and Bothwell, 1992). In skeletal muscle NT-3 mRNA is present at moderate to high levels between E11.5 and E15.5 (Schecterson and Bothwell, 1992; Griesbeck *et al.*, 1995), but message expression rapidly falls below detectable values by E17.5 (Schecterson and Bothwell, 1992). Transcripts of the neurotrophin are evidenced in the spinal cord ventral horn at high levels in mouse motoneurons from E11.5 to P1 (Schecterson and Bothwell, 1992) and in rat at E13/13.5 (Ernfors and Persson, 1991; Elkabes *et al.*, 1994).

In adult chicken a range of peripheral tissues exhibit NT-3 mRNA, including skin, muscle, intestine, heart, liver, thymus, and lung; the highest message levels are present in kidney and spleen (Maisonpierre *et al.*, 1990a). Similarly, in adult murine tissues, NT-3 transcripts are abundantly expressed in skeletal muscle, kidney glomeruli, submandibular gland, and ovary (Ernfors *et al.*, 1990b; Funakoshi *et al.*, 1993; Griesbeck *et al.*, 1995); lower levels of transcripts of the neurotrophin have been localized to sciatic nerve and spinal cord (Funakoshi *et al.*, 1993; Nitta *et al.*, 1999). In humans, NT-3 mRNA distribution and expression is also comparable to that observed in other organisms, with transcripts present at high levels in nonneuronal tissues of liver, spleen, muscle and at low levels in sciatic nerve (Yamamoto *et al.*, 1996). The presence of the neurotrophin in these tissues supports a role for target-derived NT-3 in the maintenance of adult primary sensory neurons.

2.2.2 Roles of Neurotrophin-3 in Primary Sensory Neurons

2.2.2.1 Neurotrophin and Neurotrophin Receptor Mutants

2.2.2.1.1 Neurotrophin-3 Mutants

In an attempt to better understand the biological role of NT-3, studies were conducted on mice with a targeted mutation of the NT-3 gene, engineered such that these animals lack functional neurotrophin ($NT\text{-}3^{-/-}$). At birth these animals appear normal, but show abnormal limb movements and positioning, decreased activity, no evidence of food uptake, and typically die within 24 hours (Ernfors *et al.*,

1994b; Fariñas *et al.*, 1994; Tessarollo *et al.*, 1994). Histological examinations indicate that NT-3$^{-/-}$ mice exhibit a substantial loss of peripheral sensory neurons (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994, 1998; Tojo *et al.*, 1995; ElShamy and Ernfors, 1996; Liebl *et al.*, 1997), with a 70-80% reduction in the number of lumbar DRG neurons over wild-type (Fariñas *et al.*, 1994; Liebl *et al.*, 1997; Tessarollo *et al.*, 1997). Not only are limb proprioceptive afferent fibers missing in NT-3 deficient animals (Ernfors *et al.*, 1994b; Kucera *et al.*, 1995b), they also lack the corresponding peripheral sensory receptors, muscle spindles (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994; Kucera *et al.*, 1998; Matsuo *et al.*, 2000), Golgi tendon organs (Ernfors *et al.*, 1994b), and Ia fiber projections to the spinal cord ventral horn (Fariñas *et al.*, 1994; Tessarollo *et al.*, 1994). Further, although present during early development, two subtypes of mechanoreceptors, Merkel cells and D-hair receptors, are essentially lacking in P14 NT-3$^{-/-}$ mice; other kinds of cutaneous sensory receptors appear normal (Airaksinen *et al.*, 1996). In addition, while there are no obvious alterations in the proportions of trkA- and trkB-expressing neurons in DRG (Tessarollo *et al.*, 1994), there are reported reductions in (Tojo *et al.*, 1995), or loss of (Tessarollo *et al.*, 1994), the trkC-positive subpopulation. Even though there is conspicuous limb proprioceptive dysfunction, NT-3$^{-/-}$ mice are capable of responding to tail pinch stimuli, implying normal nociceptive innervation (Tessarollo *et al.*, 1994). Moreover, there are no gross deficits in Pacinian corpuscles (Ernfors *et al.*, 1994b; Airaksinen *et al.*, 1996), deep nerve fibers to joint capsules and tendons (Ernfors *et al.*, 1994b), cutaneous afferents (Tessarollo *et al.*, 1994), or in the number and phenotype of motoneurons (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994; Tessarollo *et al.*, 1994; Kucera *et al.*, 1995a; Tojo *et al.*, 1995). NT-3$^{-/-}$ mice further genetically altered to express NT-3 only in muscle cells exhibited normal development of Ia afferents, which underscores the importance of target-derived NT-3 to these neurons (Wright *et al.*, 1997).

Interestingly, in contrast to the reported complete loss of limb proprioceptive afferents and muscle spindles in NT-3$^{-/-}$ mice (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994; Kucera *et al.*, 1998; Matsuo *et al.*, 2000), cranial proprioceptive neurons of the trigeminal mesencephalic nucleus and corresponding sensory receptors are present, although numbers are reduced by ~ 60% over wild-type (Fan *et al.*, 2000); similar but

less dramatic results have been reported for trkC$^{-/-}$ mice (Matsuo *et al.*, 2000). The presence of trigeminal mesencephalic nucleus proprioceptive neurons in NT-3$^{-/-}$ and trkC$^{-/-}$ mice may be attributed to the coexpression of trkB and trkC in wild-type animals, thereby allowing these cells to respond to multiple neurotrophins (Fan *et al.*, 2000; Matsuo *et al.*, 2000).

Mice with heterozygous expression of the mutant NT-3 gene (NT-3$^{+/-}$) do not exhibit any obvious morphological or behavioral abnormalities (Fariñas *et al.*, 1994), but microscopic examination reveals a loss of DRG neurons intermediate between wild-type and NT-3$^{-/-}$ animals (Airaksinen and Meyer, 1996; Krimm *et al.*, 2000) and an ~ 50% loss of muscle spindles (Ernfors *et al.*, 1994b). These data suggest that there is a gene-dose effect for NT-3, such that the number of nonmutated alleles reflects the severity of phenotypic alterations.

Similar to NT-3$^{-/-}$ animals, transgenic mice that overexpress NT-3 through the nestin gene, which encodes an intermediate filament widely expressed in the developing nervous system, show a severe inability to coordinate limb movement (Ringsted *et al.*, 1997). Histologically, although there is no apparent loss of DRG neurons, with both the number of trkC-positive neurons and the proportion of SP- and CGRP-positive cells consistent with wild-type, these animals lack peripheral and central Ia projections and muscle spindles (Ringsted *et al.*, 1997). That the overexpression of NT-3 results in some alterations in sensory phenotype similar to those of NT-3$^{-/-}$ mice is surprising, and may in part result from placing the control of NT-3 expression under the widely expressed nestin gene, but these results underscore the importance of NT-3 in the differentiation of proprioceptive neurons and the formation of Ia projections and their sensory receptors. Using another transgenic model of NT-3 overexpression, in which epidermal keratinocytes have been genetically engineered to heterozygously express the neurotrophin, a second research group has demonstrated that this manipulation results in a greater than 40% increase in the number of DRG neurons compared to control (Albers *et al.*, 1996). In addition, this paradigm shows that overexpression of NT-3 increases the density of both dermal and epidermal innervation, notably in association with hair follicles and touch dome mechanoreceptors (Albers *et al.*, 1996). The latter receptors are

also enlarged and have greater numbers of associated Merkel cells compared to those of control animals (Albers *et al.*, 1996). Further, these researchers have shown that overexpression of NT-3 in the skin of NT-$3^{+/-}$ mice prevents the ~ 30% loss of L_4/L_5 DRG neurons evidenced in NT-3 deficient heterozygous animals (Krimm *et al.*, 2000). Moreover, the reduction in numbers of Merkel cells associated with touch domes and the loss of associated sensory innervation witnessed in NT-$3^{+/-}$ mice are restored to values similar to those of animals modified only to overexpress NT-3 (Krimm *et al.*, 2000).

2.2.2.1.2 trkC Mutants

Mice genetically engineered to lack trkC, either through a targeted mutation in the tyrosine kinase domain of the receptor ($trkC_{kinase}^{-/-}$) (Klein *et al.*, 1994) or altered to be devoid of both the functional kinase-containing and truncated isoforms ($trkC_{all}^{-/-}$) (Liebl *et al.*, 1997; Tessarollo *et al.*, 1997), have been generated to gain insights into the biological role of this NT-3 receptor. The resulting mutant animals appear normal at birth, but are smaller than wild-type at P4, and most do not survive past P21 (Klein *et al.*, 1994; Liebl *et al.*, 1997; Tessarollo *et al.*, 1997). In addition, they show the abnormal limb movement and positioning indicative of proprioceptive deficits (Klein *et al.*, 1994; Liebl *et al.*, 1997; Tessarollo *et al.*, 1997). More specifically, as for the NT-3 mutants (see Section 2.2.2.1.1), $trkC^{-/-}$ mice lack muscle spindles (Kucera *et al.*, 1998), do not possess proprioceptive muscle afferents (Klein *et al.*, 1994; Matsuo *et al.*, 2000), and have fewer large diameter myelinated axons in the dorsal roots and posterior columns of the spinal cord (Klein *et al.*, 1994). Numbers of lumbar DRG neurons from both $trkC_{kinase}^{-/-}$ and $trkC_{all}^{-/-}$ animals show a 19% (Klein *et al.*, 1994) and a 33-38% (Liebl *et al.*, 1997; Tessarollo *et al.*, 1997) reduction over wild-type, respectively; that $trkC_{all}^{-/-}$ mice appear to exhibit a greater loss of DRG neurons may indicate an important role in sensory neuron development for the truncated isoform, different from that of $trkC_{full\text{-}length}$ (Tessarollo *et al.*, 1997).

2.2.2.1.3 Other Neurotrophin and Neurotrophin Receptor Mutants

Mice exhibiting a disrupted region in the tyrosine kinase domain of trkA ($trkA^{-/-}$), appear normal at birth, but by P10 are substantially smaller than their littermates and at P20 only about half are still surviving, with none living past P55 (Smeyne *et al.*, 1994). $trkA^{-/-}$ animals exhibit a number of sensory deficits: These mutants fail to respond to nociceptive stimuli (Smeyne *et al.*, 1994) and show extensive reductions in numbers of thoracic and lumbar DRG neurons, a 70-90% loss compared to wild-type, with a preferential loss of small cells (Smeyne *et al.*, 1994; Minichiello *et al.*, 1995; Silos-Santiago *et al.*, 1995). In addition, 3-4 weeks postnatally $trkA^{-/-}$ mice begin to develop skin abnormalities and ulcerations, the pathology of which intensifies with age (Smeyne *et al.*, 1994). Similar to $trkA^{-/-}$ animals, $NGF^{-/-}$ mice fail to survive neonatal stages, show an inability to respond to noxious mechanical stimuli, and exhibit an ~ 70% loss of primarily small-sized peptidergic DRG neurons (Crowley *et al.*, 1994). Mice heterogeneous for NGF ($NGF^{+/-}$) are capable of responding to painful stimuli, although they are not as sensitive to nociceptive stimuli as wild-type animals (Crowley *et al.*, 1994).

As is the case for $trkC^{-/-}$ and $trkA^{-/-}$ mutants, mice with a targeted mutation in the tyrosine kinase domain of trkB ($trkB_{kinase}^{-/-}$) initially appear normal, but die soon after birth (Klein *et al.*, 1993). The death of the trkB deficient animals may be exacerbated by their apparent inability to intake food (Klein *et al.*, 1993). In addition, their DRG (T_{12}-L_3) are reduced in size and exhibit a 20-30% neuronal loss compared to wild-type (Klein *et al.*, 1993; Minichiello *et al.*, 1995), and these mice do not respond to mechanical stimulation (Klein *et al.*, 1993). At birth, $BDNF^{-/-}$ mice are smaller in size than wild-type, exhibit abnormal coordination of movement and balance, and most die within 2 weeks (Ernfors *et al.*, 1994a; Jones *et al.*, 1994; Conover *et al.*, 1995). DRG from BDNF deficient animals are also smaller than normal and, as for $trkB^{-/-}$ mice, there is a loss of ~ 30% of neurons over wild-type numbers (Ernfors *et al.*, 1994a; Jones *et al.*, 1994). In contrast, the appearance and gross behavior of $NT\text{-}4^{-/-}$ mice are similar to those of wild-type at birth and, interestingly, these animals continue to survive and can produce viable offspring (Conover *et al.*, 1995; Liu *et al.*, 1995; Erickson *et al.*, 1996). Although at 2 months after birth there is no apparent difference in the number of trkB-positive neurons

between NT-4$^{-/-}$ mutants and control animals, the former exhibit an ~ 14% loss of L_4 DRG neurons (Liu *et al.*, 1995).

Double trk mutants generated through the intercrossing of single trk$^{-/-}$ mice also show distinctive patterns of neuronal loss compared to wild-type, with the effect of the double mutations apparently additive — roughly analogous to the combined neuronal loss of the corresponding single trk mutants (Minichiello *et al.*, 1995). The double mutants trkA$^{-/-}$/trkC$^{-/-}$, trkA$^{-/-}$/trkB$^{-/-}$, and trkB$^{-/-}$/trkC$^{-/-}$ exhibit 93%, 78%, and 41% loss of P1 L_4 DRG neurons, while the losses from trkA$^{-/-}$, trkB$^{-/-}$, and trkC$^{-/-}$ animals are 73%, 20%, and 17%, respectively (Minichiello *et al.*, 1995).

Mice with a targeted mutation in the ligand binding region of p75 (p75$^{-/-}$), and are therefore presumed to lack functional receptors (Lee *et al.*, 1992), allow for further investigation into the biological role of neurotrophins in sensory neurons. The p75$^{-/-}$ embryos are normal in appearance, and in contrast to the homozygous neurotrophin- and trk-knockout mice (with the exception of NT-4$^{-/-}$ animals), the mutation does not result in neonatal lethality (Lee *et al.*, 1992). Histological examination indicates that p75$^{-/-}$ mice exhibit diminished sensory fiber innervation to skin; the reduction appears to involve small diameter afferents, with fewer SP- and CGRP-positive fibers present in mutant animals than in wild-type (Lee *et al.*, 1992). In addition, these animals show marked deficits in their ability to respond to noxious heat stimuli (Lee *et al.*, 1992). Also, the majority of p75$^{-/-}$ animals develop severe skin edemas and ulcerations; these initially form in the extremities, progressing to more proximal regions, and ultimately result in secondary infections and the loss of epidermis in the areas involved (Lee *et al.*, 1992).

2.2.2.1.4 Summary of Neurotrophin and Neurotrophin Receptor Mutant Data

Germline-targeted mice, generated through rendering a specific gene nonfunctional by targeted mutagenesis, provide the opportunity to identify the biological roles of specific molecules *in vivo* (Gerlai, 1996), and studies of neurotrophin- and neurotrophin receptor-knockout mutant animals have yielded

interesting results (reviewed in: Klein, 1994; Snider, 1994; Conover and Yancopoulos, 1997).

All of the neurotrophin- and trk-knockout mice show an important similarity in that all display neuronal abnormalities that tend to occur within definable subpopulations of sensory neurons. NT-3$^{-/-}$, NGF$^{-/-}$, and BDNF$^{-/-}$ mice all exhibit specific severe neurological deficits and early postnatal death (Crowley *et al.*, 1994; Ernfors *et al.*, 1994a, 1994b; Fariñas *et al.*, 1994, 1998; Jones *et al.*, 1994; Tessarollo *et al.*, 1994, 1997; Conover *et al.*, 1995; Kucera *et al.*, 1995b, 1998; Tojo *et al.*, 1995; ElShamy and Ernfors, 1996; Liebl *et al.*, 1997; Matsuo *et al.*, 2000); NT-4/5$^{-/-}$ mutants do not die early in development and although they have some neurological defects, these do not appear to be as compromising as those of the other neurotrophin-knockout animals (Conover *et al.*, 1995; Liu *et al.*, 1995; Erickson *et al.*, 1996). Further, the data support unique roles for the trks, since distinct from trkB$^{-/-}$ mutants (Klein *et al.*, 1993; Minichiello *et al.*, 1995), trkC$^{-/-}$- and trkA$^{-/-}$-knockout mice are able to feed normally; while unlike trkA$^{-/-}$ animals (Smeyne *et al.*, 1994; Minichiello *et al.*, 1995; Silos-Santiago *et al.*, 1995), trkC$^{-/-}$ mutants are responsive to painful stimuli (Klein *et al.*, 1994; Liebl *et al.*, 1997; Tessarollo *et al.*, 1997). The data also demonstrate a relationship between the number of surviving DRG neurons and the level of neurotrophin available, with numbers for NT-3$^{+/-}$ (Ernfors *et al.*, 1994b; Airaksinen and Meyer, 1996; Krimm *et al.*, 2000) and NGF$^{+/-}$ (Crowley *et al.*, 1994) animals intermediate between those of the homozygous mutant counterparts and wild-type. In addition, experiments comparing numbers of DRG neurons between double trk mutants and the corresponding single trk$^{-/-}$ animals show that the loss is roughly additive (Minichiello *et al.*, 1995).

Despite the knowledge gained from these studies, there are negative aspects in using knockout mice: Firstly, germline-targeted studies reflect the developmental importance of the gene of interest and this may obscure its role in the adult animal. Secondly, the alteration of one gene may result in the downstream alteration of a number of other gene products in an attempt to compensate for the direct effect of the mutation — these changes may ultimately be reflected in complex phenotypic modifications that are unlike wild-type, and not related to the functioning of the selected mutant gene.

Additionally, these studies do not allow for potential interactions between neurotrophin species or between these molecules and other trophic factors (i.e. FGF, PDGF, CNTF; see Section 1.1.1). Moreover, the use of knockout models that alter the tyrosine kinase portion of trk, but retain the ligand-binding domain, still allows for a potentially functional receptor, similar to a truncated isoform (see Section 1.1.2.1.1).

To more fully understand the biological role of the neurotrophins and their receptors, data from the knockout studies must be combined with that gleaned through other means. This could include data from inducible gene targeting experiments wherein the gene of interest may be switched on or off at defined stages during development or in specific tissues; as well as from studies concerning the spatial and temporal expression patterns of both the ligands and their receptors, from tissue culture experiments characterizing the biological activities of the neurotrophins, and from the *in vivo* use of neutralizing antibodies or other means to deplete and augment neurotrophin availability.

2.2.2.2 Biological Functions of Neurotrophin-3 in Primary Sensory Neurons

NT-3 is an important neurotrophin during sensory neuron development, as evidenced through targeted mutation studies (see Section 2.2.2.1). While early in embryogenesis NT-3 and other trophic molecules have been shown to promote the *in vitro* proliferation of neural crest cells, the progenitors of sensory neurons (Kalcheim *et al.*, 1992; reviewed in: Kalcheim, 1996) (see Section 2.1.1.1), it is during gangliogenesis, at two distinct time points, that the presence of NT-3 appears critical to normal development (Wright *et al.*, 1992; Gaese *et al.*, 1994; Tessarollo *et al.*, 1994; Oakley *et al.*, 1995, 1997; ElShamy and Ernfors, 1996; Fariñas *et al.*, 1996; Lefcort *et al.*, 1996; ElShamy *et al.*, 1998; Zhou *et al.*, 1998).

In E11/11.5 mouse lumbar DRG, prior to the onset of target-dependent cell death, practically all cells, including differentiated neurons express abundant trkC mRNA (Lamballe *et al.*, 1994; Phillips and Armanini, 1996; White *et al.*, 1996) and have the potential to access local sources of NT-3 (Schecterson and Bothwell, 1992; Ernfors *et al.*, 1993; ElShamy and Ernfors, 1996; Fariñas *et al.*, 1996; Tojo *et al.*, 1996).

At this stage, NT-3 appears to increase the number of neurons, possibly through the stimulation of progenitor cell differentiation (Wright *et al.*, 1992; ElShamy and Ernfors, 1996; Fariñas *et al.*, 1996; Lefcort *et al.*, 1996; ElShamy *et al.*, 1998). Two approaches, based on the use of NT-3$^{-/-}$ mice and antiserum to trkC, have been employed to identify the role of the neurotrophin at this stage of DRG development. The reported 70-80% loss (see Section 2.2.2.1.1) of surviving primary sensory neurons in neonatal NT-3$^{-/-}$ mice compared to wild-type animals (Fariñas *et al.*, 1994; Liebl *et al.*, 1997; Tessarollo *et al.*, 1997) prompted several research groups to closely examine neuronal loss in these animals at specific developmental time points. In DRG of NT-3$^{-/-}$ mice, the loss appears to be due to an overall increase in neuronal apoptosis and an increase in neurogenesis that results in the depletion of the precursor pool, and ultimately in reduced differentiation of postmitotic neurons (ElShamy and Ernfors, 1996; Fariñas *et al.*, 1996; ElShamy *et al.*, 1998). These observations suggest that in the early stages of DRG formation NT-3 is a critical factor in precursor cell differentiation: the neurotrophin promotes the formation of mature, postmitotic primary sensory neurons, with its absence leading to precursor cell apoptosis (ElShamy *et al.*, 1998). In a second experimental paradigm adapted to understand the role of NT-3 in DRG development, daily *in ovo* injections of trkC antiserum were used to neutralize the receptor *in situ*. Beginning at the time when neural crest cell migration was complete, the injections resulted in an almost 50% depletion in numbers of neurons compared to control (Lefcort *et al.*, 1996). The deficit was attributed to either a loss of neuronal precursors or of postmitotic neurons (Lefcort *et al.*, 1996), and the work serves to further underscore the importance of NT-3 early in DRG development. In a seemingly contradictory study, early treatment of chicken embryos with NT-3 resulted in a substantial decrease in the number of DRG neurons compared to control (Ockel *et al.*, 1996a, 1996b). The loss was not restricted to any neuronal subtype and appeared to be due to a decrease in the number of proliferating neuroblasts in treated embryos (Ockel *et al.*, 1996a, 1996b). The reported neuronal loss following NT-3 treatment may be attributed to the premature differentiation of neuronal precursors which resulted in the inability of these cells to proliferate, hence a depleted precursor pool, and, therefore, ultimately in reduced numbers of neurons.

Later in DRG development, during apoptosis and continuing postnatally, trkC mRNA expression is limited to a subset of neurons (Lamballe *et al.*, 1994; Fariñas *et al.*, 1996; Phillips and Armanini, 1996; White *et al.*, 1996), and it is during this period of cell death that the influence of NT-3 appears to be restricted to the trkC-positive subpopulation (Gaese *et al.*, 1994; Tessarollo *et al.*, 1994; Oakley *et al.*, 1995, 1997; Zhou *et al.*, 1998). These neurons may have access to ganglion-derived NT-3 (Yamamoto *et al.*, 1996; Zhou *et al.*, 1999a), but the principal source seems to be provided by target tissues (see Section 2.2.1). The use of a monoclonal antibody to block the biological activity of NT-3 during the completion of gangliogenesis in quail sensory DRG has been shown to result in an ~ 30% reduction in the number of neurons (Gaese *et al.*, 1994). Likewise, in neonatal rat, administration of anti-NT-3 for 2 weeks effected the loss of a subset of large diameter DRG neurons, representing ~ 20% of the total population (Zhou *et al.*, 1998). Interestingly, also in neonatal animals, when the majority of primary sensory neurons have made contact with target tissues, the role of NT-3 is further limited, with this neurotrophin no longer being necessary for their survival (Memberg and Hall, 1995).

Experiments involving the use of NT-3, anti-NT-3, and NT-3$^{-/-}$ mice have identified the functional subset of neurons most affected by the neurotrophin prior to target innervation (Tessarollo *et al.*, 1994; Oakley *et al.*, 1995, 1997; Zhou *et al.*, 1998). For example, injection of anti-NT-3, to deplete the neurotrophin from peripheral tissues during apoptosis in embryonic chicken DRG, resulted in decreased numbers of muscle spindle afferents; the survival of cutaneous sensory neurons and motoneurons did not seem to be affected (Oakley *et al.*, 1995). In addition, the use of a fluorescent marker to retrogradely label sensory and motor neurons of skin and axial muscle in wild-type and NT-3$^{-/-}$ mice showed that, unlike wild-type, mutant animals lack demonstrable muscle spindle (Ia) afferents (Tessarollo *et al.*, 1994). That cutaneous afferents appeared normal is indicative of a selective loss of proprioceptive neurons in NT-3$^{-/-}$ mice (Tessarollo *et al.*, 1994). Further, the removal of a limb bud early in chicken development, which eliminates ~ 90% of ipsilateral trkC-positive DRG neurons, followed by daily treatments of the embryo with NT-3 late in apoptosis, resulted in the rescue of Ia afferents despite

the lack of other limb-derived signals (Oakley *et al.*, 1997). The NT-3 induced effect appeared to be specific, since treatment with the neurotrophin reestablished the normal number of trkC-expressing neurons, whereas there was no difference in the number of trkA- or trkB-positive cells between experimental and control animals (Oakley *et al.*, 1997). These findings, in conjunction with data from gene-target based experiments (see Section 2.2.2.1), point towards the importance of NT-3 in supporting proprioception. Further, in experiments that utilized two different fluorescent markers to label neurons innervating chick skin and muscle, and where these cells were subsequently cultured, NT-3 was found to promote the survival of almost twice as many muscle sensory afferents than NGF (Hory-Lee *et al.*, 1993). Conversely, NGF supported double the number of cutaneous sensory neurons (Hory-Lee *et al.*, 1993). Interestingly, even though these data indicate a bias towards the ability of specific neurotrophins to effect the viability of specific neuronal populations as defined by target tissues, the work also shows that exogenous NT-3 is able to sustain a subset of cutaneous sensory neurons (Hory-Lee *et al.*, 1993). An *in vivo* study yielded similar findings (Oakley *et al.*, 2000). Moreover, in adult rat, although trkC mRNA was detected in 73% of DRG neurons projecting to gastrocnemius muscle, 17% of cutaneous afferents (saphenous nerve) and a small subgroup of visceral afferents expressed transcripts for the receptor (McMahon *et al.*, 1994). In another study on adult rat, while 30% of DRG neurons projecting to tibial muscle were shown to exhibit NT-3-ir, additional sensory neurons displaying NT-3-ir projected to tendon, whisker hair follicles, subdermis/epidermis, and kidney (39%, 50%, 17%, and 1%, respectively) (Zhou and Rush, 1995). NT-3 is also critical to the normal development of at least two mechanoreceptor subtypes — D-hair receptors and Merkel cell touch domes (Airaksinen *et al.*, 1996; Fundin *et al.*, 1997b; Rice *et al.*, 1998; Zhou *et al.*, 1998). Thus, although many NT-3 responsive DRG neurons subserve proprioception, there are also cutaneous afferents, specifically subtypes of mechanoceptors, that depend on the neurotrophin.

In addition to supporting the survival of proprioceptive neurons and the development of at least two mechanoceptor subtypes, NT-3 has been shown to modulate

aspects of sensory neuron phenotype, affect neurite outgrowth/regeneration, and to have the potential to influence sensory neuropathies.

Several studies have examined the role of NT-3 in regulating neuropeptide expression in DRG neurons. In one experiment on adult rats, a silicone reservoir containing saline or NT-3 was attached for 3 weeks to the proximal transected sciatic nerve stump and the level of NPY in DRG neurons was compared between the two treatments (Ohara *et al.*, 1995). Exposure to the neurotrophin resulted in a reduction in the number of NPY-ir neurons and decreased staining intensities for the neuropeptide (Ohara *et al.*, 1995). Further, radioimmumoassays demonstrated that there was a 36% reduction in NPY protein levels (Ohara *et al.*, 1995). Similarly, animals with entubulated, NT-3 impregnated fibronectin mats grafted between the proximal and distal ends of transected and resected sciatic nerve showed an ~ 45% decrease in the number of NPY-ir L_4/L_5 DRG neurons compared to control (fibronectin mats not exposed to NT-3) 30 days post-injury (Sterne *et al.*, 1998). This treatment with NT-3 did not appear to alter SP, CGRP, or VIP levels (Sterne *et al.*, 1998). Nor was SP expression in DRG neurons affected after direct application of NT-3 to axotomized sciatic nerve, although a combined exposure to NT-3 and BDNF counteracted the injury-induced reduction in SP (Zhang *et al.*, 1995a). Moreover, neither SP, CGRP, VIP, nor SOM protein levels were found to be significantly changed following the treatment of adult rat DRG cultures with NT-3 (Mulderry, 1994). In general, given the size ranges of DRG neurons in which these neuropeptides are principally expressed compared to that of the trkC-positive subpopulation, these results are not necessarily unexpected.

SP and CGRP are normally associated with nociception (Klein *et al.*, 1992; Mense *et al.*, 1994; Wiesenfeld-Hallin *et al.*, 1994; Neumann *et al.*, 1996; Cao *et al.*, 1998; De Felipe *et al.*, 1998; Parker and Grillner, 1998; Nichols *et al.*, 1999; Bennett *et al.*, 2000a; Zhang *et al.*, 2001) and both are expressed in normal adult animals. Between 17-30% of DRG neurons are reported to exhibit SP transcripts and/or protein (Boehmer *et al.*, 1989; Bergman *et al.*, 1996; Ji *et al.*, 1996; Hall *et al.*, 1997; Ma and Bisby, 1998b); their perikarya are primarily small in size (Wiesenfeld-Hallin *et al.*, 1984; Dalsgaard *et al.*, 1985; Skofitsch and Jacobowitz, 1985; Ju *et al.*, 1987; Boehmer *et al.*,

1989; McCarthy and Lawson, 1989; Anand *et al.*, 1991; Villar *et al.*, 1991; Noguchi *et al.*, 1994; 1995; Verge *et al.*, 1995; Bergman *et al.*, 1996). CGRP mRNA and protein have been identified in neurons of all sizes (Rosenfeld *et al.*, 1983; Ju *et al.*, 1987; Noguchi *et al.*, 1990a, 1990b; Nothias *et al.*, 1993; Verge *et al.*, 1995), representing 35-50% of the total DRG population (Ju *et al.*, 1987; McCarthy and Lawson, 1989; Nothias *et al.*, 1993; Zhou *et al.*, 1993; Minichiello *et al.*, 1995; Bergman *et al.*, 1996; Groves *et al.*, 1996; Kashiba *et al.*, 1997a). The two peptides colocalize in a large subset of adult rat DRG neurons (Wiesenfeld-Hallin *et al.*, 1984; Skofitsch and Jacobowitz, 1985; Gibbins *et al.*, 1987; Ju *et al.*, 1987; Villar *et al.*, 1989), and virtually all SP- and most CGRP-positive cells also display high-affinity NGF binding sites (Verge *et al.*, 1989a). NGF has been shown to upregulate protein and message levels of both peptides (Kessler and Black, 1980; Otten *et al.*, 1980; Otten and Lorez, 1982; Fitzgerald *et al.*, 1985; Lindsay and Harmer, 1989; Inaishi *et al.*, 1992; Verge *et al.*, 1995; Ji *et al.*, 1996) and, because ~ 50% of trkA-positive neurons also coexpress trkC (Karchewski *et al.*, 1999), one might have expected NT-3 to have the potential to modulate SP and/or CGRP levels. Moreover, even though protein and mRNA levels of both markers decrease after nerve injury (Jessell *et al.*, 1979; Tessler *et al.*, 1985; Shehab and Atkinson, 1986; Villar *et al.*, 1989, 1991; Noguchi *et al.*, 1990b, 1993, 1994, 1995; Klein *et al.*, 1991; Inaishi *et al.*, 1992; Hökfelt *et al.*, 1993; Verge *et al.*, 1995; Groves *et al.*, 1996; Ji *et al.*, 1996; Ma and Bisby, 1998a, 1998b), SP expression appears to be induced in a relatively small subpopulation of medium to large diameter neurons (Noguchi *et al.*, 1994, 1995; Ji *et al.*, 1996; Ma and Bisby, 1998b). These cells project to the gracile nucleus (Noguchi *et al.*, 1995), part of the posterior column-medial lemniscus pathway, the principal ascending tract conveying proprioceptive information, and therefore may be responsive to NT-3 (see Section 2.2.2.1). In addition, while the majority of SP-positive DRG neurons are lacking in $trkA^{-/-}$ mice, a subset of SP-expressing, apparently trkA-independent neurons is present (Minichiello *et al.*, 1995). At least a portion of this group of neurons appear to be trkC-dependent, since there are fewer of them in $trkA^{-/-}/trkC^{-/-}$ mice than in $trkA^{-/-}$, $trkC^{-/-}$, $trkA^{-/-}/trkB^{-/-}$, $trkB^{-/-}/trkC^{-/-}$, or wild-type animals (Minichiello *et al.*, 1995). CGRP does not appear to be dependent on trkC, as the expression of this

neuropeptide is identical in wild-type and trkC$^{-/-}$ mice (Minichiello *et al.*, 1995). On the other hand, CGRP is not present in trkA$^{-/-}$ animals, or in trkA$^{-/-}$/trkB$^{-/-}$ and trkA$^{-/-}$/trkC$^{-/-}$ mice (Minichiello *et al.*, 1995). VIP and NPY are rarely expressed in DRG neurons of normal animals (Ju *et al.*, 1987; Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Ohara *et al.*, 1994; Verge *et al.*, 1995; Corness *et al.*, 1996; Ji *et al.*, 1996; Thompson *et al.*, 1998b; Li *et al.*, 1999b; Landry *et al.*, 2000), but after nerve injury VIP is detectable in many small- to medium-sized cells (Ju *et al.*, 1987; Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Ohara *et al.*, 1994; Verge *et al.*, 1995) while many large and some medium diameter neurons exhibit NPY mRNA and protein (Wakisaka *et al.*, 1991, 1992; Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Ohara *et al.*, 1994; Verge *et al.*, 1995; Corness *et al.*, 1996; Thompson *et al.*, 1998b; Cougnon-Aptel *et al.*, 1999; Li *et al.*, 1999b; Shi *et al.*, 1999; Landry *et al.*, 2000). Again, given the predominant somal size distributions of neurons expressing these neuropeptides, the NPY-positive subpopulation would be the most likely to be responsive to NT-3.

Another regulatory peptide, pituitary adenylate cyclase-activating polypeptide (PACAP), believed to function in neuronal survival, regeneration, and nociception (Morio *et al.*, 1996; Campard *et al.*, 1997; Lioudyno *et al.*, 1998), is also influenced by NT-3 (Jongsma Wallin *et al.*, 2001). In adult rat, under normal conditions, 25-30% of L_4-L_6 DRG neurons display PACAP-ir (Zhang *et al.*, 1996; Jongsma *et al.*, 2000), while ~ 50% of neurons express detectable PACAP mRNA (Jongsma Wallin *et al.*, 2001). The soma of the majority of PACAP-positive neurons are of small diameter and are heavily labeled for the neuropeptide (Zhang *et al.*, 1996; Jongsma *et al.*, 2000; Jongsma Wallin *et al.*, 2001). Sciatic nerve transection results in increased numbers of neurons exhibiting PACAP mRNA (~ 85%) (Jongsma Wallin *et al.*, 2001) and protein (50-60%) (Zhang *et al.*, 1996; Jongsma *et al.*, 2000). This subset now includes many medium and some large diameter cells (Zhang *et al.*, 1996; Jongsma *et al.*, 2000; Jongsma Wallin *et al.*, 2001), although the small PACAP-positive perikarya display reduced levels of hybridization signal compared to uninjured neurons (Jongsma Wallin *et al.*, 2001). A 1 week intrathecal infusion of NT-3, 2 weeks after axotomy resulted in lowered levels of PACAP hybridization signal, and an ~ 20% reduction in the total number of PACAP-

positive neurons compared to DRG from axotomized, non-infused animals (Jongsma Wallin *et al.*, 2001). These results are not necessarily surprising since ~ 25% of DRG neurons, representing ~ 50% of trkC-positive cells, were shown to coexpress PACAP and trkC mRNAs; these cells rarely exhibited the neuropeptide at moderate or high levels (Jongsma Wallin *et al.*, 2001). Post-axotomy, levels of trkC mRNA decreased, although the proportion of trkC-positive neurons remained similar to control values; conversely, PACAP mRNA levels were elevated following injury, and the neuropeptide was detected in most trkC-expressing neurons (Jongsma Wallin *et al.*, 2001). What is surprising is that the infusion of NT-3 reversed injury-induced message levels of the neuropeptide in both the trkC-positive and trkC-negative subpopulations, albeit most strongly in the former group (Jongsma Wallin *et al.*, 2001). Also of note, NT-3 infusion in normal, non-axotomized animals resulted in both decreased levels of PACAP mRNA and in an ~ 40% loss of PACAP-expressing neurons (Jongsma Wallin *et al.*, 2001). This NT-3 effected depression of PACAP signal levels appeared to occur in both neurons that expressed trkC and in those that lacked detectable trkC transcripts, but was most evident in the subset of trkA-positive/trkC-negative cells — those that normally express the highest levels of the neuropeptide (Jongsma Wallin *et al.*, 2001).

NT-3 has also been shown to be a potential regulator of calcium-binding proteins, including parvalbumin, calbindin, and calretinin (Copray *et al.*, 1994; Friedel *et al.*, 1997; Zhou *et al.*, 1998), which serve to restrict, redistribute, and reestablish physiological intracellular calcium levels (reviewed in: Miller, 1991, 1992; Bainbridge *et al.*, 1992). These proteins are expressed by subsets of DRG neurons (Carr *et al.*, 1989a, 1989b; Rogers, 1989; Antal *et al.*, 1990; Celio *et al.*, 1990; Zhang *et al.*, 1990; Duc *et al.*, 1993b, 1994; Ichikawa *et al.*, 1993, 1994, 1999, 2000; Kiraly *et al.*, 1993; Copray *et al.*, 1994; Ambrus *et al.*, 1998; Iino *et al.*, 1998) and are efficient regulators of calcium-dependent activity within these subpopulations (Chard *et al.*, 1993). Calbindin-, parvalbumin-, and calretinin-ir are present in 22% (Carr *et al.*, 1989b), ~ 15% (Carr *et al.*, 1989b), and less than 8% (Ambrus *et al.*, 1998) of rat DRG neurons respectively, and all of these markers predominately label large diameter perikarya (Carr *et al.*, 1989b; Antal *et al.*, 1990; Duc *et al.*, 1993b, 1994; Ichikawa *et al.*, 1993, 1994; Kiraly *et al.*,

1993). These three calcium-binding proteins exhibit distinct and overlapping distributions among DRG neurons (Carr *et al.*, 1989b; Rogers, 1989; Duc *et al.*, 1993b, 1994; Copray *et al.*, 1994; Ichikawa *et al.*, 1994): calbindin-ir and calretinin-ir frequently colocalize in large diameter cells (Rogers, 1989; Duc *et al.*, 1994), and, similarly, virtually all neurons that display parvalbumin-ir are also calbindin-positive (Carr *et al.*, 1989b; Ichikawa *et al.*, 1994). Only ~ 30% of parvalbumin-ir neurons also coexpress calretinin-ir, but ~ 70% of calretinin-positive cells exhibit parvalbumin-ir; the soma of the former subgroup are large in size, while small calretinin-ir neurons are parvalbumin-negative (Ichikawa *et al.*, 1994). Further, most calretinin-positive neurons are also calbindin-positive (Duc *et al.*, 1993b, 1994). All three markers have putative roles in mechanoeceptors, including proprioceptors (Duc *et al.*, 1993a, 1994; Ichikawa *et al.*, 1999). Several lines of evidence point to the ability of NT-3 to regulate levels of calcium-binding proteins. In one experiment, the injection of anti-NT-3 for 2 weeks in postnatal rats, to neutralize endogenous NT-3 late in sensory development, resulted in decreased levels of calbindin- and calretinin-ir and reduced numbers of parvalbumin-ir DRG neurons compared to control (Zhou *et al.*, 1998). In another study, where dissociated E16 rat DRG neurons were cultured in the presence of neurotrophins, ~ 75% of cells displayed parvalbumin-ir following NT-3 treatment, while less than 10% were positive for parvalbumin after exposure to NGF or BDNF (Copray *et al.*, 1994). In addition, embryonic DRG explants exposed to exogenous NT-3 showed significantly higher levels of parvalbumin and calretinin transcripts compared to those treated with NGF (Friedel *et al.*, 1997). The importance of NT-3 to parvalbumin expression is further demonstrated by the lack of detectable immunoreactivity for this marker in DRG of NT-$3^{-/-}$ mice (Ernfors *et al.*, 1994b). In contrast, the expression of the others may be independent, or less dependent on NT-3, given the presence of calretinin-ir in primary sensory neurons of NT-$3^{-/-}$ animals (Airaksinen and Meyer, 1996). Moreover, in dissociated embryonic DRG cultures treated with NGF, BDNF, or NT-3, all three neurotrophins supported survival and neurite outgrowth in similar percentages (10-17%) of calretinin- and calbindin-ir neurons (Copray *et al.*, 1994). That calbindin, parvalbumin, and calretinin are at least somewhat influenced by NT-3 is not surprising,

given that these markers label subsets of neurons similar in perikaryal size and in sensory function to the trkC-positive subpopulation.

The expression of two other markers, GAP-43 and Tα1 α-tubulin, have been shown to be modulated by NT-3 (Mohiuddin *et al.*, 1995). GAP-43 (B-50, F1, pp46, pp57, neuromodulin), a protein associated with axonal growth and regeneration (reviewed in: De Graan *et al.*, 1986; Basi *et al.*, 1987; Benowitz and Routtenberg, 1987; Skene, 1989), is abundantly expressed during development (Jacobson *et al.*, 1986; Basi *et al.*, 1987; Karns *et al.*, 1987); levels decline postnatally (Hoffman, 1989), but are upregulated after injury and remain high during regeneration (Skene and Willard, 1981; Hoffman, 1989; Tetzlaff *et al.*, 1989; Verge *et al.*, 1990b; Wiese *et al.*, 1992; Schreyer and Skene, 1993; Groves *et al.*, 1996; Verkade *et al.*, 1996). In adult rat lumbar DRG, under normal conditions, a subset of primarily small neurons exhibit GAP-43 mRNA and/or protein, representing 40-70% of the total population (Verge *et al.*, 1990b; Schreyer and Skene, 1993; Liabotis and Schreyer, 1995; Groves *et al.*, 1996; Andersen and Schreyer, 1999). Axotomy results in a dramatic upregulation of GAP-43, with virtually all axotomized neurons now exhibiting it at detectable levels (Hoffman, 1989; Verge *et al.*, 1990b; Tetzlaff *et al.*, 1992; Wiese *et al.*, 1992; Schreyer and Skene, 1993; Liabotis and Schreyer, 1995; Andersen and Schreyer, 1999). Like GAP-43, Tα1 α-tubulin, a tubulin isotype, is also expressed in rat at high levels during growth and following injury in developing and mature neurons (Miller *et al.*, 1987, 1989, 1991; Mathew and Miller, 1990, 1993; Ma *et al.*, 1992; Wu *et al.*, 1993). In an *in vitro* study on dissociated cultures of adult rat DRG neurons, NT-3 (10 ng/ml) treatment increased message levels for GAP-43 by over 2 fold and Tα1 α-tubulin by over 3 fold (Mohiuddin *et al.*, 1995). Interestingly, exposing the neurons to higher concentrations of NT-3 (50 ng/ml) resulted in mRNA levels for these markers being only slightly higher than those seen in untreated cultures (Mohiuddin *et al.*, 1995).

The ability of the neurotrophin to modulate GAP-43 and Tα1 α-tubulin suggests that NT-3 may not only be important in maintaining or regulating aspects of neuronal phenotype, but that it may also play a significant role in axonal growth and regeneration. Indeed, in the study described above, exogenous NT-3 was shown to enhance neurite

outgrowth in a subset of neurons with large diameter soma, although the degree of NT-3 induced outgrowth was greater at 10 ng/ml than at 50 ng/ml (Mohiuddin *et al.*, 1995). Similarly, exogenous NT-3 has been demonstrated to increase neurite outgrowth in cultures of developing sensory neurons (Maisonpierre *et al.*, 1990a; Lentz *et al.*, 1999). Despite these findings, the role of NT-3 in promoting neurite extension, as in its regulation of GAP-43 and Tα1 α-tubulin levels (Mohiuddin *et al.*, 1995), seems to be more complex. *In utero* injection of rat embryos with NT-3 appeared to either have no influence on or to inhibit axonal growth, depending on the developmental stage of the animal (Zhang *et al.*, 1994a). The degree of projection of Ia afferent axons into the spinal cord of embryos injected with NT-3 at E14 and E15, and then sacrificed at E16, was ~ 50% less than that of control animals (Zhang *et al.*, 1994a). In animals treated with the neurotrophin at E15 and E17, and then sacrificed at E19, NT-3 did not appear to influence Ia axonal projections into the spinal cord (Zhang *et al.*, 1994a). A significant difference between the two developmental stages may be that in the rat at E14, unlike E15, the Ia afferent axons have not yet penetrated the spinal cord grey matter (Snider *et al.*, 1992; Zhang *et al.*, 1994a), although the potential importance of this to the ability of NT-3 to modulate axonal growth is unclear. Exogenous NT-3 (50 ng/ml) also supported increased axonal growth in adult mouse L_4/L_5 DRG explant cultures (Edström *et al.*, 1996), but the addition of NT-3 (1, 10, or 100 ng/ml) to the distal portion of compartmentalized cultures of adult rat DRG neurons failed to induce neurite outgrowth (Kimpinski *et al.*, 1997). Neither did the treatment of parallel cultures with both NT-3 and NGF alter the growth-inducing effects of NGF alone (Kimpinski *et al.*, 1997). Likewise, NT-3 treatment of cultures of dissociated adult rat lumbar DRG neurons did not appear to influence axonal growth, although this research group found that cotreatment with NT-3 and NGF partially blocked NGF-enhanced neurite outgrowth (Gavazzi *et al.*, 1999). Together, these data indicate that although NT-3 can influence axonal growth — in a positive or negative manner, or not at all — the effect may be influenced by the concentration of the neurotrophin, the means of neurotrophin delivery, the presence of additional neurotrophic factors, and/or the level of maturity of the neuron at the time of neurotrophin exposure.

An increasing number of studies provide evidence for a role for NT-3 following injury of sensory neurons. In adult rat, spinal nerve transection results in the death of a significant number of DRG neurons, but treatment with NT-3 post-axotomy, whether through intrathecal infusion (Ljunberg *et al.*, 1999) or a reservoir attached to the proximal nerve stump (Groves *et al.*, 1999), dramatically reduced neuron loss. Similarly, delivery of NT-3 to lesioned spinal cord reversed injury-induced cell atrophy and substantially increased the number of surviving neurons compared to control (Bradbury *et al.*, 1998). Moreover, NT-3 appears to support peripheral nerve regeneration. In adult rats, entubulated fibronectin mats impregnated with NT-3 (500 ng/ml) grafted to resected sciatic nerve supported the regeneration of significantly more myelinated axons than control, nonimpregnated mat grafts, even after 8 months post-insertion (Sterne *et al.*, 1997a).

Peripheral axotomy also alters the conduction velocities of injured neurons; transection of the tibial nerve resulted in a progressive reduction in axonal conduction velocities at 1, 3, and 5 weeks post-injury (Munson *et al.*, 1997). Delayed infusion of NT-3 to the proximal nerve stump mitigated axotomy-induced changes in conduction velocities, but the effect was dose dependent (Munson *et al.*, 1997). A 2 week infusion of 6 μg/day 3 weeks post-transection had no significant consequence, whereas similar treatment with 60 μg/day reestablished conduction velocity values intermediate between those of uninjured and 5 week axotomized axons (Munson *et al.*, 1997). Moreover, conduction velocities in axons of animals treated with NT-3 for 4 weeks (60 μg/d), 1 week after nerve injury were similar to, or higher than, those recorded 1 week post-transection in untreated animals (Munson *et al.*, 1997). In addition, a 2 week infusion of trkC-IgG (60 μg/d) into muscle, to sequester endogenous NT-3 in target tissue, reflected the axotomy-induced effect of lowering axonal conduction velocities of sensory neurons (Munson *et al.*, 1997).

Another injury-induced change following peripheral axotomy is the sprouting of at least a subset of Aβ sensory fibers into laminae I and II of the spinal cord dorsal horn (Woolf *et al.*, 1992a; Shortland and Woolf, 1993; Bennett *et al.*, 1996b, 1998; Eriksson *et al.*, 1997; White, 1997b, 1998, 2000). In uninjured animals, the central axons of Aβ

neurons project to laminae III and IV (Shortland *et al.*, 1989), whereas nociceptive neurons normally terminate in lamina II (Sugiura *et al.*, 1986). The post-trauma sprouting of the Aβ fibers is important since if they can form functional synapses in lamina II then these neurons might play a role in nerve injury-induced hyperalgesia (Woolf *et al.*, 1992a). Because Aβ fibers are potentially responsive to NT-3 (see above and Sections 2.1.1.2, 2.2.2.1.1, 2.2.2.1.2) several research groups investigated whether exogenous NT-3 could prevent their sprouting (Bennett *et al.*, 1996b; Eriksson *et al.*, 1997; White, 1997b, 1998). Neither a capsule containing NT-3 (27 μg) implanted in juxtaposition to the transected sciatic nerve (Eriksson *et al.*, 1997) nor intrathecal infusion of the neurotrophin (1 mg/ml, 14 d) (Bennett *et al.*, 1996b) prevented or reduced the trauma-induced sprouting. In contrast, intrathecal infusion of NT-3 antisense oligonucleotide appeared to attenuate the Aβ fiber sprouting (White, 2000). Moreover, intrathecal infusion of NT-3 (200 ng/d, 14 d) in normal, uninjured rats mirrored the Aβ fiber trauma-induced sprouting into lamina II, albeit to a lesser degree (White, 1997b, 1998). Further, NT-3 infusion also decreased the mechanical nociceptive threshold in uninjured animals (White, 1997b, 1998), whereas the infusion of NT-3 antisense oligonucleotide had an analgesic effect (White, 2000). Both of these NT-3 effected changes in uninjured animals might occur through NPY, because the neuropeptide induces neurite outgrowth *in vitro* (White and Mansfield, 1996; White, 1997b, 1998) which is attenuated by anti-NT-3 (White, 1997b, 1998) and because treatment with exogenous NPY exacerbates injury-induced mechanical hyperalgesia (White, 1997a). These data are not supported by the reports that exogenous NT-3 downregulates NPY mRNA and protein *in vivo* (see above) (Ohara *et al.*, 1995; Sterne *et al.*, 1998). Interestingly, unlike after NT-3 treatment, aberrant Aβ fiber sprouting was significantly reduced by NGF (Bennett *et al.*, 1996b; Eriksson *et al.*, 1997). However the effect appeared to be both dose dependent and to decrease over time: 8 days post-injury the level of sprouting was greatly reduced with both 6 μg and 24 μg encapsulated NGF, but by day 16, sprouting in animals with lower dose NGF appeared similar to control (cytochrome C) and 33 days after capsule implantation, Aβ fiber sprouting under both NGF treatments was indistinguishable from control (Eriksson *et al.*, 1997). If the

Aβ sprouting into laminae I and II is induced by NPY (White and Mansfield, 1996; White, 1997b, 1998), then the capacity of NGF to reduce the injury-associated sprouting (Bennett *et al.*, 1996b; Eriksson *et al.*, 1997) may be due to the ability of NGF to mitigate axotomy-induced levels of the neuropeptide (Verge *et al.*, 1995). On the other hand, collateral sprouting of nociceptive DRG neurons requires NGF; removal of the neurotrophin stops both collateral sprouting and axonal elongation (Diamond *et al.*, 1992a; Mearow and Kril, 1995). Also of interest is that glial cell line-derived neurotrophic factor (GDNF) (Lin *et al.*, 1993, 1994) has been shown to prevent the injury-induced Aβ fiber sprouting into lamina II (Bennett *et al.*, 1998).

The CNS, unlike the PNS, appears to be an unfavorable environment for axonal regeneration but, in spite of this, the difficulties may be overcome, at least to some degree, under certain experimental conditions (McQuarrie, 1973; McQuarrie *et al.*, 1977, 1978; Oblinger and Lasek, 1984; Richardson and Issa, 1984; Bunge *et al.*, 1986, 1989; Richardson and Verge, 1987; Schnell and Schwab, 1990, 1993; Lu and Richardson, 1991; Bajrovic *et al.*, 1994; Midha *et al.*, 1994; Tuszynski and Gage, 1995; Cheng *et al.*, 1996; Torigoe *et al.*, 1996; Bradbury *et al.*, 1999; Neumann and Woolf, 1999; Oudega and Hagg, 1999; Ramer *et al.*, 2000; reviewed in: Fawcett and Keynes, 1990; Schwab, 1990; Richardson, 1991; Schwab and Bartholdi, 1996; Young, 1996). Of relevance here, is the recent finding that NT-3 can support the regrowth of injured dorsal root axons into the spinal cord of adult rats (Zhang *et al.*, 1998; Ramer *et al.*, 2000). Delayed injection of adenoviral vectors containing the genes for NT-3 or LacZ (control) into the spinal cord ventral horn of animals with axotomized dorsal roots resulted in a significant increase in the number of axons that regenerated into the spinal cord, but only in those with the introduced NT-3 gene (Zhang *et al.*, 1998). Similarly, but in a different experimental model, immediate intrathecal infusion of NT-3 (500 ng/h, 7 d) promoted the regrowth of injured dorsal root axons into the spinal cord (Ramer *et al.*, 2000). The effect was selective, with NT-3 favoring regeneration of large diameter sensory fibers and a subset of small, non-peptidergic neurons (Ramer *et al.*, 2000). Significantly, in this study, 4 weeks after axotomy of the central axons of these neurons and the initiation of NT-3 infusion, experimental animals exhibited some functional recovery, since

peripheral nerve stimulation evoked a modest postsynaptic potential, although NT-3 treatment did not influence the responsiveness of these animals to noxious mechanical and thermal stimuli (Ramer *et al.*, 2000). Further, NT-3 treatment also promoted sensory axon regeneration within ascending pathways after spinal cord injury, but again apparently in only a subset of injured neurons (Bradbury *et al.*, 1999; Oudega and Hagg, 1999).

In order to determine potential clinical relevance of the neurotrophins, their efficacy has been tested in several laboratory animal models of sensory neuropathies (reviewed in: McMahon and Priestly, 1995; Anand, 1996; McMahon, 1996; Tomlinson *et al.*, 1996; Woolf, 1996). Given the reported size distribution of trkC-positive DRG neurons (see Section 2.1.1.2), the functional population responsive to NT-3 (see above and Sections 2.2.2.1.1, 2.2.2.1.2), and the ability of NT-3 to partially reverse axotomy-induced changes in injured primary sensory neurons (see above), one might expect NT-3 to have clinical applications. Recently, a number of studies have shown this to be the case (Gao *et al.*, 1995; Rodriguez-Pena *et al.*, 1995; Tomlinson *et al.*, 1996, 1997; Helgren *et al.*, 1997; Fernyhough *et al.*, 1998; Mizisin *et al.*, 1998). Experimentally-induced large fiber sensory neuropathy is evidenced by the degeneration of large diameter DRG neurons, decreased axonal conduction velocities, and a concomitant deficit in proprioception and the capacity to detect vibration. Treatment with NT-3 appears to prevent or reverse nerve fiber degeneration, restore axonal conduction velocities, and attenuate the behavioral deficits (Gao *et al.*, 1995; Helgren *et al.*, 1997; Mizisin *et al.*, 1998). NT-3 has also been shown to be influential in treating experimentally-induced diabetes (Rodriguez-Pena *et al.*, 1995; Tomlinson *et al.*, 1996, 1997; Fernyhough *et al.*, 1998). Although NGF is the principal neurotrophin used to treat induced diabetes, one component of diabetic neuropathy appears to involve the NT-3 responsive subpopulation of primary sensory neurons (reviewed in: Anand, 1996; Tomlinson *et al.*, 1996). Induction of diabetes significantly reduces NT-3 mRNA levels in nerve (Rodriguez-Pena *et al.*, 1995), as well as in muscle, the primary target of many large fiber DRG neurons (Tomlinson *et al.*, 1996, 1997; Fernyhough *et al.*, 1998), but administration of insulin restores NT-3 mRNA levels (Tomlinson *et al.*, 1996, 1997;

Fernyhough *et al.*, 1998) and treatment with NT-3 counteracts the diabetic-induced reduction in axonal conduction velocities (Tomlinson *et al.*, 1997).

In summary, although NT-3 responsive DRG neurons project primarily to muscle, they also innervate skin and viscera, and the neurotrophin appears critical to the survival and/or normal development of proprioceptors and several other subtypes of mechanoceptors. Further, the apparent ability of NT-3 after injury to increase neuronal survival, effect regeneration, restore reduced conduction velocities, and to mitigate axotomy-induced NPY and PACAP levels points to an important role for the neurotrophin in the maintenance of normal neuronal phenotype and as a potential therapeutic molecule, for at least a subpopulation of DRG neurons.

3. MATERIALS AND METHODS

3.1 Surgery

All surgical procedures, with the exception of perfusions, were performed by Dr. V. M. K. Verge and were conducted in accordance with the animal care guidelines of the University of Saskatchewan under protocol number 9200164. Over the course of this project, 112 animals were sacrificed.

3.1.1 Axotomy

Adult Wistar rats (150-200 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The analgesic buprenorphine (0.1-0.2 mg/kg) was injected just prior to surgery, and then every 12 hours for 2-3 days. The right leg, from pelvis to knee joint, of each animal was shaved and the area wiped with 70% (v/v) ethanol. An incision was made lateral to the midline of the vertebral column, 5 mm caudal and 15 mm rostral to the right iliac crest, and a portion of the crest was rongeured to expose the sciatic nerve. The nerve was then transected with microscissors at its origin from the L_4 and L_5 spinal nerves; a 5 mm segment was resected to prevent nerve regeneration. Transections at this level should result in the axotomy of greater than 95% of L_5 DRG neurons (Swett *et al.*, 1991). Axotomies were performed on a total of 76 animals.

3.1.2 Mini-Osmotic Pumps

3.1.2.1 Pump Preparation and Assembly

3.1.2.1.1 Preparation of Pump Catheters

Two sizes of silicone tubing were used to make pump catheters: 0.635 mm inner diameter X 1.194 mm outer diameter and 0.305 mm inner diameter X 0.635 mm outer diameter (Silastic brand medical grade silicone tubing; Dow Corning Corp., Midland, MI). A razor blade was used to cut the larger diameter tubing into 0.8 cm lengths and the smaller diameter tubing into 3 cm segments. For each catheter unit, one end of the smaller diameter tubing was beveled. This end was inserted into a length of the larger diameter tubing, such that the lumen of the smaller was fully ensheathed within the larger. Silicone glue (non-sterile Silastic brand medical adhesive, silicone type A; Dow Corning Corp.) was applied around this joint and the unit was left to dry. The catheters were then placed between two pieces of thin cardboard, wrapped in foil, and autoclaved prior to pump assembly.

3.1.2.1.2 Pump Filling and Assembly

Mini-osmotic pumps (Alzat model 2001; output of 1 µl/hour for 7 days; Alza Pharmaceuticals, Palo Alto, CA) were filled and assembled under aseptic conditions 12-18 hours prior to implantation. Initial experiments were conducted by Regeneron Pharmaceuticals (Tarrytown, NY), and then by this laboratory, using 300 ng NT-3/µl of vehicle. These experiments involved a total of 29 animals (16: Regeneron Pharmaceuticals; 13: Univ. Saskatchewan) and showed that to achieve consistent results a higher concentration of the neurotrophin was needed. It was decided that the pumps should contain a working concentration of 600 ng NT-3/µl of vehicle.

Stock recombinant methionyl human NT-3 (1.1 ml aliquots of 1 mg/ml in sterile distilled water, stored at -70 °C; Regeneron Pharmaceuticals) was thawed on ice; the required volume was removed and diluted with sterile 2X vehicle (see below) to a working concentration of 600 ng/µl. The volume of NT-3 needed was calculated based on using 300 µl of diluted solution for each pump. Any remaining NT-3 stock was returned to the freezer, but on each occasion that pumps were prepared, a fresh aliquot of stock was used. To prepare 2X vehicle, 2 mg/ml rat serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO) and 20 µl/ml penicillin/streptomycin (10^3 U/ml penicillin G sodium with 10^3 µg/ml streptomycin sulfate; Gibco BRL, Grand Island, NY) were

added to sterile 0.1 M phosphate buffered saline (PBS) (Appendix). The solution was then transferred to a syringe and sterilized by passing through a 0.2 μm pore low-protein binding syringe filter (Acrodisc, Gelman Sciences Inc., Ann Arbor, MI). The fluid for filling the control pumps was made by adding the appropriate volume of 2X vehicle to an equal volume of sterile 0.1M PBS. NT-3 working solution was prepared by adding the calculated volume of NT-3 stock to the appropriate volume of sterile 2X vehicle and then passing this through a syringe and syringe filter that had been preconditioned with sterile vehicle.

Each pump was assembled from three parts: The pump body, the flow moderator, and the catheter. The flow moderator was removed from the sterile packet and the plastic end-cap was removed to reveal a short stainless steel tube. Sterile forceps were used to gently pull a sterile catheter down the length of the metal shaft and this arrangement was secured with sterile suture. The packet containing the sterilized pump body was then carefully opened to expose the top of the pump; the pump was left enclosed within the package. A 1 cc syringe (preconditioned with sterile vehicle) was filled with sterile solution and the sterile blunt-tipped filling tube (supplied with the pumps) was attached. Then, while holding the packet containing the pump body in an upright position, the body was filled with ~ 280 μl of solution and the long metal shaft of the flow moderator was fully inserted into the pump. In a properly assembled pump, the fluid was visible within the catheter after the flow moderator was in place. The assembled pump was placed in a sterile 50 cc polypropylene tube of PBS such that the body of the pump was submerged and the open end of the catheter was above the level of the buffer. Experimental and control pumps were placed in separate tubes and the tubes were stored at 4 °C until the pumps were implanted.

Pumps were filled with either vehicle alone (control) or vehicle plus NT-3 (experimental). For each run, control pumps were prepared first and then the same sterile syringe and pump needle were used to fill the experimental pumps.

3.1.2.2 Pump Implantation

Mini-osmotic pumps were implanted two weeks after axotomy (38 experimental, 2 control). Additional experimental pumps were implanted in animals that had not undergone sciatic nerve transection — when infused, but non-axotomized tissue, was desired (18 experimental, 2 control).

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the analgesic buprenorphine (0.1-0.2 mg/kg) was injected just prior to surgery, and then every 12 hours for 2-3 days. The back of each animal was shaved and the area wiped with 70% ethanol. An incision was made along the midline of the lumbosacral region and blunt tipped scissors were used to separate the skin from the muscle layer to create a pocket for the pump. A scalpel was used to cut through the back muscles on either side of the vertebral column and then, using the sacrum as a landmark, a laminectomy was performed at $sacral_2$. A cavity, 2-3 mm in diameter, was rongeured through the vertebra at the midline to expose the dura and a small slit was made through it with fine forceps. Prior to pump implantation, a sterile towel (with a central 10 cm X 3 cm hole) was placed over the animal, with the opening centered over the incision. A pump was then placed on the aseptic towel, caudal to the incision, and the catheter inserted through the opening in the dura so that a 1.5 cm portion was positioned in the subarachnoid space. While maintaining the placement of the catheter, the pump was carefully flipped laterally, positioned, and secured to the back muscles with two sutures around the pump body and one around the tubing-enclosed shaft of the flow moderator. Finally, the incision over the pump was sutured closed. Pumps were left in place for one week. At the time of sacrifice, animals were checked to ensure the correct positioning of the catheter and for visible signs of infection or inflammation; these animals were not included in further analysis.

3.1.3 Surgery Controls

Radioautographs of tissue processed for *in situ* hybridization (see Section 3.2) were qualitatively examined under dark-field illumination and it was determined that for the biochemical markers examined here, ganglia contralateral to the axotomy were comparable to DRG from non-operated animals. Therefore, in this work, unless

otherwise noted, DRG contralateral to the axotomized sciatic nerve were considered to represent "normal" or "intact" tissue.

The efficacy of the sciatic nerve transection was indicated by elevated levels of cjun, GAP-43, VIP, and/or NPY mRNAs in the injured DRG, over that of the uninjured, control tissue. It was more difficult to ascertain the efficacy of the transection in NT-3 infused animals because of potential alterations in message levels following infusion; although since SOM and VIP mRNA levels were apparently not affected by NT-3, the lack of SOM, and the presence of VIP, expression indicated that the axotomy was successful. The results from 1 animal (out of 76) were rejected from further analysis, due to an apparently poor transection.

To determine whether potential effects seen with the infusion of NT-3 were due to the vehicle components or to pump installation, radioautographs of DRG processed for *in situ* hybridization were compared between animals infused with vehicle alone or with NT-3 (Figure 3.1).

3.2 *In Situ* Hybridization

The technique of radioisotopic *in situ* hybridization, combined with radioautography, results in the formation of silver grains over tissues in which specific radioactive probes have bound to the target mRNA. Here, processing of semi-thin tissue sections allowed for the visualization of the resulting silver grains over individual cells, and computer-assisted image analyses of such tissues were used to determine the relative level of specific mRNAs for individual neurons.

3.2.1 Tissue Preparation

At the appropriate time after axotomy (3 weeks) or osmotic pump implantation (1 week), deeply anesthetized animals were perfused through the aorta in the following manner. The chest area of each animal was shaved and the area wiped with 70% ethanol. Blunt tipped scissors were used to open the chest cavity just below the rib cage and to cut along the ribs on one side; the resulting flap of tissue was clamped with a heavy pair of forceps and the area was then flipped away from the chest cavity. A hole

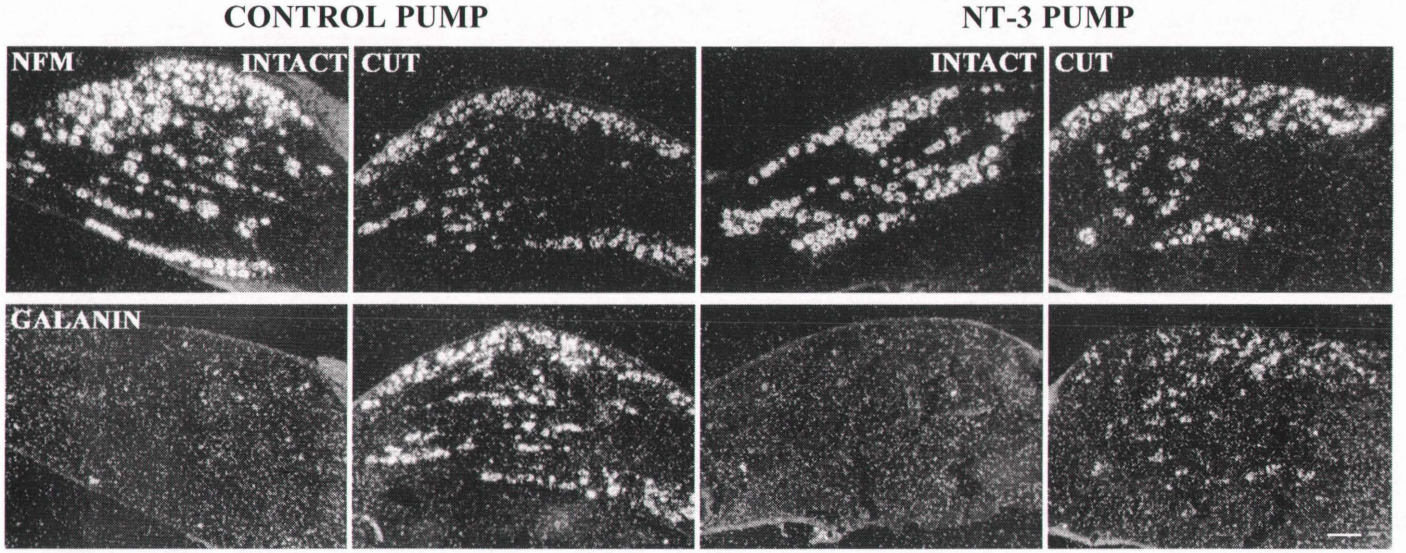

Figure 3.1 Comparison of the effect of intrathecal infusion of NT-3 plus vehicle to that of vehicle alone on mRNA levels in adult rat DRG neurons.

Scanned darkfield photomicrographs of 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization, compare the effect of a 7 day intrathecal infusion of vehicle alone (control pump) and of vehicle plus NT-3 (NT-3 pump, 600 ng/μl/h) under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut) on NFM and galanin mRNA expression. Scale bar = 200 μm.

was made in the pericardium using fine-tipped forceps and the pericardium was gently pulled away to free the heart. The heart was pushed to one side to expose the aorta, which was then clamped with a hemostat. Fatty tissue and mesentery were removed from an area ~ 3 cm from where aorta entered the heart, so that the forceps could be inserted beneath the aorta. With the forceps in position, one end of a 14 cm length of suture was grasped and pulled under the aorta. Microscissors were then used to make a small slit in the aorta in this area. A catheter, made from a 20 G needle inserted into a 13 cm length of polyethylene tubing with a beveled tip (0.86 mm inner diameter X 1.27 mm outer diameter; Intramedic PE-90, Clay Adams, Division of Becton Dickinson and Co., Parsippany, NJ), was then inserted into the aorta and tightly secured in place with the suture. A syringe containing 60 cc of cold 0.1 M PBS was attached to the catheter, a hole was cut in the right atrium, and the cold PBS was slowly pushed through the circulatory system over a 5 minute period. The ipsilateral and contralateral L_4 and L_5 DRG, which contain neurons that contribute to the sciatic nerve (Molander and Grant, 1986), were then sequentially removed. For each pair, care was taken to place the right and left ganglia in the appropriate positions in a prelabeled cryomold (Tissue Tek, Miles Labs., Elkhart, IN). The mold was then filled with OCT compound (Tissue Tek) and the tissue rapidly frozen in a beaker of isopentane cooled in a slurry of acetone and dry ice. The molds containing the frozen tissue were wrapped in aluminum foil, placed in freezer-safe storage bags, and stored at -70 °C. Prior to sectioning, blocks containing the right and left ganglia from the same lumbar region (i.e. L_4 or L_5) for both a control and an experimental animal were removed from the cryomolds and fused with OCT compound, so that further processing of that tissue would occur under identical conditions and to allow the comparison of relative levels of hybridization signal between experimental and control conditions. It was necessary to fuse OCT blocks containing control and experimental tissue, instead of placing the four ganglia into one cryomold, because RNA and protein degradation would have occurred during the time needed to dissect the DRG from the two animals. The resulting block was allowed to come to -20 to -22 °C, before being serially sectioned at 6 μm thickness at the same temperature in a cryostat (Microm HM500; Carl Zeiss Canada, Don Mills, ON). Cutting tissue at this thickness allows the

perikarya of even small diameter neurons to be present and identifiable in at least three consecutive sections. Additionally, the thinner the section the greater the probability that silver grains visualized over an individual perikaryon resulted from the probe binding to mRNA within that neuron (Salpeter *et al.*, 1969, 1978). Sections were manipulated onto cold (-22 °C) Probe-on slides (Fisher Scientific, Pittsburgh, PA) that had been previously labeled in 4H pencil, and were thaw-mounted, by briefly touching a gloved fingertip to the underside of the slide until the OCT compound around the sections visibly thawed. Care was taken to obtain serial sections and to record the beginning and end of each series for each block of tissue. While keeping the sectioned tissue in the cryostat, the slides were placed in cold desiccant-containing slide boxes, and when filled, the boxes were stored at -20 °C until tissue was processed for *in situ* hybridization. Tissue and tissue sections were subsequently stored at -70 °C and -20 °C, respectively, for up to 5 years.

3.2.2 Oligonucleotide Probes

3.2.2.1 Probes

The following oligonucleotide probes were used for *in situ* hybridization studies (synthesized by UCDA, Univ. Calgary, Calgary, AB): rat α-CGRP — bases 664-698 (Amara *et al.*, 1985), cjun — bases 778-825 (Ryseck *et al.*, 1988), rat galanin — bases 152-199 (Vrontakis *et al.*, 1987), rat GAP-43 — bases 70-117 (Karns *et al.*, 1987), rat NFM — bases 1222-1270 (Napolitano *et al.*, 1987), rat NPY — bases 1671-1714 (Larhammar *et al.*, 1987), rat p75 — bases 873-920 (Radeke *et al.*, 1987), rat SP — bases 145-192 (Krause *et al.*, 1987), chicken SNAP-25 — bases 133-179 (Catsicas *et al.*, 1991), rat SOM — nucleotides encoding amino acids 97-107 (Goodman *et al.*, 1983), rat trkA which is the counterpart of bases 1198-1245 of the human trkA sequence (Merlio *et al.*, 1992; Meakin *et al.*, 1992), rat $trkC_{all}$ — bases 1189-1236 (Merlio *et al.*, 1992), rat $trkC_{full\text{-}length}$ — bases 1654-1701 (Merlio *et al.*, 1992), rat $trkC_{no\ insert}$ — bases 2107-2154 (Valenzuela *et al.*, 1993), rat Tα1 α-tubulin — bases 1548-1594 (Lemischka *et al.*, 1981), and rat VIP — bases 347-394 (Nishizawa *et al.*, 1985). The

probes are anti-sense and, therefore, complementary to the mRNAs for which they are specific.

The probes were shipped dry and desalted and were resuspended, under RNase-free conditions, in sterile distilled water so that stock solution concentration was 400 ng/ml and the working concentration was 40 ng/ml. Probe stock solutions were stored at -20 °C indefinitely with the solution being thawed, the necessary volume removed, and the remaining fluid returned to the freezer. Diluted probes were stored at 4 °C for up to 6 months.

3.2.2.2 Three Prime-End Labeling of Probe

The following method was based on that described by Dagerlind and colleagues (1992). All equipment used was RNase-free and gloves were worn at all times during the following procedure to avoid RNase and radioactive contamination. Recipes for the solutions listed are presented in the Appendix.

The following were added in the order listed to a 10 µl aliquot of α-[^{35}S]dATP (Dupont New England Nuclear, Boston, MA) with gentle, but thorough, mixing between each: 2.5 µl 10X cobalt chloride reaction buffer (supplied with the terminal transferase), 8 µl of sterile distilled water, 2 µl of 40 ng/ml oligonucleotide probe, and 3 µl of terminal deoxynucleotidyl transferase (~24 U; Amersham Corp., Arlington Heights, IL). The vial containing terminal transferase was removed from the -20 °C freezer immediately prior to use, but was kept in a labtop cooler (Nalge Nunc International Corp., Naperville, IL) in order to avoid enzyme inactivation. After all ingredients were added, the tube was placed in a 37 °C water bath for 1.5-2 hours. The reaction was stopped by adding 500 µl 0.1 M Tris-HCl (pH 8); the solution was then vortexed and stored at 4 °C until it was purified. For each probe to be purified, an NENSORB 20 column (Dupont New England Nuclear) was prepared by holding the column upright and gently tapping it three times on the counter. The column was then clamped into a stand, a disposable beaker was positioned below, and the protective cap was removed and replaced with a syringe adapter that was supplied with the columns. The column was rinsed by adding 3 ml HPLC grade methanol and then, using constant and gentle

pressure on an air-filled 50 cc syringe, the methanol was pushed through the column bed. Throughout this, and all further steps, column flow rates were not allowed to exceed 1 drop every 2 seconds and the resin bed was not allowed to dry. Next, 3 ml of 0.1 M Tris-HCl (pH 8) was added and subsequently pushed through the column. This step was repeated with the unpurified ^{35}S-labeled probe. After the probe was loaded onto the column, the column bed was washed with 1.5 ml of 0.1 M Tris-HCl (pH 8) as before. The purified probe was eluted by loading 500 µl of freshly prepared 30% (v/v) ethanol onto the column. Pressure was then applied to the air-filled syringe; the initial drop was discarded and the following 12 drops were collected in a sterile 1.5 ml microcentrifuge tube. Dithiothetol (DTT; 1 M stock, stored at -20 °C) was added to the purified probe for a final concentration of 10 mM. After vortexing, a 2 µl sample of the probe was removed and placed in a scintillation vial. Scintillation fluid was then added, and the radioactivity of the sample was determined in order to ascertain the incorporation of ^{35}S-label (LS 6500 Liquid Scintillation System; Beckman Instruments, Inc., Fullerton, CA). The radioactivity of labeled probes used for *in situ* hybridization experiments generally ranged between 350,000 and 500,000 cpm/µl. The labeled purified probes were stored at 4 °C until they were no longer considered to generate strong signal to noise ratios. Probes under 250,000 cpm/µl were not used for *in situ* hybridization studies.

3.2.2.3 Probe Controls

The specificity of the hybridization signal for each probe was determined by hybridizing adjacent sections under the following conditions. A 400-1000 fold excess of a dissimilar unlabeled probe, of the same length and of similar guanine and cytosine content, was combined with the 10^7 cpm/ml hybridization solution of labeled experimental probe (Figure 3.2). For each probe used, no change in the hybridization pattern was observed between that of the experimental probe alone and when an excess of unlabeled dissimilar probe was used in competition. Signal was depleted when tissue was hybridized with solution containing a 400-1000 fold excess of unlabeled probe and 10^7 cpm/ml of the same ^{35}S-labeled probe.

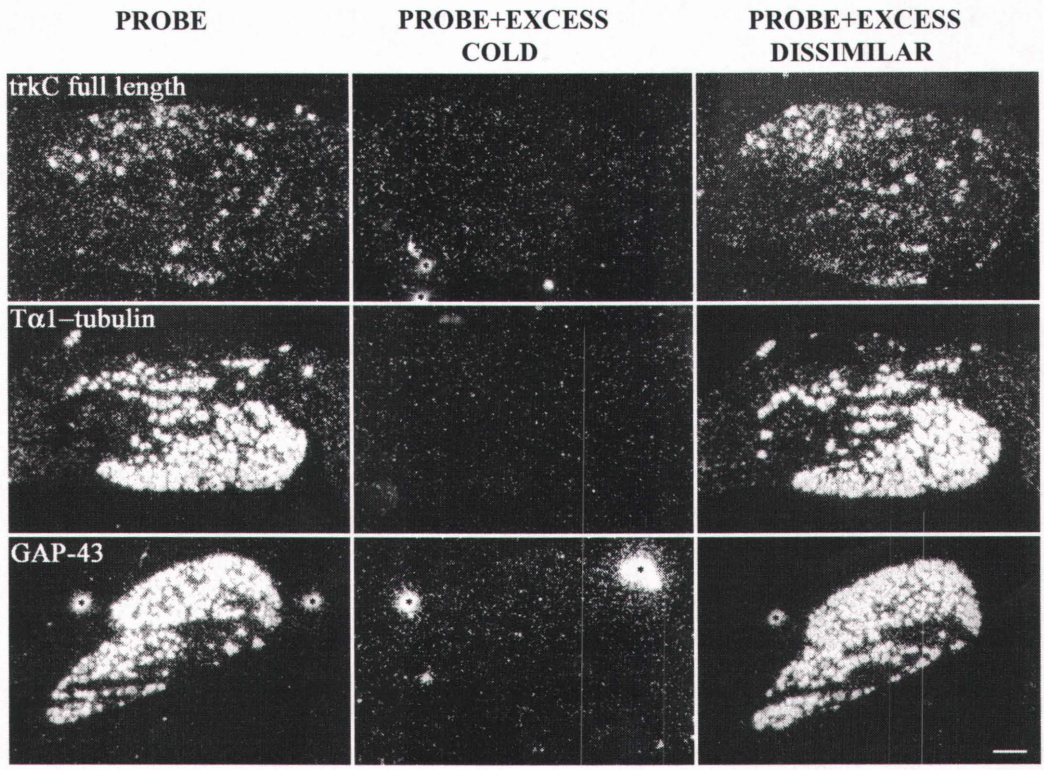

Figure 3.2 Oligonucleotide probe controls.

Scanned darkfield photomicrographs of 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect trkC, Tα1 α-tubulin, and GAP-43 mRNA plus either a 400-1000 fold excess of the same, but unlabeled, probe or a 400-1000 fold excess of a dissimilar unlabeled probe, of the same length and of similar guanine and cytosine content. Scale bar = 200 μm. Asterisks identify artifacts on the slide.

Also, the sequences of all probes were checked against Genbank entries (website: www.ncbi.nlm.nih.gov/BLAST) prior to use, and again prior to thesis preparation, to verify no greater than 65% homology with any other known mRNA sequences.

In addition, the stringency used for the *in situ* experiments (e.g. wash temperature, salt concentration) was such that greater than 90% identity was required for the probe to remain hybridized to the mRNA transcript (Dagerlind *et al.*, 1992).

3.2.3 Hybridization

The following method was based on that described by Dagerlind and colleagues (1992). All equipment used was RNase-free and gloves were worn at all times during the following procedures to avoid RNase and radioactive contamination. Glassware was pretreated with diethyl-pyrocarbonate (DEPC, Appendix) and equipment was treated with RNase ZAP according to manufacturer's directions (Sigma). Recipes for the solutions listed below are presented in the Appendix.

For each probe, the necessary volume of hybridization solution containing ^{35}S-labeled probe was calculated based on 200 µl for each slide pair. At least one slide was included in each run as a hybridization control for each probe used. For each milliliter of solution, 900 µl of hybridization cocktail (43 °C), 50 µl of 10 mg/ml denatured salmon sperm DNA stock (prior to use the vial of frozen stock was placed in boiling water and then submerged in ice water, for 5 minutes each), 10^7 cpm of labeled probe, and 40 µl of 5 M DTT were added in the order as listed and the solution was vortexed between each addition. The hybridization solution was then placed in a 43 °C water bath. Hybridization trays were lined with blotting paper and the paper was then saturated with 1X SSC. Slide boxes containing tissue sections were removed from the freezer, placed in a cold cryostat (-20 to -22 °C), and the desired slides were removed and allowed to equilibrate to room temperature (~ 20 minutes). In a laminar flow hood, and working on a raised surface, 180 µl of hybridization solution was pipetted near the tissue on one slide. A second slide was then gently layered on top of the first so that the DRG sections on each slide were exposed to an even layer of the hybridization solution; the paint on the corners of the Probe-on slides permitted two slides to be "sandwiched" together

during *in situ* hybridization processing. This technique minimized probe usage and evaporation and did not result in tissue damage. When all slides were paired, or the hybridization tray was full, the lid was placed in position and sealed tightly with tape to construct a humidified chamber. The tray was then placed into a 43 °C oven for 16-18 hours. Following hybridization, spatula-tipped forceps were used to transfer a slide pair to a 250 ml beaker of 55 °C 1X SSC. The slides were gently separated and transferred to a glass slide rack submerged in a glass staining dish filled with 55 °C 1X SSC. This procedure was repeated, and when the rack was full it was transferred to another staining dish containing fresh hot (55 °C) 1X SSC and then placed in a 55 °C water bath. The fluid from the original staining dish and beaker was discarded as radioactive waste and the vessels were refilled with hot 1X SSC. This process was continued until all slides were submerged in SSC. Slides were incubated in the water bath for 15 minutes and then the slide racks were transferred to staining dishes containing fresh hot 1X SSC and the dishes replaced in the 55 °C water bath. After four 15 minute incubations in 1X SSC at 55 °C, the dishes containing the slides were removed from the water bath and left at room temperature for an additional 15 minutes. Racks containing slides were then dipped into two staining dishes filled with triple distilled water four times (no longer than 4 seconds in total), followed by nine dips over 9 seconds in 60% ethanol, and then twelve dips over 12 seconds in 95% ethanol. Slides were air-dried for 1-2 hours and then placed in slide boxes containing packets of desiccant. Slides were not overcrowded; one box or more was used for each probe and at least one box was used for each radiography development time point for the hybridization control slides. The slide boxes were stored at room temperature overnight, prior to coating in radiosensitive emulsion.

3.2.4 Radioautography

In a light-tight darkroom and under sodium vapour lamp illumination (Thomas Duplex Super Safelight; Thomas Instruments Co., Inc., Charlottesville, VA), slides processed for *in situ* hybridization were coated with radiosensitive emulsion (Kodak NTB_2, Eastman Kodak Co., Rochester, NY; diluted 1:1 with triple distilled water) in the following manner. The emulsion was melted in a 43 °C water bath, gently

mixed, poured into a dipping container, and returned to the water bath. Control slides and identical probe-labeled slides were processed in consecutive series. In a smooth and steady movement, each slide was dipped into the emulsion and then placed to dry on a rack. After all slides were coated with emulsion, racks were covered to avoid dust contamination and slides were left to dry for 2.5-3 hours. The slides were then replaced in the desiccant-containing slide boxes and the boxes inserted into pre-labeled light-tight bags, which were sealed and incubated at 4 °C for 15 hours to 7-30 days, depending on the probe.

In order to determine the optimal degree of exposure for the radioautography, control slides for each probe were developed at 1, 3, or 7 days; the duration of incubation having been predetermined for each probe. Microscopic examination of the developed control slides for each run of each probe was conducted to determine the time point for the development of the corresponding experimental slides. Development times were chosen to maximize silver grain densities, while allowing for minimal grain overlap, and to permit the identification of those neuronal populations exhibiting low levels of labeling. To develop the radioautography, the emulsion-coated slides were allowed to come to room temperature (20-30 minutes). In a darkroom, under sodium vapour lamp illumination, slide boxes were removed from the light-tight bags and the slides were loaded into glass slide carriers. The carriers were then placed in staining dishes of cooled (18-20 °C), filtered D-19 developer (Eastman Kodak Co.) for 3 minutes, dipped several times in 20 °C running tap water, fixed for 5-6 minutes in filtered Rapid Fixer (Eastman Kodak Co.), and left to rinse for 20 minutes in a basin of 20 °C running tap water. When rinsing was complete, the basin containing the submerged slides was removed from the darkroom. Individual slides were removed from the water, the emulsion scraped from the back of the slide with a single edged razor blade, and the slide placed to dry in a dust-free environment. Dried slides were stored at room temperature. For dark-field photomicroscopy (dark-field stage adapter; Micro Video Instruments, Inc., Avon, MA) sections were mounted in glycerine. If rephotographed under bright-field conditions, the coverslip was removed and tissue was lightly stained with 0.5 (w/v) toluidine blue, dehydrated, cleared in xylene, and mounted with permount and a coverslip.

Alternatively, digital images of the stained ganglia were obtained (Northern Eclipse, Version 2.0; Empix Imaging, Inc., Mississauga, ON). Radioautographs of ganglia on individual slides were qualitatively examined under dark- and/or bright-field illumination for relative differences in silver grain density levels.

3.2.5 Quantification of Relative Levels of mRNA

Radioautographs were examined under dark-field illumination to qualitatively determine potential differences or similarities in relative mRNA levels of a number of proteins between control and experimental conditions. For each probe or series of probes under deliberation, a minimum of 2 representative slides, or consecutive slide series (if colocalization or trilocalization was to be undertaken), were chosen for quantification of relative neuronal grain densities. Care was taken to choose radioautographs that exhibited a compromise between minimizing grain overlap over those neurons with high silver grain densities and maximizing the positive-label to background ratio for neurons expressing low levels of hybridization signal. Care was also taken to choose ganglia with a similar plane of section, so that there was a comparable distribution of neurons throughout each of the sections that were to be compared, in order to minimize potential differences in the topography of subpopulations of DRG neurons.

Photomicrographs (25X, bright-field) of lightly stained DRG sections were taken in series and the resulting enlarged prints were compiled to create montages. For each serial set of montages, those neurons present in all sections were identified and consecutively numbered (on average 229 per ganglion section). Then, as described by Richardson and coworkers (1989), a computer-assisted image analysis system, with a software program developed by W. G. Tatton (Department of Neurology, Mt. Sinai School of Medicine, New York, NY), and modified by P. M. Richardson (Division of Neurosurgery, Montreal General Hospital and McGill Univ., Montreal, PQ), was used to determine relative silver grain densities for the probes under consideration. A digital camera, connected to a microscope and a computer monitor, was used to obtain black and white images of each of the identified neurons under 63X oil immersion

microscopy. If the staining of the tissue was sufficiently dark as to interfere with silver grain visualization, the coverslip was removed after soaking the slide in xylene and sections were then destained in a downgraded ethanol series (100-75%). When the desired staining intensity was reached, sections were dehydrated and coversliped as before. As each neuronal image was obtained, background light was subtracted and the image was "stretched" to maximize the grey scale value range. Then the perikaryal area covered by silver grains for each of the identified cells was determined. To do this, a grain overlay was created for each neuron. Since under bright-field illumination silver grains appear as dark spots, it was necessary to select a minimal grey-shade pixel value so that the resulting grain overlay fitted the majority of grains over an individual neuron. The differential cytoplasmic staining intensity of neurons was the most important factor in determining the need for this adjustment. Although it was necessary to adjust the grain overlay threshold value for each neuron, it was critical that the degree of grain overlay was consistent for all neurons in all of the DRG sections in which relative levels of hybridization signal were to be compared. After the grain overlay threshold was adjusted, the periphery of the neuron, and then the nucleus, were traced and the software program determined the percentage of these areas covered by silver grains. When individual neurons were identified in adjacent sections, the largest perikaryal area was used to determine the diameter of the cell body; calculations were based on the presumption that the perikaryon was spherical. Background labeling was determined by averaging grain counts over 5 areas of neuropil lacking positive-labeled cell bodies. Data files were edited using WordStar 5.5 (MicroPro International, San Rafael, CA) and were later transferred to Microsoft Excel (Microsoft Excel 97 SR-1; Microsoft Corp., 1985-1997) to allow for further analyses. To determine the density of silver grains over perikaryal cytoplasm, "f", the area of the nucleus multiplied by the density of silver grains over the nucleus was subtracted from the area of the cell body multiplied by the density of silver grains over the cell body. The resulting number was then divided by the area of the nucleus subtracted from the area of the cell body. Since the image analysis program calculated grain coverage, a grain overlap correction factor, f/(1 - f), was included in the analysis (Richardson *et al.*, 1989). The corrected parameter, termed

labeling density, reflects the relative level of target mRNA. To avoid negative numbers, total labeling was used without the subtraction of background labeling. Instead, the corrected labeling density was divided by the corrected background density and this value was termed the labeling index of the neuron. Cells with labeling indices of greater than three to ten times background levels were said to be positive for the marker under consideration; for each probe and for each set of ganglia this "cut off" value was chosen to reflect both the level of positive hybridization signal and the degree of nonspecific binding. Scatterplots of labeling indices of the identified neurons were constructed (SPSS for Windows, Release 6.1; SPSS Inc., 1989-1994) to depict the relationship between perikaryal diameter and probe labeling density, or between the labeling indices of two probes, for the entire identified DRG population or for defined subpopulations. For each probe, neuronal labeling indices were compared only between ganglia situated on the same slide because, with the exception of the time taken for dissection and freezing, any further processing of these tissues would have been under identical conditions. For individual slides, differences such as the thickness of the radiosensitive emulsion, or the temperature and/or duration of the post-hybridization washes, result in different densities of silver grains (Verge *et al.*, 1990b; Tetzlaff *et al.*, 1992). No attempt was made to convert individual neuronal labeling indices to actual cellular mRNA levels. This conversion would have necessitated the inclusion of variables that were not considered in this work, such as the degree of message degradation during tissue processing, the ability of the probe to access cellular mRNA, and the standardization of the hybridizations with known amounts of the appropriate mRNA (Verge *et al.*, 1990b; Tetzlaff *et al.*, 1992).

3.2.5.1 Statistical Analyses

While *in situ* hybridization allows for the localization of mRNA at the cellular level, there are certain disadvantages to the technique. Aspects of the protocol, including radioisotopic labeling of the probe, hybridization and wash conditions, and emulsion thickness, are likely to result in different silver grain densities, making the comparison between grain labeling indices, and therefore relative mRNA

levels of tissues processed separately, inadvisable. To highlight the trends in changes of relative message levels seen following NT-3 infusion, and to combine the data from replicate analyses for each marker, summary graphs were constructed (SPSS). Since labeling index values for specific markers in the L_5 DRG neuronal population could not be assumed to be normally distributed, median, not mean, values were used as a measure of central tendency. The median of a set of measurements is defined as the midpoint value when the measurements are arranged in order of magnitude. In addition, the median, unlike the mean, is a resistant measure of center; that is to say that the median is not sensitive to the influence of extreme values and it does not respond strongly to changes in a few observations, no matter how large those changes may be. These qualities are important here, because the intuitive component of the data collection methodology (see Section 3.2.5) may have increased the possibility of the inclusion of occasional outlier values. To depict the variability of the data, the 25th and 75th percentile values were also plotted for each treatment. The 25th percentile is the labeling index value such that 25% of the measurements are less than, and 75% are greater than, that value; while the 75th percentile is the labeling index value such that 75% of the measurements are less than, and 25% are greater than, that value. Hence, labeling index values falling between the 25th and 75th percentiles encompass 50% of the measurements and the median labeling index value.

In order to be consistent in statistical analyses, and since it could not be assumed that the population distribution of the labeling indices for the various biochemical markers examined were normally distributed, nonparametric analyses were conducted. Within each replicate, and to determine if the population distribution of labeling indices between axotomized and axotomized/NT-3 infused or between normal and normal/NT-3 infused treatments were identical, a Mann-Whitney U Test was performed. The threshold probability value (α) was set at 0.05. Therefore if the calculated probability value (P) was less than 0.05, the null hypothesis was rejected and the difference in labeling indices between the two populations was deemed statistically significant.

Statistical analyses, including calculation of medians and of statistical significance, were performed in SPSS.

3.3 Detection of High-Affinity NGF Binding Sites

In order to determine the effect of NT-3 infusion on the density of high-affinity NGF binding sites, 40 pM radiolabeled NGF was employed in binding experiments on DRG tissue sections using a modification of the technique described by Richardson and colleagues (1989). Iodination was achieved by the lactoperoxidase method, originally described by Sutter and coworkers (1979), which allows the ^{125}I-NGF to maintain essentially the same biological activity as unlabeled NGF (Richardson *et al.*, 1993). Recipes are presented in the Appendix.

3.3.1 Tissue Preparation

L_4 and L_5 DRG were obtained and processed for cryostat sectioning as described in Section 3.2.1. Serial sections (6 μm thick) were thaw-mounted onto cold gelatin-subbed glass microscope slides for ^{125}I-NGF binding. Additional sections were mounted onto cold Probe-on slides; these sections were reserved for trkA *in situ* hybridization and were consecutive in series to tissue processed for ^{125}I-NGF binding. Since both techniques were isotopic, it was necessary to use adjacent DRG sections to colocalize ^{125}I-NGF binding sites and trkA message expression in individual neurons (Verge *et al.*, 1992a). Only freshly cut, frozen tissue was used in binding experiments.

3.3.2 Iodination of NGF

In an iodination hood, and with the appropriate precautions (i.e. double gloved, masked, behind a lead brick wall), the following ingredients were added in the order listed to 20 μl of 2 mCi ^{125}I (Amersham): 20 μl 0.1 M phosphate buffer (PB) (pH 7.4), 10 μl NGF stock [15 mg in 0.2 (v/v) acetic acid, pH 5; gift of W. Mobely, UCSF], 10 μl lactoperoxidase stock (0.5 mg in 0.1 M PB), and 10 μl of 0.003% (v/v) hydrogen peroxide (in 0.1 M PB). The ingredients were added to the ^{125}I in the radioisotope shipping vial and mixed thoroughly *via* trituration between each addition. After 30 minutes, an additional 10 μl of 0.003% hydrogen peroxide was added and the mixture incubated for an extra 30 minutes. Prior to the next step, but before the end of the final

incubation period, a Sep-Pak Waters Accell CM cation-exchange cartridge (Waters, a division of Millipore Corp., Milford, MA) was equilibrated with 5 cc of 0.05 M sodium acetate (pH 4.0, 4 °C). When the final incubation was finished, a 10 µl aliquot of the ^{125}I-NGF was removed, added to 3 ml of 0.1 M PB (pH 7.4) with 1 mg/ml bovine serum albumin (BSA, Fraction V; Sigma), and placed on ice. This sample was for trichloroacetic acid (TCA) precipitation. In order to separate ^{125}I-NGF from free ^{125}I, the remaining iodination mixture was applied to the equilibriated cartridge and then loaded by pushing the fluid through the cartridge with an air-filled syringe. A 1 cc syringe containing 0.05 M sodium acetate buffer (pH 4.0) was then attached to one end of the cartridge and an empty syringe to the other. The cartridge was washed by drawing the sodium acetate solution back and forth five times through the cartridge. The syringes were removed, and one containing 20 cc of 0.05 M Tris-HCl (pH 7.6) was attached to the cartridge and the buffer was pushed through into a waste container. Finally, 10 cc of 0.05 M Tris-HCl (pH 9.0) with 1 M sodium chloride and 0.5 mg/ml BSA was used to elute the ^{125}I-NGF into six microcentrifuge tubes; the first aliquot was 0.5 ml and the remaining aliquots were 1 ml each. Aliquots were stored on ice, or at 4 °C. To determine radioactivity, each aliquot was vortexed and a 10 µl subsample was removed and analyzed with a scintillation counter as described in Section 3.2.2.2. The ^{125}I-NGF was used within 24 hours of preparation.

3.3.2.1 TCA Precipitation

The tube containing the 10 µl aliquot of ^{125}I-NGF in the 3 ml of 0.1 M PB (pH 7.4) with 1 mg/ml BSA was vortexed, a 10 µl sample removed, and the radioactivity of the sample was determined as described in Section 3.2.2.2. This value was designated "C" and used in the calculation of percentage of isotope incorporation. A 200 µl sample was then removed, added to 400 µl of freshly prepared 20% (w/v) TCA solution, vortexed, and subdivided into equal subsamples in four 1.5 ml microcentrifuge tubes. These subsamples were vortexed and then incubated at 4 °C for 2 hours. Following incubation, the subsamples were centrifuged (10,000 g, 10 minutes, 4 °C) and then, avoiding the precipitate, 10 µl was removed from each tube, and the radioactivity

of each determined. The counts for the four 10 µl subsamples were averaged to obtain the value "T", which represented free ^{125}I; the pellet contained iodinated and non-iodinated NGF.

3.3.2.2 Calculation of Available ^{125}I-NGF

The percentage of ^{125}I incorporation was calculated as follows: 100% - T (cpm/10 µl) * 300 / C (cpm/10 µl). Incorporation for the two binding experiments undertaken was 60%. The total counts of bound ^{125}I (cpm/µg) was calculated using the following equation: C (cpm/10 µl) * 300 * v * i / total NGF; where "v" was the volume of the iodination mixture prior to removal of samples and "i" represented the incorporation of ^{125}I, expressed as a decimal. Specific activity was 31.7 µCi/µg. Of the 6 microcentrifuge tubes of ^{125}I-NGF, the sample with the highest level of radioactivity was selected as the source of ^{125}I-NGF for binding experiments. The amount of available ^{125}I-NGF (ng/µl) was calculated by dividing the total cpm/µl of the chosen sample by total counts of bound ^{125}I.

3.3.3 ^{125}I-NGF Binding

Immediately prior to use, the necessary volume of radioligand solution was prepared by adding 1.5 mg/ml cytochrome C (Sigma), 0.083 mg/ml phenylmethyl-sulphonyl fluoride solution (solid dissolved in 500 µl 100% ethanol; Sigma), 4.0 µg/ml leupeptin (Sigma), 0.5 mM magnesium chloride, and 40 pM ^{125}I-NGF (molecular weight of NGF dimer is 26,000 g/l). This concentration of NGF had been previously shown to provide the best ratio of neuronal to background labeling (Richardson *et al.*, 1986; Verge *et al.*, 1989a). For each run, it was necessary to prepare controls to distinguish between specific and nonspecific binding. To accomplish this, slides were incubated in a Coplin jar of radioligand solution containing 40 pM ^{125}I-NGF to which excess non-iodinated NGF (2560 pM) had been added.

After preparation of the radioligand solution, the slides were removed from the -20 °C freezer or cryostat and allowed to come to room temperature (20-30 minutes). Slides were incubated in Coplin jars containing radioligand solution for 90 minutes; jars

were placed on a rocker table for the duration of the incubation period. Slides were then rinsed in six changes over 3 minutes of cold 0.1 M PBS. Sections were fixed for 10 minutes in cold 2% (w/v) formaldehyde with 2% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.4), rinsed in two changes over 1 minute in PBS, 30 seconds in distilled water, 1 minute in 60% ethanol, 2 minutes in 95% ethanol, and 1 minute in 100% ethanol. Slides were air-dried, placed in slide boxes containing packets of desiccant, stored overnight at 4 °C, and then dipped in radiosensitive emulsion (see Section 3.2.4). Radioautographs were tested for degree of ^{125}I exposure 2 days after dipping. Optimal emulsion exposure time was chosen to maximize the ratio of specific ^{125}I-NGF to nonspecific (background) binding. Two runs of ^{125}I-NGF binding were undertaken.

3.3.4 Quantification of ^{125}I-NGF Binding

In order to quantify the density of NGF binding sites and trkA message levels, serial sections of tissue processed for ^{125}I-NGF binding, ^{125}I-NGF plus 2560 pM non-iodinated NGF binding, and trkA *in situ* hybridization were examined following the method described in Section 3.2.5. The corrected grain densities, labeling indices, were linearly related to the concentration of bound ^{125}I-NGF (Richardson *et al.*, 1989; Verge *et al.*, 1992a). Scatterplots of labeling indices of the 191-276 identified neurons in each ganglion were constructed to depict the relationship between perikaryal diameter and labeling index or between the labeling indices of ^{125}I-NGF binding and trkA *in situ* hybridization or ^{125}I-NGF and ^{125}I-NGF plus excess cold NGF binding. To highlight the trends seen following NT-3 infusion, and to combine the data from replicate analyses, summary graphs were constructed as described in Section 3.2.5.1.

3.4 Preparation of Photo-plates

Darkfield and brightfield photomicrographs were prepared using standard darkroom techniques. These were later scanned (Hewlett-Packard ScanJet 6100 C/T, Hewlett-Packard Co., Palo Alto, CA); the resulting images were then appropriately sized, compiled to form a plate, and contrast-adjusted as necessary for best output in Adobe Photoshop Version 5.5 (Adobe Systems Inc., San Jose, CA). Contrast adjustment

at this time did not interfere with the determination of neuron labeling indices, but was for photo-plate preparation only. Finally, plate text and figure headings were added in Adobe Pagemaker Version 6.5.

4. THE ROLE OF NT-3 IN THE MAINTENANCE OF PHENOTYPE OF ADULT PRIMARY SENSORY NEURONS: CHARACTERIZATION OF THE trkC-POSITIVE SUBPOPULATION

4.1 Introduction

DRG neurons innervate skin, muscle, joints, or viscera and serve in a somatosensory capacity as nociceptors, mechanoreceptors, or proprioceptors. Mirroring the diversity of function, this heterogeneous population may also be differentiated according to perikaryal and axon fiber diameter and physiology. Moreover, these neurons may be divided into a number of subpopulations based on their expression of the neurotrophin receptors trkA, trkB, and trkC and then further characterized *vis-à-vis* the expression of biochemical markers — including neuropeptides, cytoskeletal elements, enzymes, and other proteins.

As many as 10,500-17,900 neuronal perikarya are estimated to be housed in individual adult rat L_4 or L_5 DRG (Schmalbruch, 1987; Klein *et al.*, 1991; Swett *et al.*, 1991; Tandrup, 1993). The best characterized subgroup of these neurons is that responsive to NGF, which represents 40-45% of the total neuronal population (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1990a, 1990b, 1992a; Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Bennett *et al.*, 1996a; Kashiba *et al.*, 1996, 1997a, 1998; Karchewski *et al.*, 1999), and displays high affinity NGF binding sites and expresses both p75 and trkA mRNAs (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1989b, 1990a, 1990b, 1992a). The group includes virtually all SP- and most CGRP-, but not SOM-, expressing DRG neurons (Verge *et al.*, 1989a; Kashiba *et al.*, 1996); all express GAP-43 mRNA (Verge *et al.*, 1990a). They are predominately small to medium in size (Richardson *et al.*, 1986; Verge *et al.*, 1989a,

1989b, 1992a, 1992b; Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Kashiba *et al.*, 1996; Karchewski *et al.*, 1999) and are thought to function in nociception (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). While the NGF-responsive subpopulation is well characterized, the same cannot be said for the subsets of DRG neurons capable of responding to the other neurotrophins.

Micro-injection of sciatic nerve with iodinated NT-3 labels a subset of principally large-sized DRG neurons in a receptor-mediated fashion (DiStefano *et al.*, 1992; Helgren *et al.*, 1997), and NT-3 is potentially bioavailable to these cells from target (Ernfors *et al.*, 1990b; Maisonpierre *et al.*, 1990a; Funakoshi *et al.*, 1993; Griesbeck *et al.*, 1995; Yamamoto *et al.*, 1996), nerve (Funakoshi *et al.*, 1993; Yamamoto *et al.*, 1996; Nitta *et al.*, 1999), spinal cord (Funakoshi *et al.*, 1993; Nitta *et al.*, 1999), and local autocrine/paracrine (Zhou *et al.*, 1999a) sources. Since among the trks, NT-3 preferentially binds to trkC (Lamballe *et al.*, 1991; Ip *et al.*, 1993a), characterization of the trkC-positive population is critical to discerning the role(s) of this neurotrophin in maintaining the phenotype of adult primary sensory neurons. Although the majority of DRG neurons that express trkC mRNA are reported to be medium to large in size (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Kashiba *et al.*, 1996, 1997a; Helgren *et al.*, 1997; Karchewski *et al.*, 1999) and are believed to be proprioceptive in function (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994; Klein *et al.*, 1994; Tessarollo *et al.*, 1994; Kucera *et al.*, 1998; Matsuo *et al.*, 2000), there is disagreement as to the number of trkC-positive cells. Several research groups have indicated that for adult rat DRG between 10-20% of DRG neurons display trkC transcripts (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Kashiba *et al.*, 1996, 1997a). However, in this laboratory, *in situ* hybridization experiments with an oligonucleotide probe for trkC mRNA demonstrate that 30-35% of the total population are strongly trkC-positive, with an additional 10% exhibiting low levels of signal (Karchewski *et al.*, 1999). Moreover, ~ 50% of the trkC-positive subset coexpress trkA mRNA (Karchewski *et al.*, 1999).

Peripheral nerve injury may result in loss of DRG neurons, but those that survive undergo characteristic alterations in response to the insult and in preparation for axonal

regeneration (see Section 1.2.3.1). Included among these changes are the upregulation of microtubule-associated proteins (Oblinger and Lasek, 1988; Skene, 1989; Hoffman *et al.*, 1993; Moskowitz *et al.*, 1993); GAP-43 (Skene, 1989; Schreyer and Skene, 1991, 1993; Verge *et al.*, 1990b; Wiese *et al.*, 1992), and the transcription factor cJUN (Gold *et al.*, 1993a, 1994). Further, additional axotomy-induced biochemical changes within DRG neurons include the differential regulation of neuropeptides: with levels of α- and β-CGRP (Shehab and Atkinson, 1986; Noguchi *et al.*, 1990b, 1993; Klein *et al.*, 1991), SP (Jessell *et al.*, 1979; Tessler *et al.*, 1985; Villar *et al.*, 1989; Klein *et al.*, 1991; Hökfelt *et al.*, 1993; Noguchi *et al.*, 1994, 1995) and SOM (Noguchi *et al.*, 1993) decreasing; while NPY (Wakisaka *et al.*, 1991, 1992; Frisén *et al.*, 1992; Noguchi *et al.*, 1993; Zhang *et al.*, 1994c; Ohara *et al.*, 1994, 1995), galanin (Hökfelt *et al.*, 1987, 1993; Villar *et al.*, 1989; Klein *et al.*, 1991; Noguchi *et al.*, 1993; Verge *et al.*, 1993b), and VIP (Villar *et al.*, 1989; Klein *et al.*, 1991; Noguchi *et al.*, 1993) are upregulated.

Upon gaining a better understanding of the phenotype(s) of normal and injured DRG neurons that may be responsive to NT-3, unique subgroups may be identified. This knowledge can be used to manipulate specific neuronal populations, through the introduction of exogenous trophic factor, and ultimately such studies will yield valuable information that could lead to advances in the treatment of neurological diseases or trauma.

Here, the subpopulation of DRG neurons potentially responsive to NT-3 is characterized using serial adult rat DRG sections processed for *in situ* hybridization to allow for the colocalization or trilocalization of the following mRNAs with trkC: the neurotrophin receptors trkA and p75; the cytoskeletal elements NF medium (160 kDa) subunit (NFM) and Tα1 α-tubulin; the neuropeptides SP, α–CGRP, SOM, galanin, NPY, and VIP; the injury/regeneration-associated markers GAP-43 and cJUN; and the synaptosomal-associated protein SNAP-25. Computer-assisted image analysis of radioautographs has been used to determine the relative transcript levels for trkC and the preceding markers coexpressed by individual neurons, both under normal conditions and following sciatic nerve transection for injury-induced markers.

4.2 Results

4.2.1 Technical Considerations

Labeling index, determined through computer-assisted image analysis of radioautographs, refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of the target mRNA (see Section 3.2.5). Labeling index values between the probe-positive "cut off" value and fifteen times background are considered to represent low levels of hybridization signal, while those above fifteen times background denote moderate to heavy signal levels. To allow for the quantification of relative mRNA levels following radioisotopic *in situ* hybridization, radioautographs must be developed so that silver grain overlap is minimized. In order to achieve this result, but at the same time permit the identification of neuronal populations with low transcript levels, development times were chosen to maximize silver grain densities and minimize grain overlap. Because of this, in cases where there is heterogeneous mRNA expression for specific markers, there may be instances where neurons with low transcript levels are not considered to exhibit detectable message for that marker. Another consideration that must be taken into account when analyzing radioautographs is the inability to compare actual labeling index values for individual probes between slides; for each marker, neuronal labeling indices may only be compared between ganglia present on the same slide (see Section 3.2.5). This is due to constraints inherent to the technique that affect the formation of silver grains. Only relative labeling indices, not the actual values, are meaningful.

To ascertain neuronal size, the image analysis software program (see Section 3.2.5) uses the largest perikaryal area of each individual neuron identified in serial sections to determine the diameter of the cell body; calculations are based on the presumption that the perikaryon is spherical; diameters less than 30 μm, between 30-45 μm, and greater than 45 μm represent small-, medium-, and large-sized neurons, respectively. Since some of the image analyses presented here involve the trilocalization of specific markers, there may be a bias against small diameter neurons because of the difficulty in identifying perikarya of this size in all three of the serial 6 μm thick DRG sections.

4.2.2 Neurotrophin Receptors

4.2.2.1 The Expression of trkC mRNA in the Intact State and Following Axotomy

Qualitative assessment of radioautographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect transcripts of either the full-length ($trkC_{full\text{-}length}$), or both the full-length and truncated ($trkC_{all}$), forms of the trkC receptor shows that the two probes exhibit virtually identical patterns of neuronal expression (Figure 4.1). In some cases, a $trkC_{no\ insert}$ probe (see Section 3.2.2.1) was used instead of the $trkC_{full\text{-}length}$ probe to identify transcripts of the full-length trkC receptor. DRG sections processed with these probes show similar patterns of labeling and percentages of probe-positive neurons (Karchewski *et al.*, 1999), therefore from this point in this work, the term "trkC" is used to represent *in situ* hybridization results obtained by using either the $trkC_{full\text{-}length}$ or the $trkC_{no\ insert}$ probe.

In intact DRG, trkC mRNA is detectable in many neurons and these trkC-positive perikarya are distributed throughout the ganglion (Figure 4.2). The majority of trkC-expressing neurons are medium to large in size, exhibiting moderate to high levels of hybridization signal. Radioautographs from intact animals compared to those from animals sacrificed 3 weeks after sciatic nerve transection, show a perceptible axotomy-induced decrease in trkC hybridization signal levels (Figure 4.2).

Scatterplots (e.g. Figure 4.3) constructed from data obtained through quantitative analysis of labeling indices and perikaryal diameter of individual neurons identified in sections of adult rat L_5 DRG (18 DRG sections/treatment, average 227 neurons/section) processed for *in situ* hybridization, and subsequent radioautography, to detect trkC transcripts show that in the intact state trkC mRNA is expressed by ~ 40% of neurons, with ~ 30% of the total DRG population exhibiting moderate to high levels of hybridization signal compared to the remaining ~ 10% (see Section 4.2.1). Further, these analyses show that ~ 85% of trkC-positive neurons possess medium and large diameter perikarya. Small cells tend to express low levels of hybridization signal for trkC, while larger diameter neurons exhibit the full range of labeling indices. Those neurons with the

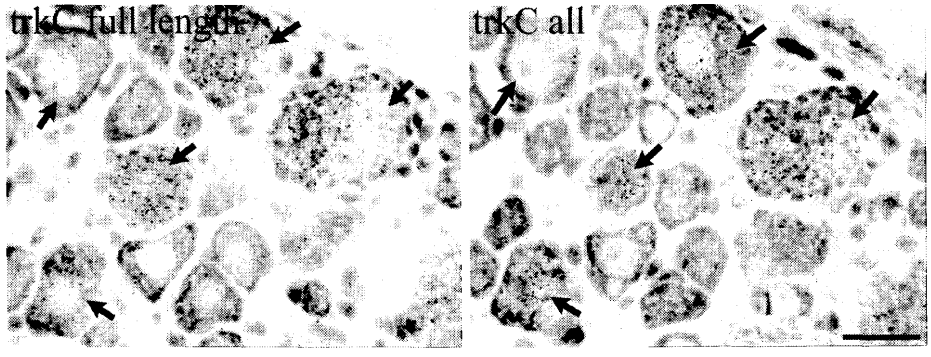

Figure 4.1 Comparison between the expression of $trkC_{full\text{-}length}$ and $trkC_{all}$ transcripts in adult rat DRG neurons.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect mRNA transcripts of the full-length ($trkC_{full\text{-}length}$), and both the full-length and the truncated ($trkC_{all}$), forms of the trkC receptor. Arrows indicate that both probes hybridize to transcripts in the same neurons. Scale bar = 30 μm.

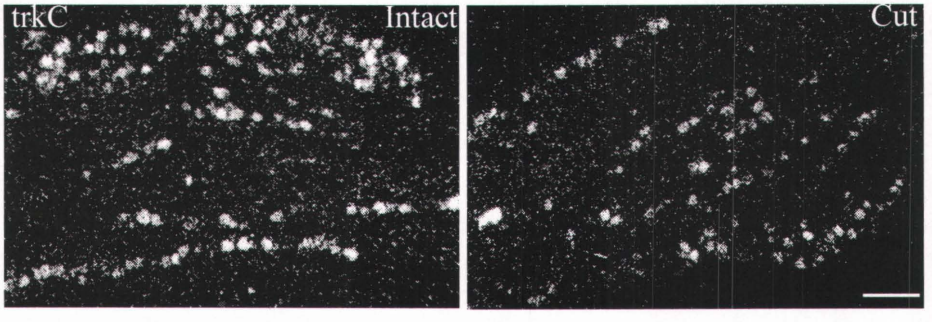

Figure 4.2 Expression of trkC mRNA under normal and axotomized conditions.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect trkC mRNA, under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Scale bar = 200 μm.

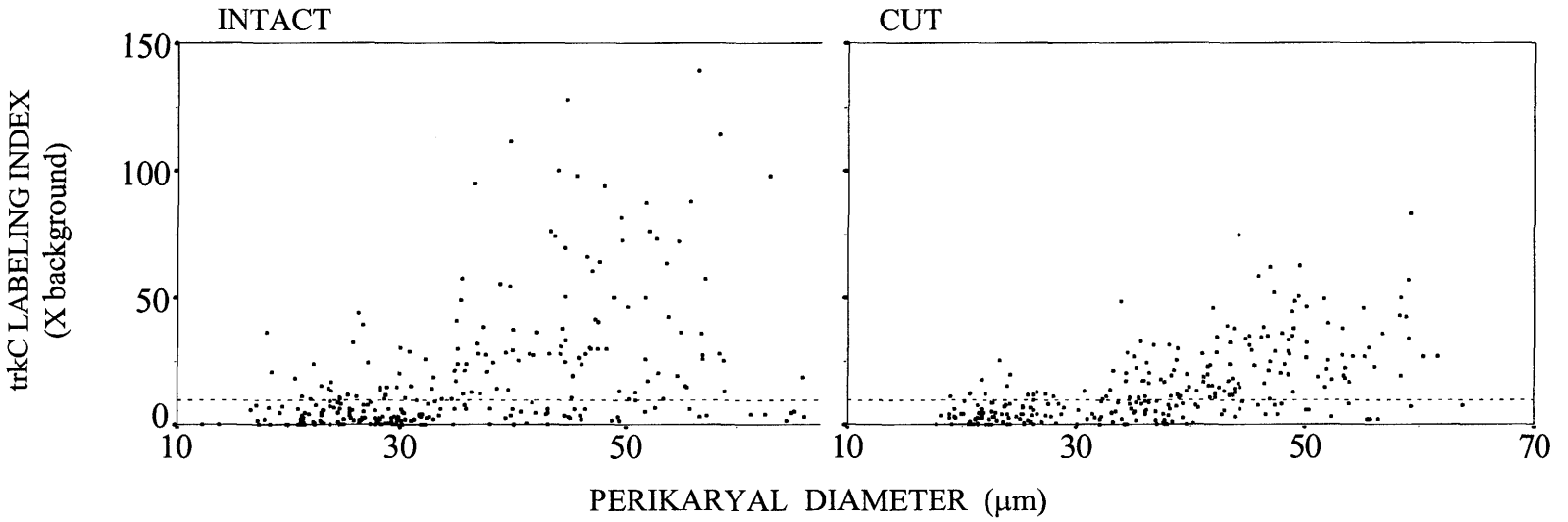

Figure 4.3 Relationship between perikaryal diameter and the relative level of trkC mRNA under normal and axotomized conditions.

Scatterplots of labeling indices of 283-291 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC mRNA, depict the relationship between perikaryal diameter (x axis) and trkC labeling indices (y axis) under normal (intact) and 3 week axotomized (cut) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of trkC mRNA. Neuronal profiles above the dashed horizontal line are considered positive for trkC mRNA.

highest level of trkC mRNA are of larger diameter. The majority of small-sized neurons, and a subpopulation of medium to large diameter cells, do not express detectable levels of trkC mRNA. Three weeks following sciatic nerve transection trkC mRNA levels decrease, although axotomy does not appear to greatly affect the proportion of trkC-positive cells within the population of surviving neurons. However, there may be a reduced ability, in the axotomized verses the intact state, to detect trkC mRNA in neurons with low trkC levels.

4.2.2.2 The Expression of trkA mRNA in the Intact State

Microscopic analysis of serial L_5 DRG sections processed for *in situ* hybridization to detect trkC or trkA mRNA show that the mRNAs for the two neurotrophin receptors are abundantly and heterogenously expressed in DRG neurons (Figure 4.4). Message for trkA is primarily localized to small and medium diameter perikarya and may colocalize with trkC mRNA; there are also neurons in which only trkA or trkC transcripts are detectable.

Scatterplots (e.g. Figure 4.5), comparing the relationship between trkA and trkC mRNA expression for individual neurons (6 DRG sections/probe, 265 neurons/section), demonstrate that while ~ 40% of neurons express trkA transcripts, there is a subpopulation of cells in which the message for both of these high-affinity neurotrophin receptors is coexpressed. This trkA/trkC-positive subset represents ~ 10% of the total DRG neuronal population, and approximately one quarter of trkC- or of trkA-expressing neurons. They tend to exhibit moderate levels of hybridization signal for trkA and low to moderate signal levels for trkC mRNA, but there are also small subpopulations of these neurons with either high trkA/low trkC or low trkA/high trkC labeling indices.

Scatterplots (e.g. Figure 4.6), depicting the relationship between perikaryal diameter and trkA or trkC mRNA expression for individual neurons (10 DRG sections/probe, average 250 neurons/section), indicate that trkA-positive neurons are primarily small in size, although there are a number with medium, and fewer with large, diameter perikarya. The level of hybridization signal for trkA ranges from low to high, with smaller neurons expressing all ranges of labeling, while larger diameter neurons

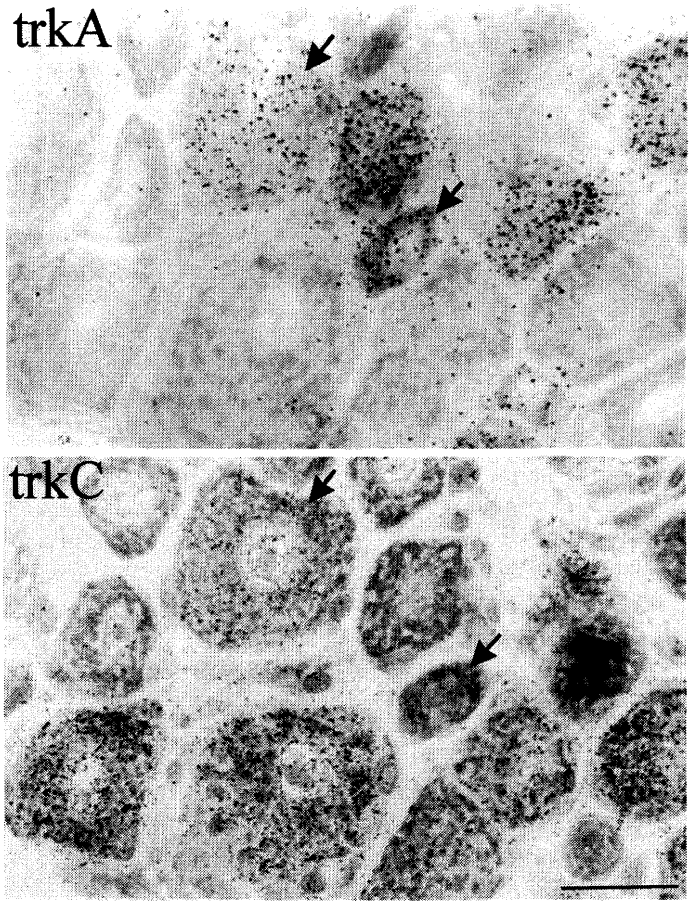

Figure 4.4 Colocalization of trkA and trkC mRNA under normal conditions.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect trkA (upper panel) and trkC (lower panel) mRNA under normal conditions. Arrows indicate neurons positive for both markers. Scale bar = 30 μm.

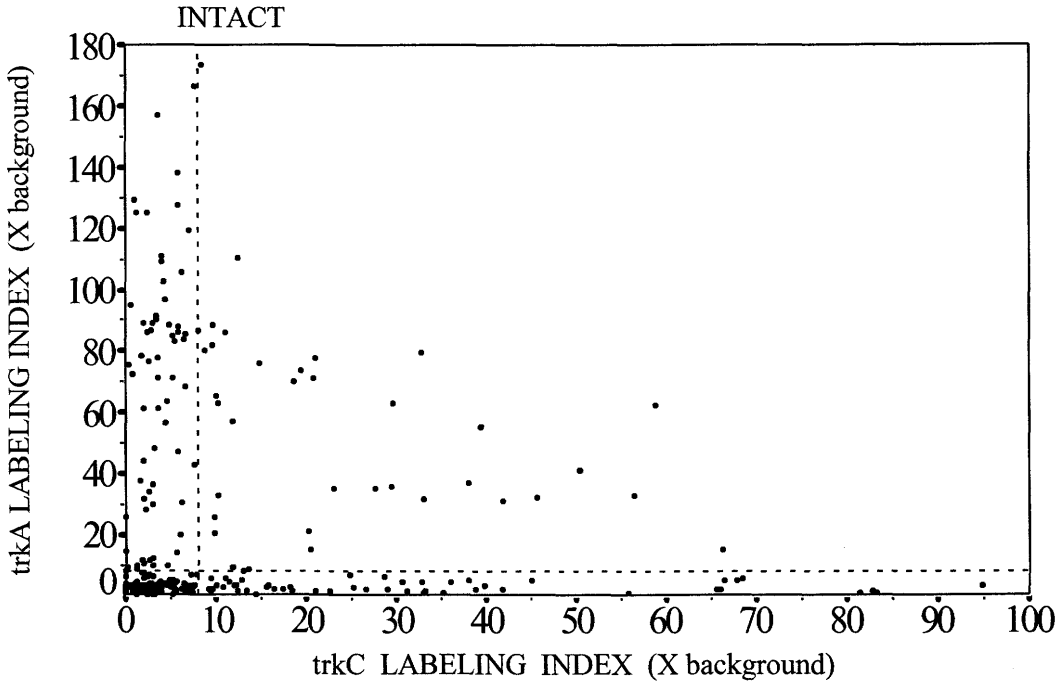

Figure 4.5 Relationship between relative levels of trkC and trkA mRNA under normal conditions.

Scatterplot demonstrates the relationship between trkC and trkA labeling indices for 252 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or trkA (y axis) mRNA under normal (intact) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkC and trkA mRNA, respectively. The upper right quadrant encompasses neurons labeled for both markers.

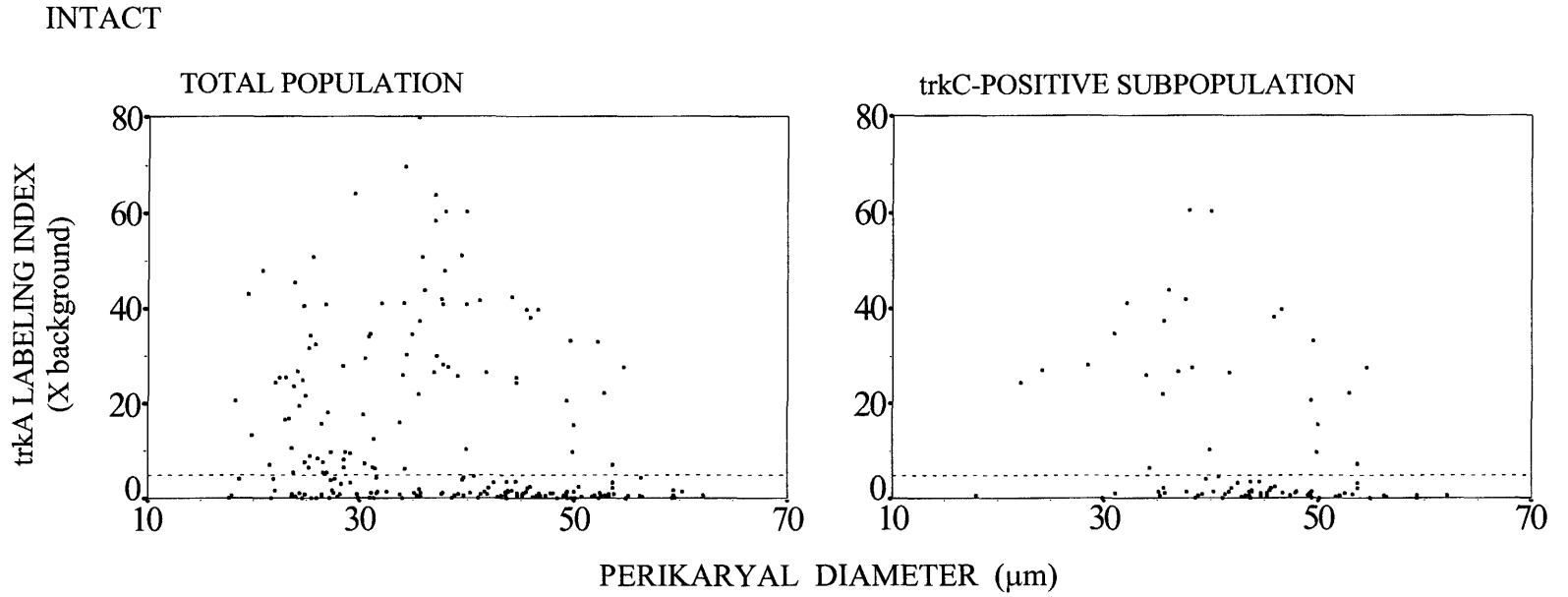

Figure 4.6 Relationship between perikaryal diameter and the relative level of trkA mRNA under normal conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 210 individual neurons (of which 87/210 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and trkA labeling indices (y axis), for the total population and for the trkC-positive subpopulation under normal conditions (intact). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of trkA mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for trkA mRNA.

exhibit moderate levels of trkA labeling. Neurons lacking detectable levels of trkA mRNA do not fit any specific size distribution, but represent all size classes; those coexpressing trkA and trkC message are primarily of medium size, yet include some large neurons.

4.2.2.3 The Expression of p75 mRNA in the Intact State

Serial L_5 DRG sections, processed for *in situ* hybridization to detect trkC or p75 mRNA, show that there is colocalization of the mRNA for the two neurotrophin receptors (Figure 4.7). All, or virtually all, trkC-positive neurons appear to also express p75, although there are many p75-positive neurons that do not express trkC mRNA and a subpopulation that do not express detectable p75 or trkC hybridization signal.

Scatterplots (e.g. Figure 4.8), illustrating the relationship between trkC and p75 mRNA expression for individual neurons (4 DRG sections/probe, average 255 neurons/section), demonstrate that virtually all trkC-positive cells exhibit detectable levels of p75 transcripts. Although p75 mRNA is heterogenously expressed throughout the trkC-positive subpopulation, neurons with the highest levels of trkC mRNA tend to possess lower p75 message levels. Additionally, ~ 20% of DRG neurons do not exhibit detectable p75 mRNA.

Scatterplots (e.g. Figure 4.9), comparing the relationship between perikaryal diameter and p75 mRNA expression for individual neurons (4 DRG sections/probe, average 255 neurons/section) in the total DRG and for the trkC-positive subpopulation, indicate that there is no obvious correlation between p75 mRNA levels, neuronal size, and trkC expression — although a subgroup of medium-sized cells that exhibit the highest levels of p75 transcripts and do not appear to express detectable levels of trkC mRNA. Further, neurons lacking detectable p75 transcripts tend to have small diameter perikarya.

Scatterplots (e.g. Figure 4.10), demonstrating the relationship between p75, trkC, and trkA mRNA expression for individual neurons (4 DRG sections/probe, average 255 neurons/section), show that there is a tendency for trkC/trkA-positive cells to display

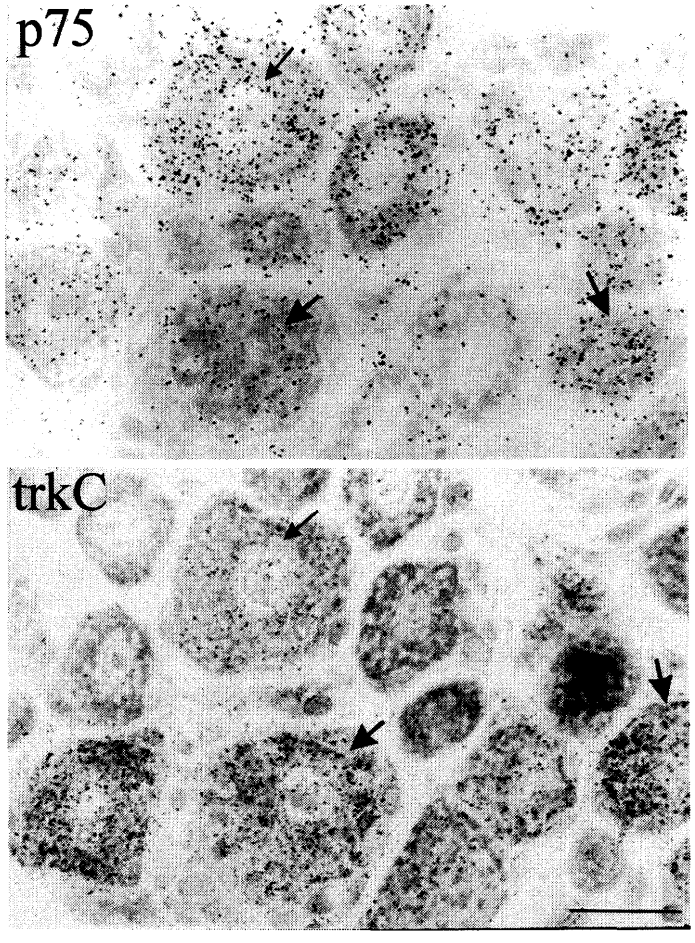

Figure 4.7 Colocalization of p75 and trkC mRNA under normal conditions.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect p75 (upper panel) and trkC (lower panel) mRNA under normal conditions. Arrows indicate neurons positive for both markers. Scale bar = 30 μm.

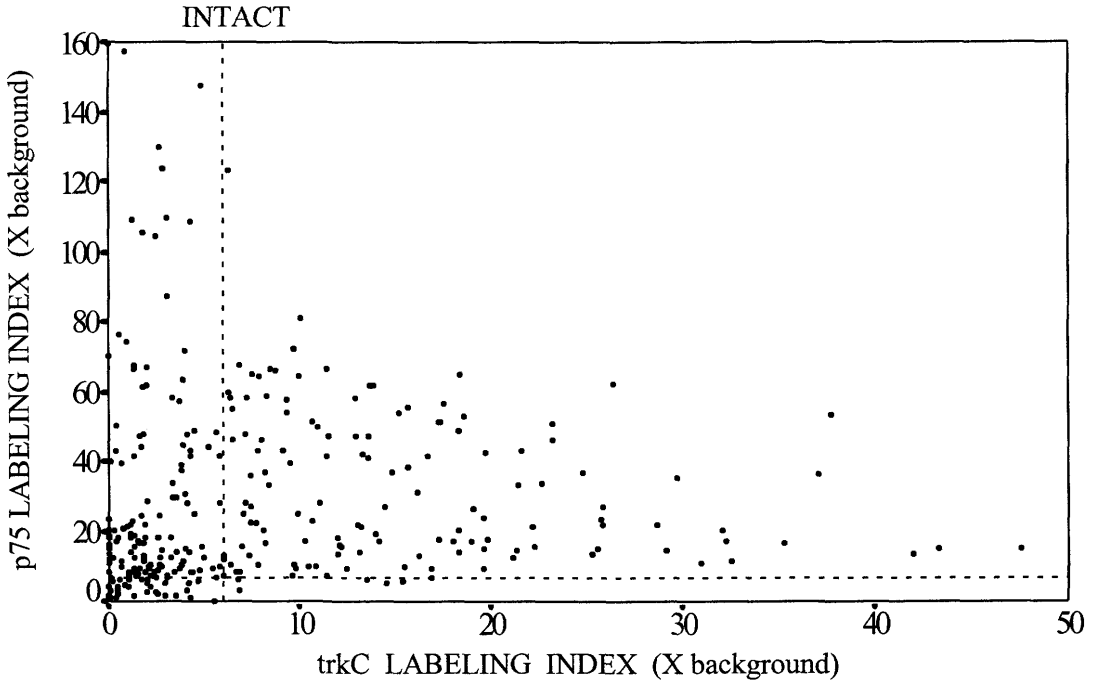

Figure 4.8 Relationship between relative levels of trkC and p75 mRNA under normal conditions.

Scatterplot demonstrates the relationship between trkC and p75 labeling indices for 296 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or p75 (y axis) mRNA under normal (intact) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkC and p75 mRNA, respectively. The upper right quadrant encompasses neurons labeled for both markers.

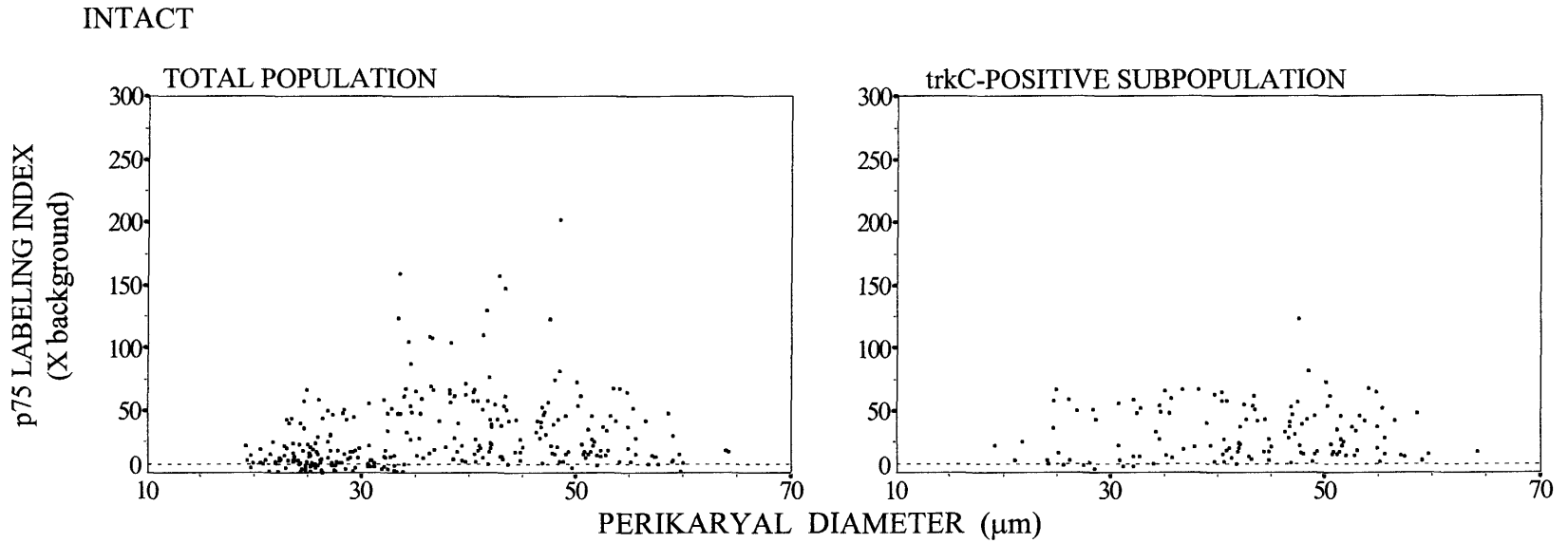

Figure 4.9 Relationship between perikaryal diameter and the relative level of p75 mRNA under normal conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 296 individual neurons (of which 133/296 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect p75 and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and p75 labeling indices (y axis), for the total population and for the trkC-positive subpopulation under normal conditions (intact). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of p75 mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for p75 mRNA.

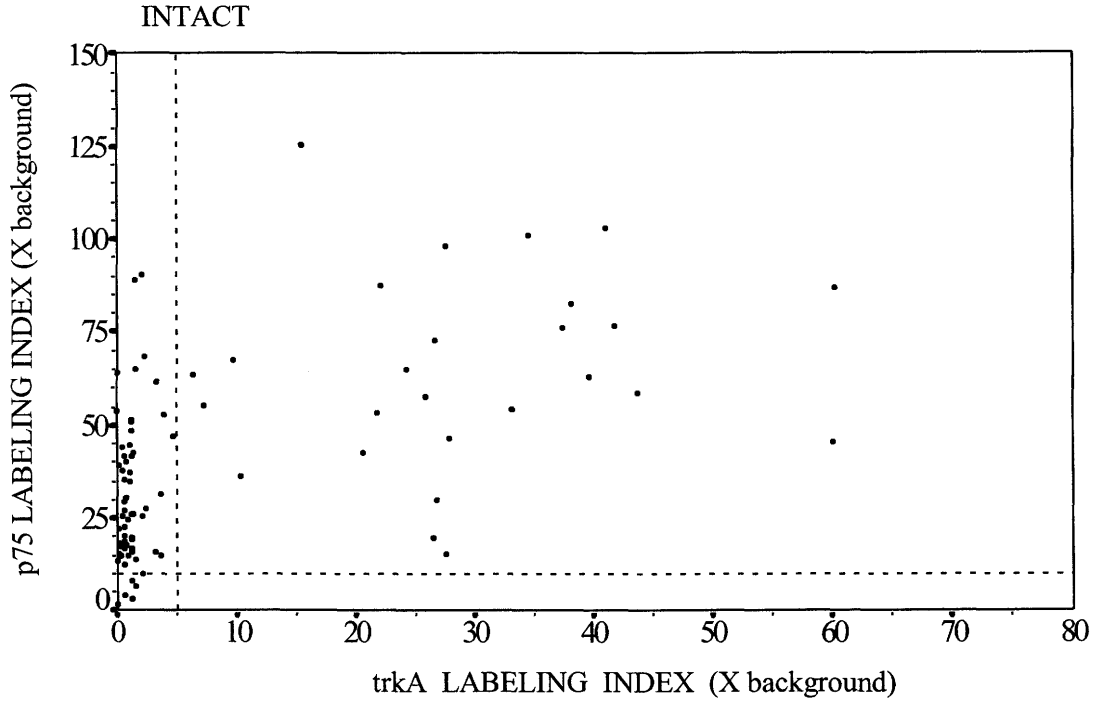

Figure 4.10 Relationship between relative levels of trkA and p75 mRNA under normal conditions in the trkC-positive subpopulation.

Scatterplot demonstrates the relationship between trkA and p75 labeling indices for 87/210 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA (x axis), p75 (y axis), or trkC mRNA under normal (intact) conditions in the subpopulation of neurons coexpressing trkC. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkA and p75 mRNA, respectively. The upper right quadrant encompasses neurons labeled for all three markers.

higher p75 labeling indices than the low to moderate levels of hybridization signal for p75 exhibited by trkC-positive neurons that do not coexpress trkA.

4.2.3 Cytoskeletal Elements

4.2.3.1 The Expression of NFM mRNA in the Intact State

NFM mRNA is detectable in virtually all normal DRG neurons (Figure 4.11) and those cells that express trkC tend to exhibit high levels of NFM message.

Scatterplots (e.g. Figure 4.12), comparing the relationship between NFM and trkC mRNA expression for individual neurons (2 DRG sections/probe, 333 neurons/section), demonstrate that virtually all trkC-positive neurons also exhibit detectable NFM transcripts. Neurons with high levels of hybridization signal for trkC tend to express high signal levels for NFM, and while there is a group of cells with low trkC/high NFM labeling indices, there is also a subset with low levels of trkC, and low to moderate levels of NFM, hybridization signal. The largest proportion of neurons that do not express detectable trkC mRNA, exhibit low to moderate levels of hybridization signal for NFM.

Scatterplots (e.g. Figure 4.13), depicting the relationship between perikaryal diameter and NFM mRNA expression for individual neurons (2 DRG sections/probe, average 333 neurons/section) in the total DRG and for the trkC-positive subpopulation, indicate that there appears to be a positive correlation between perikaryal size and NFM transcript level, with larger diameter cells expressing higher levels of hybridization signal for NFM. This correlation is maintained within the subset of neurons expressing trkC message.

4.2.3.2 The Expression of Tα1 α-tubulin mRNA in the Intact State and Following Axotomy

Under normal conditions, hybridization signal for Tα1 α-tubulin is exhibited by almost all DRG neurons at moderate to high levels; axotomy results in increased message levels (Figure 4.14).

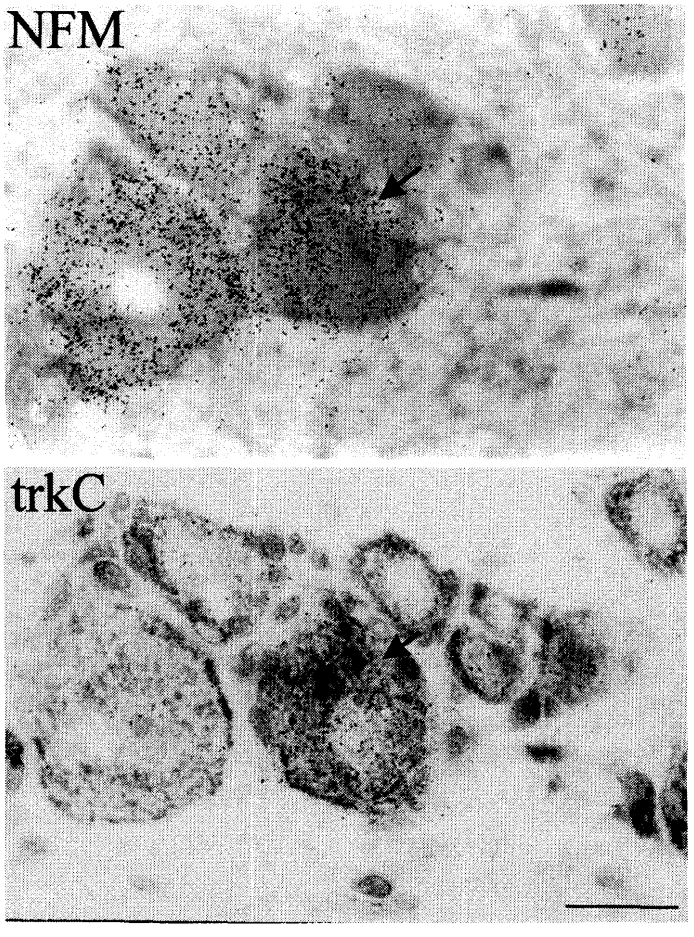

Figure 4.11 Colocalization of NFM and trkC mRNA under normal conditions.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect NFM (upper panel) and trkC (lower panel) mRNA under normal conditions. Arrows indicate neurons positive for both markers. Scale bar = 30 μm.

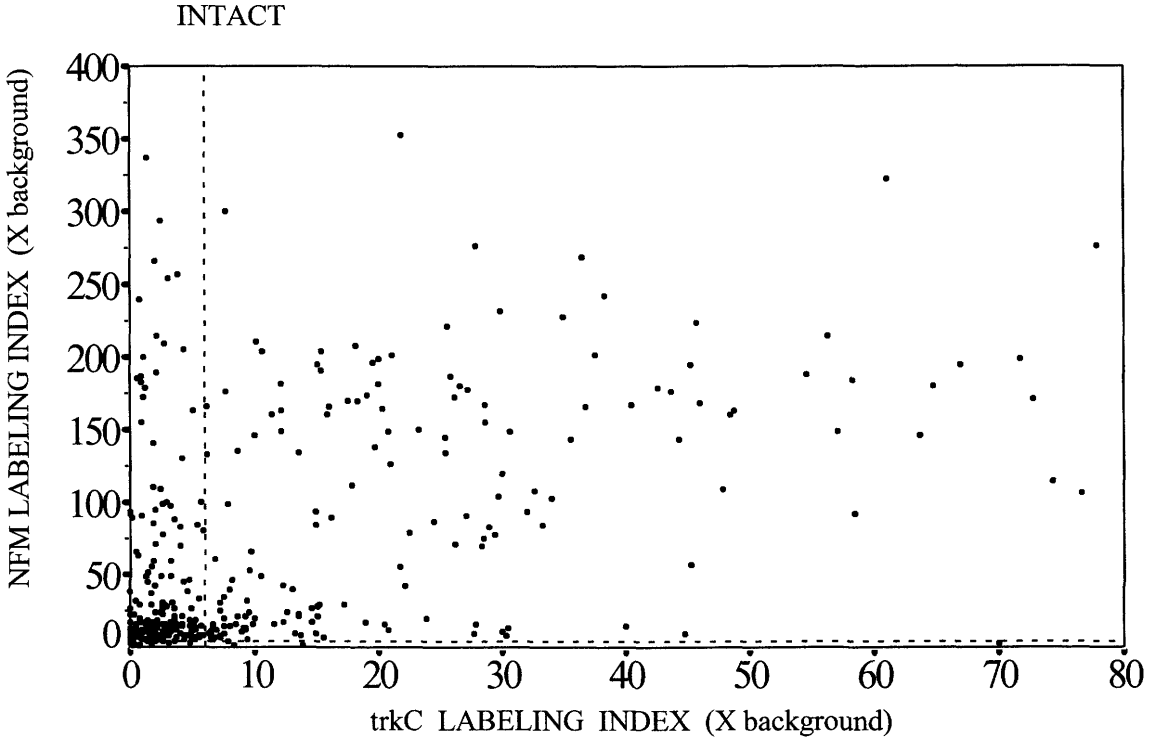

Figure 4.12 Relationship between relative levels of trkC and NFM mRNA under normal conditions.

Scatterplot demonstrates the relationship between trkC and NFM labeling indices for 373 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or NFM (y axis) mRNA under normal conditions (intact). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkC and NFM mRNA, respectively. The upper right quadrant encompasses neurons labeled for both markers.

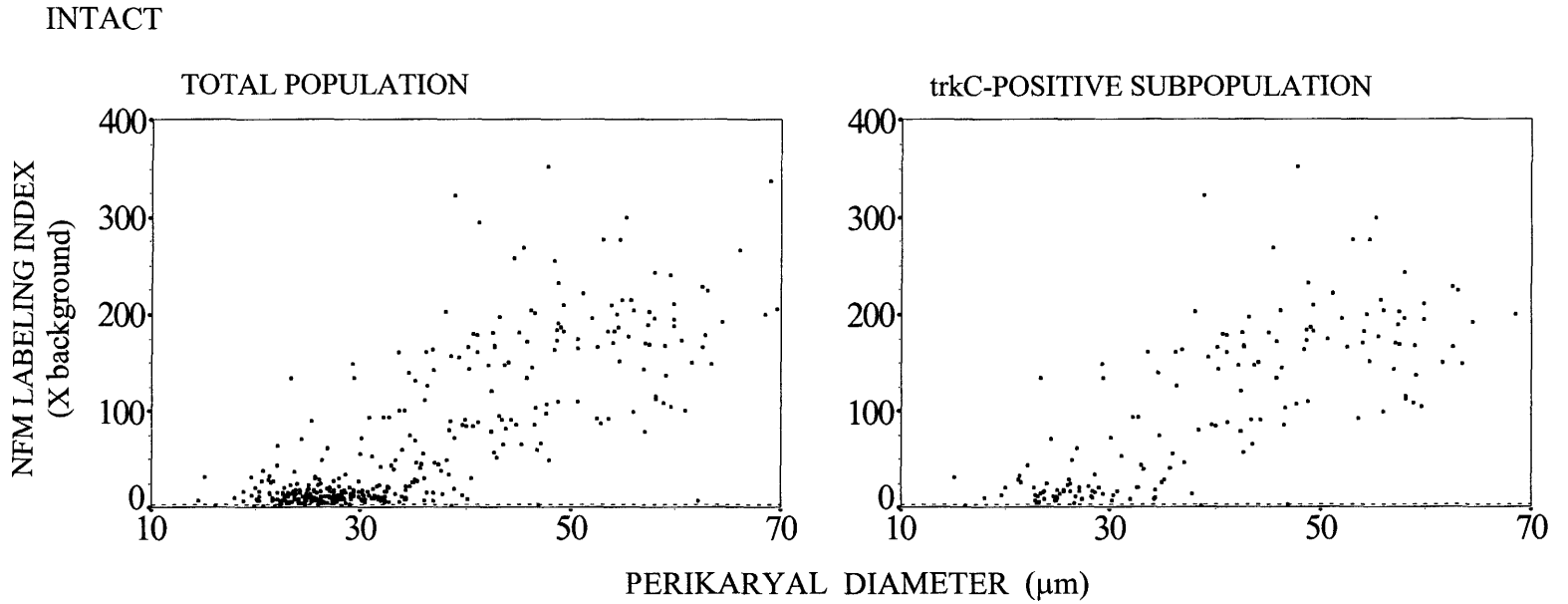

Figure 4.13 Relationship between perikaryal diameter and the relative level of NFM mRNA under normal conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 373 individual neurons (of which 165/373 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect NFM and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and NFM labeling indices (y axis), for the total population and for the trkC-positive subpopulation under normal conditions (intact). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of NFM mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for NFM mRNA.

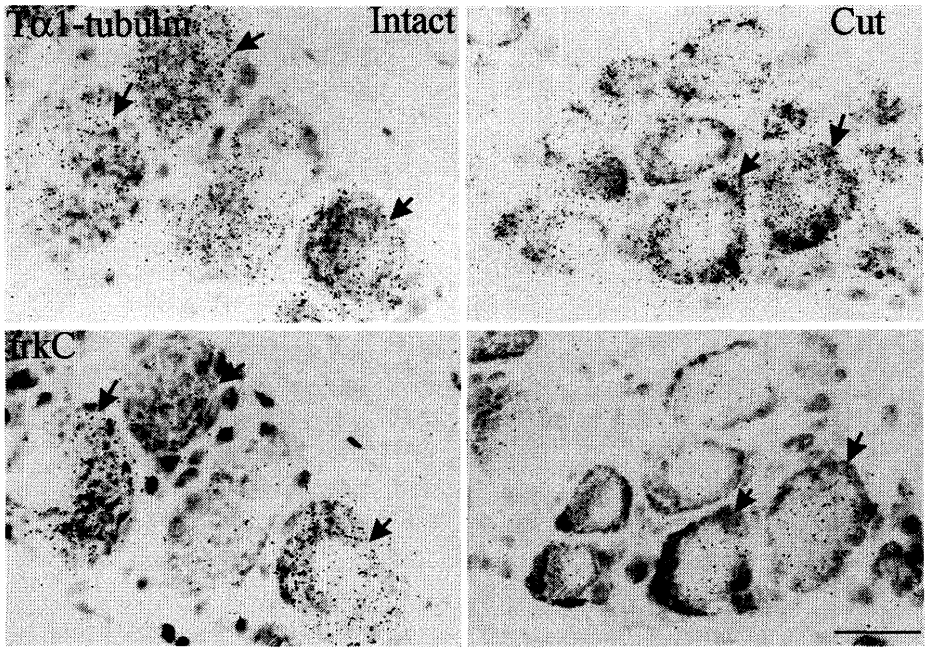

Figure 4.14 Colocalization of Tα1 α-tubulin and trkC mRNA under normal and axotomized states.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect Tα1 α-tubulin (upper panel) and trkC (lower panel) mRNA under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Arrows indicate neurons positive for both markers. Scale bar = 30 μm.

Scatterplots (e.g. Figure 4.15), illustrating the relationship between trkC and Tα1 α-tubulin mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 257 neurons/section), demonstrate that under normal conditions all DRG neurons exhibit detectable message levels of Tα1 α-tubulin. Although trkC transcripts are heterogenously expressed, this subpopulation tends to display moderate labeling indices for Tα1 α-tubulin, with the exception of a small subgroup that exhibits low levels of trkC, and high levels of Tα1 α-tubulin, hybridization signal. The highest basal levels of message for Tα1 α-tubulin are expressed in a subset of neurons that do not exhibit detectable levels of trkC mRNA. Three weeks post-axotomy, message levels of Tα1 α-tubulin increase, seemingly in all DRG neurons, although the subpopulation possessing the highest levels of Tα1 α-tubulin mRNA is still represented by neurons lacking detectable levels of trkC message.

Scatterplots (e.g. Figure 4.16), comparing the relationship between perikaryal diameter and Tα1 α-tubulin mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 257 neurons/section) in the total DRG and for the trkC-positive subpopulation, indicate that under normal conditions there is a loose negative correlation between Tα1 α-tubulin labeling indices and perikaryal size, with small diameter cells exhibiting the highest levels of hybridization signal for Tα1 α-tubulin. This correlation is upheld in the trkC-positive subpopulation. Axotomy results in increased Tα1 α-tubulin message levels throughout the neuronal population.

4.2.4 Neuropeptides

4.2.4.1 The Expression of α-CGRP mRNA in the Intact State

In intact DRG, α-CGRP mRNA is detectable in many small and medium diameter neurons and, in some cases, transcripts for this neuropeptide colocalize with those for trkC (Figure 4.17).

Scatterplots (e.g. Figure 4.18), depicting the relationship between perikaryal diameter and α-CGRP mRNA expression for individual neurons (2 DRG sections/probe, average 200 neurons/section), indicate that ~ 55% of DRG neurons express detectable α-

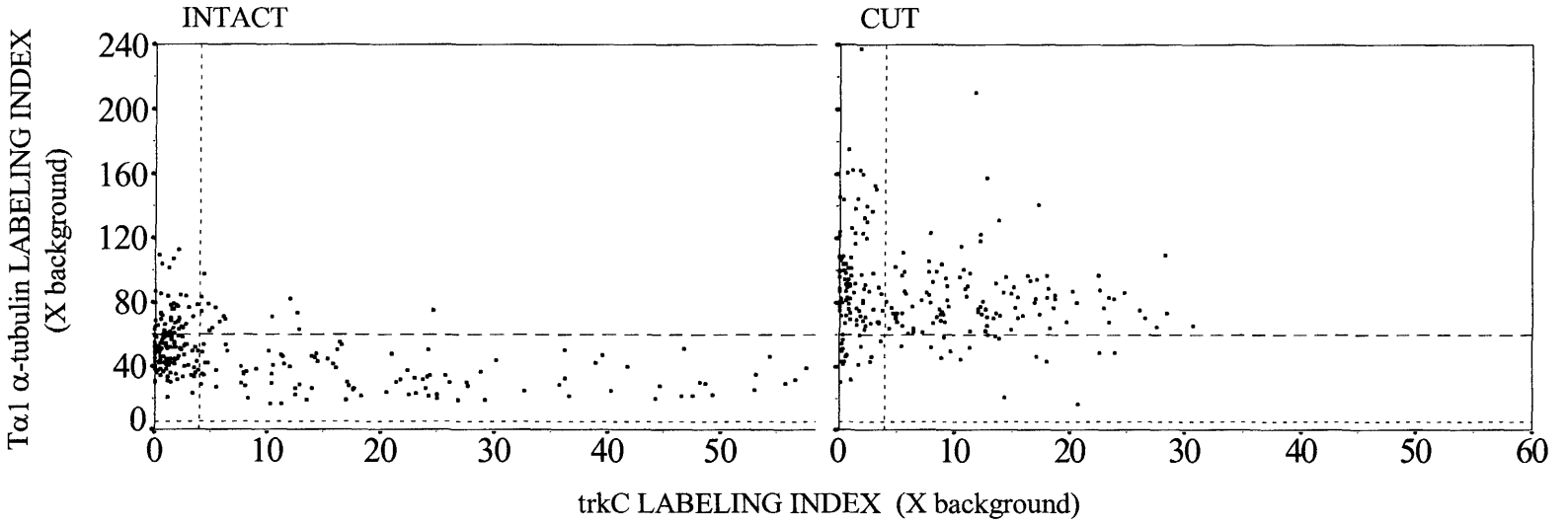

Figure 4.15 Relationship between relative levels of trkC and Tα1 α-tubulin mRNA under normal and axotomized states.

Scatterplots demonstrate the relationship between trkC and Tα1 α-tubulin labeling indices for 262-283 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or Tα1 α-tubulin (y axis) mRNA under normal (intact) and 3 week axotomized (cut) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles to the right of the short-dashed vertical line, and above the short-dashed horizontal line, are considered positive for trkC and Tα1 α-tubulin mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

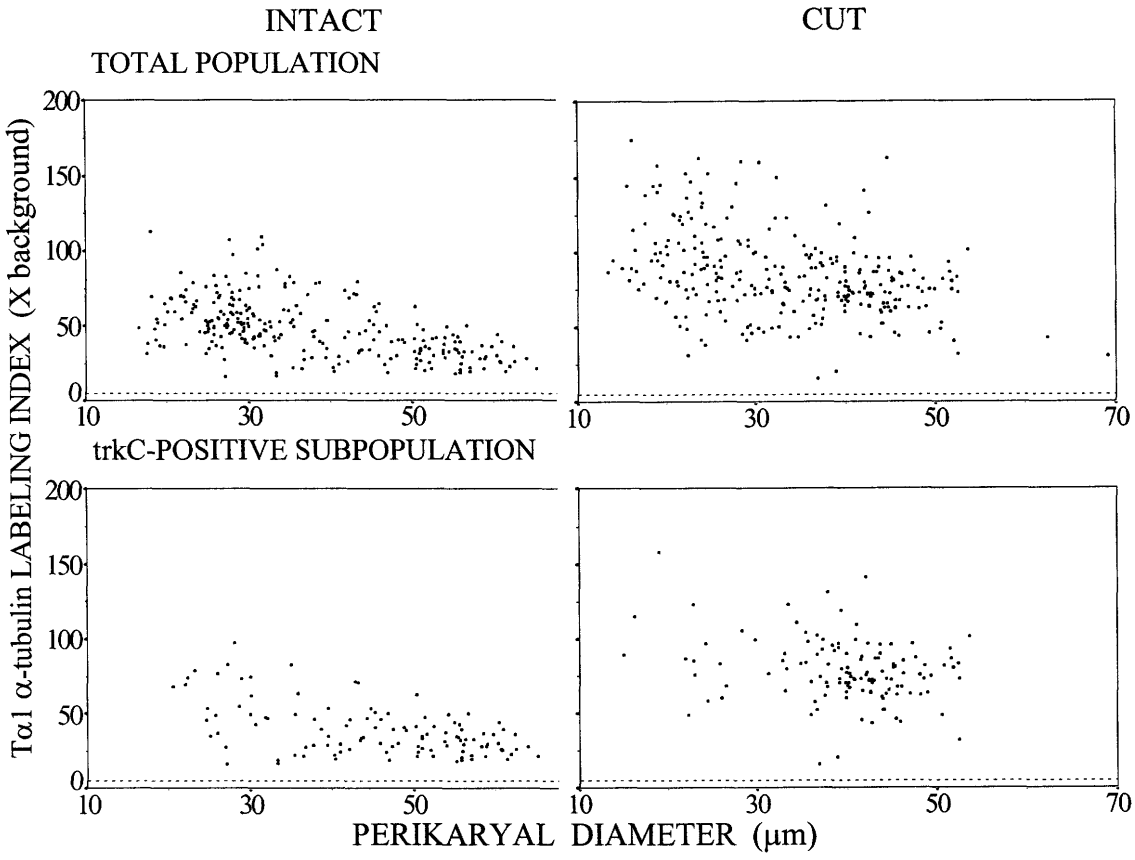

Figure 4.16 Relationship between perikaryal diameter and the relative level of Tα1 α-tubulin mRNA under normal and axotomized conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 262 (intact) and 283 (cut) individual neurons (of which 112/262 and 126/283 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect Tα1 α-tubulin and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and Tα1 α-tubulin labeling indices (y axis), for the total population and for the trkC-positive subpopulation. Panels show a comparison of data under normal conditions (intact) and 3 week axotomized (cut). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of Tα1 α-tubulin mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for Tα1 α-tubulin mRNA.

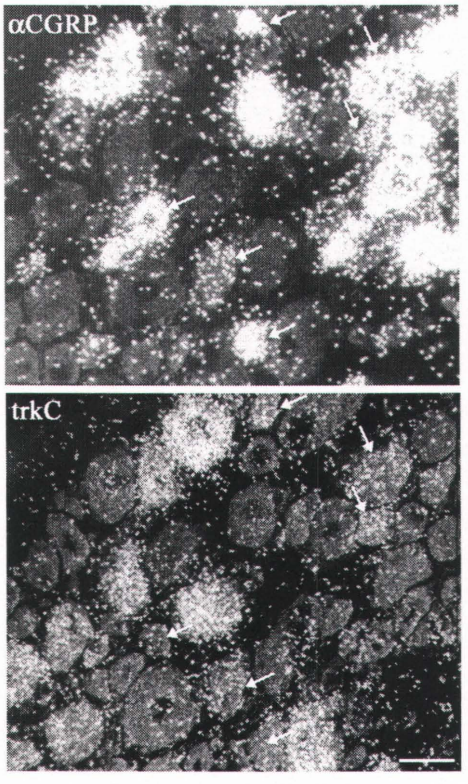

Figure 4.17 Colocalization of α-CGRP and trkC mRNA under normal conditions.

Scanned stained darkfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect α-CGRP (upper panel) and trkC (lower panel) mRNA under normal conditions. Arrows indicate neurons positive for both markers. Scale bar = 40 μm.

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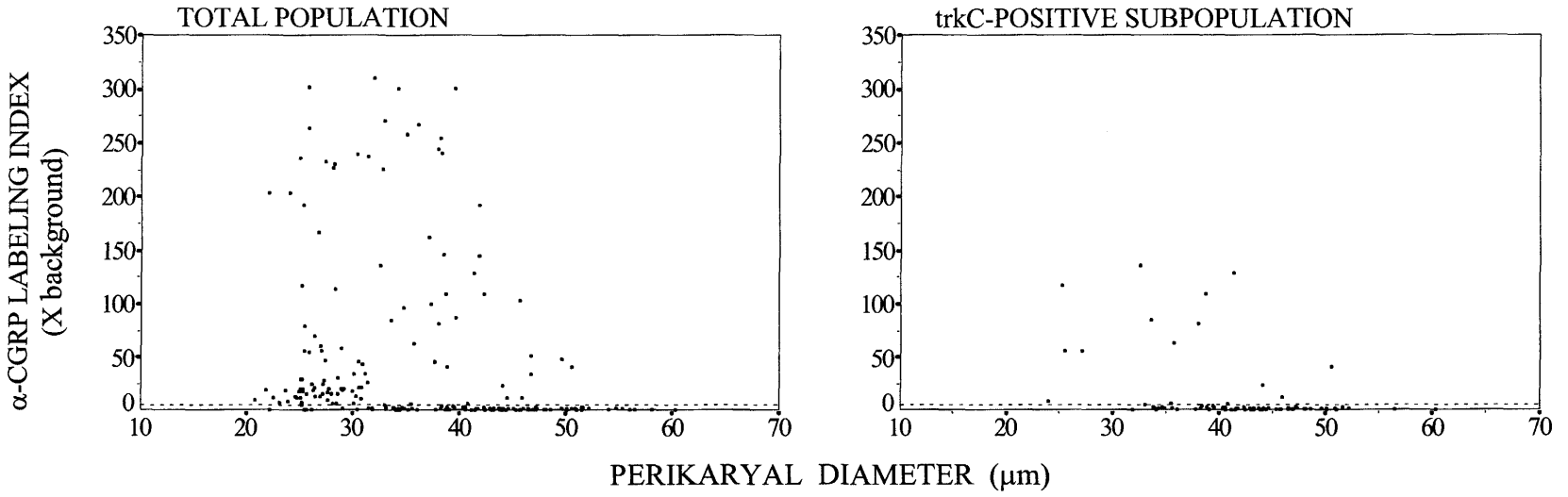

Figure 4.18 Relationship between perikaryal diameter and the relative level of α-CGRP mRNA under normal conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 200 individual neurons (of which 78/200 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect α-CGRP and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and α-CGRP labeling indices (y axis), for the total population and for the trkC-positive subpopulation under normal conditions (intact). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of α-CGRP mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for α-CGRP mRNA.

CGRP message. Within the α-CGRP positive population, there is a subset of small diameter cells that tend to express low to moderate levels of hybridization signal for the peptide and a group of predominately medium-sized neurons that exhibit high signal levels for α-CGRP. The trkC-positive population includes a small group of α-CGRP positive cells, which represent ~ 8% of DRG, ~ 16% of α-CGRP, and ~ 22% of trkC-expressing neurons. The α-CGRP/trkC-positive neurons range in size from small to large and tend to express moderately high levels of hybridization signal for α-CGRP.

Scatterplots (e.g. Figures 4.19 and 4.20), comparing the relationship between α-CGRP, trkA, and trkC mRNA expression for individual neurons (2 DRG sections/probe, 200 neurons/section), demonstrate that the majority of cells that express α-CGRP mRNA are trkA-positive and that this subgroup represents ~ 63% of α-CGRP and ~ 90% of trkA-positive cells. Neurons with higher levels of hybridization signal for trkA typically exhibit higher levels of α-CGRP signal and those with low trkA hybridization signal levels tend to express moderate levels of α-CGRP signal. For the most part, the α-CGRP/trkC-positive subpopulation (~ 8% of DRG neurons) show low trkC, and moderately high α-CGRP, labeling indices. The subset in which all three markers are detectable represents ~ 6% of DRG neurons; ~ 11% of α-CGRP, ~ 16% of trkA-, ~ 15% of trkC-positive cells; and ~ 17% of α-CGRP/trkA- and ~ 70% of α-CGRP/trkC-expressing neurons. They exhibit moderate to moderately high α-CGRP and trkA, and low trkC, hybridization signal levels.

4.2.4.2 The Expression of SP mRNA in the Intact State and Following Axotomy

Under normal conditions, many small diameter perikarya exhibit medium to high hybridization signal levels for SP and although SP and trkC transcripts colocalize, these occurrences are limited (Figure 4.21). Following axotomy, there is a substantial reduction in levels of hybridization signal for SP; low levels of hybridization signal for the peptide are now detectable in a subpopulation of medium-sized, trkC-positive neurons.

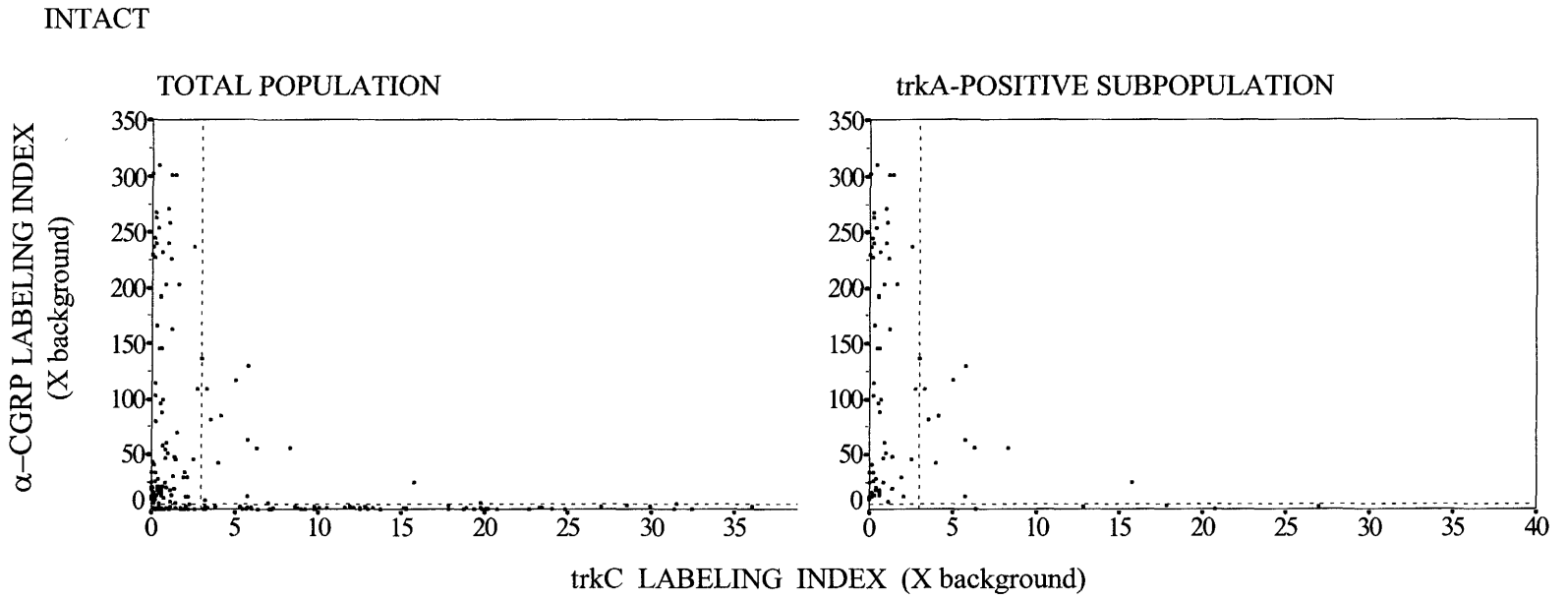

Figure 4.19 Relationship between relative levels of trkC and α–CGRP mRNA in the total DRG population and in the trkA-positive subpopulation.

Left scatterplot demonstrates the relationship between trkC and α–CGRP labeling indices for 200 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or α–CGRP (y axis), or trkA mRNA under normal (intact) conditions. The right scatterplot demonstrates the same relationship between trkC and α-CGRP, in the same section, but is specific for those neurons identified as expressing trkA mRNA (76/200). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkC and α–CGRP mRNA, respectively.

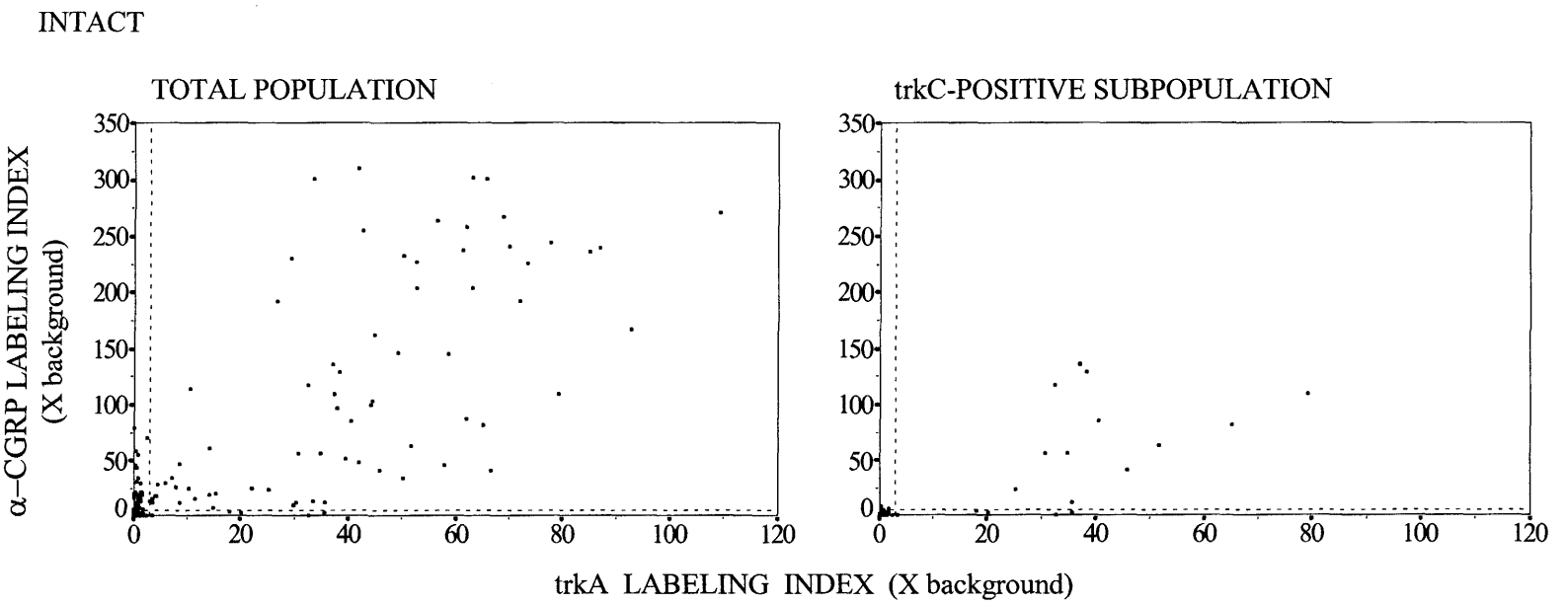

Figure 4.20 Relationship between relative levels of trkA and α–CGRP mRNA in the total DRG population and in the trkC-positive subpopulation.

Left scatterplot demonstrates the relationship between trkA and α–CGRP labeling indices for 200 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA (x axis) or α–CGRP (y axis), or trkC mRNA under normal (intact) conditions. The right scatterplot demonstrates the same relationship between trkA and α-CGRP, in the same section, but is specific for those neurons identified as expressing trkC mRNA (78/200). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkA and α–CGRP mRNA, respectively.

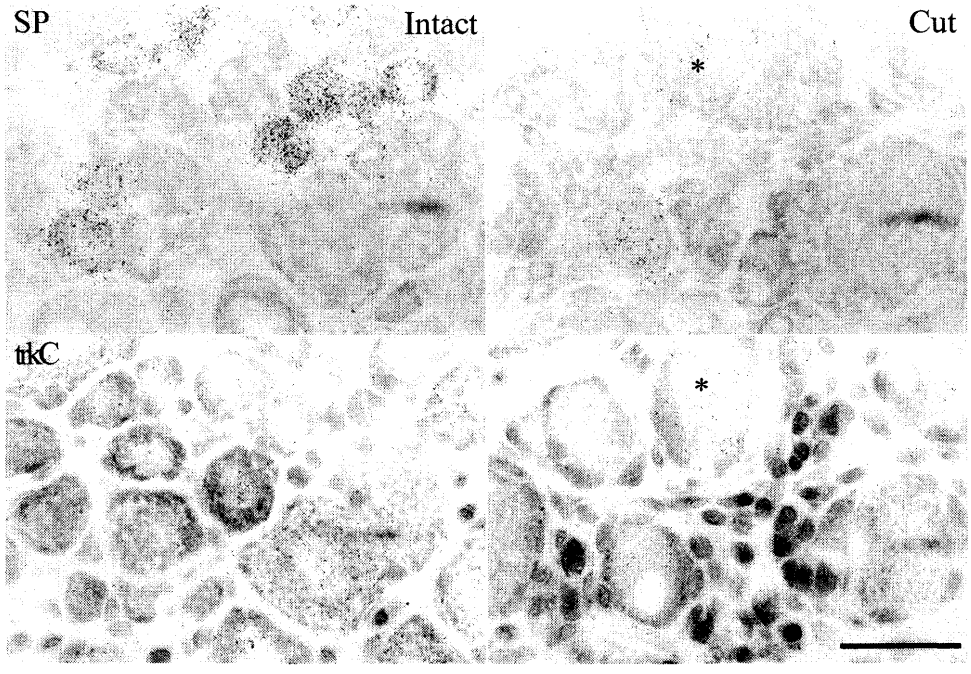

Figure 4.21 Colocalization of SP and trkC mRNA under normal and axotomized states.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect SP (upper panel) and trkC (lower panel) mRNA under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Asterisks indicate neurons positive for both markers. Scale bar = 40 μm.

Scatterplots (e.g. Figure 4.22), illustrating the relationship between SP and trkC mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 215 neurons/section) under normal conditions and 3 weeks post-axotomy, demonstrate that while ~ 20% of DRG neurons heterogenously express SP message in the intact state, only ~ 4% of these cells coexpress trkC mRNA. Cells in which SP and trkC transcripts colocalize represent ~ 20% of SP-positive, and ~ 12% of trkC-positive, neurons; the group includes a small subpopulation with moderate trkC, and low to moderate SP, hybridization signal levels and a subset of cells with low trkC, and moderate to high SP, signal levels. Following sciatic nerve transection SP transcript levels decrease, with the majority of SP-positive neurons now exhibiting only low levels of hybridization signal. Interestingly, although the percentage of SP-expressing cells remains the same (~ 20%), there is a trauma-induced shift in the character of the subpopulations of SP-positive neurons. Although SP mRNA is coexpressed in a group of trkC-positive neurons in the intact state (~ 20% of SP-positive neurons), axotomy appears to trigger the induction of detectable message levels of SP in a second subset of trkC-expressing neurons; SP and trkC mRNA now colocalize in ~ 15% of the surviving cells, representing ~ 78% of the SP-positive, and ~ 28% of the trkC-positive, subpopulations. In addition to exhibiting low levels of hybridization signal for SP, the post-transection SP/trkC population expresses low levels of signal for trkC.

Scatterplots (e.g. Figure 4.23), comparing the relationship between perikaryal diameter and SP mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 215 neurons/section) in the total DRG and for the trkC-expressing subpopulation, indicate that in the intact state the majority of SP-positive neurons are small in size (~ 72%) and only a subset of these small cells coexhibit trkC mRNA (~ 12%). There is also a group of medium and large diameter trkC-positive cells that coexpress low levels of SP hybridization signal (~ 10% of SP-positive neurons). Axotomy appears to result in a substantial reduction in the number of small neurons exhibiting detectable SP message and in the induction of low levels of hybridization signal for SP in a subpopulation of larger diameter neurons; following transection ~ 88 % of the cells with detectable SP mRNA are medium to large in size.

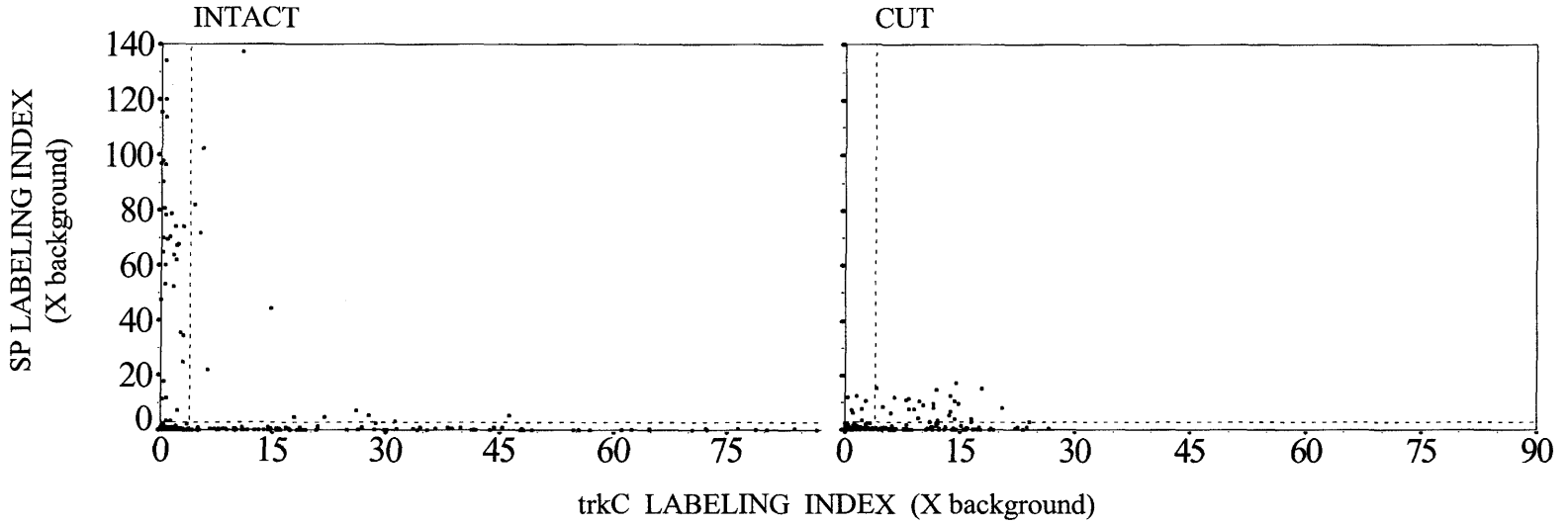

Figure 4.22 Relationship between relative levels of trkC and SP mRNA under normal and axotomized states.

Scatterplots demonstrate the relationship between trkC and SP labeling indices for 192-283 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or SP (y axis) mRNA under normal (intact) and 3 week axotomized (cut) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkC and SP mRNA, respectively. The upper right quadrant of each plot encompasses neurons labeled for both markers.

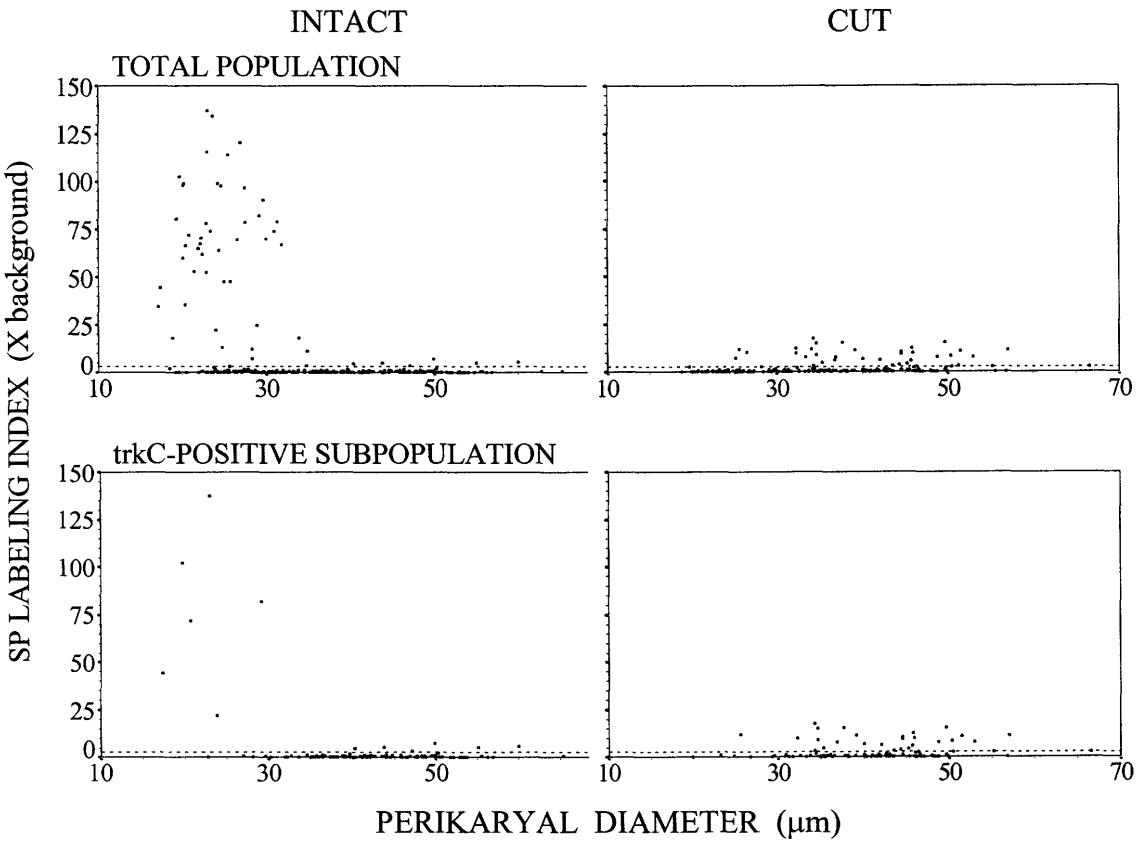

Figure 4.23 Relationship between perikaryal diameter and the relative level of SP mRNA under normal and axotomized conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 283 (intact) and 192 (cut) individual neurons (of which 107/283 and 99/192 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect SP and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and SP labeling indices (y axis), for the total population and for the trkC-positive subpopulation. Panels show a comparison of data under normal conditions (intact) and 3 week axotomized (cut). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of SP mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for SP mRNA.

The post-trauma SP-positive population is principally composed of cells expressing trkC message, representing ~ 78% of SP-expressing neurons.

Scatterplots (e.g. Figure 4.24), comparing the relationship between SP and trkA mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 192 neurons/section) in the total DRG and for the trkC-positive subpopulation, indicate that in the intact state ~ 75% of SP-positive neurons coexpress trkA, with only ~ 12% of the SP population exhibiting detectable levels of both trkA and trkC transcripts. This group, in which all of the markers trilocalize, expresses variable trkC, and moderate to high SP and trkA, hybridization signal levels. Following axotomy, only ~ 10% of the neurons that display SP mRNA coexpress trkA, with the majority of SP-positive neurons now exhibiting trkC message (~ 78%); ~ 6% of the SP population express all three markers.

4.2.4.3 The Expression of SOM mRNA in the Intact State

High levels of hybridization signal for SOM are exhibited by a small subpopulation of small- to medium-sized DRG neurons (Figure 4.25). There appears to be limited, if any, colocalization between this neuropeptide and trkC mRNA.

4.2.4.4 The Expression of Galanin mRNA in the Intact State and Following Axotomy

In the intact state, galanin mRNA is detectable in only a small number of small-sized DRG neurons (Figure 4.26). Sciatic nerve transection results in a dramatic increase in both the number of galanin-positive neurons and the level of hybridization signal. Three weeks post-axotomy, neurons of all sizes now express the peptide and there is significant overlap between these cells and the trkC-positive subpopulation.

4.2.4.5 The Expression of NPY mRNA in the Intact State and Following Axotomy

Normal DRG neurons do not exhibit detectable NPY mRNA (Figure 4.27). Three weeks following sciatic nerve transection, hybridization signal for

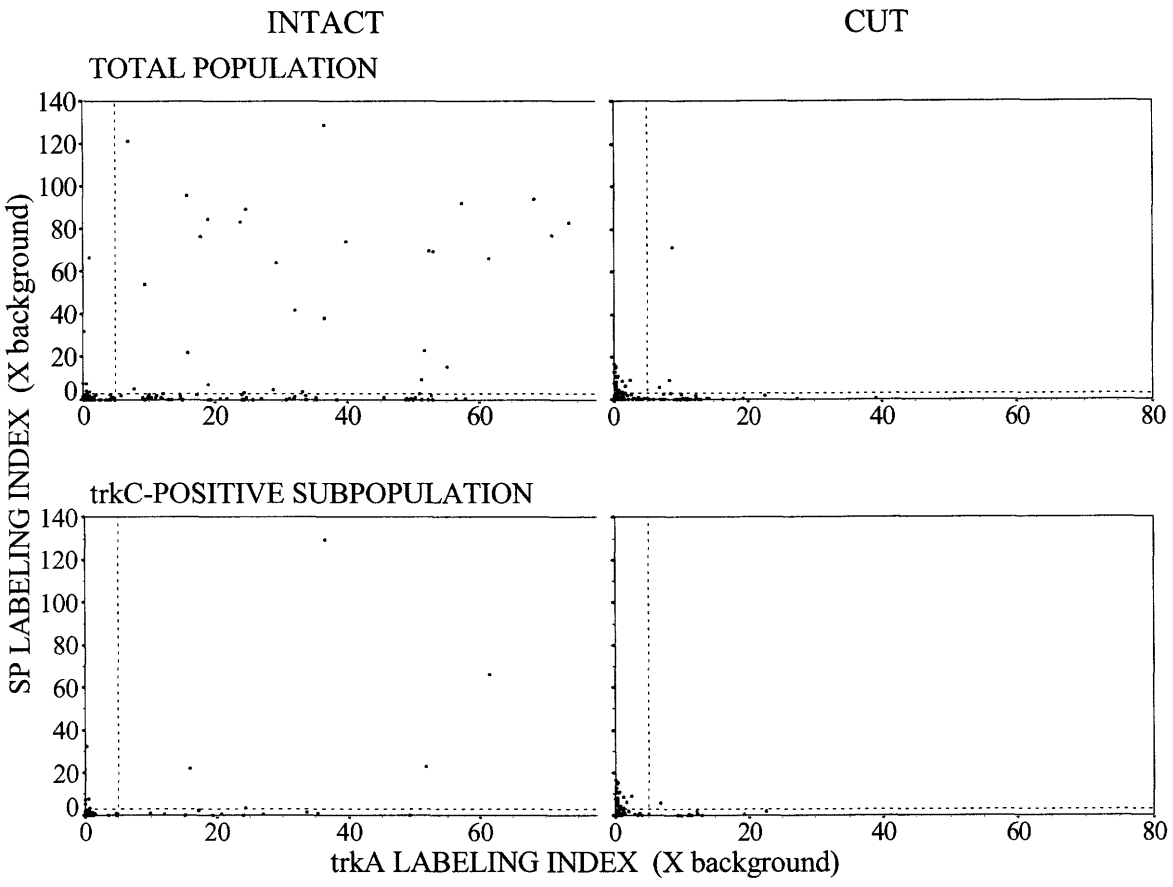

Figure 4.24 Relationship between the relative levels of trkA and SP mRNA under normal and axotomized conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 228 (intact) and 156 (cut) individual neurons (of which 79/228 and 82/156 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA, SP, and trkC mRNA. Graphs depict the relationship between trkA (x axis) and SP labeling indices (y axis), for the total population and for the trkC-positive subpopulation. Panels show a comparison of data under normal conditions (intact) and 3 week axotomized (cut). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles to the right of the the dashed vertical line and above the dashed horizontal line are considered positive for trkA and SP mRNA, respectively.

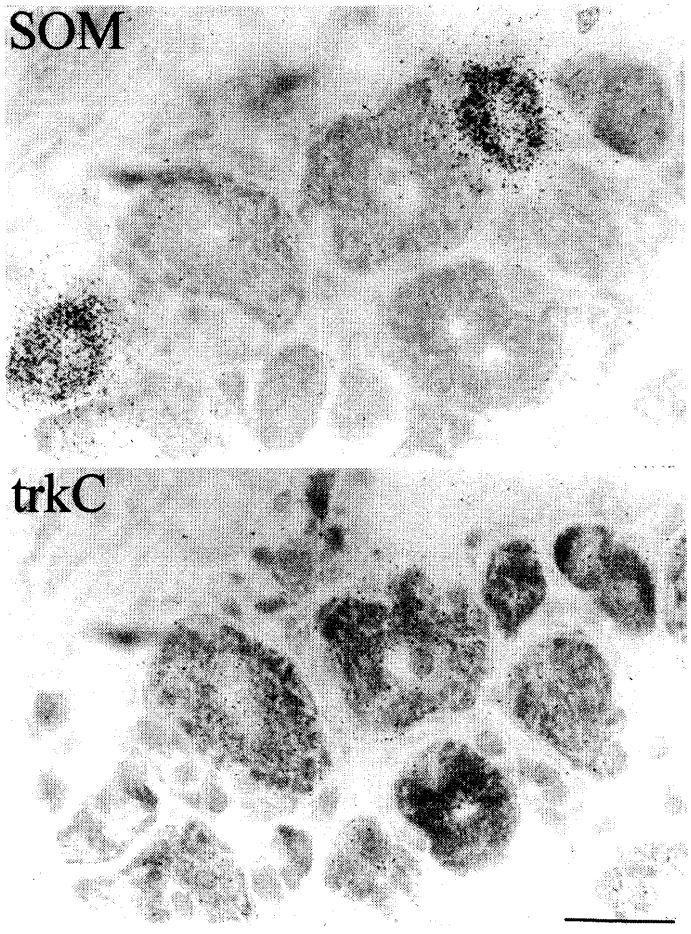

Figure 4.25 Colocalization of SOM and trkC mRNA under normal conditions.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect SOM (upper panel) and trkC (lower panel) mRNA under normal conditions. Scale bar = 30 μm.

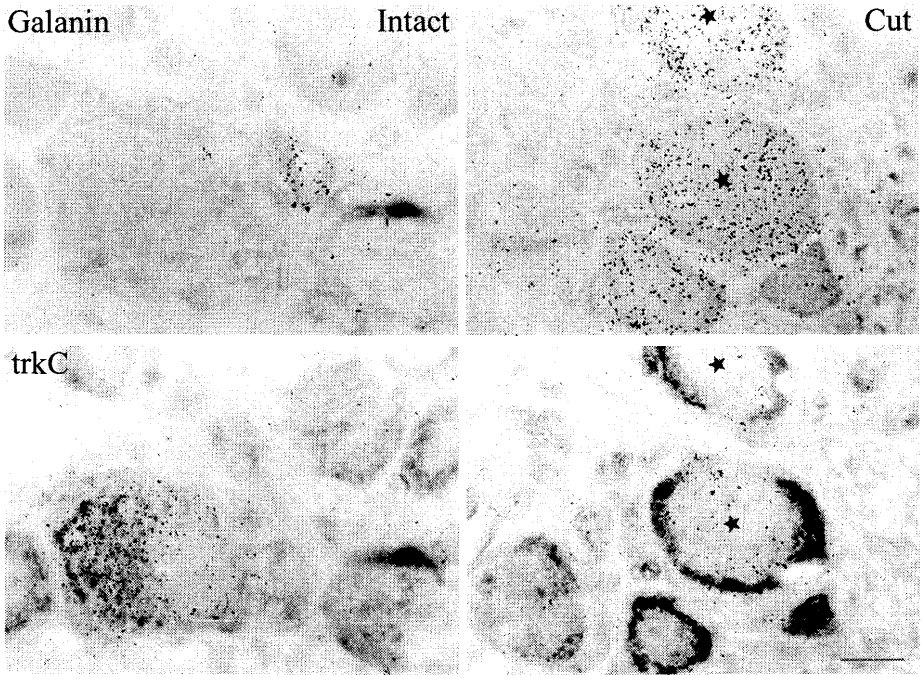

Figure 4.26 Colocalization of galanin and trkC mRNA under normal and axotomized states.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect galanin (upper panel) and trkC (lower panel) mRNA under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Stars indicate neurons positive for both markers. Scale bar = 20 μm.

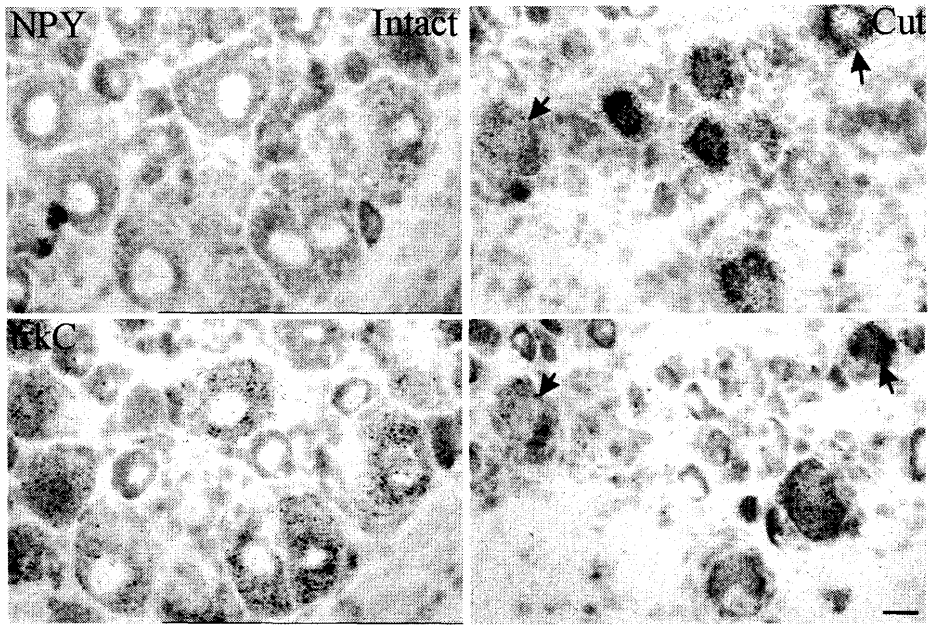

Figure 4.27 Colocalization of NPY and trkC mRNA under normal and axotomized states.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect NPY (upper panel) and trkC (lower panel) mRNA under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Arrows indicate neurons positive for both markers. Scale bar = 30 μm.

NPY is evident at moderate to high levels in a subpopulation of medium and large neurons, many of which also coexpress trkC mRNA.

4.2.4.6 The Expression of VIP mRNA in the Intact State and Following Axotomy

In the intact state, VIP mRNA is not detectable in DRG neurons (Figure 4.28). Even though 3 weeks post-axotomy many neurons, representing all size classes, exhibit moderate to high levels of hybridization signal for this peptide there is no apparent overlap with the trkC-positive subpopulation.

4.2.5 Injury and Regeneration Associated Markers

4.2.5.1 The Expression of GAP-43 mRNA in the Intact State and Following Axotomy

Hybridization signal detecting GAP-43 mRNA is present at moderate to high levels in many intact DRG neurons and there is a limited overlap between the GAP-43 positive and trkC-positive subpopulations (Figure 4.29). Following axotomy message levels appear to increase dramatically, with virtually all neurons expressing detectable levels of this marker.

Scatterplots (e.g. Figure 4.30), illustrating the relationship between trkC and GAP-43 mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 243 neurons/section), demonstrate that under normal conditions ~ 60% of DRG neurons exhibit detectable GAP-43 transcripts and that ~ 15% of neurons coexpress GAP-43 and trkC mRNA. The GAP-43/trkC-positive subpopulation represents ~ 25% of GAP-43 expressing, and ~35% of trkC-expressing, cells: There is no apparent correlation between labeling index values for the two markers. There is also a subset of neurons that do not exhibit detectable levels of either marker. Three weeks post-axotomy, there is a notable increase in both the levels of GAP-43 hybridization signal and in the number of neurons expressing GAP-43; transcripts are detectable in virtually all neurons. The highest GAP-43 message levels are present in a subset of cells in which trkC transcripts are not detectable and in a subset of cells with low levels of

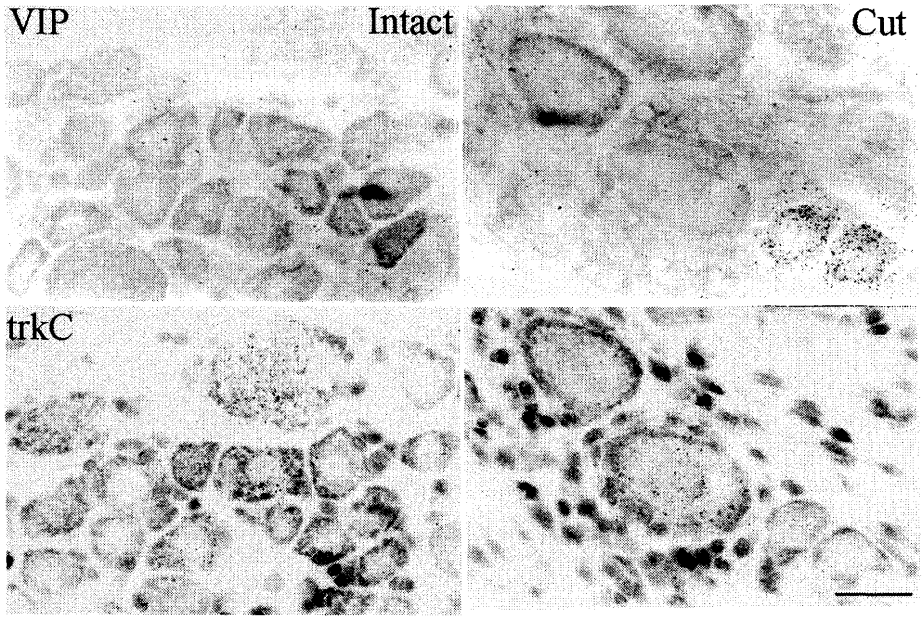

Figure 4.28 Colocalization of VIP and trkC mRNA under normal and axotomized states.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect VIP (upper panel) and trkC (lower panel) mRNA under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Scale bar = 30 μm.

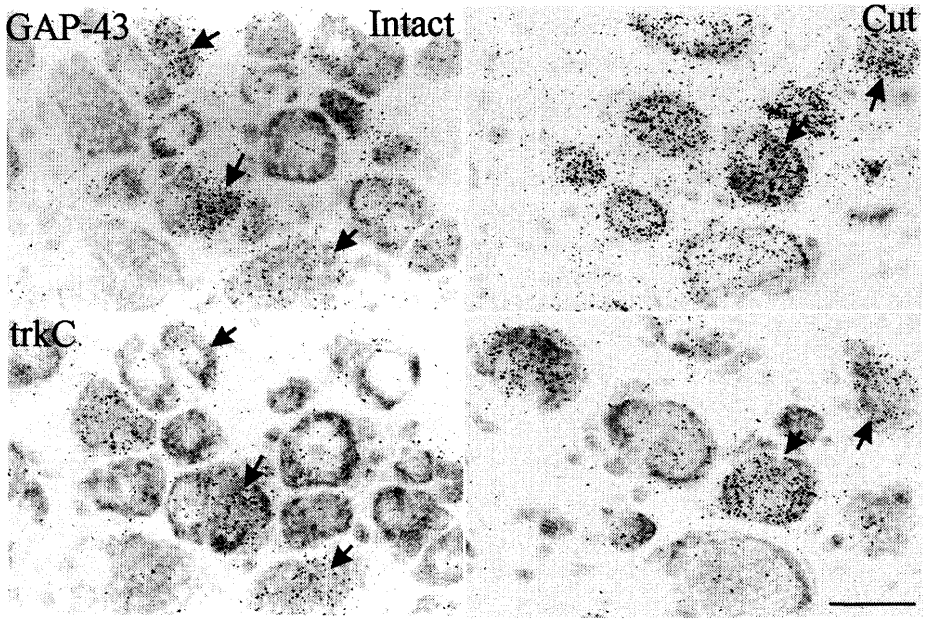

Figure 4.29 Colocalization of GAP-43 and trkC mRNA under normal and axotomized states

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect GAP-43 (upper panel) and trkC (lower panel) mRNA under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Arrows indicate neurons positive for both markers. Scale bar = 30 μm.

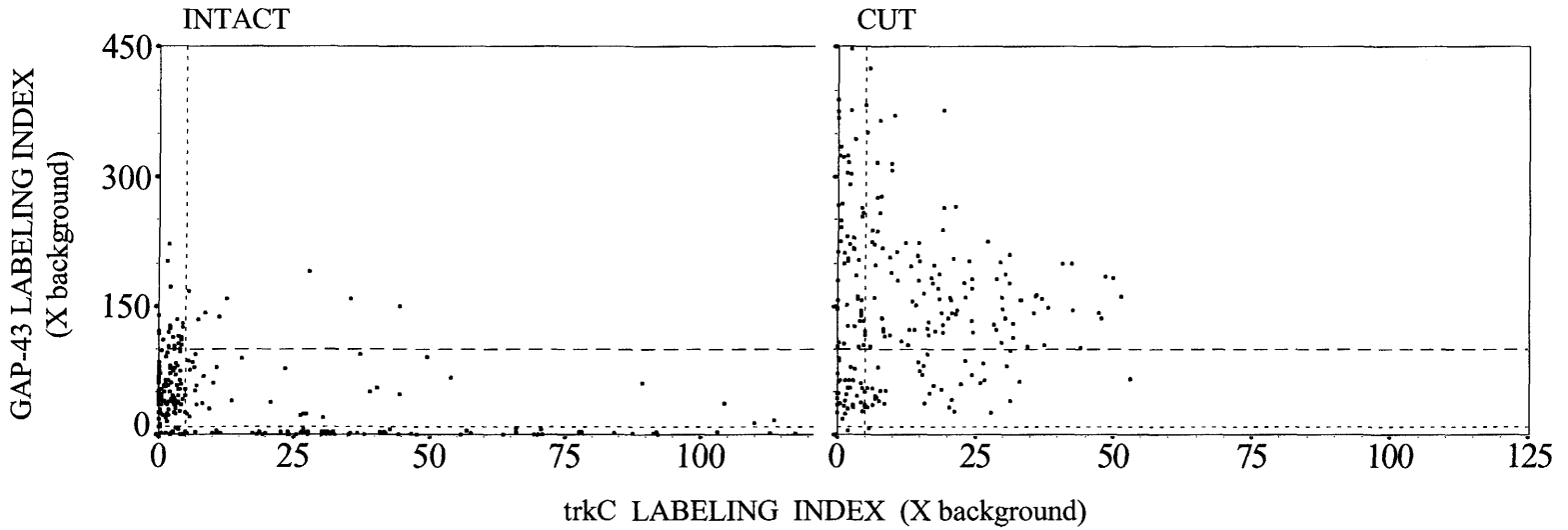

Figure 4.30 Relationship between relative levels of trkC and GAP-43 mRNA under normal and axotomized states.

Scatterplots demonstrate the relationship between trkC and GAP-43 labeling indices for 233-246 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or GAP-43 (y axis) mRNA under normal (intact) and axotomized (cut) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles to the right of the short-dashed vertical line, and above the short-dashed horizontal line, are considered positive for trkC and GAP-43 mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

hybridization signal for trkC.

Scatterplots (e.g. Figure 4.31), comparing the relationship between perikaryal diameter and GAP-43 mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 243 neurons/section) in the total DRG and for the trkC-positive subpopulation, indicate that in the intact state the majority of small, a subset of medium, and fewer large diameter neurons express GAP-43 mRNA. Three weeks following sciatic nerve transection GAP-43 transcripts are detectable in almost all neurons and although the mRNA is heterogenously expressed, a subset of small cells display the highest levels of hybridization signal. GAP-43 mRNA levels in the trkC-positive subpopulation, under both normal conditions and post-axotomy, reflect the range of levels present in the DRG population as a whole.

4.2.5.2 The Expression of cjun mRNA in the Intact State and Following Axotomy

Qualitative assessment of serial DRG sections show that in the intact state the majority of neurons exhibit low levels of hybridization signal for cjun (Figure 4.32). Following axotomy, there is a substantial increase in cjun mRNA levels.

Scatterplots (e.g. Figure 4.33), illustrating the relationship between trkC and cjun mRNA expression for individual neurons (3 DRG sections/treatment/probe, average 206 neurons/section), demonstrate that under normal conditions ~ 55% of neurons possess detectable levels of cjun transcripts and message expression is homogenous. Approximately 30% of the DRG population coexpress cjun and trkC mRNA; this subpopulation represents ~ 53% of cjun-positive, and ~ 72% of trkC-positive, cells. Since there is little variation in cjun labeling index values within the trkC-positive subset, there is no apparent correlation between levels of cjun and trkC transcripts. Three weeks following axotomy, there is a substantial upregulation of basal cjun message levels and virtually all neurons now express mRNA for the marker. Although the post-trauma message levels of cjun are elevated compared to those of the intact state, labeling index values remain more or less homogenous — except for a subset of cells lacking detectable trkC transcripts that exhibits higher levels of cjun hybridization signal.

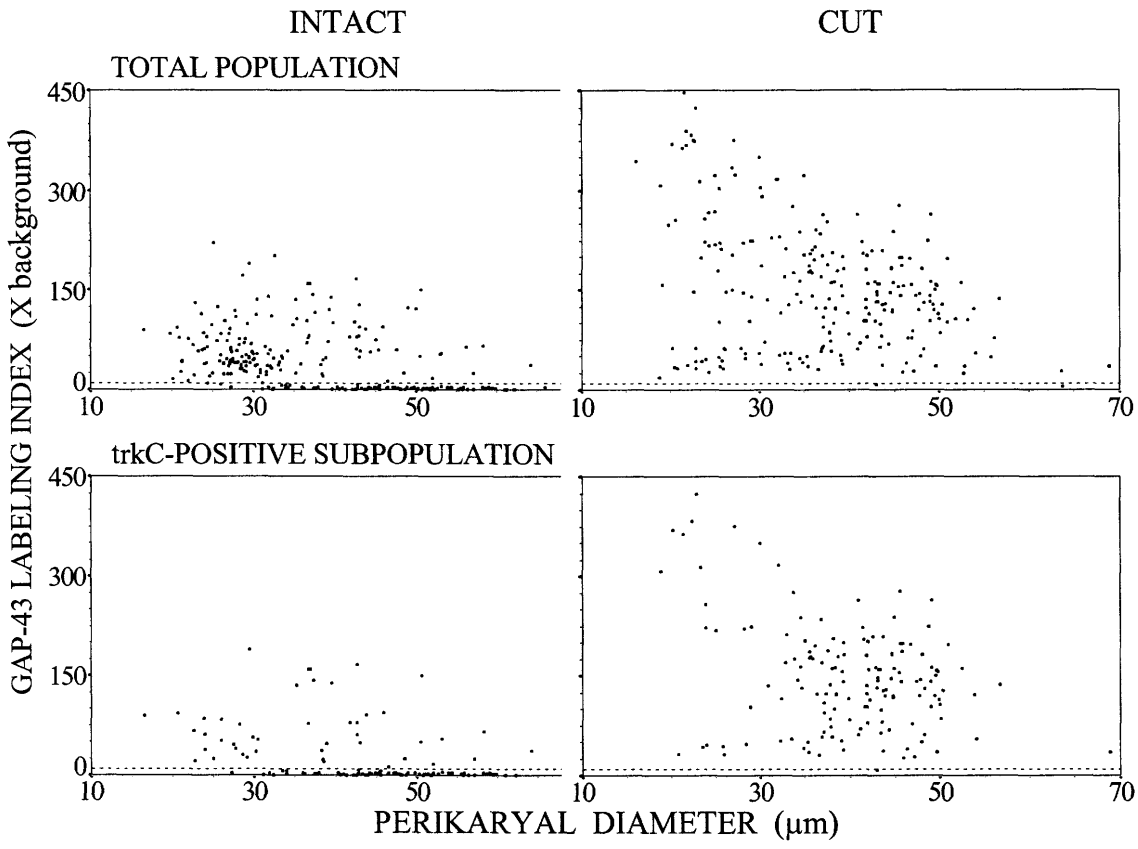

Figure 4.31 Relationship between perikaryal diameter and the relative level of GAP-43 mRNA under normal and axotomized conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 246 (intact) and 233 (cut) individual neurons (of which 116/246 and 147/233 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect GAP-43 and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and GAP-43 labeling indices (y axis), for the total population and for the trkC-positive subpopulation. Panels show a comparison of data under normal conditions (intact) and 3 week axotomized (cut). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of GAP-43 mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for GAP-43 mRNA.

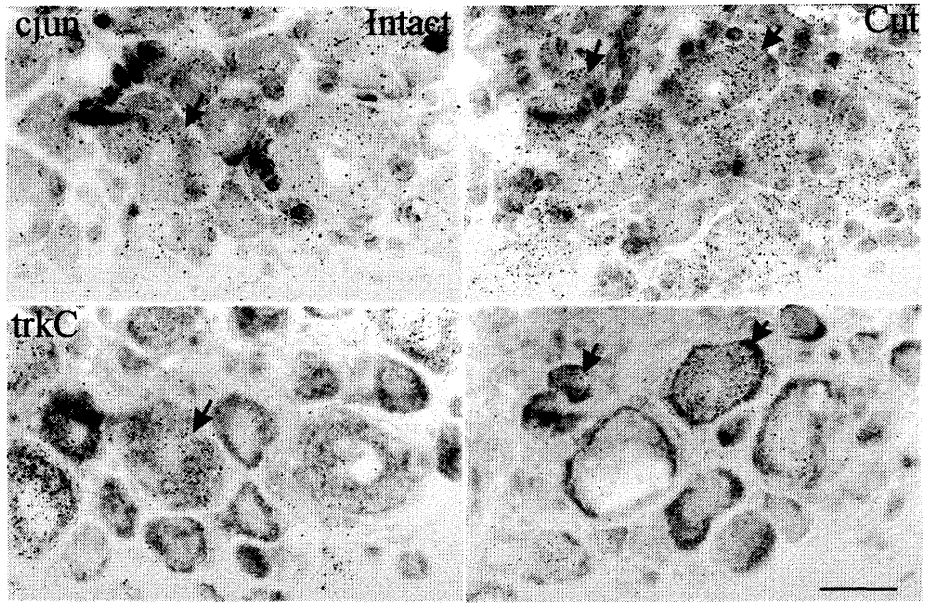

Figure 4.32 Colocalization of cjun and trkC mRNA under normal and axotomized states.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect cjun (upper panel) and trkC (lower panel) mRNA under normal conditions (intact) and 3 weeks following sciatic nerve transection (cut). Arrowheads indicate neurons positive for both markers. Scale bar = 30 μm.

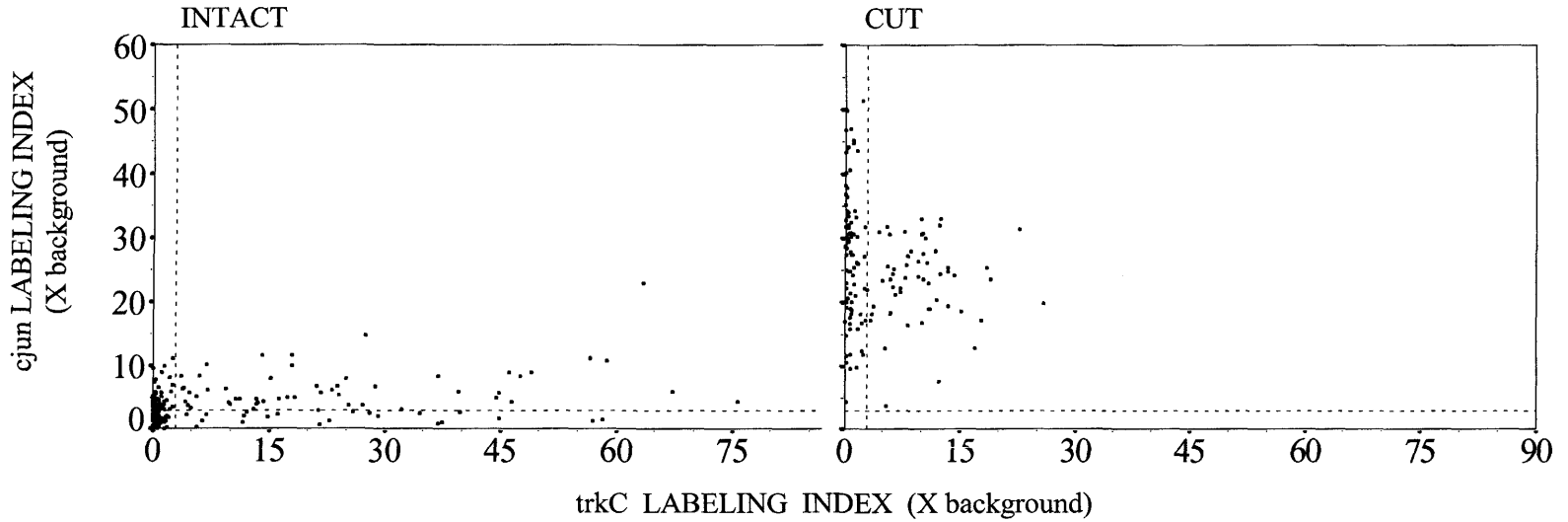

Figure 4.33 Relationship between relative levels of trkC and cjun mRNA under normal and axotomized states.

Scatterplots demonstrate the relationship between trkC and cjun labeling indices for 156-188 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or cjun (y axis) mRNA under normal (intact) and 3 week axotomized (cut) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkC and cjun mRNA, respectively. The upper right quadrant of each plot encompasses neurons labeled for both markers.

Scatterplots (e.g. Figure 4.34), comparing the relationship between perikaryal diameter and cjun mRNA expression for individual neurons (3 DRG sections/treatment/probe, average 206 neurons/section) in the total DRG and for the trkC-positive subpopulation, indicate that in the intact state cells exhibiting cjun transcripts represent all sizes of neurons and that there are more small-sized than large diameter perikarya that lack detectable cjun mRNA. After axotomy, virtually all DRG neurons display moderate to high levels of hybridization signal for cjun. Within each perikaryal size range neurons express a range of cjun hybridization signal levels, although a greater number of small diameter, non-trkC-expressing cells appear to display higher labeling indices for cjun. Within the trkC-positive subpopulation, cjun labeling index values are representative of those exhibited by the DRG population as a whole.

4.2.6 The Expression of SNAP-25 mRNA in the Intact State

SNAP-25 transcripts are abundantly expressed in most DRG neurons, although some neurons exhibit higher levels of hybridization signal than others — these cells tend to coexpress trkC mRNA (Figure 4.35).

Scatterplots (e.g. Figure 4.36), delineating the relationship between trkC and SNAP-25 mRNA expression for individual neurons (2 DRG sections/probe, average 208 neurons/section), demonstrate that under normal conditions all, or virtually all, neurons exhibit detectable, though variable, levels of SNAP-25 hybridization signal. While trkC-positive neurons display high levels of SNAP-25 hybridization signal, regardless of trkC mRNA levels, a subset of these cells display SNAP-25 labeling indices higher than those of the general DRG population. In contrast, a subgroup of neurons that lack detectable trkC mRNA exhibit the lowest relative levels of SNAP-25 hybridization signal.

Scatterplots (e.g. Figure 4.37), depicting the relationship between perikaryal diameter and SNAP-25 mRNA expression for individual neurons (2 DRG sections/probe, average 208 neurons/section), show that small diameter cells, typically lacking detectable trkC mRNA, tend to express low SNAP-25 labeling indices; medium and large neurons principally exhibit higher levels of SNAP-25 hybridization signal. There is no apparent correlation between perikaryal diameter and SNAP-25 message

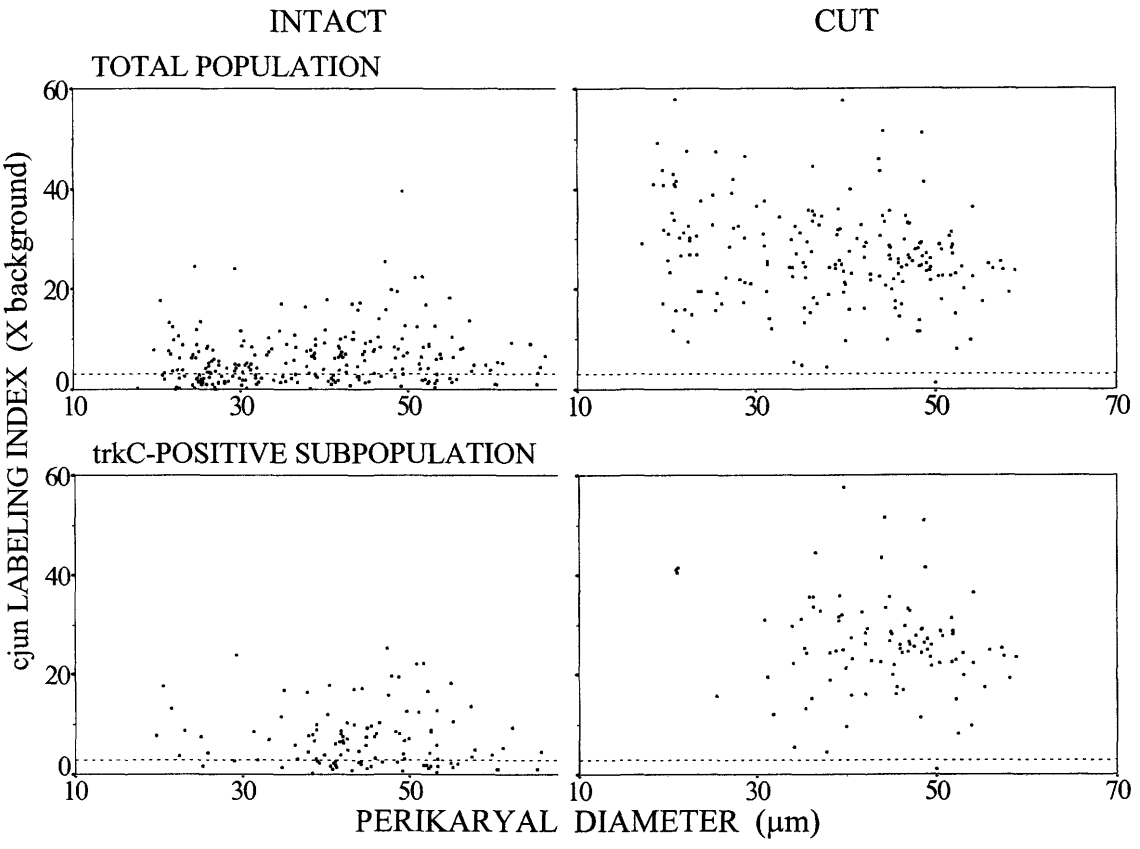

Figure 4.34 Relationship between perikaryal diameter and the relative level of cjun mRNA under normal and axotomized conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 246 (intact) and 198 (cut) individual neurons (of which 112/246 and 99/198 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect cjun and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and cjun labeling indices (y axis), for the total population and for the trkC-positive subpopulation. Panels show a comparison of data under normal conditions (intact) and 3 week axotomized (cut). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of cjun mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for cjun mRNA.

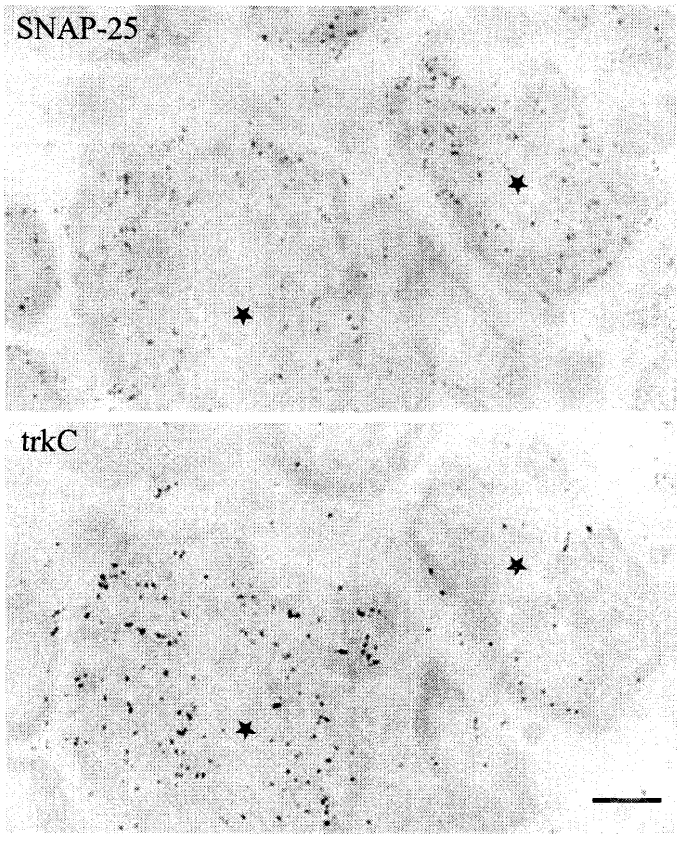

Figure 4.35 Colocalization of SNAP-25 and trkC mRNA under normal conditions.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect SNAP-25 (upper panel) and trkC (lower panel) mRNA under normal conditions. Stars indicate neurons positive for both markers. Scale bar = 10 μm.

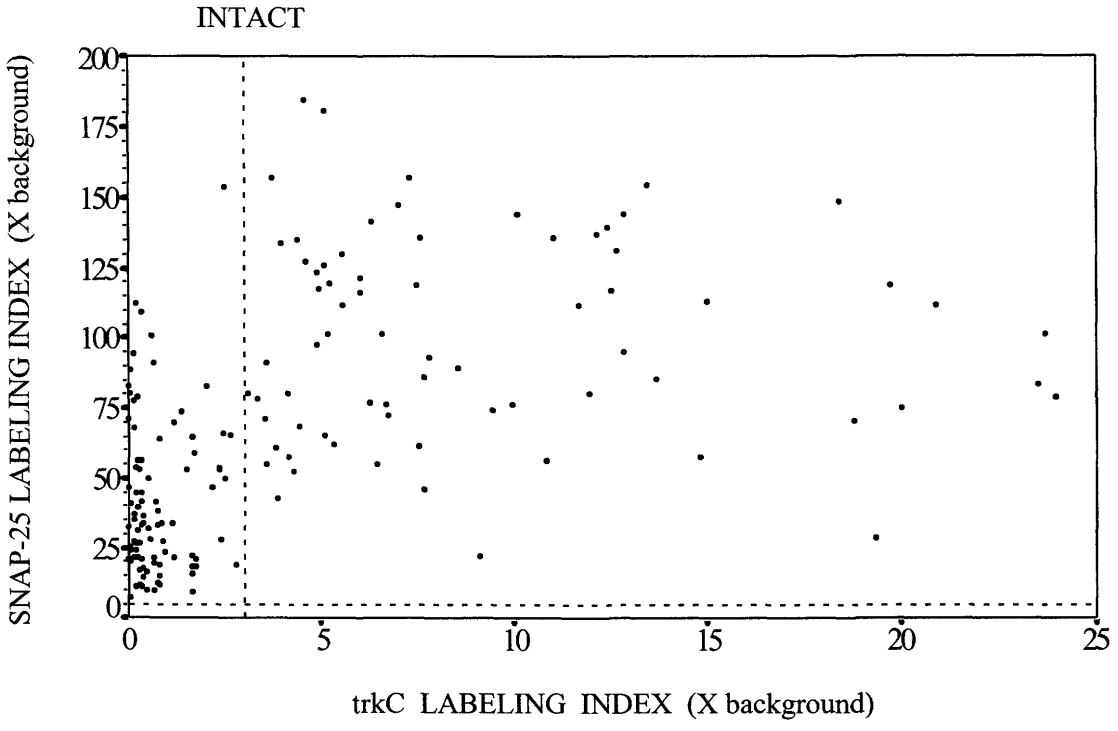

Figure 4.36 Relationship between relative levels of trkC and SNAP-25 mRNA under normal conditions.

Scatterplot demonstrates the relationship between trkC and SNAP-25 labeling indices for 165 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or SNAP-25 (y axis) mRNA under normal (intact) conditions. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Neuronal profiles to the right of the dashed vertical line, and above the dashed horizontal line, are considered positive for trkC and SNAP-25 mRNA, respectively, with the upper right quadrant encompassing neurons labeled for both markers.

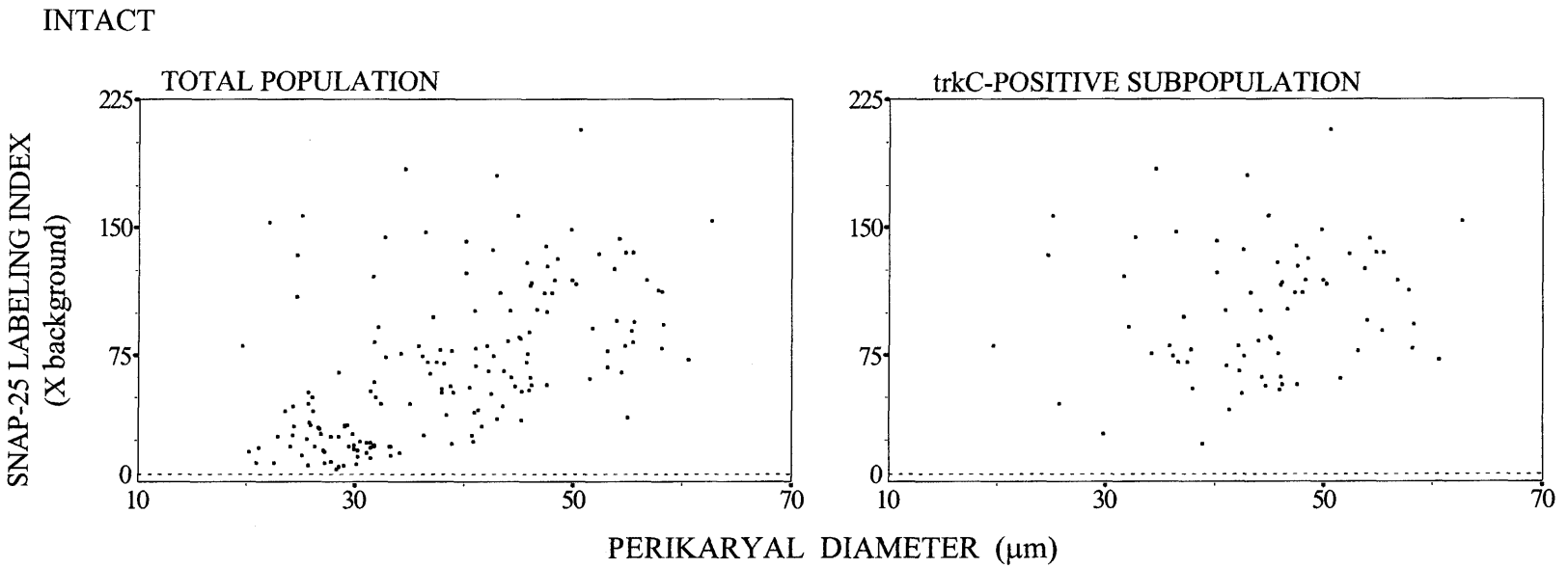

Figure 4.37 Relationship between perikaryal diameter and the relative level of SNAP-25 mRNA under normal conditions for the total DRG and for the trkC-positive subpopulation.

Scatterplots of labeling indices of 165 individual neurons (of which 72/165 are trkC-positive) identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect SNAP-25 and trkC mRNA. Graphs depict the relationship between perikaryal diameter (x axis) and SNAP-25 labeling indices (y axis), for the total population and for the trkC-positive subpopulation under normal conditions (intact). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of SNAP-25 mRNA. For each plot, neuronal profiles above the dashed horizontal line are considered positive for SNAP-25 mRNA.

levels in the trkC-positive subpopulation, with the majority of these cells expressing high labeling index values for SNAP-25.

4.3 Discussion

4.3.1 trkC mRNA Expression In Adult Rat Lumbar DRG Neurons

Although transcripts of the NT-3 receptor, trkC, have been identified in a subset of adult rat lumbar DRG neurons, reported percentages differ greatly, with from 10% (Mu *et al.*, 1993), to 15-20% (McMahon *et al.*, 1994; Wetmore and Olson, 1995; Kashiba *et al.*, 1996, 1997a), and up to 45% (Karchewski *et al.*, 1999) of the total population deemed to express this marker. Results here, indicating that ~ 40% of adult rat lumbar DRG neurons exhibit detectable trkC mRNA, correspond most closely with the latter findings (Karchewski *et al.*, 1999), not a surprising observation since the same animal Strain (Wistar), protocol, materials, and modes of analyses were used in both cases. These findings are also supported by a study that indicates that of the ~ 40% of lumbar DRG neurons that display NT-3-ir (Zhou and Rush, 1995; Chen *et al.*, 1996), trkC-ir colocalizes in ~ 95% of these cells (Chen *et al.*, 1996).

The apparent discrepancies in the percentages of trkC-positive DRG neurons identified by different research groups may be due to a number of factors. Most importantly, even though all data were obtained through *in situ* hybridization studies (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Kashiba *et al.*, 1996, 1997a; Karchewski *et al.*, 1999), the protocols followed may have differed, with some processing methods more sensitive to the detection of mRNA than others (Tecott *et al.*, 1987). Furthermore, when radioisotopic probes are used the length of time that the radioautographs are exposed to emulsion before developing is also important in distinguishing between cells with low levels of trkC transcripts and nonspecific, background labeling. Several groups have reported uniformity of labeling intensities among the trkC-positive subpopulation (Wetmore and Olson, 1995; Wright and Snider, 1995), which may reflect a technical limitation in their abilities to identify neurons with low to near background levels of hybridization signal. Here, as for the findings of Karchewski and coworkers (1999), ~ 10% of trkC-expressing L_5 DRG neurons are

shown to exhibit relatively low signal levels for the receptor — a factor that may account for the apparent inability of some researchers to detect trkC transcripts within these cells. The small disparity between the number of trkC-positive neurons identified here compared to that reported by Karchewski and colleagues (1999) (40% *versus* 45%, respectively) may be attributed to the need in this work to colocalize transcripts of one or more markers with trkC mRNA in consecutive sections and to select DRG with a similar plane of section for all experimental treatments examined in order to ensure comparable ganglionic topography. In some instances, these considerations may have necessitated the use of tissue with suboptimal radioautograph exposure to preserve the series, which may have resulted in slightly reduced numbers of neurons exhibiting trkC hybridization signal above detectable levels.

In spite of the interstudy variability in the reported percentages of trkC-positive DRG neurons, all groups concur that the perikarya of these cells are primarily large-sized (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Kashiba *et al.*, 1996, 1997a; Helgren *et al.*, 1997; Karchewski *et al.*, 1999). Here, in agreement with the observations of Karchewski and coworkers (1999), ~ 85% of trkC-expressing cells possess medium to large diameter soma. Further, there is a tendency for small neurons to exhibit relatively low levels of hybridization signal for trkC mRNA and, although larger perikarya display variable labeling intensities, those with the highest levels of labeling are of large diameter. These data parallel the findings of a study wherein large diameter soma were moderately to heavily labeled with radioiodinated NT-3 that had been injected into sciatic nerve, but small diameter perikarya exhibited only moderate levels of labeling (Helgren *et al.*, 1997). Also, I have shown that three weeks following sciatic nerve transection levels of trkC hybridization signal decrease, but the proportion of surviving neurons that are trkC-positive remains comparable to that of the intact state, inferring a lack of bias for the loss or survival of trkC-positive neurons. Similar data have been reported for the same and earlier post-axotomy time points (McMahon *et al.*, 1994; Jongsma Wallin *et al.*, 2001).

These results, together with the data reported by Karchewski and colleagues (1999), indicate that a far greater proportion (40-45%) of adult rat lumbar DRG neurons

are trkC-positive than had been previously believed, and that these cells express a range of *in situ* hybridization labeling intensities and include neurons of all sizes. Moreover, while axotomy results in reduced levels of trkC expression in individual neurons, it does not appear to alter the percentage of cells that exhibit transcripts for the receptor.

4.3.2 Colocalization of trkC with trkA and p75

In this work ~ 40% of adult rat L_5 DRG neurons are shown to exhibit detectable trkA mRNA — a finding supported by the literature, wherein 40-45% of lumbar neurons are reported to possess high-affinity NGF binding sites (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1989b, 1990b, 1992a) or to express trkA message (Verge *et al.*, 1992a, 1995; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Karchewski *et al.*, 1999). Further, as also shown here, there is consensus that the perikarya of these cells are primarily small to medium in size (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1992a, 1992b; Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Kashiba *et al.*, 1996; Karchewski *et al.*, 1999).

There are a limited number of studies that concern the coexpression of trks in adult DRG neurons. One experiment (DiStefano *et al.*, 1992) demonstrated that although retrogradely transported iodinated NT-3, NGF, and BDNF labeled neurotrophin-specific subsets of neurons, the range of perikaryal sizes that each identified raises the possibility of trk species colocalization. Similarly, in a study comparing the number of trk-positive neurons in adult rat DRG sections hybridized with a cocktail containing probes for all three known trks (66%) to the cumulative number of neurons labeled with the same probes but for the individual trks (% trkA + % trkB + % trkC = 88%), the greater number of neurons identified in the latter analysis implies that overlapping expression of the trks exists (McMahon *et al.*, 1994). Moreover, two research groups have directly demonstrated that a subset of primary sensory neurons coexpress trkA and trkC. Wright and Snider (1995) describe a small subgroup of thoracic DRG neurons in which mRNAs for the two receptor species colocalize. In a more detailed study, Karchewski *et al.* (1999) showed that in adult rat L_4/L_5 DRG ~ 20% of neurons are trkA/trkC-positive; these cells represent ~ 50% of both the trkA- and trkC-expressing subpopulations.

Further, they showed that within the trkA/trkC-positive subset, mRNAs for both markers were expressed at variable levels, although many exhibited low levels of trkC hybridization signal. Here, ~ 10% of L_5 DRG neurons, representing ~ 25% of the trkA- and trkC-positive subpopulations, are shown to coexpress variable levels of trkA and trkC transcripts. That a lower percentage of trkA/trkC-positive neurons has been reported in this work is most likely a reflection of a failure to detect at least some cells with low message levels of either marker (see Section 4.3.1). This is especially relevant in the case of neurons that coexpress trkA and trkC, since a subset appear to exhibit relatively low levels of hybridization signal for the latter receptor (Karchewski *et al.*, 1999). In agreement with the findings of Wright and Snider (1995), trkA/trkC-expressing neurons are primarily medium-sized, with larger perikarya than most other trkA-positive cells.

In addition to the trkA/trkC-expressing subset, trkC/trkB- and trkA/trkB-positive subpopulations have been identified and represent ~ 18% and ~ 10% of L_4/L_5 DRG neurons, respectively (Karchewski *et al.*, 1999). In fact, their data indicate that the colocalization of trks occurs more often than the expression of individual trk species in adult rat lumbar DRG neurons (Karchewski *et al.*, 1999). Moreover, the three trk species trilocalize in 3-4% of these cells (Karchewski *et al.*, 1999). These observations may represent important phenomena in defining the NT-3 responsive subpopulation of DRG neurons, given the reports of that neurotrophin binding to trkA and/or trkB under certain conditions (Cordon-Cardo *et al.*, 1991; Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991) (see Section 1.1.2.1.1).

Also of interest is the report that an isoform of trkA ($trkA_{insert}$) that appears to bind NT-3 with high affinity in sympathetic neurons (Barker *et al.*, 1993; Clary and Reichardt, 1994) (see Section 1.1.2.1.1), is present in at least 90% of lumbar DRG neurons that express trkA; relative levels of expression of both isoforms seem to be positively correlated (Karchewski *et al.*, 1999). These findings further increase the potential that NT-3 can directly influence all, or most, trkA-positive neurons.

Here, in agreement with previous findings (Wetmore and Olson, 1995; Karchewski *et al.*, 1999), p75 mRNA is shown to be heterogenously expressed in ~ 80%

of adult rat lumbar DRG neurons. Moreover, the reported tight correlation between trk and p75 mRNA expression (Verge *et al.*, 1992a; Wright and Snider, 1995; Karchewski *et al.*, 1999) appears to be maintained for trkC and p75, with the colocalization of p75 transcripts in virtually all trkC-positive neurons. These findings conflict with those of Wright and Snider (1995), wherein only 50% of trkC-expressing neurons were shown to coexhibit p75 mRNA in adult rat thoracic DRG. Their use of a combination of isotopic and nonisotopic probes to colocalize the two receptor species may have resulted in a reduced ability to detect low levels of transcripts. Results here (e.g. Figure 4.8) show that many trkC-positive cells, especially those with the highest levels of trkC hybridization signal, exhibit relatively low labeling indices for p75 — these neurons may therefore possess message levels below the detection limit of the digoxigenin-labeled p75 probe used by Wright and Snider (1995). Their report that the majority of trkA- or trkB-positive thoracic DRG neurons coexpress p75 does not contradict this observation, since data here indicate that trkC/trkA-expressing cells tend to exhibit higher levels of p75 hybridization signal than trkC-positive neurons that do not coexpress trkA. Additionally, because *in situ* hybridization studies demonstrate p75 transcripts in ~ 80% of DRG neurons (Wetmore and Olson, 1995; Karchewski *et al.*, 1999), while only ~ 60% of neurons have been shown to display detectable p75-ir in less sensitive immunohistochemical analyses (Zhou *et al.*, 1993, 1996a), indicates that some neurons express relatively low levels of p75.

The colocalization of trkC and p75 transcripts and the heterogeneous expression of mRNA for these receptors in subsets of adult rat lumbar DRG neurons allow for the possibility that individual neurons, or at least specific subpopulations of these cells characterized on the presence and level of the receptors, may uniquely respond to NT-3. Moreover, the potential for the coexistence of trkC, p75, trkA, and $trkA_{insert}$ transcripts further subdivides DRG neurons, creating micropopulations that potentially permit population-specific responses to the neurotrophins; presumably the same would hold true for the trkC/p75/trkB- and trkC/p75/trkB/trkA-positive subsets identified by Karchewski and colleagues (1999). Such responses could be attributed to a number of factors: The presence of p75 may influence the ability of ligand to bind to trk (Barker

and Shooter, 1994; Kahle *et al.*, 1994; Twiss *et al.*, 1998; Wang *et al.*, 1998) in either a positive or negative manner, depending on neurotrophin concentration and the duration of ligand availability (see Section 1.1.2.2.2). Further, at least for trkA and potentially for the other trks, the ratio between trk and p75 appears to be important to establishing the efficacy of the former receptor (see Section 1.1.2.2.2). Also, the presence in individual neurons of multiple neurotrophin receptors that can bind NT-3 may allow for parallel and/or synergistic downstream signal transduction pathways and effects or, conversely, the NT-3 induced activation of multiple trk species may be redundant (see Section 1.1.2.1.2).

4.3.3 Characterization of the trkC-Positive Subpopulation with Respect to the Expression of the Cytoskeletal Elements NFM and Tα1 α-tubulin

Neurofilaments are a class of intermediate filaments specific to neurons and are an important component of the neuronal cytoskeleton (reviewed in: Fliegner and Liem, 1991). They are assembled from three polypeptide subunits, defined according to molecular weight and designated NF low/light (NFL), NF middle/medium (NFM), and NF high/heavy (NFH), although the actual molecular weight of each varies between species. In mammals, the molecular weights for NFL, NFM, and NFH range from 60-70 kDa, 130-170 kDa, and 180-200 kDa, respectively (reviewed in: Fliegner and Liem, 1991). A positive correlation has been demonstrated to exist between NF level and perikaryal size (Sharp *et al.*, 1982; Verge *et al.*, 1990a; Tetzlaff *et al.*, 1992), with even the smallest diameter DRG neurons expressing low, but detectable levels of NFM transcripts (Verge *et al.*, 1990a). Similar observations are reported in this study. Further, although there does not appear to be a correlation between the numbers of high-affinity NGF-binding sites and NFM levels in primary sensory neurons (Verge *et al.*, 1990a; Tetzlaff *et al.*, 1992), there is some degree of correlation between trkC and NFM message levels. Here, small trkC-positive neurons are shown to express low levels of trkC and NFM mRNAs, and although larger diameter neurons exhibit a range of trkC labeling indices, those with the highest levels of hybridization signal for trkC tend to have larger soma and higher NFM labeling indices. Moreover, because there is a tight

correlation between NF level and axonal diameter (Friede and Samorajski, 1970; Weiss and Mayr, 1971; Hoffman *et al.*, 1987) and between perikaryal volume and fiber conduction velocity (Harper and Lawson, 1985a, 1985b; Lee *et al.*, 1986), these data support the role of large diameter, high NFM- and trkC-expressing neurons as mechanoceptors, and more specifically as proprioceptors (see Sections 2.2.2.1, 2.2.2.2).

Tubulin, which polymerizes to form microtubules, is itself formed from chains of heterodimers of α- and β-tubulin (reviewed in: Baas, 1997). Neurons constitutively express two isotypes of α-tubulin, T26 and Tα1, although levels of the former remain unchanged throughout development and during regeneration (Miller *et al.*, 1987, 1989), while those of the latter are upregulated under such circumstances (Miller *et al.*, 1987, 1989, 1991; Mathew and Miller, 1990, 1993; Ma *et al.*, 1992; Wu *et al.*, 1993). β-tubulin has also been shown to be upregulated in neurons following peripheral nerve injury (Hoffman *et al.*, 1987, 1993; Hoffman, 1989; Oblinger *et al.*, 1989; Wong and Oblinger, 1990; Moskowitz *et al.*, 1993). In this work, all L_5 DRG neurons are shown to express Tα1 α-tubulin mRNA in the intact state. This corresponds to a report indicating that 100% of DRG neurons, at all spinal levels, express α-tubulin (Boehmer *et al.*, 1989), and to the important role of microtubules in intracellular transport, axonal growth, and in the neuronal cytoskeleton (reviewed in: Baas, 1997). Moreover, in this study the trkC-positive subpopulation has been characterized as to Tα1 α-tubulin expression: Even though in normal animals trkC transcripts are heterogenously expressed, Tα1 α-tubulin mRNA expression in trkC-positive cells tends to be relatively homogenous, exhibiting moderate labeling index values. A small subset of neurons, with relatively low levels of hybridization signal for trkC and high levels of Tα1 α-tubulin message, is a notable exception to this observation. Neurons with the highest Tα1 α-tubulin transcript levels lack detectable trkC mRNA. Two weeks after sciatic nerve transection, Tα1 α-tubulin message levels increase in all L_5 DRG neurons, although smaller, trkC-negative neurons still tend to exhibit the highest labeling indices for Tα1 α-tubulin.

4.3.4 Characterization of the trkC-Positive Subpopulation with Respect to Neuropeptide Expression

Although it is often thought that large diameter, potentially trkC-positive primary sensory neurons do not tend to express neuropeptides, this study examined the potential colocalization between several of these markers and trkC.

The neuropeptides CGRP and SP exhibit both a commonality of function and a high degree of colocalization. Including a putative role in nociception (Wiesenfeld-Hallin *et al.*, 1994; Bennett *et al.*, 2000a; Zhang *et al.*, 2001) shared with SP (Klein *et al.*, 1992; Mense *et al.*, 1994; Neumann *et al.*, 1996; Cao *et al.*, 1998; De Filipe *et al.*, 1998; Parker and Grillner, 1998; Nichols *et al.*, 1999), CGRP can also potentiate the algesic actions of SP (Wiesenfeld-Hallin *et al.*, 1994). In addition, the neuropeptide has been shown to inhibit bone resorption (Valentijn *et al.*, 1997; Ballica *et al.*, 1999) and to stimulate endothelial cell proliferation (Haegerstrand *et al.*, 1990), two processes potentially important in recovering from injury. Under normal conditions, 35-50% of DRG neurons have been shown to express CGRP transcripts and/or protein (Ju *et al.*, 1987; McCarthy and Lawson, 1989; Nothias *et al.*, 1993; Zhou *et al.*, 1993; Minichiello *et al.*, 1995; Bergman *et al.*, 1996; Groves *et al.*, 1996; Kashiba *et al.*, 1997a; Bennett *et al.*, 1998), and this subset includes neurons of all sizes (Rosenfeld *et al.*, 1983; Ju *et al.*, 1987; Noguchi *et al.*, 1990a, 1990b; Nothias *et al.*, 1993; Verge *et al.*, 1995). Moreover, depending upon the probe used, these observations may represent two distinct subpopulations of neurons, since through alternative processing the gene encodes two forms of the neuropeptide, α- and β-CGRP (Amara *et al.*, 1985). The two are differentially expressed in adult rat (Noguchi *et al.*, 1990a, 1990b; Verge *et al.*, 1995), with α-CGRP labeling ~ 36% of L_5 DRG neurons of all sizes and ~ 30% of primarily small- and medium-sized cells exhibiting β-CGRP mRNA (Noguchi *et al.*, 1990a, 1990b). Here, ~ 55% of adult rat L_5 DRG neurons were found to possess transcripts specific for α-CGRP; the greater number of labeled neurons in this study may be due to increased sensitivity of the *in situ* hybridization technique (see Section 2.1.1.2). The population includes many small and medium diameter neurons, with small cells expressing low to moderate labeling indices and medium-sized neurons displaying higher levels of hybridization signal for the neuropeptide. Further, trkC and α-CGRP were shown to colocalize in a subset of neurons with small to large diameter perikarya,

representing ~ 8% of DRG, ~ 16% of α-CGRP, and ~ 22% of trkC-positive neurons. These cells tended to exhibit low levels of hybridization signal for trkC and moderate levels for α-CGRP.

SP functions as an excitatory neuropeptide in nociception (Klein *et al.*, 1992; Mense *et al.*, 1994; Neumann *et al.*, 1996; Cao *et al.*, 1998; De Felipe *et al.*, 1998; Parker and Grillner, 1998; Nichols *et al.*, 1999), but may also play a role in wound healing since the neuropeptide has been shown to stimulate the proliferation of arterial smooth muscle cells and skin fibroblasts *in vitro* (Nilsson *et al.*, 1985). SP, and the preprotachykinin gene that encodes it (Krause *et al.*, 1987), have been detected in 20-25% of DRG neurons (Boehmer *et al.*, 1989; Bergman *et al.*, 1996; Ji *et al.*, 1996; Hall *et al.*, 1997; Ma and Bisby, 1998b); their perikarya are predominately small-sized (Wiesenfeld-Hallin *et al.*, 1984; Dalsgaard *et al.*, 1985; Skofitsch and Jacobowitz, 1985; Ju *et al.*, 1987; Boehmer *et al.*, 1989; McCarthy and Lawson, 1989; Anand *et al.*, 1991; Villar *et al.*, 1991; Noguchi *et al.*, 1994; 1995; Verge *et al.*, 1995; Bergman *et al.*, 1996). These observations on the SP-positive subset of DRG neurons correspond to the findings reported in this study. Further, ~ 4% of L_5 DRG neurons coexpress SP and trkC, which represents ~ 20% of the SP-positive and ~ 12% of the trkC-positive subpopulations. These neurons tend to have small soma and fall into two major groups — those with moderate trkC and low to moderate SP labeling indices, and those with low trkC and moderate to high SP labeling indices.

Virtually all SP-and most CGRP-positive cells have also been shown to display high-affinity NGF binding sites (Verge *et al.*, 1989a). Here, ~ 63% of α-CGRP- and ~ 75% of SP-positive neurons coexpress trkA mRNA, and trkC and trkA transcripts colocalize in ~ 11% of α-CGRP- and ~ 12% of SP-expressing neurons. Although no attempt was made to identify SP/α-CGRP/trkC-positive neurons, SP and α-CGRP may coexist in at least a subset of these cells because the neuropeptides have been shown to colocalize in many adult rat primary sensory neurons (Wiesenfeld-Hallin *et al.*, 1984; Skofitsch and Jacobowitz, 1985; Gibbins *et al.*, 1987; Ju *et al.*, 1987; Villar *et al.*, 1989). Moreover, there appears to be a few SP/trkC-positive, trkA-negative neurons (see Figure 4.24). These cells could represent the subset of SP-expressing neurons that appear to be

trkC-dependent, but trkA-independent, described from experiments on $trk^{-/-}$ and double $trk^{-/-}$ mice (Minichiello *et al.*, 1995). Interestingly, even though a few α-CGRP/trkC-positive, trkA-negative neurons were identified here (see Figure 4.20), α-CGRP transcripts were not detected in $trkA^{-/-}$, $trkA^{-/-}/trkC^{-/-}$, or $trkA^{-/-}/trkB^{-/-}$ animals and α-CGRP expression in both $trkC^{-/-}$ and $trkB^{-/-}$ mice resembled that of wild-type (Minichiello *et al.*, 1995).

Following peripheral nerve injury, levels of SP and CGRP protein and mRNA have been shown to decrease (Jessell *et al.*, 1979; Tessler *et al.*, 1985; Shehab and Atkinson, 1986; Villar *et al.*, 1989, 1991; Noguchi *et al.*, 1990b, 1993, 1994, 1995; Klein *et al.*, 1991; Inaishi *et al.*, 1992; Hökfelt *et al.*, 1993; Verge *et al.*, 1995; Groves *et al.*, 1996; Ji *et al.*, 1996; Ma and Bisby, 1998a, 1998b). Contrary to, but concomitant with this phenomenon, SP expression appears to be induced, albeit at low levels, in a relatively small subpopulation of medium to large diameter neurons (Noguchi *et al.*, 1994, 1995; Ji *et al.*, 1996; Ma and Bisby, 1998a). In this study, the subset of neurons that appear to exhibit injury-induced SP coexpress trkC mRNA, but seem to lack detectable trkA transcripts. That such cells have been shown to project to the gracile nucleus (Noguchi *et al.*, 1995), part of the posterior column-medial lemniscus pathway, further supports the identification of these neurons as part of the trkC-positive subpopulation and their role in proprioception under normal conditions. Recently, similar observations were made for a subset of medium to large diameter DRG neurons in which CGRP mRNA is upregulated after sciatic nerve transection (Miki *et al.*, 1998). I failed to detect such a subpopulation in this study (data not shown). This is most likely because under normal conditions many neurons that express α-CGRP exhibit very high levels of hybridization signal for the neuropeptide, and in this project it was necessary to strike a compromise between minimizing silver grain overlap and detecting cells with low levels of α-CGRP transcripts in order to allow for the calculation of labeling indices (see Section 4.2.1). Presumably in having the injured ganglion section on the same slide, and therefore exposed to the emulsion for the same length of time as normal tissue, very low message levels of α-CGRP were not discernible from background, and the subset of α-CGRP positive neurons was overlooked.

Other neuropeptides are expressed by DRG neurons: A subset of mainly small neurons display SOM protein and/or transcripts (Hökfelt *et al.*, 1976; Price, 1985; Tuchscherer and Seybold, 1985; Ju *et al.*, 1987) and represent between 5-10% of lumbar DRG neurons in normal animals (Boehmer *et al.*, 1989; Verge *et al.*, 1995; Bergman *et al.*, 1996; Kashiba *et al.*, 1997a; Bennett *et al.*, 1998). This study yielded similar results, but as appears to be the case for trkA (Verge *et al.*, 1995), I was unable to colocalize trkC and SOM mRNAs. Somatostatinergic neurons have recently been shown to coexpress receptor components for a trophic factor unrelated to the neurotrophins, GDNF (Bennett *et al.*, 1998).

In normal lumbar DRG, galanin mRNA and/or protein has been detected in 3-7% of neurons (Hökfelt *et al.*, 1987; Bergman *et al.*, 1996; Ji *et al.*, 1996; Thompson *et al.*, 1998b), primarily with small soma (Hökfelt *et al.*, 1987; Zhang *et al.*, 1993a; Wiesenfeld-Hallin *et al.*, 1994; Verge *et al.*, 1995; Ji *et al.*, 1996; Thompson *et al.*, 1998b; Hao *et al.*, 1999; Landry *et al.*, 2000). Results presented here are in accord with those described above, with the additional observation that there is no apparent overlap between the trkC- and galanin-expressing subpopulations. Peripheral nerve injury effects an upregulation in galanin (Hökfelt *et al.*, 1987; Villar *et al.*, 1989, 1991; Anand *et al.*, 1991; Noguchi *et al.*, 1993; Wiesenfeld-Hallin *et al.*, 1994; Corness *et al.*, 1996; Thompson *et al.*, 1998b; Shi *et al.*, 1999; Landry *et al.*, 2000), such that signal levels are increased and the neuropeptide is expressed in many neurons, of all sizes (Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Verge *et al.*, 1995; Corness *et al.*, 1996; Ji *et al.*, 1996; Hao *et al.*, 1999). The number of galanin-positive DRG neurons increases over time post-trauma; 3 days following sciatic nerve transection ~ 25% of cells have been shown to express galanin (Ji *et al.*, 1996), by day 7 ~ 35% exhibit galanin mRNA (Noguchi *et al.*, 1993), but 2 weeks after axotomy galanin message has been detected in ~ 80% of neurons (Verge *et al.*, 1995). Again, results from this study concur with previous reports. Further, unlike in normal animals, 3 weeks post-axotomy galanin and trkC transcripts are shown here to frequently colocalize. The principal role for galanin appears to be in inhibiting nociception and preventing allodynia (Villar *et al.*, 1989; Wiesenfeld-Hallin *et al.*, 1994; Hao *et al.*, 1999; Shi *et al.*, 1999; Xu *et al.*, 2000; reviewed in: Bartfai *et al.*,

1992, 1993; Wiesenfeld-Hallin *et al.*, 1992; Bedecs *et al.*, 1995). In addition, the neuropeptide also has been shown to function in learning and memory (Ogren *et al.*, 1992).

NPY is rarely detected in DRG neurons of normal animals (Zhang *et al.*, 1993a; Ohara *et al.*, 1994; Verge *et al.*, 1995; Corness *et al.*, 1996; Ji *et al.*, 1996; Thompson *et al.*, 1998b; Li *et al.*, 1999b; Landry *et al.*, 2000). But following peripheral axotomy, many large and some medium diameter neurons exhibit NPY mRNA and protein (Wakisaka *et al.*, 1991, 1992; Frisén *et al.*, 1992; Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Ohara *et al.*, 1994; Verge *et al.*, 1995; Corness *et al.*, 1996; Ji *et al.*, 1996; Thompson *et al.*, 1998b; Cougnon-Aptel *et al.*, 1999; Li *et al.*, 1999b; Marchand *et al.*, 1999; Shi *et al.*, 1999; Landry *et al.*, 2000); 25-50% of DRG neurons have been shown to express NPY message 3 days to 2 weeks post-injury (Noguchi *et al.*, 1993; Verge *et al.*, 1995; Corness *et al.*, 1996; Ji *et al.*, 1996). Here, similar findings are reported, with the additional observation that 3 weeks after sciatic nerve transection many NPY-positive neurons now are labeled for trkC mRNA. NPY appears to act as an analgesic (Walker *et al.*, 1988; Duggan *et al.*, 1991; Hua *et al.*, 1991; Mantyh *et al.*, 1994; Naveilhan *et al.*, 2001), potentially through the inhibition of SP release (Walker *et al.*, 1988; Duggan *et al.*, 1991). Moreover, the neuropeptide may be important to neuronal regeneration (White and Mansfield, 1996).

Like NPY, VIP mRNA and protein have been detected in a minimal number of DRG neurons in normal animals (Ju *et al.*, 1987; Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Wiesenfeld-Hallin *et al.*, 1994; Verge *et al.*, 1995), but as early as 3 days post-axotomy many small and medium diameter perikarya now exhibit VIP (Ju *et al.*, 1987; Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Wiesenfeld-Hallin *et al.*, 1994; Verge *et al.*, 1995) and by 2 weeks post-injury they represent 20-35% of the total DRG population (Noguchi *et al.*, 1993; Verge *et al.*, 1995). Data from this study support the previous reports but, in addition, indicate that VIP and trkC mRNAs do not appear to be coexpressed in injured DRG neurons. VIP has been identified as having roles in such varied processes as nociception (Wiesenfeld-Hallin *et al.*, 1994; Dickinson *et al.*, 1999), stimulation of neuronal regeneration (Shehab and Atkinson, 1986; White and Mansfield,

1996), and memory and learning (Magistretti *et al.*, 1998; Blondel *et al.*, 2000).

As is the case for SP and CGRP (see above and Section 2.2.2.2), other neuropeptides are expressed in both overlapping and unique subpopulations of sensory neurons (Hökfelt *et al.*, 1976, 1994b; Dalsgaard *et al.*, 1982; Leah *et al.*, 1985a, 1985b; Gibbins *et al.*, 1987; Ju *et al.*, 1987; Villar *et al.*, 1989; Kashiba *et al.*, 1992b, 1994; Zhang *et al.*, 1993a; Heppelmann *et al.*, 1994; Wiesenfeld-Hallin *et al.*, 1994; Verge *et al.*, 1995; Landry *et al.*, 2000). In adult rat DRG neurons, SP and CGRP have been reported to colocalize with each other, and with galanin and VIP (Ju *et al.*, 1987; Villar *et al.*, 1989; Verge *et al.*, 1995). Furthermore, CGRP- and SOM-ir have been identified in the same neuron (Ju *et al.*, 1987), but there are conflicting reports as to whether SOM colocalizes with SP (Ju *et al.*, 1987) or not (Hökfelt *et al.*, 1976; Verge *et al.*, 1995). SOM has also been shown to be coexpressed with VIP (Verge *et al.*, 1995), but not with galanin (Ju *et al.*, 1987). In addition, although galanin colocalizes with CGRP (Ju *et al.*, 1987; Villar *et al.*, 1989), SP (Ju *et al.*, 1987), VIP (Kashiba *et al.*, 1992b; Wiesenfeld-Hallin *et al.*, 1994), and NPY (Zhang *et al.*, 1993a; Hökfelt *et al.*, 1994b; Kashiba *et al.*, 1994; Landry *et al.*, 2000); NPY has only been shown to be coexpressed with galanin (Zhang *et al.*, 1993a; Hökfelt *et al.*, 1994b; Kashiba *et al.*, 1994; Landry *et al.*, 2000). In spite of the putative roles of many of these neuropeptides in nociception, there does not appear to be a clear relationship between sensory function and peptide expression, but subsets of neurons, defined according to peptide content, can be linked to target projection fields. A study comparing the expression patterns of four peptides associated with nociceptive pathways (SP, CGRP, CCK, dynorphin) in DRG of guinea-pig showed that subpopulations of neurons exhibiting various combinations of these markers projected to specific peripheral targets (Gibbins *et al.*, 1987): For the most part, SP/CGRP/CCK/dynorphin-positive cells projected to skin; SP/CGRP/CCK-positive, dynorphin-negative neurons to small blood vessels of skeletal muscle; SP/CGRP/dynorphin-positive, CCK-negative neurons to pelvic viscera and airways; and SP/CGRP-positive, CCK/dynorphin-negative cells innervating the heart and blood vessels to viscera and sympathetic ganglia. Small subpopulations of neurons that expressed only SP, CGRP, or CCK were also identified (Gibbins *et al.*, 1987).

Neuropeptides may also colocalize with neuropeptide receptors in sensory neurons (Zhang *et al.*, 1994b, 1995b, 1997; Xu *et al.*, 1996; Landry *et al.*, 2000, Naveilhan *et al.*, 2001). For example, of two major receptors for NPY (reviewed in: Wahlestedt and Reis, 1993; Blomqvist and Hertzog, 1997), Y1 and Y2 are primarily expressed in small- and medium-sized (Naveilhan *et al.*, 1998, 2001; Landry *et al.*, 2000) and medium to large diameter (Zhang *et al.*, 1997; Landry *et al.*, 2000) neurons, respectively. Y1 and SP frequently colocalize (Naveilhan *et al.*, 2001), and both Y1 and Y2 are often expressed by CGRP-, galanin-, and NPY-positive neurons (Zhang *et al.*, 1994b, 1995b, 1997; Landry *et al.*, 2000). Further, transcripts for one type of galanin receptor, gal-R1, are found in ~ 60% of neurons that exhibit CGRP message and the two markers colocalize in over 95% of the gal-R1 expressing subpopulation (Xu *et al.*, 1996). Moreover, since trkC has been shown here to colocalize with SP, CGRP, galanin, and NPY there is the potential for multiple neuropeptides and/or neuropeptide receptors to be coexpressed by individual trkC-positive cells, with the variable peptide expression within the NT-3 responsive population possibly reflecting differences in sensory function between subsets of these neurons.

4.3.5 Characterization of the trkC-Positive Subpopulation with Respect to the Expression of the Injury and Regeneration Associated Markers GAP-43 and cjun

GAP-43 (B-50, F1, pp46, pp57, neuromodulin) is an intracellular protein with a distribution restricted to the nervous system (Basi *et al.*, 1987; Karns *et al.*, 1987; Benowitz *et al.*, 1988; Meiri *et al.*, 1988), expressed not only in neurons, but also in CNS and PNS glia (Deloulme *et al.*, 1990; da Cunha and Vitkovic, 1990; Curtis *et al.*, 1992; Woolf *et al.*, 1992b; Scherer *et al.*, 1994). The marker is linked to axonal growth and regeneration (reviewed in: De Graan *et al.*, 1986; Basi *et al.*, 1987; Benowitz and Routtenberg, 1987; Skene, 1989). It is abundantly expressed during development (Jacobson *et al.*, 1986; Basi *et al.*, 1987; Karns *et al.*, 1987); levels decrease postnatally (Hoffman, 1989), but are upregulated after peripheral nerve injury and remain high during regeneration (Skene and Willard, 1981; Hoffman, 1989; Tetzlaff *et al.*, 1989; Verge *et al.*, 1990b; Wiese *et al.*, 1992; Schreyer and Skene, 1993; Groves *et al.*, 1996;

Verkade *et al.*, 1996; Andersen and Schreyer, 1999). Although GAP-43-ir is distributed throughout the neuron, the highest levels are in growth cones (Meiri *et al.*, 1988; Coggeshall *et al.*, 1991), where it appears to be associated with the cell membrane, not the cytoskeleton (Skene and Virág, 1989; Meiri and Gordon-Weeks, 1990). GAP-43 is important in neurite outgrowth: *in vitro* it has been shown to play roles in growth cone formation (Aigner and Caroni, 1993), promoting neurite and growth cone adhesion (Aigner and Caroni, 1995), potentiating the growth cone response to extrinsic signals (Igarashi *et al.*, 1995), and in the regulation of G_0, a GTP-binding protein and major growth cone component (Strittmatter *et al.*, 1990). It has also been implicated in the regulation of neurotransmitter release (Dekker *et al.*, 1989), endocytosis and synaptic vesicle recycling (Neve *et al.*, 1998), and in influencing neuronal survival (Gagliardini *et al.*, 2000).

In normal rat lumbar DRG, 40-70% of neurons, primarily with small diameter perikarya, exhibit detectable levels of GAP-43 (Verge *et al.*, 1990b; Schreyer and Skene, 1993; Liabotis and Schreyer, 1995; Groves *et al.*, 1996; Andersen and Schreyer, 1999), but following peripheral axotomy all injured neurons express the marker (Hoffman, 1989; Verge *et al.*, 1990b; Tetzlaff *et al.*, 1992; Wiese *et al.*, 1992; Schreyer and Skene, 1993; Liabotis and Schreyer, 1995; Andersen and Schreyer, 1999). Results presented in this work are in agreement with these observations. Moreover, findings reported here indicate that in the intact state, trkC colocalizes with GAP-43 in ~ 15% of L_5 DRG neurons, many of which exhibit low trkC and high GAP-43 labeling indices. At least a portion of this subset may also coexpress trkA, since the majority of neurons expressing high GAP-43 mRNA levels exhibit high-affinity NGF binding sites (Verge *et al.*, 1990b; Tetzlaff *et al.*, 1992), and because the two trks colocalize in 25-50% of trkA- and/or trkC-positive neurons (see Section 4.3.2). Others have shown the population of intact sensory neurons expressing NGF receptors to be highly plastic, readily undergoing collateral sprouting in response to NGF (Diamond *et al.*, 1992a); the observation here that ~ 85% of uninjured trkC-positive DRG neurons lack detectable GAP-43 transcripts may reflect an apparent inability of NT-3 to influence neurite outgrowth (Kimpinski *et al.*, 1997).

Some immediate early genes (IEGs) encode transcription factors that upon site-specific binding to target gene DNA, can activate or repress gene expression, and although IEG transcription is induced both rapidly and transiently after noxious cellular stimulation, their effects may contribute to long-term changes within the cell (reviewed in: Sheng and Greenberg, 1990; Herdegen and Zimmermann, 1994b; Morgan and Curran, 1995; Herdegen *et al.*, 1997). One such IEG is cjun, which upon activation by ATF-2 encodes the protein, cJUN, that serves in the regulation of apoptosis and cellular differentiation, and is the primary transcription factor expressed following neurodegenerative disorders — including multiple sclerosis, Alzheimer's disease, ischemic insult, and nerve injury (reviewed in: Herdegen *et al.*, 1997). Following the activation of cJUN through cJUN NH_2-terminal kinases (JNKs) (Hibi *et al.*, 1993; reviewed in: Kyriakis *et al.*, 1994; Herdegen *et al.*, 1997), the protein forms a heterodimeric complex with one of several other IEG products, creating the transcription factor, AP-1 (activator protein-1) (Curran and Franza, 1988; reviewed in: Herdegen *et al.*, 1997). This complex binds to the AP-1 DNA consensus element in the promoter region of a target gene, therein regulating its transcription; the functional specificity of the complex — including DNA binding affinity, stability of AP-1, and whether positively or negatively regulating transcription — is dependent on the specific combination of the components (Hai and Curran, 1991; Kerppola and Curran, 1994; reviewed in: Herdegen and Zimmermann, 1994a; Herdegen *et al.*, 1997).

Low basal levels of cjun mRNA and protein have been detected in normal adult rat L_4/L_5 DRG (De León *et al.*, 1995). Here, over half were found to exhibit transcripts for the marker, with over half of these coexpressing trkC, and with no apparent difference in cjun levels between the two populations. This number corresponds to the ~ 60% of mouse L_4 DRG neurons reported to exhibit cJUN-ir (Gold *et al.*, 1994). Following peripheral nerve injury, as described in the literature (Jenkins and Hunt, 1991; Herdegen *et al.*, 1992; De León *et al.*, 1995) and demonstrated in this study, there is a dramatic upregulation in cjun expression, notable as early as 2 hours post-axotomy (Herdegen *et al.*, 1992). Here, 3 weeks after sciatic nerve transection cjun transcripts are detectable in all DRG neurons, with no apparent overall difference in levels of the

marker between those that coexpress trkC and the majority of cells that lack the receptor. The presence of a small subset of trkC-negative neurons that exhibit the highest cjun labeling index values (see Figure 4.32) suggests that these cells are more susceptible to stress (reviewed in: Herdegen *et al.*, 1997) than the others, including those that express trkC.

4.3.6 Characterization of the trkC-Positive Subpopulation with Respect to SNAP-25 Expression

SNAP-25 (synaptosomal-associated protein, 25 kDa) (reviewed in: Hodel, 1998), is an integral membrane protein that is widely, but differentially, expressed by diverse subpopulations of mammalian neurons (Oyler *et al.*, 1989). Although its distribution is almost exclusively neural, SNAP-25 is also present in some endocrine cells, including neutrophils and pinealocytes (Redecker *et al.*, 1996, 1997; Yamada *et al.*, 1996; Nabokina *et al.*, 1997). The protein plays a key role in vesicle-membrane fusion and exocytosis as part of the SNARE [soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptor] complex in conjunction with syntaxin 1 and VAMP-2 (vesicle-associated membrane protein) (Hao *et al.*, 1997; O'Conner *et al.*, 1997; reviewed in: Söllner and Rothman, 1994; Emr and Malhotra, 1997; Bürgoyne and Morgan, 1998; Robinson and Martin, 1998). The presence of highly concentrated SNAP-25-ir at nerve terminals (Catsicas *et al.*, 1991; Li *et al.*, 1996), presumably mirrors, through its participation in the SNARE complex, its critical role in the release of neurotransmitters (O'Conner *et al.*, 1997; reviewed in: Mochida, 2000), neuropeptides (Banerjee *et al.*, 1996; Purkiss *et al.*, 2000), and neurotrophins (Blochl, 1998). SNAP-25 also appears to be important in membrane expansion, and therefore in axonal elongation and potentially synaptic plasticity (Catsicas *et al.*, 1991; Osen-Sand *et al.*, 1993; Haruta *et al.*, 1997; Igarashi *et al.*, 1997; Roberts *et al.*, 1998). Its biological importance is underscored by the high degree of sequence conservation between species (Catsicas *et al.*, 1991), and the presence of several SNAP-25 homologues. SNAP-23, and its murine equivalent syndet, performs a role similar to that of SNAP-25, but is expressed by lymphocytes, fibroblasts, muscle,

and adipocytes (Wang *et al.*, 1997; Wong *et al.*, 1997; Guo *et al.*, 1998; Leung *et al.*, 1998; Rea *et al.*, 1998); Vam7p is associated with vacuolar membranes and participates in the vacuolar SNARE complex (Sato *et al.*, 1998; Ungermann and Wickner, 1998).

In this study, SNAP-25 is shown to be heterogeneously expressed and detectable in virtually all L_5 DRG neurons. Further, while all trkC-positive neurons exhibit high mRNA levels for the marker, a subset of medium to large diameter cells have the highest SNAP-25 labeling index values; neurons that lack trkC transcripts express the lowest message levels for SNAP-25. The presence of high levels of SNAP-25 mRNA in a subset of trkC neurons may be a reflection of their sensory function. Rapidly adapting mechanoceptors, including those that innervate muscle spindles, discharge rapidly and/or frequently (Carr *et al.*, 1989a), and because of this would presumably require high levels of SNAP-25 for neurotransmitter release. Moreover, at least some of these neurons are responsive to NT-3 (see Sections 2.2.2.1.1, 2.2.2.1.2, 2.2.2.2). Further to this, since calcium plays an important role in vesicle release (Söllner and Rothman, 1994; Sheng and Kim, 1996; Bürgoyne and Morgan, 1998), this subset of trkC-positive neurons might represent the NT-3 responsive subpopulation that expresses one or more of the calcium-binding proteins parvalbumin, calbindin, and calretinin (see Section 2.2.2.2).

4.3.7 Summary

Determining the role of NT-3 in adult primary sensory neurons necessitates an understanding of the phenotype of the putative NT-3 responsive population — the trkC-positive subset, which represents ~ 40% of DRG neurons. Of the thirteen markers examined in this study, eleven colocalized, at least in part, with transcripts for trkC (Table 4.1). Coexpression ranged from including virtually all trkC-positive cells (i.e. for p75, NFM, and Tα1 α-tubulin) to less than 15% of trkC-expressing neurons (i.e. for α-CGRP and SP), but even a small percentage equates to a significant population, since it is estimated that 10,500-17,900 neuronal perikarya are housed in individual adult rat L_4/L_5 DRG (Schmalbruch, 1987; Klein *et al.*, 1991; Swett *et al.*, 1991; Tandrup, 1993). Together, these results indicate that whether under normal or axotomized conditions, beyond the presence of trkC transcripts, no single marker can be

Table 4.1 Summary of selected phenotypic markers expressed by DRG neurons with and without detectable levels of trkC mRNA in the adult rat.

	INTACT		AXOTOMIZED	
MARKER	% total DRG neurons	% total DRG neurons coexpressing trkC	% total DRG neurons	% total DRG neurons coexpressing trkC
trkA	~40% (40-45%)[1]	~10% (~20%)[2]	ND	ND
p75	~80% (~80%)[3]	~100%	ND	ND
NFM	~100% (~100%)[4]	~100%	ND	ND
Tα1 α-tubulin	~100% (~100%)[5]	~100%	~100%	~100%
α-CGRP	~55% (35-50%)[6]	~8%	ND	ND
SP	~20% (20-25%)[7]	~4%	~20%	~15%
SOM	#, % ND (5-10%)[8]	*, % ND	ND	ND
galanin	#, % ND (3-7%)[9]	*, % ND	#, % ND (~80%)[10]	#, % ND
NPY	*, % ND (*)[11]	*, % ND	#, % ND (25-50%)[12]	#, % ND
VIP	*, % ND (*)[13]	*, % ND	#, % ND (20-35%)[14]	*, % ND
GAP-43	~60% (40-70%)[15]	~15%	~100% (~100%)[16]	~100%
cjun	~55% (~60%)[17]	~30%	~100%	~100%
SNAP-25	~100%	~100%	ND	ND

Table illustrates the percentages of primary sensory neurons expressing selected phenotypic markers and the percentage of neurons in which that marker colocalizes with trkC, under both normal (intact) and axotomized conditions. The first number or symbol in each cell represents findings of this project (rat L_5 DRG) and the second, enclosed in parentheses, those reported in the literature (murine DRG). "#" indicates that at least some neurons express that marker; "*" indicates that neurons rarely exhibit message for that marker or that transcripts were not detected; "ND" indicates that data was not determined. [1] Verge *et al.*, 1992a, 1995; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Karchewski *et al.*, 1999. [2] Karchewski *et al.*, 1999. [3] Wetmore and Olson, 1995; Karchewski *et al.*, 1999. [4] Verge *et al.*, 1990a; Tetzlaff *et al.*, 1992. [5] Boehmer *et al.*, 1989. [6] Ju *et al.*, 1987; McCarthy and Lawson, 1989; Nothias *et al.*, 1993; Zhou *et al.*, 1993; Minichiello *et al.*, 1995; Bergman *et al.*, 1996; Groves *et al.*, 1996; Kashiba *et al.*, 1997a; Bennett *et al.*, 1998. [7] Boehmer *et al.*, 1989; Bergman *et al.*, 1996; Ji *et al.*, 1996; Hall *et al.*, 1997; Ma and Bisby, 1998b. [8] Boehmer *et al.*, 1989; Verge *et al.*, 1995; Bergman *et al.*, 1996; Kashiba *et al.*, 1997a; Bennett *et al.*, 1998. [9] Hökfelt *et al.*, 1987; Bergman *et al.*, 1996; Ji *et al.*, 1996; Thompson *et al.*, 1998b.Boehmer. [10] Verge et al., 1995. [11] Zhang *et al.*, 1993a; Ohara *et al.*, 1994; Verge *et al.*, 1995; Corness *et al.*, 1996; Ji *et al.*, 1996; Thompson *et al.*, 1998b; Li *et al.*, 1999b; Landry *et al.*, 2000. [12] Noguchi *et al.*, 1993; Verge *et al.*, 1995; Corness *et al.*, 1996; Ji *et al.*, 1996. [13] Ju *et al.*, 1987; Noguchi *et al.*, 1993; Zhang *et al.*, 1993a; Wiesenfeld-Hallin *et al.*, 1994; Verge *et al.*, 1995. [14] Noguchi *et al.*, 1993; Verge *et al.*, 1995. [15] Verge *et al.*, 1990b; Schreyer and Skene, 1993; Liabotis and Schreyer, 1995; Groves *et al.*, 1996; Andersen and Schreyer, 1999. [16] Hoffman, 1989; Verge *et al.*, 1990b; Tetzlaff *et al.*, 1992; Wiese *et al.*, 1992; Schreyer and Skene, 1993; Liabotis and Schreyer, 1995; Andersen and Schreyer, 1999. [17] Gold *et al.*, 1994.

used to define the subgroup of primary sensory neurons responsive to NT-3. Rather, the trkC-positive subset is made up of phenotypically unique micropopulations. Moreover, understanding the importance of NT-3 to mature DRG neurons is even more complex because of its promiscuous nature — that of potentially binding to receptors other than trkC (see Section 1.1.2.1.1). But do these subsets reflect functionally unique groups of neurons? Even though trkC has classically been only associated with proprioception, several other mechanoreceptor subtypes have been shown to be dependent upon NT-3 for normal formation (see Sections 2.2.2.1.1, 2.2.2.1.2, 2.2.2.2) and these sensory neurons have been reported to exhibit complex phenotypes (Fundin *et al.*,1997a; Rice *et al.*,1997). Although much has been accomplished here in gaining insight into the phenotypic makeup of trkC-positive DRG neurons, further studies might involve refining the definition of the micropopulations, that is to say examining potential overlap between the markers and attempting to define their functional significance. A technical advance in data analysis developed after the completion of this project, and therefore used infrequently here, allows for the direct comparison of markers X, Y, and Z in individual cells, instead of the previous limitation of comparing X to Y, X to Z, and Y to Z, which can obscure important data. For example, Figure 4.19 compares α-CGRP and trkC labeling indices for individual neurons in the total DRG and for the trkA-positive subset. These two graphs demonstrate that virtually all of those neurons that coexpress α-CGRP and trkC also express trkA, and that trkC/trkA/ α-CGRP-positive cells exhibit low to moderate trkC, and high α-CGRP, mRNA levels. Moreover, they indicate that the highest levels of α-CGRP transcripts are present in trkA-positive/trkC-negative neurons, and that while there are trkA-positive/trkC-negative cells with α-CGRP levels comparable to those of trkA/trkC- positive neurons, the lowest levels of α-CGRP are localized to a subset of trkA-positive cells that lack detectable trkC transcripts. Similar trilocalization analyses should be performed for all markers of trkC-positive neurons in order to better phenotypically define the different micropopulations that express trkC.

5. THE EFFECT OF NT-3 INFUSION ON INJURED DRG NEURONS

5.1 Introduction

As demonstrated in this thesis (see Sections 4.2, 4.3), the subset of trkC-positive DRG neurons may be divided into micropopulations, defined through the variable expression of phenotypic markers. However, although the data provide a characterization of trkC-expressing neurons, there is limited published information concerning the *in vivo* functioning of its ligand, NT-3, in adult primary sensory neurons (see Section 2.2.2.2). One way to gain insight into the biological roles of the neurotrophins is to deprive DRG neurons of their principal source of endogenous trophic factors *via* peripheral axotomy and to then supply specific exogenous neurotrophin, comparing alterations in phenotypic markers between the two conditions, and with uninjured tissue. Following peripheral nerve transection, the surviving neurons undergo a series of predictable structural, physiological, and biochemical changes (see Section 1.2.3.1), while successful regeneration results in a complete to partial return towards the normal state, purportedly due to a successful re-establishment of the retrograde transport of target-derived trophic molecules (see Section 1.2.3.2). There is abundant experimental evidence that reintroduction of NGF to axotomized DRG neurons mitigates many of the trauma-induced alterations, in at least some cells (see Section 1.2.3.2). But, since NGF high-affinity binding sites or trkA are present in only ~ 40% of adult DRG neurons (Richardson *et al.*, 1986; Verge *et al.*, 1989a, 1989b, 1990a, 1990b, 1992a; Mu *et al.*, 1993; McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Bennett *et al.*, 1996a; Kashiba *et al.*, 1996, 1997a; Karchewski *et al.*, 1999), those non-trkA-expressing/trk-positive cells (McMahon *et al.*, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Kashiba *et al.*, 1996, 1997a; Karchewski *et al.*, 1999)

presumably respond to other molecules and, because an individual neuron may possess more than one trk species (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wright and Snider, 1995; Karchewski *et al.*, 1999), one can expect to encounter both unique and overlapping forms of cellular phenotype regulation by the various neurotrophins. Indeed, it has been shown that a number of unidentified genes are differentially regulated by NGF and NT-3 (Friedel *et al.*, 1997). Here, a similar experimental paradigm, a 2 week peripheral axotomy followed by a 1 week intrathecal infusion of NT-3, will be employed in order to examine the role of this neurotrophin in the maintenance of adult primary sensory neuron phenotype.

5.2 Results

5.2.1 Technical Considerations

See Section 4.2.1.

5.2.2 Neurotrophin Receptors

5.2.2.1 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on trkC mRNA Levels

Qualitative assessment of radioautographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect trkC mRNA show that while hybridization signal intensity decreases 3 weeks following sciatic nerve transection, a delayed 7 day 600 ng/μl/h intrathecal infusion of NT-3 is at least partially effective in counteracting the reduction in neuronal expression of trkC (Figure 5.1).

Scatterplots (e.g. Figure 5.2) constructed from data obtained through quantitative analysis of labeling indices of individual neurons identified in 6 μm thick DRG sections (16 DRG sections/treatment, average 218 neurons/section) processed for *in situ* hybridization, and subsequent radioautography, illustrate the relationship between perikaryal diameter and trkC mRNA expression in the intact state and under axotomized and axotomized/NT-3 infused conditions. These graphs show that while levels of hybridization signal for trkC range from low to high in the intact state, 3 weeks following sciatic nerve transection trkC signal levels decrease. A 7 day intrathecal

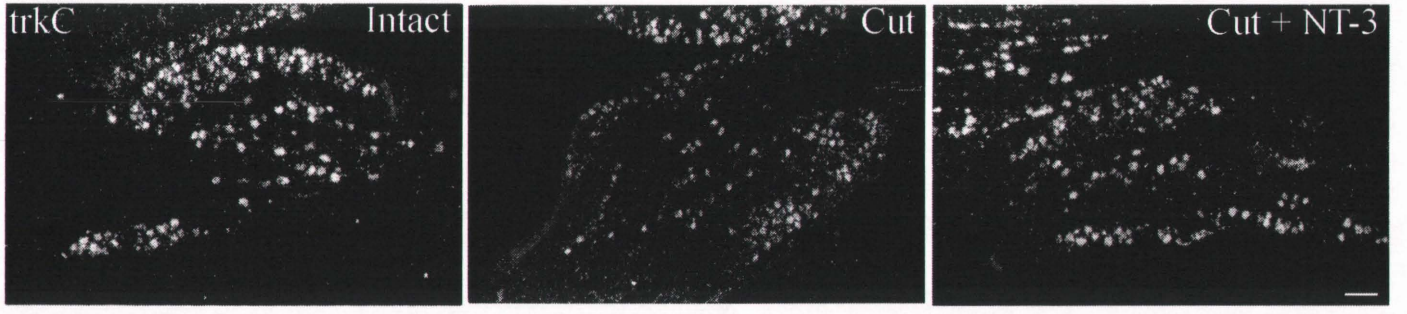

Figure 5.1. Expression of trkC mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect trkC mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm.

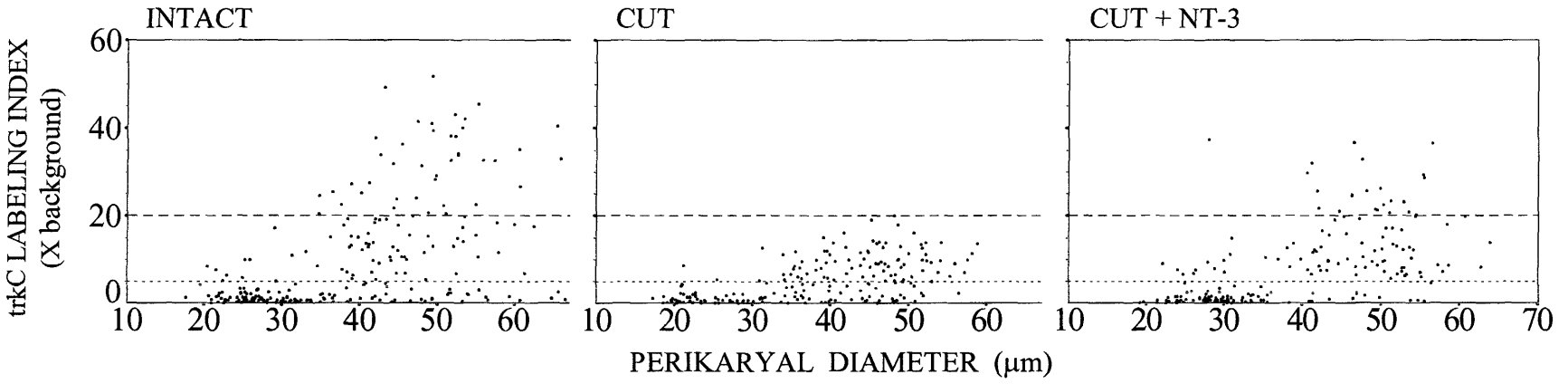

Figure 5.2 Relationship between perikaryal diameter and the relative level of trkC mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots of labeling indices of 186-246 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC mRNA, depict the relationship between perikaryal diameter (x axis) and trkC labeling indices (y axis). Panels show a comparison of data under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for trkC mRNA. The long-dashed lines serve as references to facilitate data interpretation.

infusion of NT-3, 2 weeks following axotomy, appears to mitigate the injury-reduced levels of trkC mRNA, although message levels for this receptor do not reach those observed in the intact state.

Figure 5.3 highlights the trend in changes in levels of trkC mRNA expression following axotomy and the effect of a post-trauma, 7 day intrathecal infusion of NT-3 for six replicate data sets. Whereas properties inherent to the technique of *in situ* hybridization do not allow for the comparison of silver grain densities between replicates (see Section 3.2.5), these graphs provide a summary of the data seen in the panel of scatterplots for each replicate set (e.g. Figure 5.2). The median and the 25^{th} and 75^{th} percentile values for trkC mRNA labeling are plotted for each treatment condition (intact, cut, cut plus NT-3) for each replicate. Although the return towards normal trkC mRNA levels is clearly evident in the graph representing trkC labeling indices for all identified trkC-positive neurons, the effect of NT-3 infusion appears more pronounced when only those neurons with moderate to heavy levels of hybridization signal for trkC are considered. In five out of six of cases, the difference in trkC labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.05$). Data from the six replicates represent analyses of tissues from four different animals; replicates two, four, and six represent analyses of tissue sections from the same animal.

5.2.2.2 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on p75 mRNA Levels

The low-affinity neurotrophin receptor is heterogenously expressed by a large number of DRG neurons (Figure 5.4). Axotomy results in decreased p75 mRNA levels and a delayed 7 day intrathecal infusion of NT-3 appears to partially reverse this effect, within at least a subpopulation of neurons.

Scatterplots (e.g. Figure 5.5), illustrating the relationship between perikaryal diameter and p75 mRNA expression for individual neurons identified in DRG sections (4 DRG sections/treatment, average 220 neurons/section), show that in the intact state ~ 80% of DRG neurons exhibit detectable p75 message levels. Although p75 mRNA is

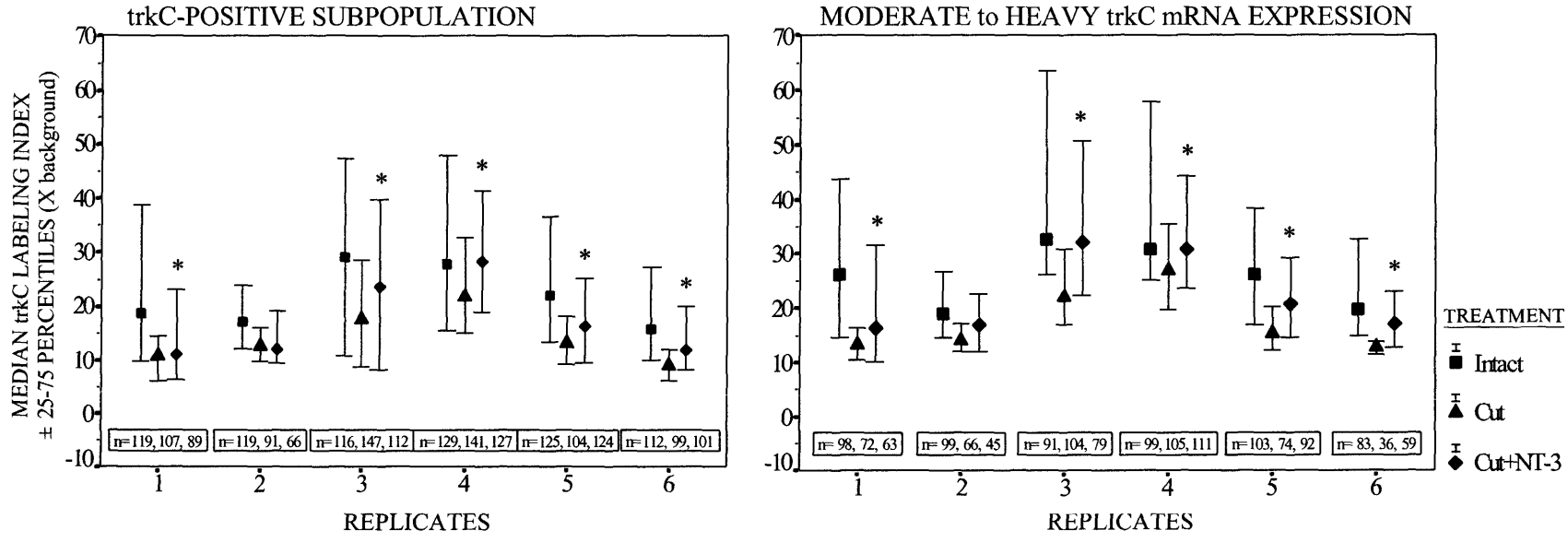

Figure 5.3 The effect of intrathecal infusion of NT-3 on trauma-reduced trkC mRNA levels.

Left graph depicts the relative differences in trkC labeling indices between normal (intact), 3 week axotomized (cut), and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 (cut + NT-3) treatments, for those neurons expressing trkC mRNA. Right graph shows the relative differences in trkC labeling indices for the same data sets, but only for neurons with moderate to heavy trkC mRNA levels. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, trkC labeling indices for individual neurons were determined from image analysis of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-6 (intact, cut, cut + NT-3) are as follows: 283, 192, 200; 298, 195, 175; 246, 233, 207; 291, 283, 256; 304, 195, 328; 246, 198, 186.

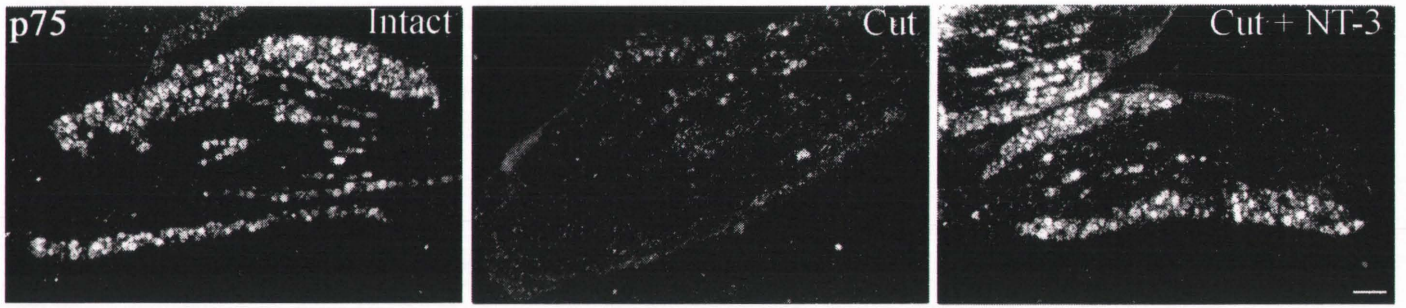

Figure 5.4 Expression of p75 mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect p75 mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm.

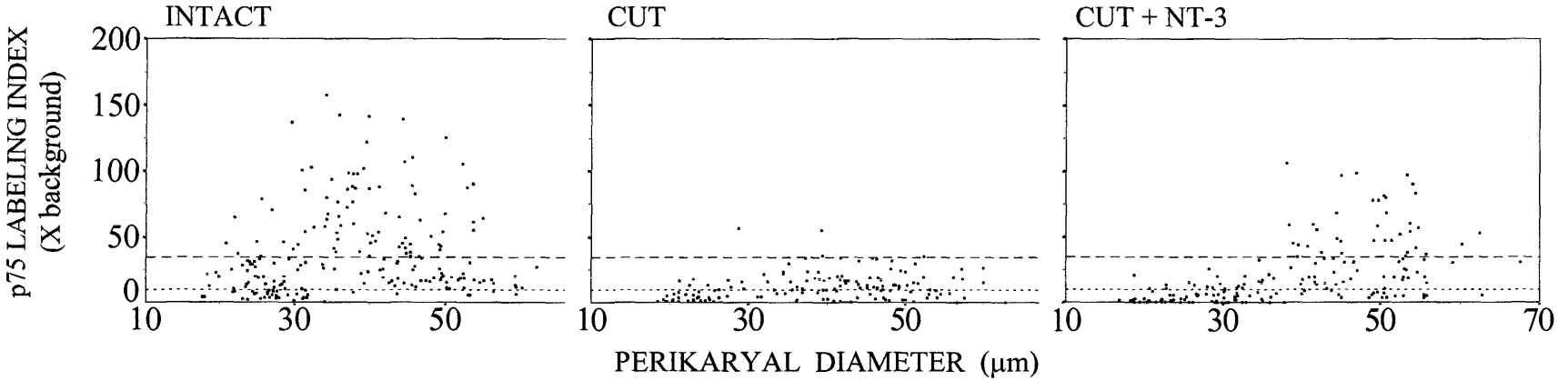

Figure 5.5 Relationship between perikaryal diameter and the relative level of p75 mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots of labeling indices of 131-210 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect p75 mRNA, depict the relationship between perikaryal diameter (x axis) and p75 labeling indices (y axis). Panels show a comparison of data under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of p75 mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for p75 mRNA. The long-dashed lines serve as references to facilitate data interpretation.

heterogenously expressed by neurons of all size ranges, those with the highest level of hybridization signal tend to be medium-sized, with small and large diameter perikarya exhibiting lower p75 labeling indices. Three weeks after axotomy p75 mRNA levels decrease dramatically, with virtually all p75-positive cells now expressing low to moderate levels of hybridization signal. Additionally, there is a trauma-induced reduction in the number of neurons with detectable hybridization signal; ~ 55% of surviving neurons exhibit p75 mRNA. Following sciatic nerve transection, in addition to the small-sized cells observed under normal conditions, a subpopulation of medium and large diameter neurons do not express detectable levels of hybridization signal for p75. Exogenous NT-3, supplied 2 weeks after injury, appears to result in heightened p75 message levels and this increase is most conspicuous in medium and large diameter cells. The infusion also seems to increase the percentage of surviving neurons with detectable p75 hybridization signal (~ 62%). Neither the labeling indices for p75, nor the percentage of p75-positive neurons, return to normal, pre-axotomy values.

Scatterplots (e.g. Figure 5.6), demonstrating the relationship between trkC and p75 mRNA expression for individual neurons (4 DRG sections/treatment/probe, average 220 neurons/section), indicate that there is a subset of neurons that coexpress the message for both of these NT-3 receptors. Under normal conditions, the majority of trkC-positive neurons express detectable p75 mRNA. There is also a subgroup of cells that exhibit p75 message but do not express hybridization signal for trkC and one that lacks detectable transcripts for either receptor. Message levels for both markers decrease following axotomy. A 1 week infusion of NT-3 appears to off set the injury-induced reduction in p75 and trkC mRNA levels; the increase in p75 message expression seems to be most evident in the trkC-positive subpopulation.

Figures 5.7 highlights the trend in changes in p75 mRNA expression following axotomy and the effect of a post-trauma, 7 day intrathecal infusion of NT-3 for the subpopulation of trkC-positive neurons in 4 replicate data sets. Following sciatic nerve transection there is a dramatic reduction in the median and 25th and 75th percentile values for p75 labeling indices. Post-injury, delayed infusion of NT-3 results in median labeling index values equal to, or greater than, those recorded for the intact state, in three out of

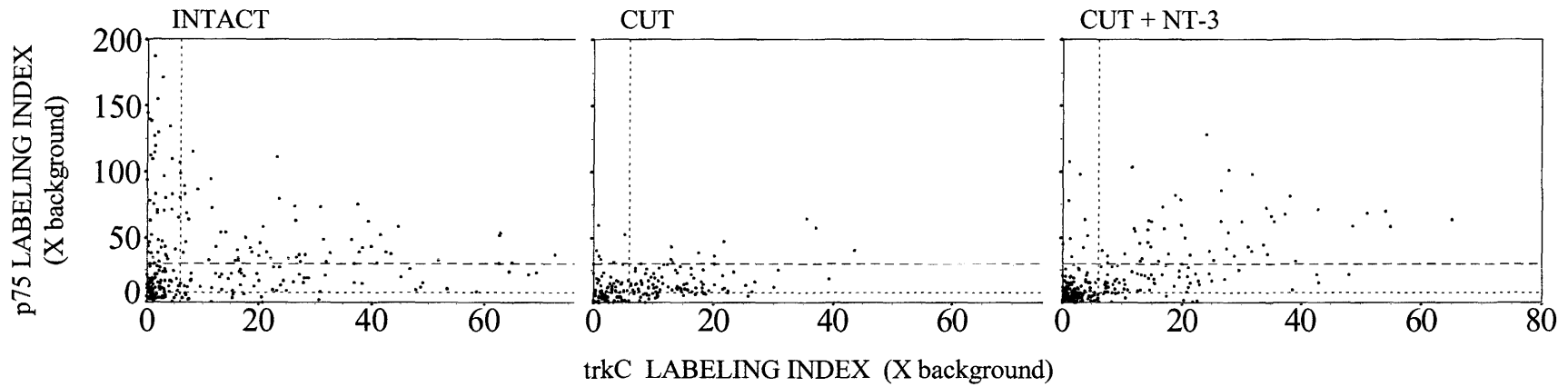

Figure 5.6 Relationship between the relative levels of trkC and p75 mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkC and p75 mRNA labeling indices for 195-328 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or p75 (y axis) mRNA under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neurons to the right of the short-dashed vertical line, and those above the short-dashed horizontal line, are considered positive for trkC and p75 mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

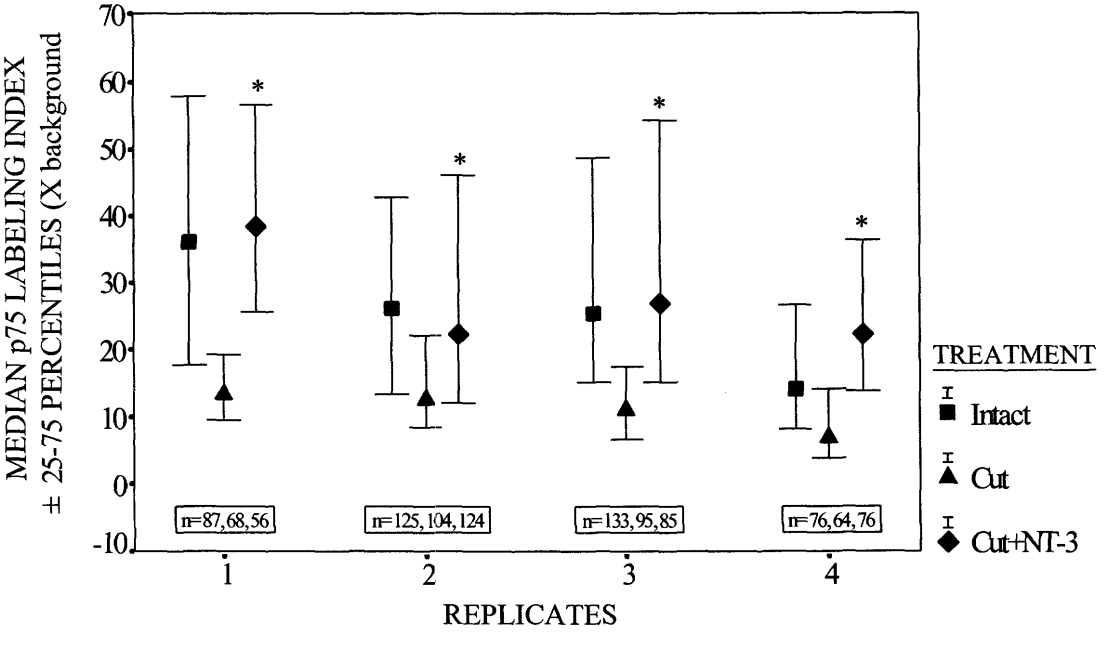

Figure 5.7 The effect of intrathecal infusion of NT-3 on trauma-reduced p75 mRNA levels in neurons expressing trkC mRNA.

Graph depicts the relative differences in p75 mRNA labeling indices between intact, 3 week axotomized, and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments, for those neurons expressing trkC mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, p75 and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC or p75 mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.001$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-4 (intact, cut, cut + NT-3) are as follows: 210, 146, 131; 304, 195, 329; 296, 194, 233; 209, 174, 165.

four replicates. In all cases, for the trkC-positive subpopulation, the difference in p75 labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.001$). Data from the four replicates represent analyses of tissues from four different animals. For the same set of replicates, but considering the subpopulation of neurons lacking detectable trkC mRNA (Figure 5.8), there is a substantial decrease in p75 transcript levels after axotomy, but median p75 labeling index values following NT-3 infusion are greater than those of transection alone in only one of four cases. This is also the only replicate in which the difference in p75 labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.05$).

5.2.3 Cytoskeletal Elements

5.2.3.1 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on NFM mRNA Levels

In the intact state, virtually all DRG neurons express moderate to high levels of hybridization signal for NFM; a dramatic decrease in NFM mRNA expression is evident 3 weeks following axotomy (Figure 5.9). Delayed NT-3 infusion appears to counteract this decrease, although normal NFM message levels are not reestablished.

Scatterplots (e.g. Figure 5.10) are used to illustrate the relationship between perikaryal diameter and NFM mRNA expression for individual neurons (2 DRG sections/treatment, average 264 neurons/section) under normal conditions and in axotomized and axotomized/NT-3 infused animals. In the intact state, NFM mRNA is detectable in virtually all DRG neurons. The marker is heterogenously expressed, and there seems to be a positive correlation between perikaryal diameter and NFM message level. Three weeks following sciatic nerve transection, there is a notable decrease in NFM mRNA expression and the number of neurons exhibiting detectable levels of NFM transcripts is reduced by ~ 10%. Exogenous NT-3 appears to partially counteract the axotomy-induced reduction in NFM mRNA levels and seem to influence the return in the number of cells with detectable NFM message towards normal.

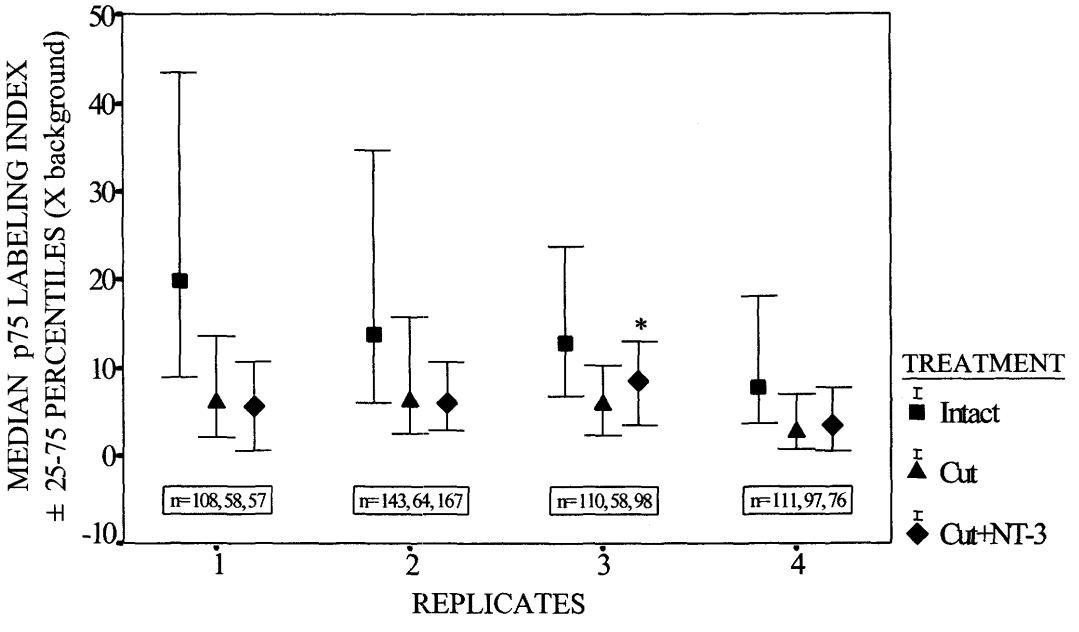

Figure 5.8 The effect of intrathecal infusion of NT-3 on trauma-reduced p75 mRNA levels in neurons not expressing detectable trkC mRNA.

Graph depicts the relative differences in p75 mRNA labeling indices between intact, 3 week axotomized, and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments, for those neurons with near to below background labeling for trkC mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, p75 and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect p75 or trkC mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-4 (intact, cut, cut + NT-3) are as follows: 210, 146, 131; 304, 195, 329; 296, 194, 233; 209, 174, 165.

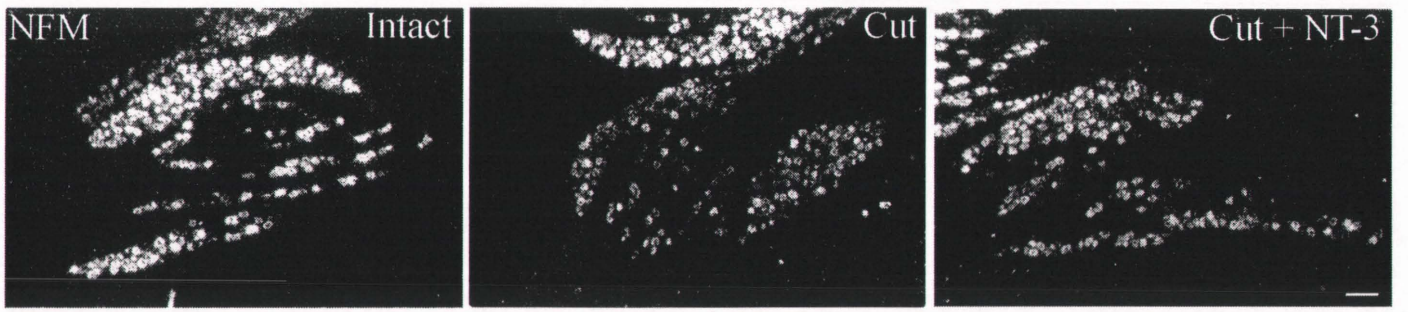

Figure 5.9 Expression of NFM mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect trkC mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm.

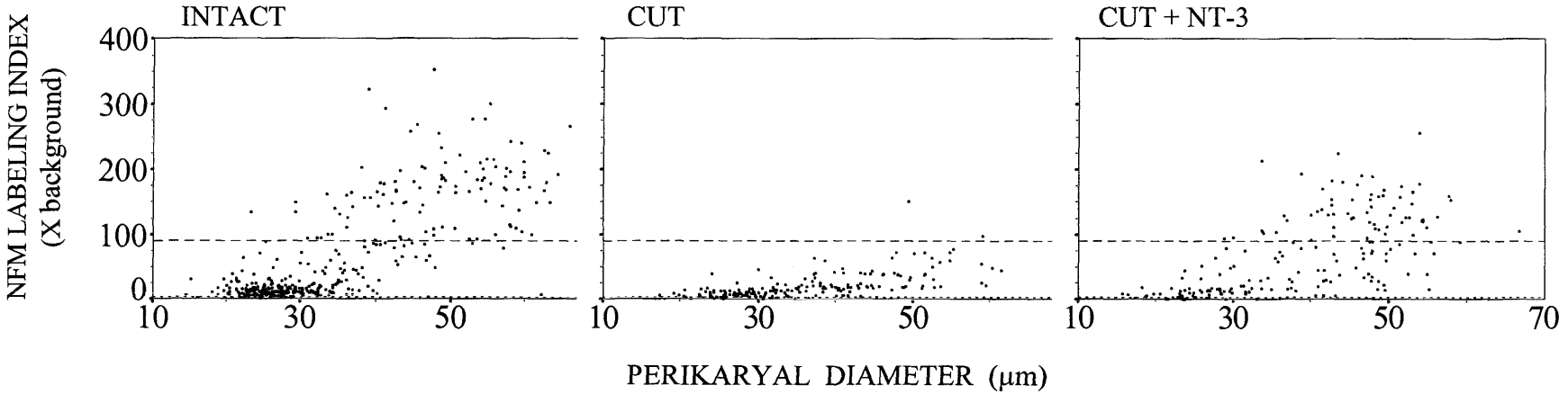

Figure 5.10 Relationship between perikaryal diameter and the relative level of NFM mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots of labeling indices of 187-373 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect NFM mRNA, depict the relationship between perikaryal diameter (x axis) and NFM labeling indices (y axis). Panels show a comparison of data under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of NFM mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for NFM mRNA. The long-dashed lines serve as references to facilitate data interpretation.

Scatterplots (e.g. Figure 5.11), demonstrating the relationship between trkC and NFM mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 264 neurons/section), indicate that in the intact state NFM message is heterogenously expressed by both neurons lacking detectable trkC mRNA and in the trkC-positive subpopulation. Axotomy results in a striking reduction in the level of NFM transcripts. This loss in NFM mRNA expression can be at least partially reversed by delayed infusion of NT-3 and this phenomenon appears to occur in both trkC-positive cells and in at least a subset of neurons that do not exhibit detectable trkC message.

Figure 5.12 highlights trends in alterations in NFM mRNA expression in the trkC-positive subpopulation following axotomy and with a post-trauma, 7 day intrathecal infusion of NT-3 for 2 replicate data sets. Three weeks following sciatic nerve transection, a dramatic reduction in the median and 75th percentile NFM labeling index values is evident; exogenous NT-3 effects an increase in both of these values. The difference in NFM labeling indices between axotomized and axotomized/NT-3 infused tissues for neurons expressing detectable levels of trkC mRNA is statistically significant for both replicates (Mann-Whitney U Test, $p<0.001$). Since in the intact state neurons lacking detectable trkC transcripts tend to exhibit low to moderate levels of NFM hybridization signal (Figure 5.11), the post-trauma diminished NFM message levels appear less conspicuous here than for the cells that express trkC. Data from the two replicates represent analyses of tissues from two different animals. For the same set of replicates depicted in Figure 5.12, but considering only neurons that do not exhibit detectable trkC mRNA, axotomy results in only a relatively modest reduction in median NFM labeling index values (Figure 5.13). Infusion of NT-3 appears to influence a return towards normal median NFM labeling index values. In one of the two cases, the NFM labeling index 75th percentile value for injured/NT-3 infused tissues is greater than that for the intact state, and it is for the same replicate that the difference in NFM labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.05$).

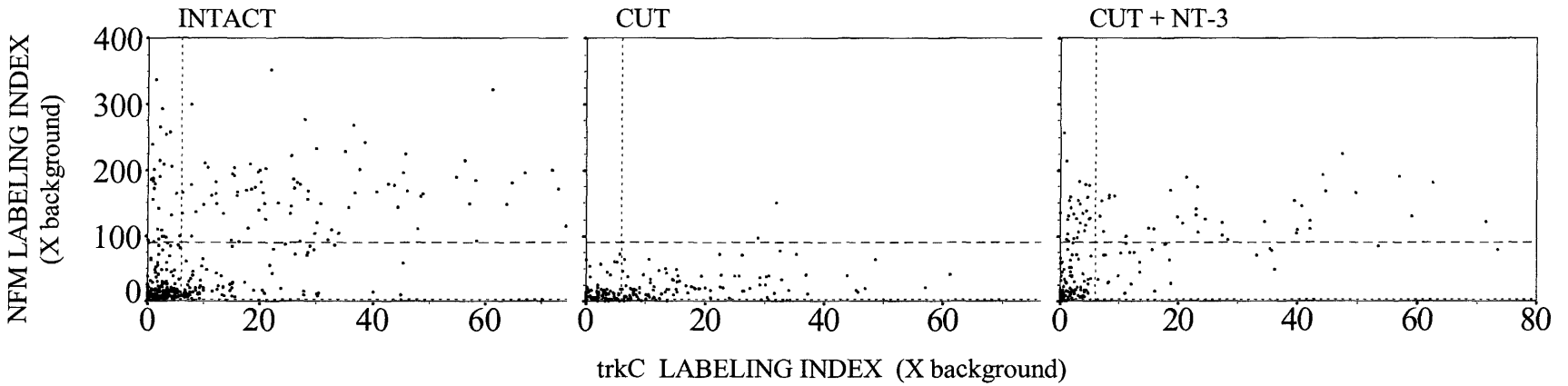

Figure 5.11 Relationship between the relative levels of trkC and NFM mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkC and NFM mRNA labeling indices for 187-373 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or NFM (y axis) mRNA under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neurons to the right of the short-dashed vertical line, and those above the short-dashed horizontal line, are considered positive for trkC and NFM mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

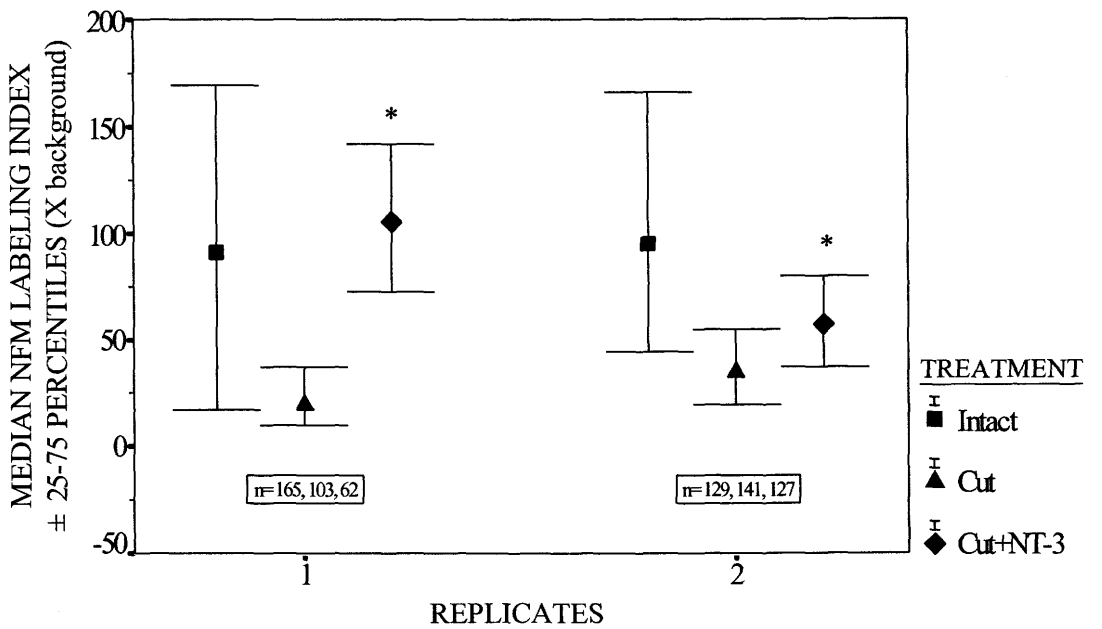

Figure 5.12 The effect of intrathecal infusion of NT-3 on trauma-reduced NFM mRNA levels in neurons expressing trkC mRNA.

Graph depicts the relative differences in NFM mRNA labeling indices between normal (intact), 3 week axotomized (cut), and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 (cut + NT-3) treatments, for the trkC-positive subpopulation of neurons. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, NFM and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC or NFM mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.001$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1 and 2 (intact, cut, cut + NT-3) are as follows: 373, 195, 187; 291, 283, 256.

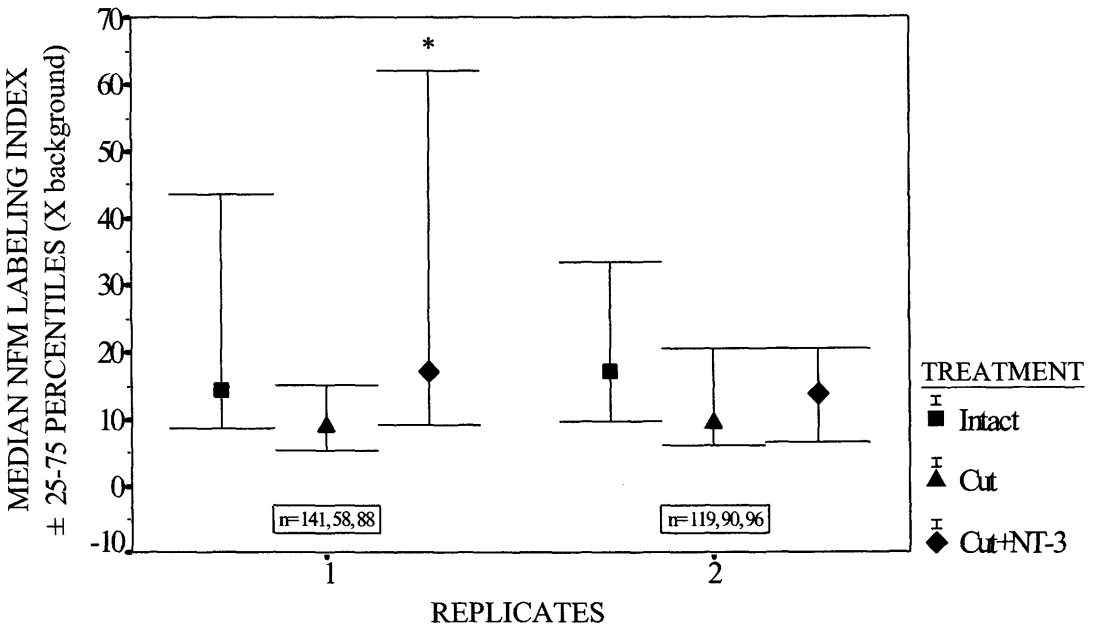

Figure 5.13 The effect of intrathecal infusion of NT-3 on trauma-reduced NFM mRNA levels in neurons not expressing detectable trkC mRNA.

Graph depicts the relative differences in NFM mRNA labeling indices between intact, 3 week axotomized, and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments, for those neurons with near to below background labeling for trkC mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, NFM and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect NFM or trkC mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1 and 2 (intact, cut, cut + NT-3) are as follows: 373, 195, 187; 291, 283, 256.

5.2.3.2 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on Tα1 α-tubulin mRNA Levels

Although hybridization signal for Tα1 α-tubulin is expressed at moderate to high levels in most DRG neurons in the intact state, 3 weeks post-axotomy signal levels increase (Figure 5.14). Delayed intrathecal infusion of NT-3 appears to result in a decrease in trauma-induced Tα1 α-tubulin message levels.

Scatterplots (e.g. Figure 5.15), depicting the relationship between perikaryal diameter and Tα1 α-tubulin mRNA expression for individual neurons (4 DRG sections/treatment, average 197 neurons/section), indicate that all DRG neurons exhibit detectable Tα1 α-tubulin transcripts and that there seems to be a loose negative correlation between Tα1 α-tubulin labeling indices and perikaryal diameter. Although axotomy results in a general increase in Tα1 α-tubulin mRNA expression, small cells still tend to possess the highest levels of hybridization signal for Tα1 α-tubulin, and there is no change in the percentage of surviving neurons that express Tα1 α-tubulin message. Intrathecal infusion of NT-3 appears to result in a reduction of Tα1 α-tubulin mRNA levels in all sizes of cells.

Scatterplots (e.g. Figure 5.16), illustrating the relationship between trkC and Tα1 α-tubulin mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 238 neurons/section), show that under normal conditions the majority of trkC-positive cells display relatively similar levels of Tα1 α-tubulin hybridization signal, regardless of the trkC message expression, and that the subgroup of neurons with the highest Tα1 α-tubulin mRNA levels do not typically exhibit detectable trkC transcripts. Following axotomy, message levels of Tα1 α-tubulin appear to increase in all neurons, although a subpopulation of cells lacking detectable trkC mRNA still expresses the highest levels of Tα1 α-tubulin transcripts. Exogenous NT-3 partially mitigates injury-induced Tα1 α-tubulin message levels in the trkC-expressing population and also, to some extent, in neurons that do not exhibit detectable trkC mRNA.

Figure 5.17 highlights the trend in changes in Tα1 α-tubulin mRNA expression following axotomy and the effect of a post-trauma, 7 day intrathecal infusion of NT-3 for 4 replicate data sets. Three weeks following sciatic nerve transection, the median and

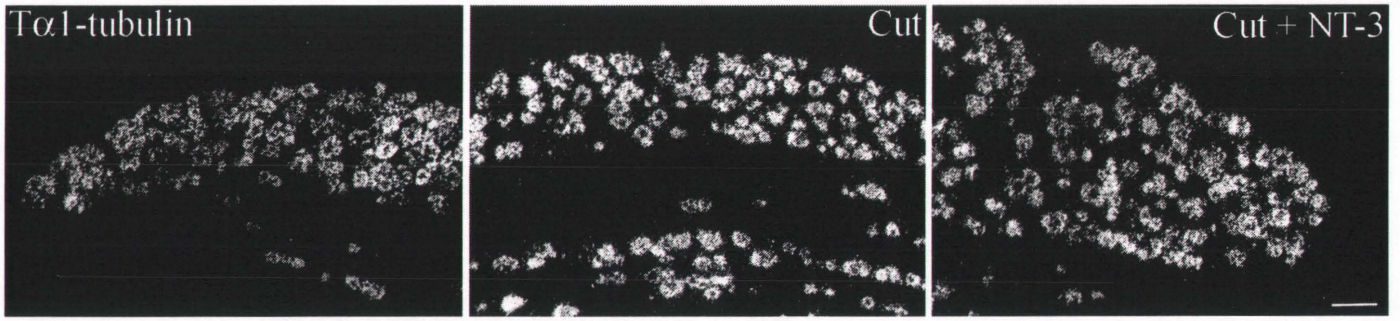

Figure 5.14 Expression of Tα1 α-tubulin mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect Tα1 α-tubulin mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 100 μm.

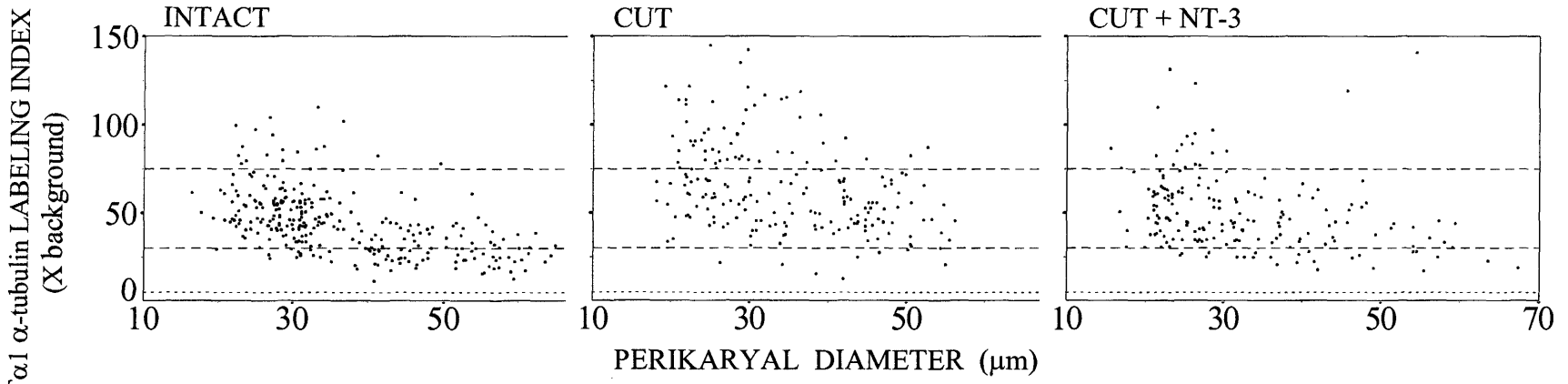

Figure 5.15 Relationship between perikaryal diameter and the relative level of Tα1 α-tubulin mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots of labeling indices of 184-286 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect Tα1 α-tubulin mRNA, depict the relationship between perikaryal diameter (x axis) and Tα1 α-tubulin labeling indices (y axis). Panels show a comparison of data under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of Tα1 α-tubulin mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for Tα1 α-tubulin mRNA. The long-dashed lines serve as references to facilitate data interpretation.

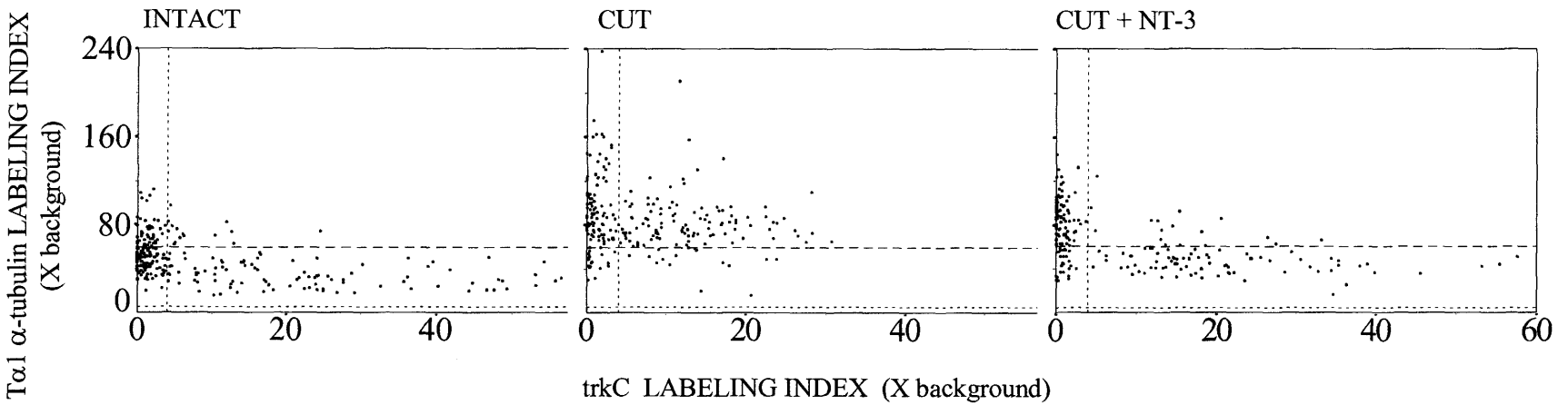

Figure 5.16 Relationship between the relative levels of trkC and Tα1 α-tubulin mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkC and Tα1 α-tubulin mRNA labeling indices for 213-283 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or Tα1 α-tubulin (y axis) mRNA under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of Tα1 α-tubulin mRNA. For each plot, neurons to the right of the short-dashed vertical line, and those above the short-dashed horizontal line, are considered positive for trkC and Tα1 α-tubulin mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

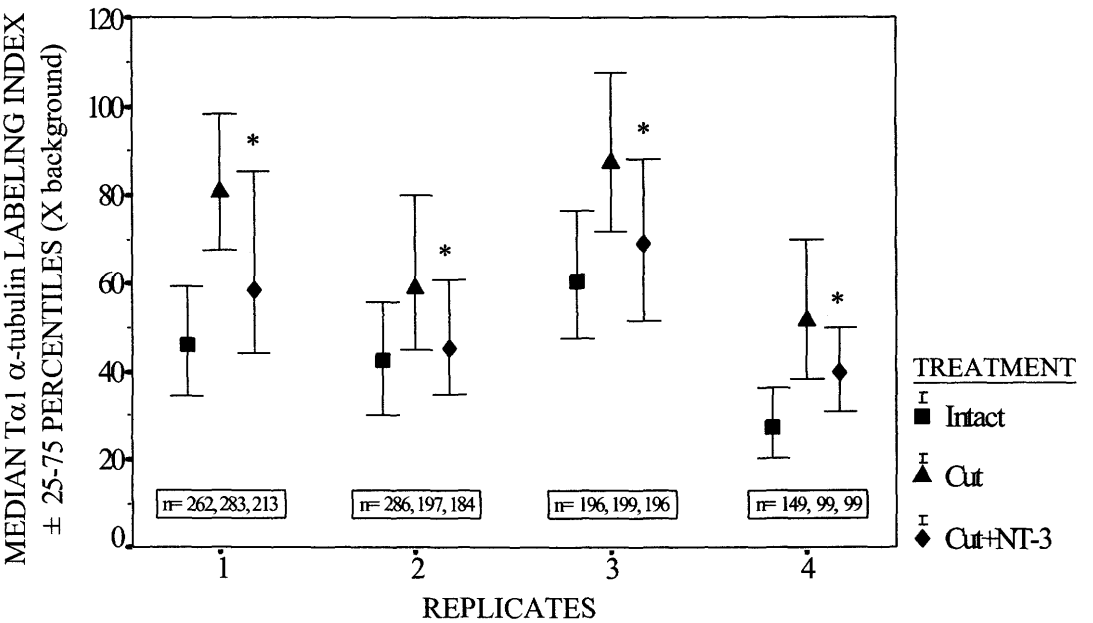

Figure 5.17 The effect of intrathecal infusion of NT-3 on trauma-induced Tα1 α–tubulin mRNA levels.

Graph depicts the relative differences in Tα1 α-tubulin mRNA labeling indices between intact, 3 week axotomized, and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the four replicates, Tα1 α-tubulin labeling indices for individual neurons were determined from image analysis of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect Tα1 α-tubulin mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.001$). Sample sizes (number of neurons) for each treatment are indicated below each replicate.

the 25th and 75th percentile labeling index values for Tα1 α-tubulin increase over those observed in the intact state. Delayed infusion of NT-3 partially mitigates the trauma-induced levels of these values. In all cases, the difference in Tα1 α-tubulin labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.001$). Data from the four replicates represent analyses of tissues from two different animals; replicates one and three represent analyses of tissue sections from the same animal. A summary of the effect of axotomy and the post-trauma infusion of NT-3 on Tα1 α-tubulin message levels within the trkC-positive subpopulation (Figure 5.18) shows that these data reflect the findings for the general population (Figure 5.17). The same may be said for the data when only those cells lacking detectable trkC hybridization signal are considered (Figure 5.19), although the ability of NT-3 to reduce Tα1 α-tubulin message levels appears to be less dramatic than that seen in trkC-positive neurons. In both cases, for the subset of neurons exhibiting trkC mRNA, the difference in Tα1 α-tubulin labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.001$), while for neurons not expressing detectable trkC transcripts, the difference in Tα1 α-tubulin labeling indices is statistically significant for only one of the two replicate sets (Mann-Whitney U Test, $p<0.05$). Data from the two replicates represent analyses of tissues from two different animals.

5.2.4 Neuropeptides

5.2.4.1 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on Galanin mRNA Levels

Only a small number of DRG neurons exhibit detectable levels of galanin mRNA in the intact state (Figure 5.20) and there does not appear to be overlap between this subset and the trkC-positive subpopulation (Figure 5.21). Three weeks after sciatic nerve transection there is a notable increase in both the number of galanin-positive neurons and the level of galanin message expression; post-axotomy there is substantial colocalization of galanin and trkC mRNA. NT-3 infusion appears to partially

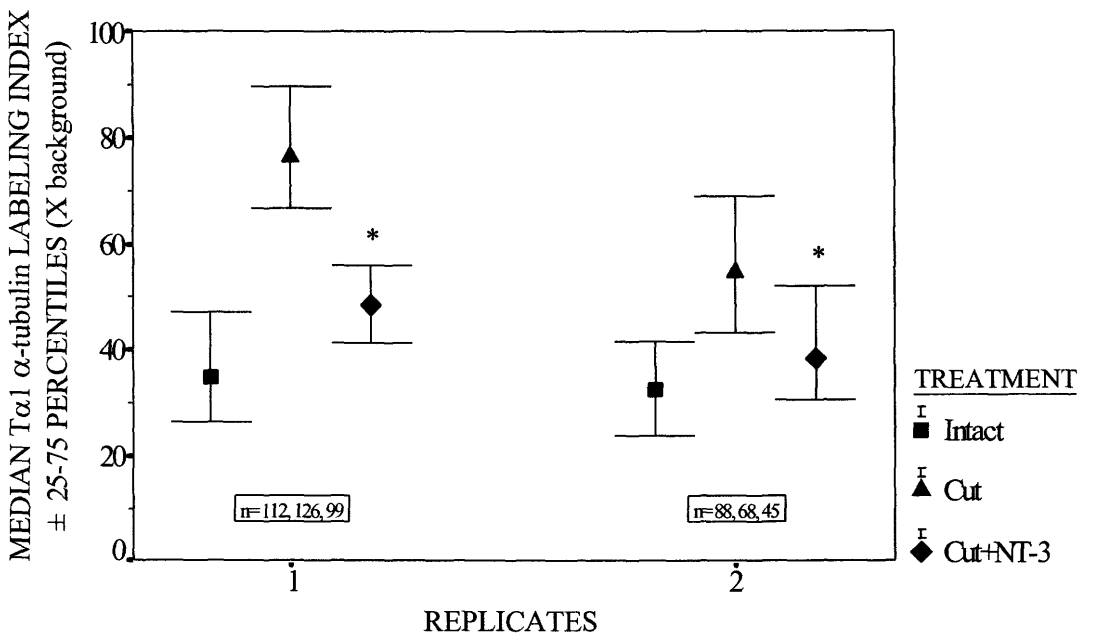

Figure 5.18 The effect of intrathecal infusion of NT-3 on trauma-induced Tα1 α-tubulin mRNA levels in neurons expressing trkC mRNA.

Graph depicts the relative differences in Tα1 α-tubulin mRNA labeling indices between normal (intact), 3 week axotomized (cut), and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 (cut + NT-3) treatments, for those neurons coexpressing trkC mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each replicate, Tα1 α-tubulin and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC or Tα1 α-tubulin mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.001$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-2 (intact, cut, cut + NT-3) are as follows: 262, 283, 213; 286, 197, 184.

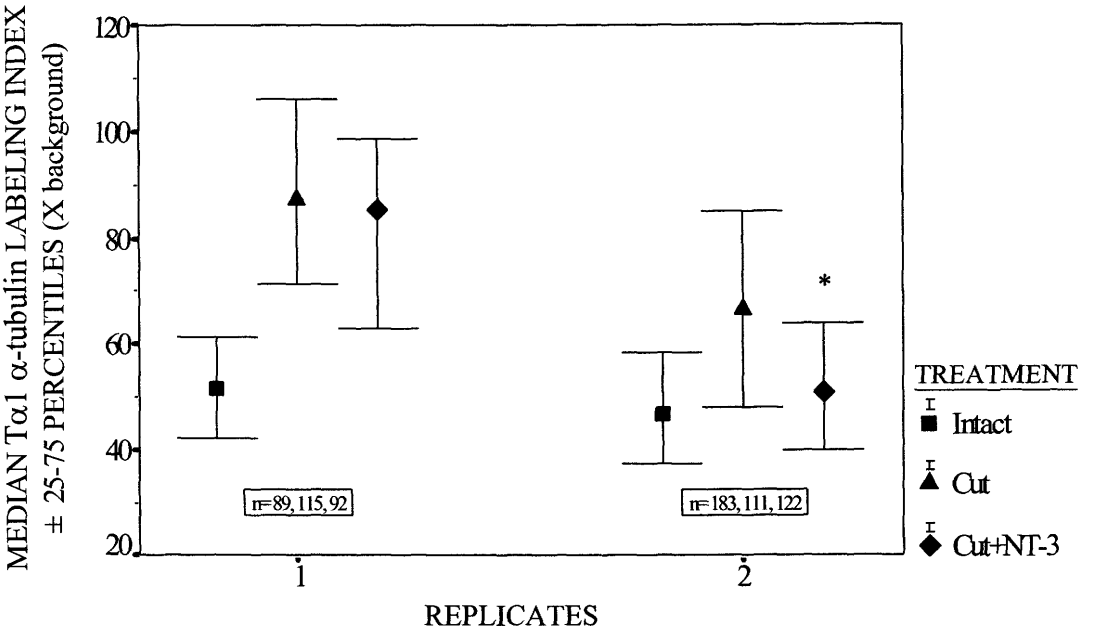

Figure 5.19 The effect of intrathecal infusion of NT-3 on trauma-induced Tα1 α-tubulin mRNA levels in neurons not expressing detectable trkC mRNA.

Graph depicts the relative differences in Tα1 α-tubulin mRNA labeling indices between intact, 3 week axotomized, and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments, for those neurons with near to below background labeling for trkC mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each replicate, Tα1 α-tubulin and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect Tα1 α-tubulin or trkC mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-2 (intact, cut, cut + NT-3) are as follows: 262, 283, 213; 286, 197, 184.

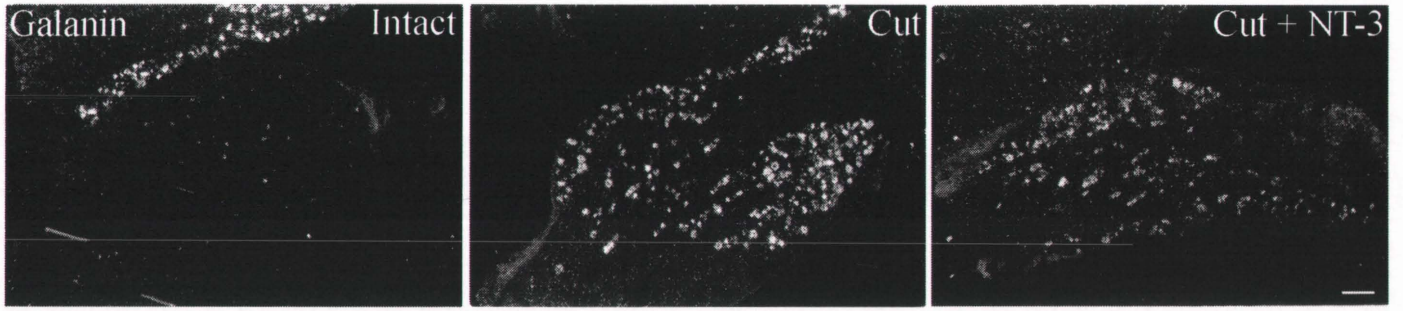

Figure 5.20 Expression of galanin mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect galanin mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm.

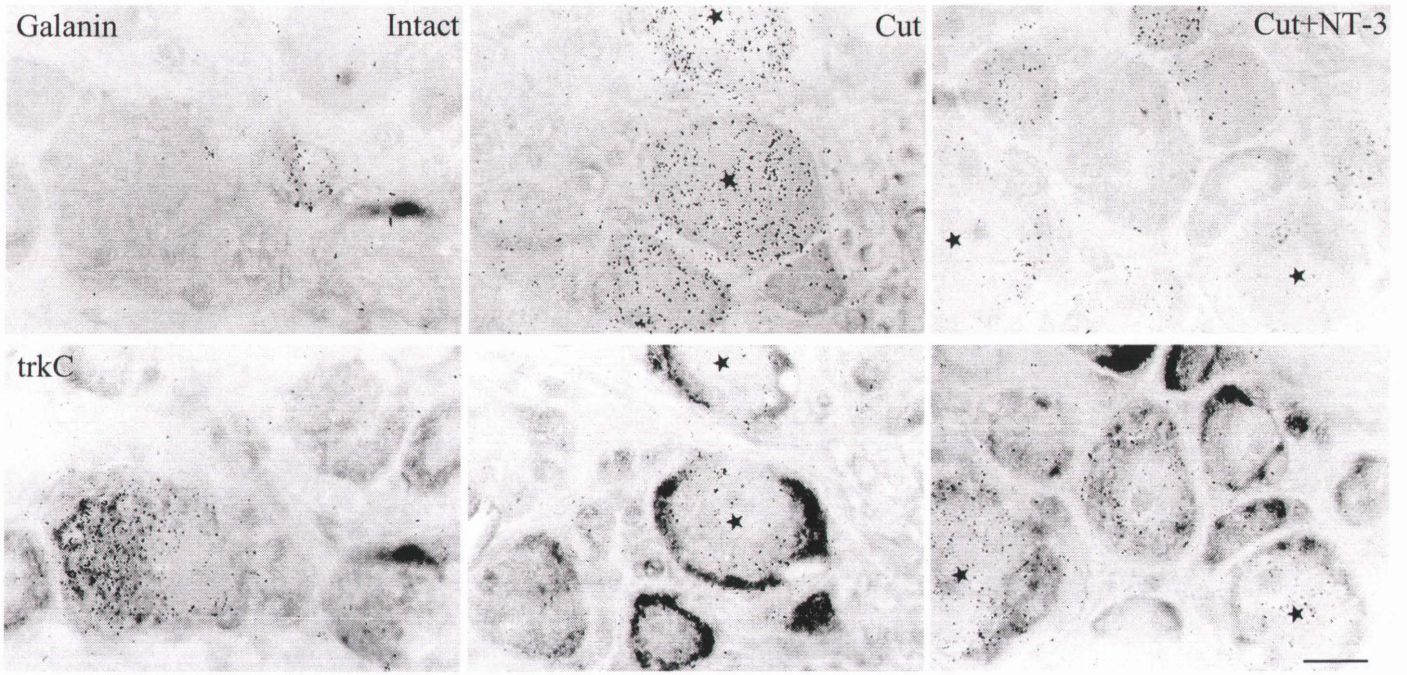

Figure 5.21 Colocalization of galanin and trkC mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect galanin (upper panel) and trkC (lower panel) mRNA under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Stars indicate neurons positive for both markers. Scale bar = 20 μm.

counteract the axotomy-induced upregulation of this neuropeptide, with the decrease in galanin transcripts seemingly most dramatic within neurons coexpressing trkC mRNA.

5.2.4.2 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on NPY mRNA Levels

Under normal conditions few, if any, DRG neurons express detectable levels of NPY mRNA (Figure 5.22). Three weeks post-axotomy, NPY message is upregulated in many medium and large diameter neurons, and mRNA for the neuropeptide strongly colocalizes with trkC message (Figure 5.23). Delayed intrathecal infusion of NT-3 for 7 days, 2 weeks after injury, partially mitigates the trauma-induced levels of NPY transcripts. The decrease in NPY message seen following NT-3 infusion in axotomized animals is most apparent in neurons that coexpress trkC mRNA.

5.2.4.3 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on VIP mRNA Levels

VIP transcripts are not detectable in normal DRG (Figure 5.24). After axotomy, many small and medium size neurons exhibit moderate to high levels of hybridization signal for the peptide, although there is no apparent overlap between VIP and trkC mRNA expression (Figure 5.25). Exogenous NT-3 does not appear to affect the injury-induced levels of VIP mRNA.

5.2.4.4 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on SOM mRNA Levels

A small number of DRG neurons express detectable SOM mRNA under normal conditions and axotomy results in reduced message levels (Figure 5.26). SOM-positive neurons are small to medium in size and transcript colocalization does not appear to occur between this neuropeptide and trkC (Figure 5.27). NT-3 infusion has no apparent influence on injury-reduced SOM expression.

5.2.5 Injury and Regeneration Associated Markers

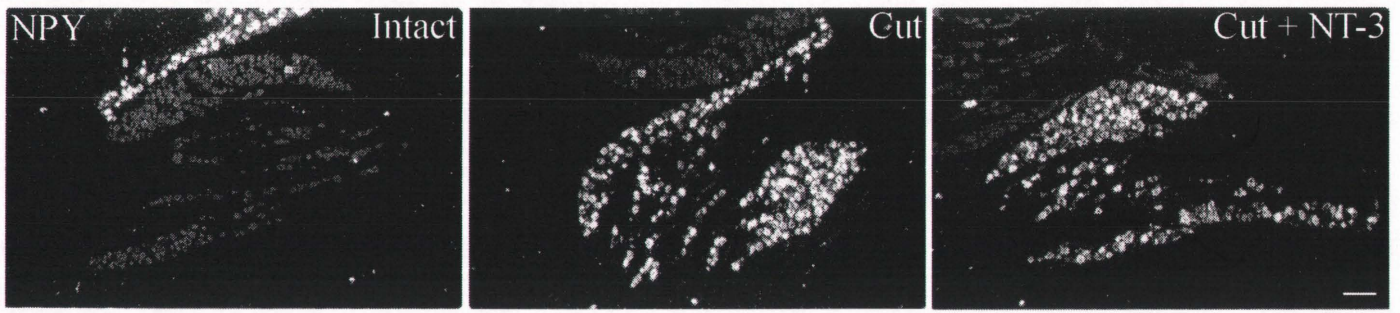

Figure 5.22 Expression of NPY mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned stained darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect NPY mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm.

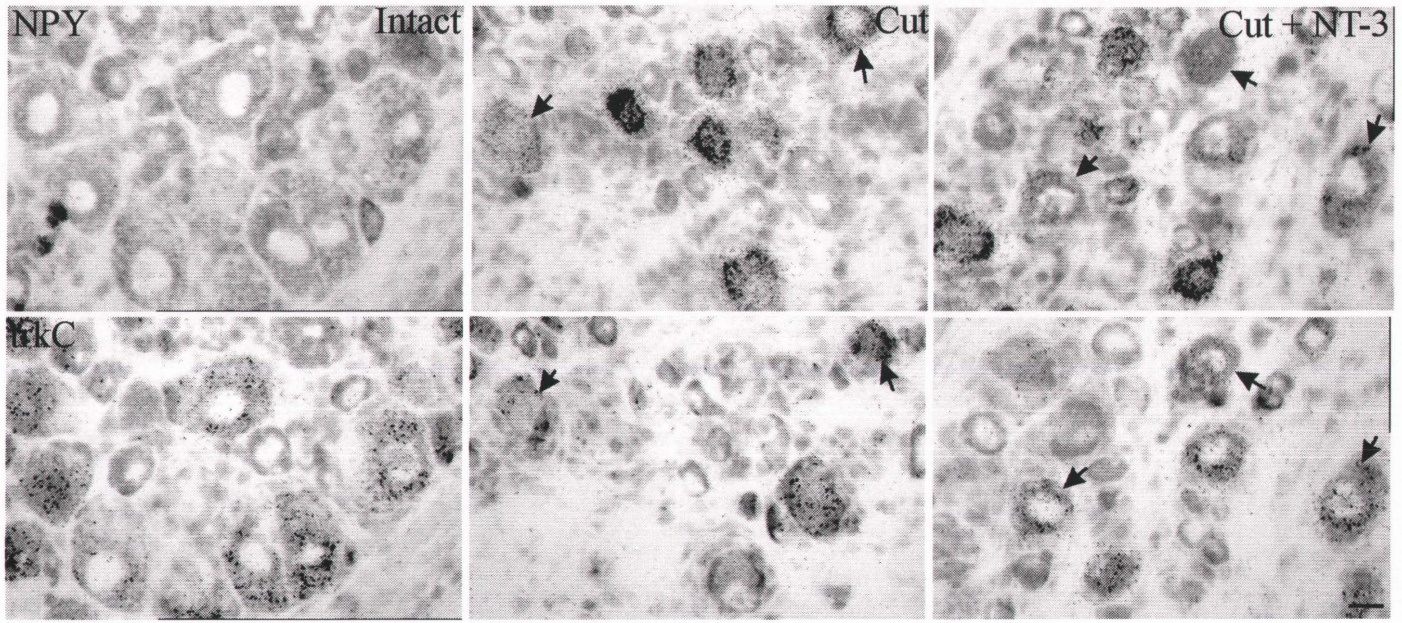

Figure 5.23 Colocalization of NPY and trkC mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect NPY (upper panel) and trkC (lower panel) mRNA under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Arrows indicate neurons positive for both markers. Scale bar = 30 μm.

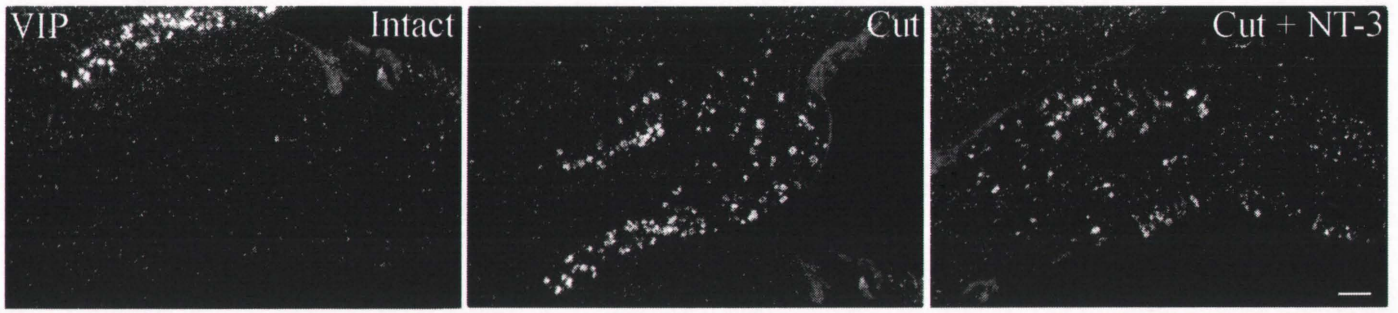

Figure 5.24 Expression of VIP mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect VIP mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm.

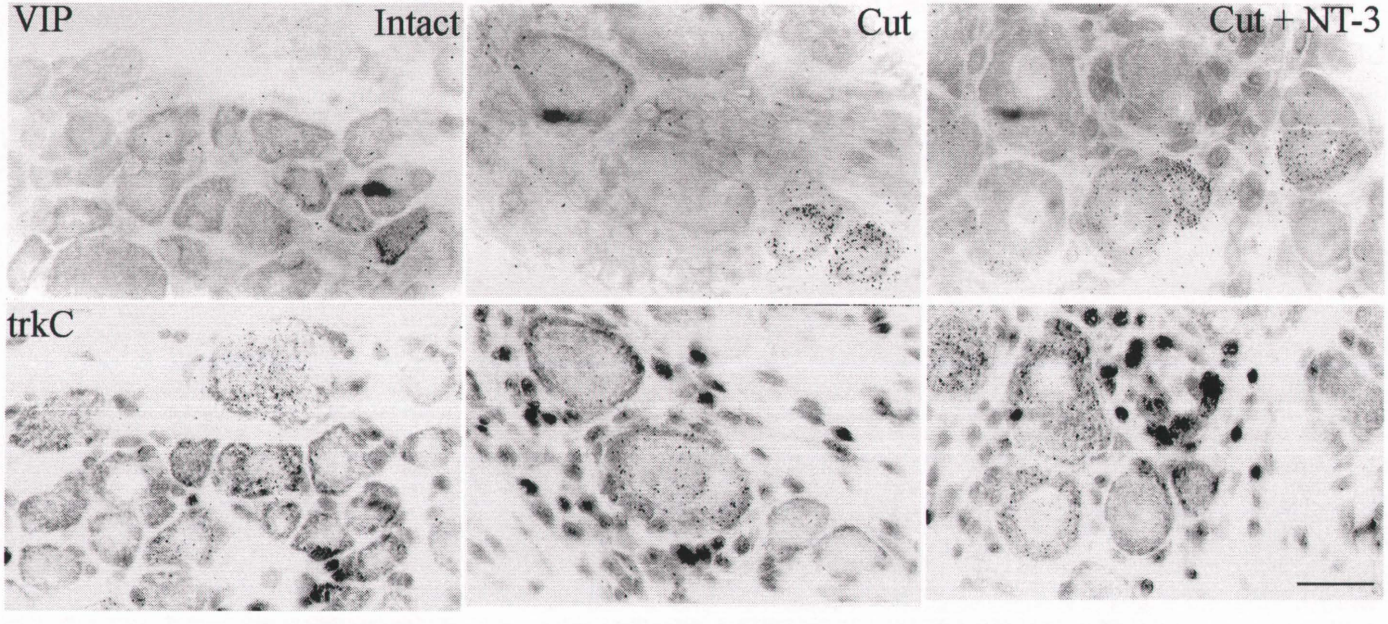

Figure 5.25 Colocalization of VIP and trkC mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect VIP (upper panel) and trkC (lower panel) mRNA under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 30 μm.

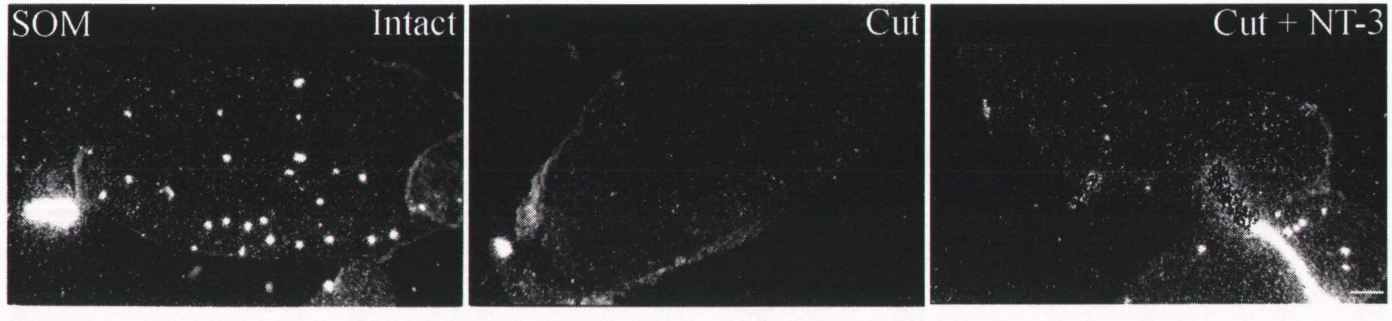

Figure 5.26 Expression of SOM mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect SOM mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm.

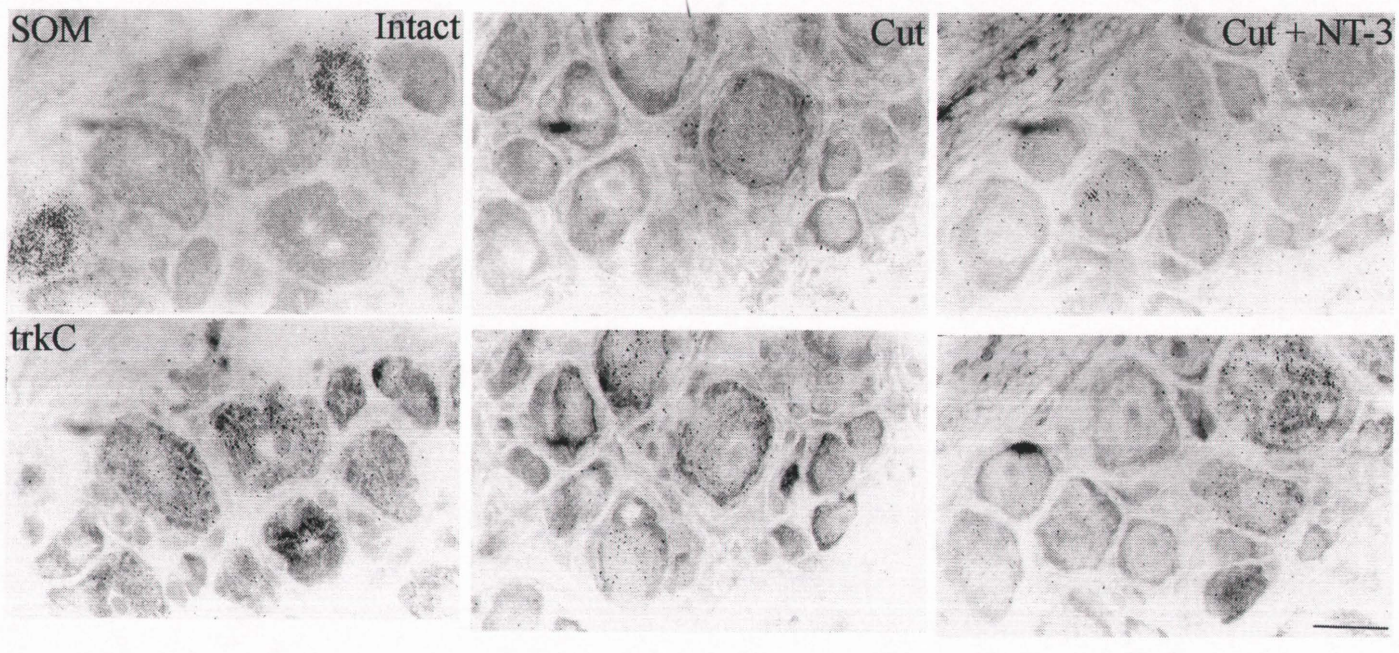

Figure 5.27 Colocalization of SOM and trkC mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect SOM (upper panel) and trkC (lower panel) mRNA under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 30 μm.

5.2.5.1 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on GAP-43 mRNA Levels

Although under normal conditions GAP-43 mRNA is detectable in many neurons, 3 weeks following sciatic nerve transection message levels increase dramatically, with virtually all neurons now expressing this marker (Figure 5.28). NT-3 infusion, 2 weeks post-axotomy, appears to result in decreased levels of GAP-43 transcripts.

Scatterplots (e.g. Figure 5.29), depicting the relationship between perikaryal diameter and GAP-43 mRNA expression for individual neurons (2 DRG sections/treatment, average 226 neurons/section), indicate that ~ 60% of DRG neurons express detectable message levels of GAP-43 in the intact state. The majority of small diameter cells exhibit GAP-43 transcripts, while a high proportion of large neurons lack detectable GAP-43 mRNA. Virtually all neurons display GAP-43 hybridization signal after axotomy. Although the post-trauma infusion of NT-3 results in a partial reduction in injury-induced message levels, the proportion of GAP-43 expressing neurons is not altered. The regulatory influence of NT-3 on GAP-43 mRNA levels does not appear to be restricted to any specific neuronal size class.

Scatterplots (e.g. Figure 5.30), illustrating the relationship between trkC and GAP-43 mRNA expression for individual neurons (2 DRG sections/treatment/probe, average 226 neurons/section), show that under normal conditions ~ 15% of DRG neurons display detectable levels of both trkC and GAP-43 mRNA. These GAP-43/trkC-expressing neurons tend to exhibit low to moderate levels of GAP-43, and low levels of trkC, hybridization signal. Expression of trkC transcripts appears heterogenous in GAP-43 negative/trkC-positive neurons. Three weeks following sciatic nerve transection, virtually all surviving neurons express GAP-43 mRNA; a subset of non-trkC expressing cells tend to display the highest GAP-43 labeling indices. Treatment with NT-3 appears to allay injury-induced message levels for GAP-43. The effect of NT-3 on GAP-43 mRNA expression is notable in the trkC-positive subpopulation, but the influence of the neurotrophin is not always evident in neurons that lack detectable trkC transcripts.

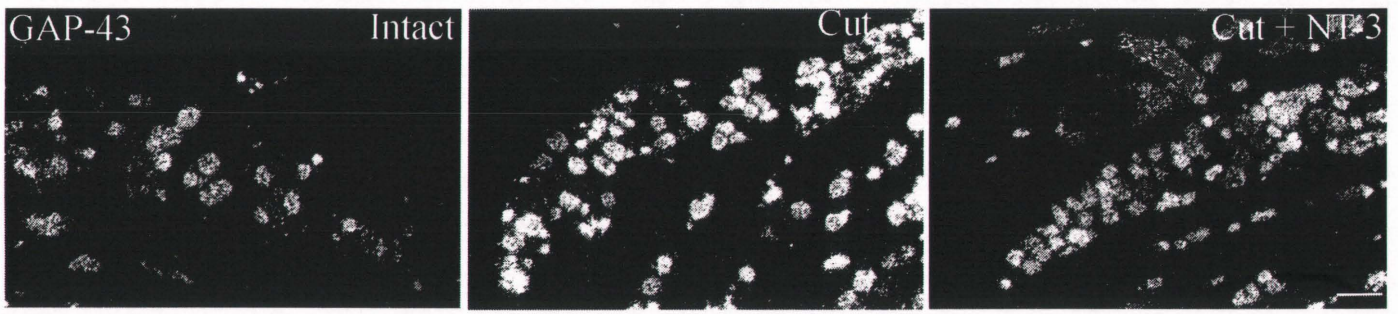

Figure 5.28 Expression of GAP-43 mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect trkC mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 100 μm.

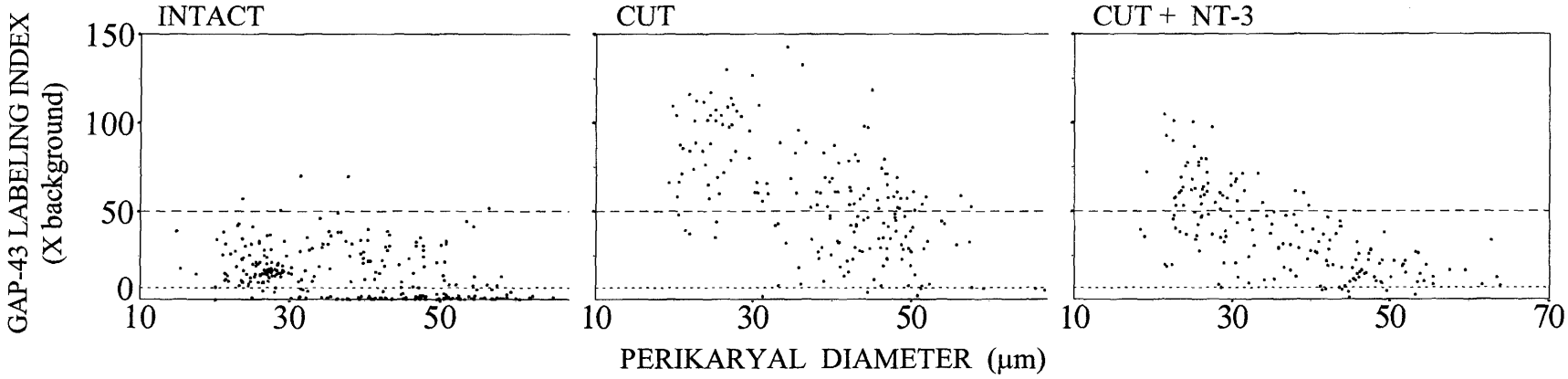

Figure 5.29 Relationship between perikaryal diameter and the relative level of GAP-43 mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots of labeling indices of 175-298 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect GAP-43 mRNA, depict the relationship between perikaryal diameter (x axis) and GAP-43 labeling indices (y axis). Panels show a comparison of data under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of GAP-43 mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for GAP-43 mRNA. The long-dashed lines serve as references to facilitate data interpretation.

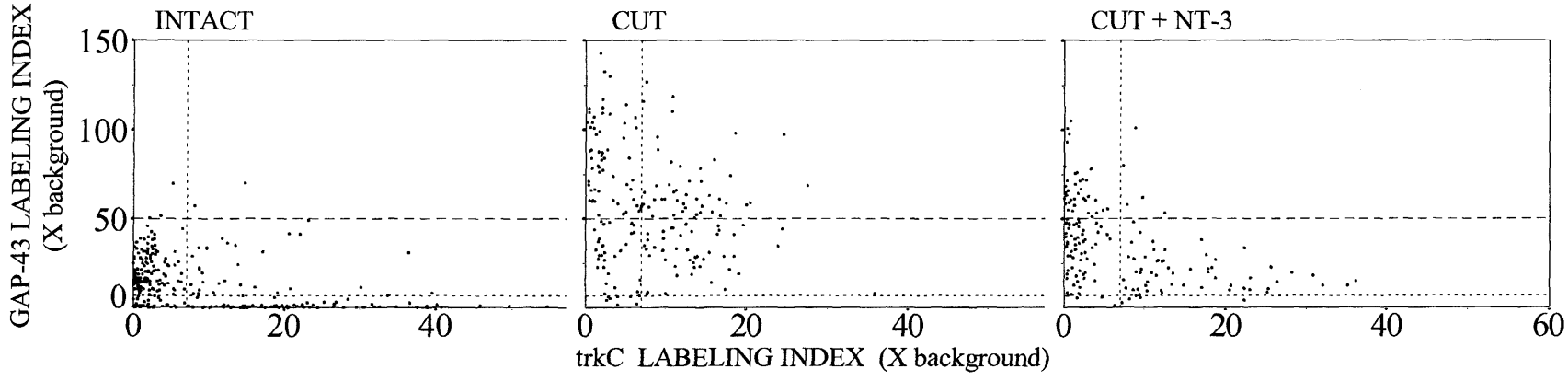

Figure 5.30 Relationship between the relative levels of trkC and GAP-43 mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkC and GAP-43 mRNA labeling indices for 175-298 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or GAP-43 (y axis) mRNA under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neurons to the right of the short-dashed vertical line, and those above the short-dashed horizontal line, are considered positive for trkC and GAP-43 mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

Figures 5.31 and 5.32 highlight the trend in changes in GAP-43 mRNA expression following axotomy and the effect of a delayed 7 day intrathecal infusion of NT-3, in trkC-positive neurons for 2 replicate data sets. The axotomy-induced increase, and the post-infusion reduction, in median GAP-43 labeling index values is clear, although the influence of NT-3 on these values appears to be more robust in cells with moderate to high levels of trkC hybridization signal. The difference in GAP-43 mRNA labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant for both replicates for the subset of neurons exhibiting moderate to heavy levels of trkC hybridization signal (Mann-Whitney U Test, $p<0.005$). Data from the two replicates represent analyses of tissues from two different animals. Figure 5.33 represents the same data, but examines the effect of exogenous NT-3 on those cells lacking detectable trkC mRNA. The graph mirrors the trkC versus GAP-43 scatterplot observations, in that the effect of NT-3 infusion on GAP-43 message expression in this subpopulation is unclear. NT-3 appears to result in an elevated GAP-43 median labeling index value in one replicate and a lower value in the second.

5.2.5.2 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on cjun mRNA Levels

In the intact state many DRG neurons exhibit low levels hybridization signal for cjun and levels increase dramatically after axotomy (Figure 5.34). Infusion of NT-3 appears to result in the return towards normal cjun message levels.

Scatterplots (e.g. Figure 5.35), demonstrating the relationship between perikaryal diameter and cjun mRNA expression for individual neurons (3 DRG sections/treatment, average 204 neurons/section), show that in the intact state cjun message is detectable in ~ 55% of cells, at more or less homogenous levels. There is no clear correlation between neuronal size and the level of hybridization signal for cjun. Three weeks following sciatic nerve transection cjun message increases over basal levels, and now virtually all neurons display moderate to high levels of cjun hybridization signal. Although the proportion of cjun-positive cells remains the same, infusion of NT-3 appears to result in

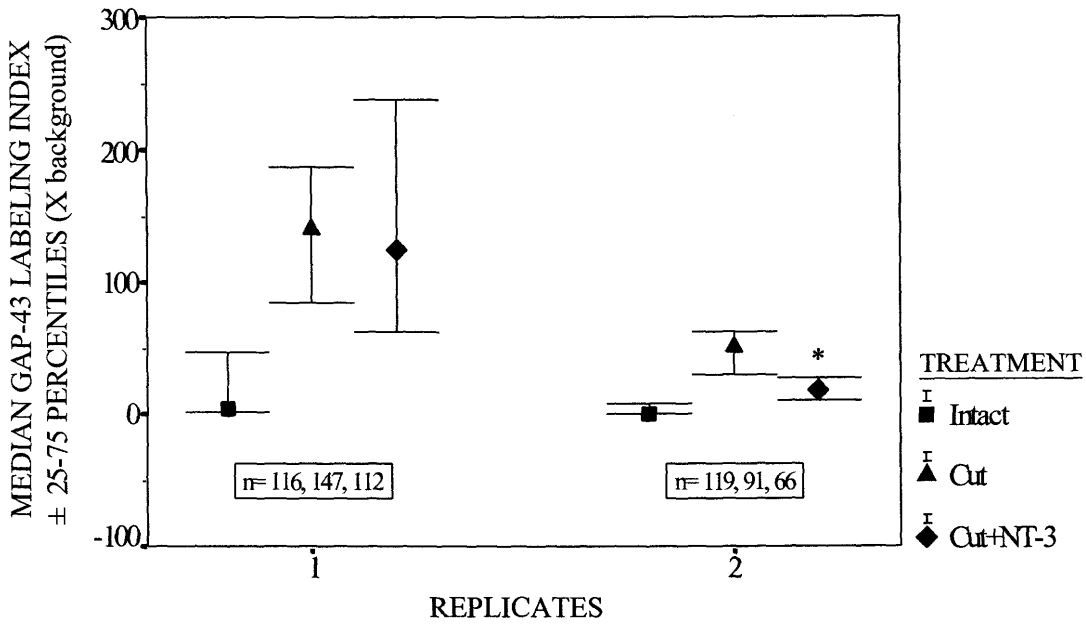

Figure 5.31 The effect of intrathecal infusion of NT-3 on trauma-induced GAP-43 mRNA levels in the trkC-positive subpopulation of neurons.

Graph depicts the relative differences in GAP-43 mRNA labeling indices between normal (intact), 3 week axotomized (cut), and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 (cut + NT-3) treatments, for those neurons with positive trkC mRNA labeling. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, GAP-43 and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC or GAP-43 mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1 and 2 (intact, cut, cut + NT-3) are as follows: 246, 233, 207; 298, 195, 175.

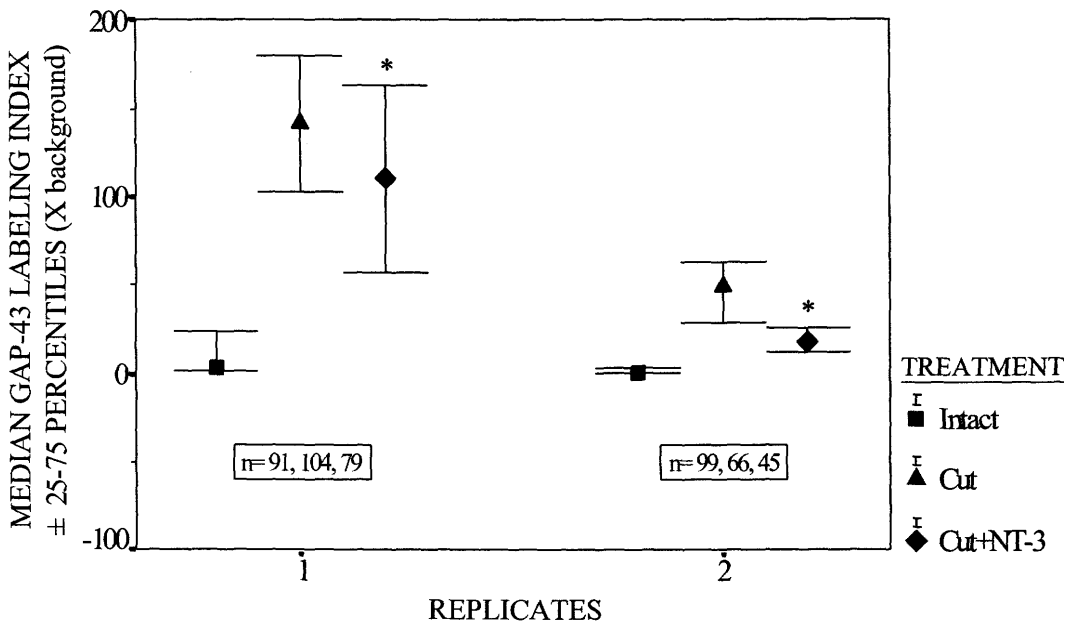

Figure 5.32 The effect of intrathecal infusion of NT-3 on trauma-induced GAP-43 mRNA levels in neurons with moderate to heavy trkC mRNA expression.

Graph depicts the relative differences in GAP-43 mRNA labeling indices between normal (intact), 3 week axotomized (cut), and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 (cut + NT-3) treatments, for those neurons with moderate to heavy trkC mRNA labeling. In each replicate, GAP-43 and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC or GAP-43 mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.005$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1 and 2 (intact, cut, cut + NT-3) are as follows: 246, 233, 207; 298, 195, 175.

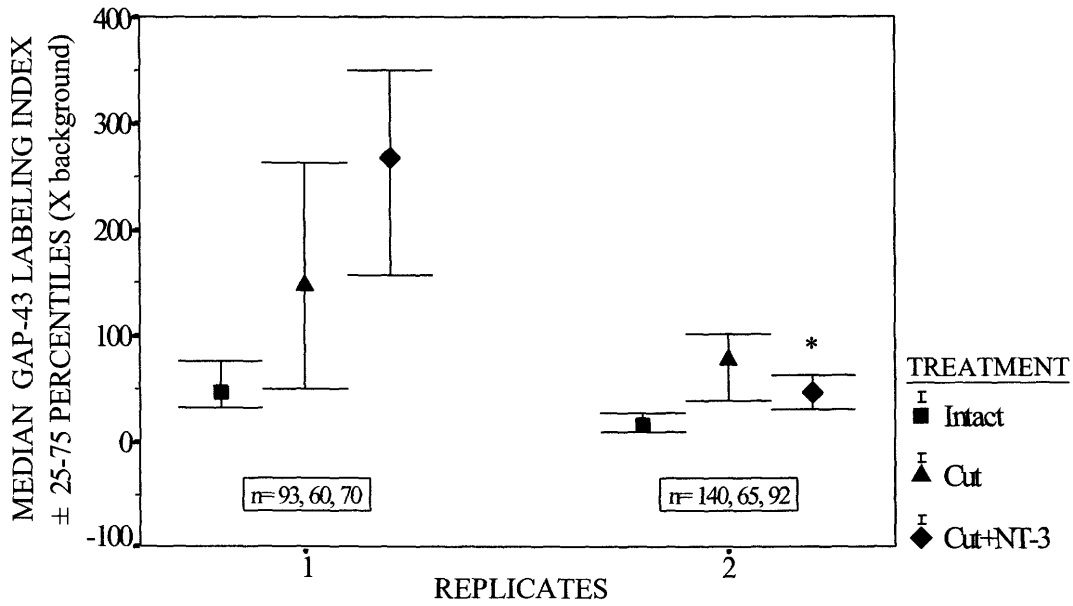

Figure 5.33 The effect of intrathecal infusion of NT-3 on trauma-induced GAP-43 mRNA levels in neurons not expressing detectable trkC.

Graph depicts the relative differences in GAP-43 mRNA labeling indices between intact, 3 week axotomized, and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments, for those neurons with near to below background labeling for trkC mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, GAP-43 and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect GAP-43 or trkC mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1 and 2 (intact, cut, cut + NT-3) are as follows: 246, 233, 207; 298, 195, 175.

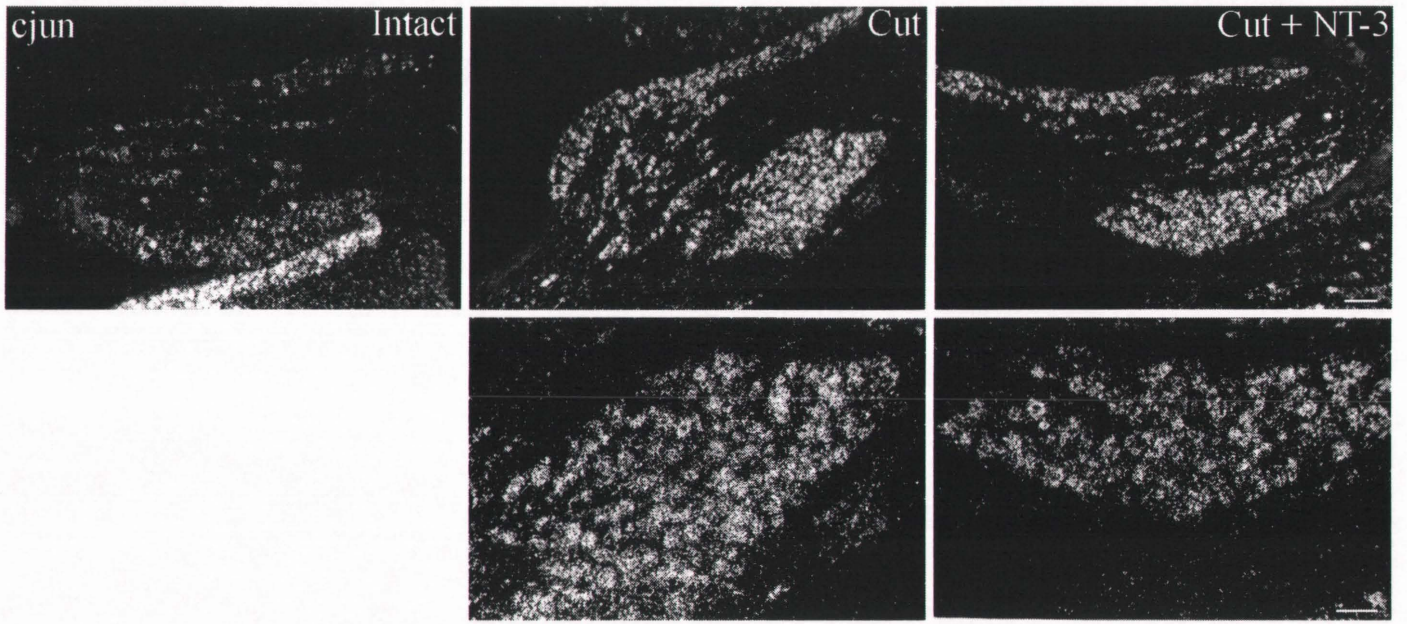

Figure 5.34 Expression of cjun mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Upper panel: Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect cjun mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 200 μm. Lower panel: Corresponding enlarged portion of upper photomicrograph. Scale bar = 100 μm.

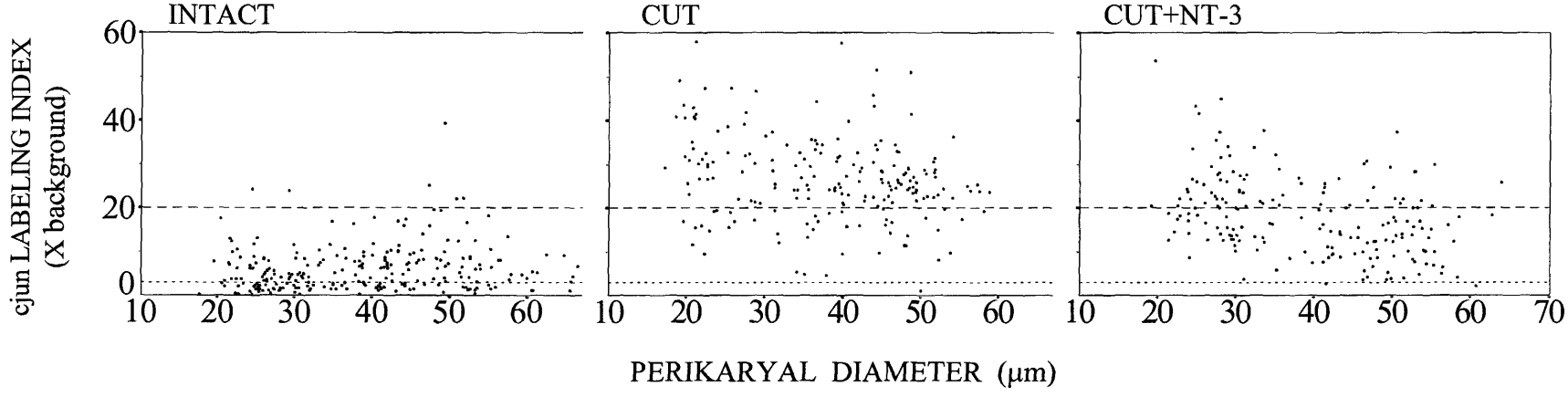

Figure 5.35 Relationship between perikaryal diameter and the relative level of cjun mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots of labeling indices of 186-246 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect cjun mRNA, depict the relationship between perikaryal diameter (x axis) and cjun labeling indices (y axis). Panels show a comparison of data under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of cjun mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for cjun mRNA. The long-dashed lines serve as references to facilitate data interpretation.

a decrease in axotomy-induced levels of cjun mRNA. The lessening in cjun message levels is evident in neurons of all sizes, but the effect may be more conspicuous in large diameter cells.

Scatterplots (e.g. Figure 5.36), depicting the relationship between trkC and cjun mRNA expression for individual neurons (3 DRG sections/treatment/probe, average 204 neurons/section), indicate that under normal conditions trkC and cjun transcripts colocalize in ~ 30% of DRG neurons, representing ~ 72% of trkC-positive, and ~ 53% of cjun-positive, cells. Message expression for cjun is more or less homogenous and this observation holds true for trkC-expressing neurons, regardless of trkC message level. After axotomy, the level of cjun hybridization signal increases and now ~ 100% L_5 DRG neurons exhibit moderate to high labeling index values for this marker. Infusion of NT-3, two weeks following the transection, appears to result in decreased cjun message levels, although ~ 100% of neurons still express cjun transcripts. The modulation in trauma-induced cjun mRNA levels seems to be most distinct in the trkC-positive subpopulation, and there appears to be a negative correlation between the levels of trkC and cjun hybridization signal; neurons with higher trkC labeling indices tend to exhibit lower levels of cjun hybridization signal.

Figure 5.37 highlights the trend in changes in cjun mRNA expression following axotomy and the effect of a delayed 7 day intrathecal infusion of NT-3 in trkC-positive neurons for 3 replicate data sets. Sciatic nerve transection results in a conspicuous increase in the median and the 25^{th} and 75^{th} percentile labeling index values for cjun. Infusion of NT-3 effects a reduction in these values within the trkC-positive subpopulation, such that the difference in cjun labeling indices between axotomized and axotomized/NT-3 infused tissues is statistically significant for all replicates (Mann-Whitney U Test, $p<0.05$). Data from the three replicates represent analyses of tissues from three different animals. The effect of axotomy on the median and the 25^{th} and 75^{th} percentile cjun labeling index values for those neurons that lack detectable trkC mRNA is similar to that observed in the trkC-positive subpopulation (Figure 5.38), and although NT-3 induced alterations in these values are less dramatic, the difference in cjun labeling

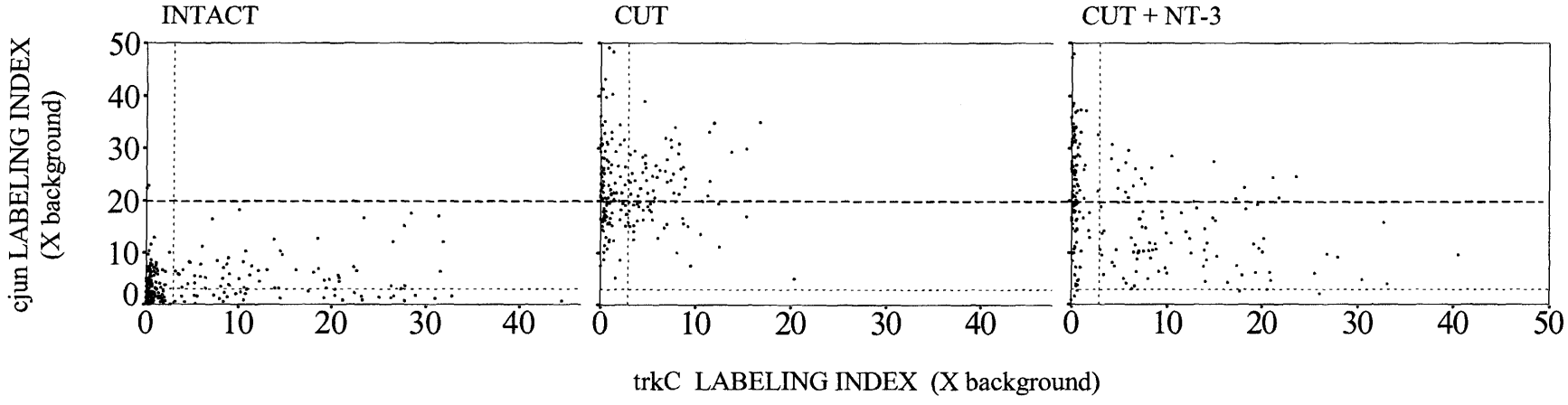

Figure 5.36 Relationship between the relative levels of trkC and cjun mRNA under the following conditions: normal, axotomized, and axotomized with delayed intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkC and cjun mRNA labeling indices for 198-248 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or cjun (y axis) mRNA under normal conditions (intact), 3 week axotomized (cut), and 3 week axotomized with intrathecal infusion of 600 ng/μl/h NT-3 for the final 7 days (cut + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neurons to the right of the short-dashed vertical line, and those above the short-dashed horizontal line, are considered positive for trkC and cjun mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

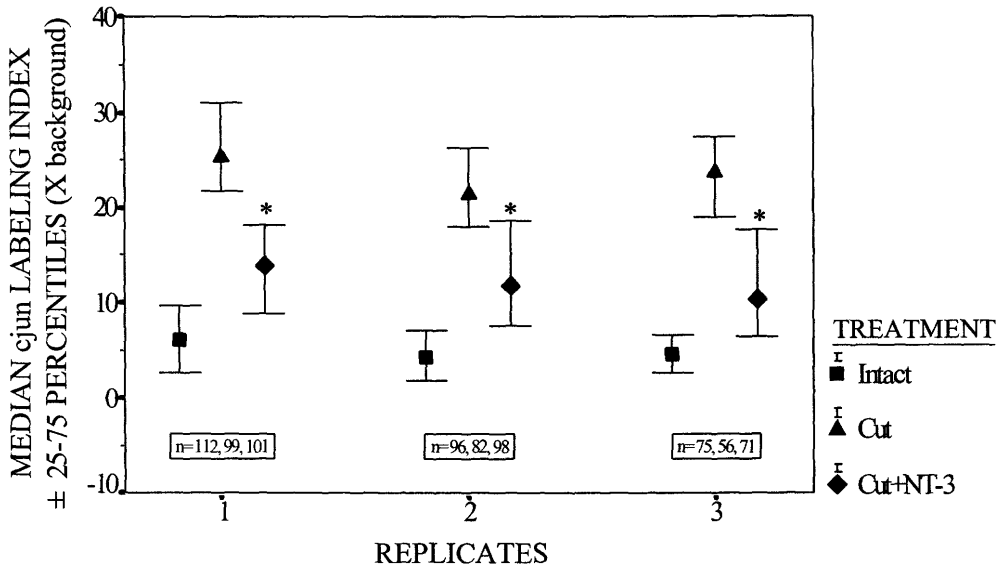

Figure 5.37 The effect of intrathecal infusion of NT-3 on trauma-induced cjun mRNA levels in neurons expressing trkC mRNA.

Graph depicts the relative differences in cjun mRNA labeling indices between normal (intact), 3 week axotomized (cut), and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments (cut + NT-3), for those neurons with trkC mRNA labeling. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the three replicates, cjun and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC or cjun mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-3 (intact, cut, cut + NT-3) are as follows: 246, 198, 186; 248, 198, 218; 188, 156, 148.

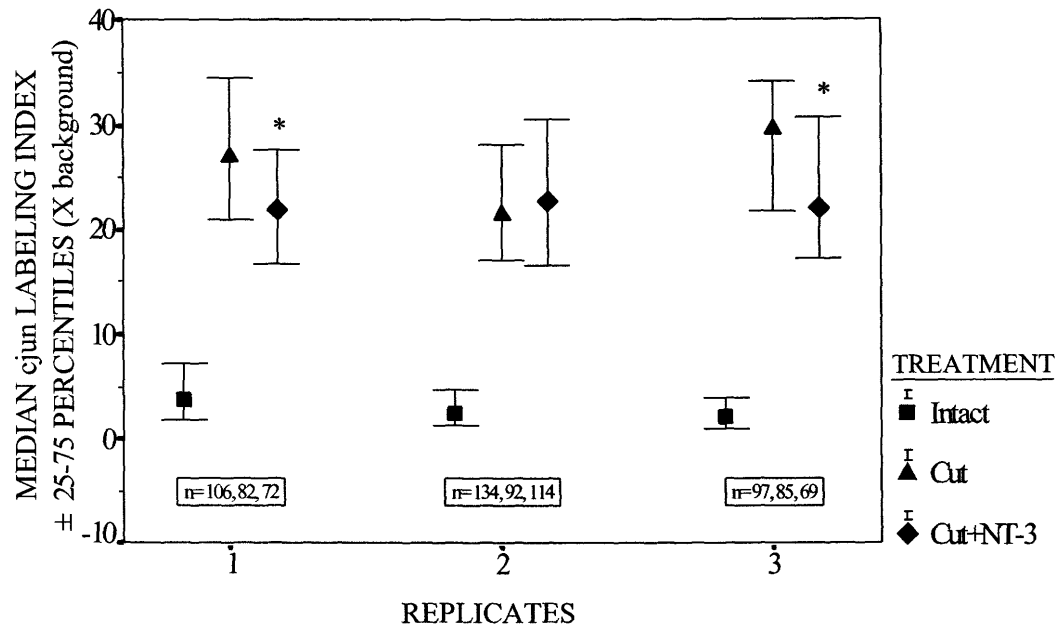

Figure 5.38 The effect of intrathecal infusion of NT-3 on trauma-induced cjun mRNA levels in neurons not expressing detectable trkC mRNA.

Graph depicts the relative differences in cjun mRNA labeling indices between normal (intact), 3 week axotomized (cut), and 3 week axotomized with delayed 7 day intrathecal infusion of NT-3 treatments (cut + NT-3), for those neurons with near to below background labeling for trkC mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the three replicates, cjun and trkC labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect cjun or trkC mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. Asterisk indicates significant difference between axotomized and axotomized plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-3 (intact, cut, cut + NT-3) are as follows: 246, 198, 186; 248, 198, 218; 188, 156, 148.

indices between axotomized and axotomized/NT-3 infused tissues is statistically significant in two of the three cases (Mann-Whitney U Test, $p<0.05$).

5.2.6 The Effect of Axotomy and Subsequent Delayed Intrathecal Infusion of NT-3 on SNAP-25 mRNA levels.

Under normal conditions, SNAP-25 mRNA is abundantly, although heterogenously, expressed in all DRG neurons (Figure 5.39); with larger diameter, trkC-positive cells typically expressing higher levels of hybridization signal for the marker (Figure 5.40). Three weeks following sciatic nerve transection, SNAP-25 message levels decrease; a 1 week intrathecal infusion of NT-3 appears to result in the upregulation of injury-reduced levels of SNAP-25 mRNA towards normal, but this increase appears to be primarily restricted to a subpopulation of medium and large diameter neurons that coexpress trkC transcripts.

5.3 Discussion

5.3.1 The Influence of Exogenous NT-3 on Injury-Induced Changes in Neurotrophin Receptor Expression

Consistent with the literature (Johnson *et al.*, 1987; Raivich and Kreutzberg, 1987; Verge *et al.*, 1989b, 1992a; Ernfors *et al.*, 1993; Funakoshi *et al.*, 1993; Sebert and Shooter, 1993; McMahon *et al.*, 1994; Zhou *et al.*, 1996; Kashiba *et al.*, 1998; Jongsma Wallin *et al.*, 2001), results here demonstrate that axotomy induces a reduction in trk and p75 levels in DRG neurons. Despite the loss of peripherally-derived NT-3 following sciatic nerve transection (see Section 2.2.1.3), primary sensory neurons may access local sources of the neurotrophin (see Section 2.2.1.2): NT-3 mRNA, present in satellite cells surrounding some neurons, is upregulated in the ipsilateral ganglion within 48 hours of axotomy and remains so for at least 2 months (Zhou *et al.*, 1999a). But, even though the neurotrophin is present in DRG after injury, this source is not sufficient to prevent the trauma-induced alterations in neuronal phenotype. It would appear that peripherally-sourced neurotrophin is critical to the maintenance of normal phenotype of primary sensory neurons. Moreover, data presented herein indicate that a 7

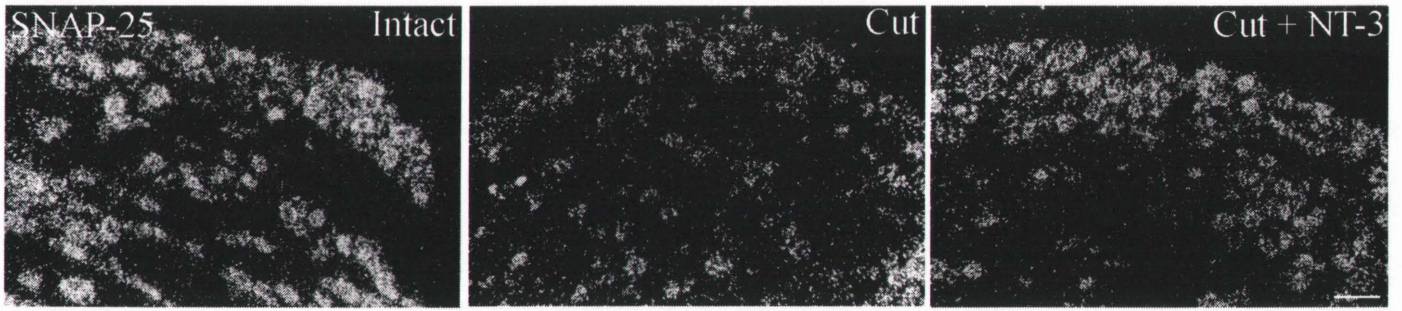

Figure 5.39 Expression of SNAP-25 mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned darkfield photomicrographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect SNAP-25 mRNA, under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Scale bar = 100 μm.

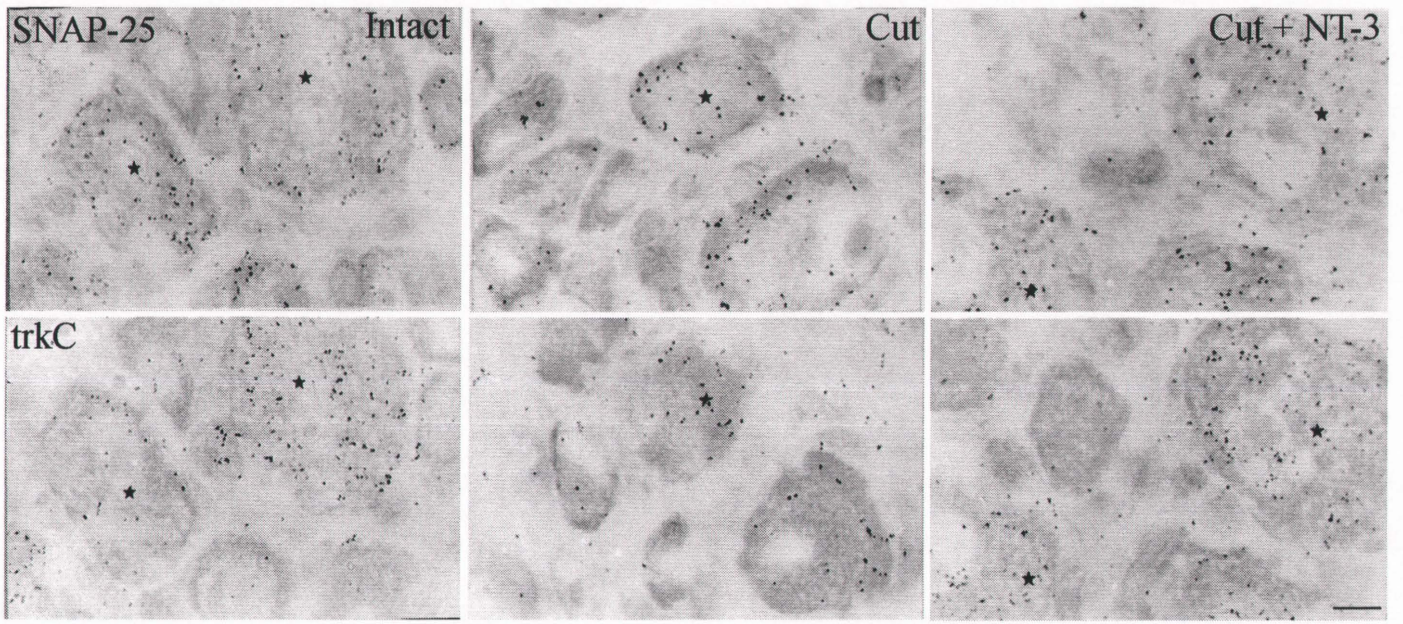

Figure 5.40 Colocalization of SNAP-25 and trkC mRNA under normal and axotomized conditions and following intrathecal infusion of NT-3.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect SNAP-25 (upper panel) and trkC (lower panel) mRNA under normal conditions (intact), 3 weeks following sciatic nerve transection (cut), and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3, 2 weeks post-axotomy (cut + NT-3). Asterisks indicate neurons positive for both markers. Scale bar = 10 μm.

day intrathecal infusion of NT-3 (600 ng/μl/h) 14 days post-injury, is successful in partially mitigating the trauma-induced reduction in both trkC and p75 mRNA expression within these cells. These data are not surprising, since NGF has long been shown to regulate high-affinity NGF binding sites/trkA and p75 in primary sensory neurons *in vivo* (Verge *et al.*, 1989b, 1992a; Jongsma Wallin *et al.*, 2001). Furthermore, a recent study (Wyatt *et al.*, 1999) has reported that NT-3 influences trkC expression in dissociated and explant cultures of embryonic mouse trigeminal ganglia. It is of interest to note that because exogenous NT-3 was more effective in upregulating trkC in explant cultures, the regulation of trkC message levels might involve additional factors present in the ganglion but not available to neurons in monocultures. This observation is also important to keep in mind when comparing data between *in vivo* and *in vitro* based experimental paradigms. The same study (Wyatt *et al.*, 1999) also showed that trkC expression in cultured sympathetic neurons was not altered following NT-3 treatment, indicating that the ability of the neurotrophin to regulate receptor expression may be dependent on cell type. This may be due in part to the low level of trkC transcripts in sympathetic neurons (Belliveau *et al.*, 1997; Dechant *et al.*, 1997), with any NT-3 influenced elevation in trkC levels potentially below the detection limit of the technique used in that study, or because the degree of change in trkC expression might be dependent on the initial level of receptor expression. Here (see Section 5.2.2.1, Figure 5.3), although NT-3 is effective in mitigating the injury-induced reduction in trkC levels in virtually all trkC-positive neurons, the infusion-effect is most pronounced within the subset of cells with moderate to high trkC labeling indices, further supporting the concept that the influences of NT-3 on receptor levels may be dependent on receptor concentration.

In addition to potential dependence on NT-3 effected regulation of receptor levels, various effects attributed to neurotrophins have been shown to be dose-dependent. As reported here (see Section 3.1.2.1.2) and in the literature, alterations in aspects of cellular phenotype vary with neurotrophin concentration (Ma *et al.*, 1992; Belliveau *et al.*, 1997; Bennett *et al.*, 1998). Similar results have been described for neurotrophin-influenced regulation of axonal elongation (Lentz *et al.*, 1999), level of trk

tyrosine phosphorylation (Belliveau *et al.*, 1997; Senger and Campenot, 1997), neurotransmitter release (Blochl and Sirrenberg, 1996), and degree of neuroprotective effects (Helgren *et al.*, 1997).

Another important factor to note regarding neurotrophin regulation of receptors concerns p75. In this study (see Section 5.2.2.2; Figures 5.6, 5.7 5.8), the NT-3 effected upregulation of p75 appears to be specific for the subgroup of neurons in which p75 and trkC transcripts colocalize. Similar parallel results were described for NGF and p75, wherein post-axotomy infused NGF mitigated the injury-induced decrease in p75 levels only in trkA/p75-positive sensory neurons (Verge *et al.*, 1992a). These data are interesting because all of the neurotrophins bind to p75 (see Section 1.1.2.2) and because up to 80% of lumbar DRG neurons express the receptor (see Sections 2.1.3, 4.2.2.3, 4.3.1), suggesting that p75 mRNA regulation does not occur *via* the neurotrophin binding to that receptor, but through binding to the appropriate trk.

5.3.2 The Influence of Exogenous NT-3 on Injury-Induced Changes in Expression of the Cytoskeletal Elements NFM and Tα1 α-tubulin

Under normal conditions all, or virtually all, adult rat lumbar DRG neurons, including the trkC-positive population, express transcripts for the cytoskeletal elements NFM and Tα1 α-tubulin (see section 4.3.3), with message levels for the former exhibiting a positive correlation and the latter a loose negative correlation with perikaryal diameter. Three weeks following sciatic nerve transection NFM message levels fall, while those for Tα1 α-tubulin rise (see Sections 4.2.3.1, 4.2.3.2, 4.3.3), but here a 2 week delayed, 7 day intrathecal infusion of NT-3 partially mitigates the axotomy-induced changes, bringing transcript levels for both markers towards normal values. With regard to NF expression these results are not surprising, since NGF has been reported to upregulate the message levels of all three NF subunits in cultured PC12 cells (Lindenbaum *et al.*, 1988) and in adult rat DRG neurons following axotomy (Verge *et al.*, 1990a; Gold *et al.*, 1991; Tetzlaff *et al.*, 1992). Since there is a positive correlation between NF levels and perikaryal and fiber diameter (Friede and Samorajski, 1970; Weiss and Mayr, 1971; Sharp *et al.*, 1982; Hoffman *et al.*, 1987; Verge *et al.*, 1990a;

Tetzlaff *et al.*, 1992) (see Section 4.2.3.1), and since axotomy results in both a reduction in NF expression (Greenberg and Lasek, 1988; Oblinger and Lasek, 1988; Verge *et al.*, 1990b; Wong and Oblinger, 1987, 1990, 1991; Ernfors *et al.*, 1993; Hoffman *et al.*, 1993) and somal and fiber size (Gold *et al.*, 1991; Hoffman *et al.*, 1993), one might expect that reestablishment of NF levels might also restore neuronal volume. Although investigation of this was not rigorously pursued in this thesis, results here suggest that exogenous NT-3 successfully restores near-normal perikaryal diameter. Similar data, collected under a variety of experimental conditions, have been reported after treatment with either NT-3 (Zhang *et al.*, 1994a; Gao *et al.*, 1995; Albers *et al.*, 1996; Belliveau *et al.*, 1997; Mizisin *et al.*, 1998; Lentz *et al.*, 1999) or NGF (Gold *et al.*, 1991; Belliveau *et al.*, 1997; Goodness *et al.*, 1997). Moreover, axonal conduction velocity, which decreases following injury (Munson *et al.*, 1997) and is determined by axonal caliber (Harper and Lawson, 1985a, 1985b; Lee *et al.*, 1986), has also been reported to be restored with NT-3 treatment (Munson *et al.*, 1997) (see Section 2.2.2.2).

NGF has also been shown to regulate Tα1 α-tubulin expression in PC12 cells (Miller *et al.*, 1987) and neonatal sympathetic neurons (Matthew and Miller, 1990; Ma *et al.*, 1992) *in vitro*, but in all of these cases transcript levels of the marker increased after exposure to the neurotrophin. In contrast, data from a study involving exposure of the proximal nerve stump of adult rats to NGF showed that this treatment failed to mitigate the injury-induced levels of Tα1 α-tubulin mRNA, although neither was the expression of NFL reported to be altered (Wong and Oblinger, 1991). As is the case for NGF, *in vitro* treatment with NT-3 also appears to result in higher levels of Tα1 α-tubulin expression. Two studies, using dissociated cultures of adult rat DRG neurons (Mohiuddin *et al.*, 1995) or neonatal sympathetic neurons (Belliveau *et al.*, 1997), report that exogenous NT-3 effected increased levels of Tα1 α-tubulin mRNA. These results conflict with my findings; this may be due to a potential dose-dependent effect (see Section 5.3.1.) or may be a consequence of the mode of neurotrophin delivery. Support for the former argument comes from a study on adult primary sensory neurons (Mohiuddin *et al.*, 1995), wherein exposure to higher levels of NT-3 yielded Tα1 α-tubulin message levels only slightly elevated above control values. Support for the latter

hypothesis may be derived from experiments involving comparisons between two treatment methods, including where or how the molecule is delivered. For instance, in adult rats intrathecal infusion of NGF allowed for more consistent results than did its infusion into the cut nerve stump (Verge *et al.,* 1990b), while peripheral injection of chicken embryos with NT-3 specific antiserum resulted in decreased numbers of muscle spindle afferents, but injection of the same protein into the spinal cord failed to produce a demonstrable change (Oakley *et al.,* 1995). These data suggest that the differences between my finding and those of Mohiuddin and colleagues (1995) may be due to the differences between supplying NT-3 *via* intrathecal infusion versus bathing the entire cell in neurotrophin. Further, the rate of delivery appears to be important in delineating the neuronal response to the neurotrophin: BDNF delivered slowly to hippocampal slices failed to incite synaptic potentiation, whereas fast delivery was reflected by increased potentiation rates (Kang *et al.,* 1996).

Interestingly, in the cases of NFM and Tα1 α-tubulin, unlike for p75 (see Section 5.3.1), NT-3 effected alterations in mRNA levels appear to occur throughout the population of DRG neurons, although changes are most pronounced in those cells that express detectable levels of trkC. Reexamination of data comparing the ability of NGF to mitigate the post-axotomy reduction in NFM levels in neurons with or without high–affinity NGF binding sites (Verge *et al.,* 1990a; Tetzlaff *et al.,* 1992) also appears to indicate that the NGF-effected increase in NFM mRNA levels is not restricted to neurons with high densities of NGF binding sites. This suggests that the regulation of cytoskeletal elements is under a more general control than is p75 — one that is influenced by the neurotrophin, but ultimately overseen by a more broad-acting factor. Or, the regulation of NFM and Tα1 α-tubulin gene expression may be determined through the activation of different parallel, convergent, or divergent signal transduction pathways associated with the trks (see Section 1.1.2.1.1). Another possibility is that NT-3 effects an upregulation in NFM and a concomitant downregulation of Tα1 α-tubulin levels by binding not only to trkC, but to the other trk species (see Section 1.1.2.1.1). Moreover, in addition to potentially activating trkA and trkB, NT-3 has been shown to bind to $trkA_{ei6}$ (see Section 1.1.2.1.1), an isoform of trkA that exhibits heightened

responsiveness to NT-3 (Barker *et al.,* 1993; Clary and Reichardt, 1994) and which colocalizes with non-insert trkA in over 90% of trkA adult rat lumbar DRG neurons (Karchewski *et al.,* 1999). But this premise does not explain the ability of NGF to also regulate NFM mRNA levels in non-trkA expressing neurons, since that ligand, unlike NT-3, has only been reported to bind specifically to trkA (see Section 1.1.2.1.2). Alternately, regulation of the expression of the two cytoskeletal markers may be through the activation of p75, which is expressed in up to 80% of adult primary sensory neurons (see Sections 4.2.2.3, 4.3.2).

5.3.3 The Influence of Exogenous NT-3 on Injury-Induced Changes in Neuropeptide Expression

The neuropeptides galanin and SOM are expressed by small subsets of small diameter DRG neurons, but both the levels and the number of cells exhibiting the former increase post-axotomy (see Sections 4.2.4.3, 4.2.4.4, 4.3.4), while those of the latter decrease (Noguchi *et al.*, 1993; Verge *et al.*, 1995). NPY and VIP are rarely detected in uninjured neurons, but peripheral nerve transection results in the expression of both markers in many medium to large and small to medium diameter neurons, respectively (see Sections 4.2.4.5, 4.2.4.6, 4.3.4). My data are consistent with these findings, but, in addition, demonstrate that there is limited, if any, overlap between trkC and SOM or VIP and a significant degree of coexpression of the receptor with galanin or NPY post-injury. Further, because of their expression, or lack thereof, of these neuropeptides in the trkC-positive subpopulation, it is not surprising to note here that while NT-3 infusion mitigates the axotomy-induced levels of galanin and NPY mRNA, NT-3 treatment fails to alter SOM and VIP message levels.

Several studies have examined the role of NT-3 in regulating neuropeptide expression in DRG neurons (see Section 2.2.2.2), and these data support my findings: A continual 3 week delivery of NT-3 to the proximal transected sciatic nerve stump resulted in a reduction in the number of NPY-ir neurons and decreased staining intensities for the neuropeptide, with an overall 36% reduction in NPY protein levels (Ohara *et al.*, 1995). Similarly, there was ~ 45% decrease in the number of NPY-ir L_4/L_5

DRG neurons in animals 30 days after NT-3 impregnated, entubulated fibronectin mats were grafted between the proximal and distal ends of the transected/resected sciatic nerve; the presence of exogenous NT-3 did not appear to alter VIP levels (Sterne *et al.*, 1998). Additionally, neither VIP nor SOM protein levels were found to be significantly changed following the treatment of adult rat DRG neuron cultures with NT-3 (Mulderry, 1994). Interestingly, the neurotrophin appears to affect these neuropeptides in an opposite manner in CNS neurons. NT-3 treatment of neonatal rat cortex (Barnea *et al.*, 1995, 1996) and hippocampal slice (Marty and Onteniente, 1999) cultures resulted in increased NPY and SOM protein levels. Moreover, culturing vagal/glossopharyngeal visceral afferent neurons in the presence of NT-3 effected an upregulation in the number of VIP-positive cells, whereas anti-NT-3 treatment prevented this increase (Helke and Verdier-Pinard, 2000).

NT-3 is not the only neurotrophic factor known to influence neuropeptide expression in primary sensory neurons — in fact, the regulation of neuropeptide levels appears to be complex. For example, NGF has been shown to modulate levels of SP, CGRP, NPY, galanin, VIP, and CCK (Kessler and Black, 1980; Otten *et al.*, 1980; Otten and Lorez, 1982; Lindsay *et al.*, 1989; Verge *et al.*, 1995; Bennett *et al.*, 1998; Corness *et al.*, 1998; Ramer *et al.*, 1998). In addition, GDNF mitigates the post-axotomy levels of both SOM and SP (Bennett *et al.*, 1998; Ogun-Muyiwa *et al.*, 1999; Issa *et al.*, 2001), as does aFGF for galanin and bFGF for SP (Ji *et al.*, 1996).

Besides the negative regulation of galanin levels in injured primary sensory neurons effected by NT-3 (see Section 5.2.4.1), NGF (Verge *et al.*, 1995; Corness *et al.*, 1998; Ramer *et al.*, 1998), and aFGF (Ji *et al.*, 1996), other trophic factors influence galanin levels in a positive manner. The injury-induced cytokines, IL-6 (interleukin-6) and LIF (leukemia inhibitory factor) (Curtis *et al.*, 1994; Murphy *et al.*, 1995, 1999a, 1999b; Kurek *et al.*, 1996a, 1996b; Sun and Zigmond, 1996; Arruda *et al.*, 1998), appear to be responsible for the upregulation of the neuropeptide after nerve injury (Corness *et al.*, 1998; Ramer *et al.*, 1998; Thompson *et al.*, 1998b; Kerekes *et al.*, 1999; Murphy *et al.*, 1999b). Two studies have provided insight into how these molecules may interact in regulating galanin expression. Cultures of E13 DRG neurons, initially exposed to both

NGF and LIF, exhibited elevated galanin message levels upon withdrawal of NGF, while the addition of LIF to the NGF-deprived cultures resulted in even higher levels of galanin expression; culturing these cells in the presence of both neurotrophic factors did not result in galanin levels significantly different from control values (Corness *et al.*, 1998). Moreover, application of LIF directly to intact superior cervical ganglion neurons did not alter galanin levels, but injection of anti-NGF coupled with LIF treatment stimulated galanin expression (Shadiack *et al.*, 1998). These observations suggest that the availability of NGF (or NT-3 or aFGF), is potentially more critical to maintaining normal galanin levels than is the presence of the cytokine. Similar interactions may occur in the trophic factor regulation of the other neuropeptides.

5.3.4 The Influence of Exogenous NT-3 on Injury-Induced Changes in Expression of the Injury and Regeneration Associated Markers GAP-43 and cjun

GAP-43 and cjun transcripts are expressed in many DRG neurons, including some that are trkC-positive, in the intact state, but peripheral axotomy triggers a dramatic upregulation in both levels of mRNA expression and in the numbers of neurons exhibiting detectable levels of these the markers (see Sections 4.2, 4.3.5, 5.2.5). Here, intrathecal infusion of NT-3 has been shown to effect a reduction in injury-induced GAP-43 and cjun message levels within trkC-expressing neurons. Downregulation of GAP-43 mRNA levels appears to be most notable in those neurons with moderate to high trkC labeling indices; the degree of cjun mRNA regulation appears to be negatively correlated with trkC expression, with post-infusion cjun levels lowest in cells with the highest labeling indices for the receptor.

Although to my knowledge there is no published record of NT-3 modulating cjun expression in primary sensory neurons, intrathecal infusion of NGF has also been reported to mitigate injury-induced cJUN-ir levels in adult rat L_5 DRG neurons (Gold *et al.*, 1993a). Further to this, removal of access to target-derived NGF through injection of antiserum to NGF resulted in elevated cJUN-ir, both in the number of cJUN-positive neurons and the staining intensity (Gold *et al.*, 1993a). These data suggest a similar control mechanism for cjun expression in neurotrophin-responsive primary sensory

neurons. Contrary to these findings, another group found that in cultures of DRG neurons and glia neither NGF nor BDNF inhibited cjun expression (De Filipe and Hunt, 1994).

In an experiment in which dissociated cultures of adult rat DRG neurons were treated with 10 ng/ml NT-3, there was a reported 2 fold increase in GAP-43 mRNA levels (Mohiuddin *et al.*, 1995) (see Section 2.2.2.2). These data contrast sharply with my results, but the same study also noted that a higher concentration of NT-3 (50 ng/ml) effected an upregulation in GAP-43 message levels only slightly elevated compared to those of untreated cultures. This observation suggests that the influence of the neurotrophin on GAP-43 mRNA regulation is dose-dependent and may also be a reflection of the method used to deliver the neurotrophin (see Sections 5.3.1, 5.3.2). Unlike for NT-3, intrathecal infusion of NGF did not appear to significantly alter injury-induced GAP-43 mRNA levels, implying that a peripherally-derived signal other than NGF regulates the post-trauma expression of GAP-43 (Verge *et al.*, 1990b). Interestingly, the positive correlation that exists between GAP-43 mRNA expression and the presence of high-affinity NGF binding sites under normal conditions suggests a role for NGF in maintaining the high GAP-43 message levels within this subpopulation (Verge *et al.*, 1990b). Moreover, in a different study, *in vivo* NGF treatment resulted in elevated GAP-43 mRNA levels compared to control, which was thought to reflect the role of that neurotrophin in collateral sprouting (Mearow, 1998). Further, exposure of cultures of adult rat DRG neurons to anti-NGF prevented the normal increased levels of GAP-43 expression in sprouting neurons (Mearow and Kril, 1995).

Although it is unclear whether NT-3 treatment influences GAP-43 expression in neurons that lack detectable levels of trkC transcripts, the neurotrophin effects a downregulation of cjun mRNA levels in this subset, albeit a less notable one than that in trkC-positive neurons. It may be that at least in the case of cjun, similar to that reported here for NFM and Tα1 α-tubulin (see Section 5.3.2), regulation of gene expression is under a more general control. The source of this regulation could involve retrograde signals derived from the site of the lesion (Murphy *et al.*, 1999a), or more generally those derived from immune cells participating in the injury response (Brown *et al.*,

1991; Richardson and Lu, 1994; Lu and Richardson, 1995). In this sense the modulation of GAP-43 and cjun expression by NT-3 would be an indirect one, through influencing the production of factors in glia and immune cells, which normally positively regulate the injury phenotype. This, coupled with a direct influence on the trkC subpopulation, could be responsible for the stronger modulation observed in this subgroup. This could also account for the *in vivo/in vitro* differences, since key glia and immune cells would be missing from these cultures.

The ability of the neurotrophin to modulate GAP-43 and Tα1 α-tubulin expression (see Sections 5.2.3.2, 5.3.2) suggests that NT-3 may not only be important in maintaining or regulating aspects of neuronal phenotype, but that it may also play a significant role in regulating axonal growth and regeneration. A number of studies have examined the ability of NT-3 to influence neurite extension and the resulting data form a complex picture.

NT-3 was shown to enhance neurite outgrowth in a subset of adult rat DRG neurons *in vitro*, although the degree of NT-3 induced outgrowth was greater at 10 ng/ml than at 50 ng/ml (Mohiuddin *et al.*, 1995). Exogenous NT-3 (50 ng/ml) also supported increased axonal growth in adult mouse L_4/L_5 DRG explant cultures (Edström *et al.*, 1996), but the addition of NT-3 (1, 10, or 100 ng/ml) to the distal portion of compartmentalized cultures of adult rat DRG neurons failed to induce neurite outgrowth (Kimpinski *et al.*, 1997). Neither did the treatment of parallel cultures with both NT-3 and NGF alter the growth-inducing effects of NGF alone (Kimpinski *et al.*, 1997). Likewise, treatment of cultures of dissociated adult rat lumbar DRG neurons with NT-3 did not appear to influence axonal growth, although cotreatment with NT-3 and NGF partially blocked NGF-enhanced neurite outgrowth (Gavazzi *et al.*, 1999).

NT-3 has been shown to effect peripheral nerve regeneration *in vivo*. In adult rats, entubulated fibronectin mats impregnated with NT-3 (500 ng/ml) grafted to resected sciatic nerve supported the regeneration of significantly more myelinated axons than control, nonimpregnated mat grafts, even at 8 months post-insertion (Sterne *et al.*, 1997a). In contrast, *in utero* injection of rat embryos with NT-3 appeared to either have

no influence on, or to inhibit, axonal growth, depending on the developmental stage of the animal (Zhang *et al.*, 1994a).

Together, these data indicate that although NT-3 can influence axonal growth — in a positive or negative manner, or not at all — the effect may be influenced by the concentration of the neurotrophin, the means of neurotrophin delivery, the presence of additional neurotrophic factors, and/or the level of maturity of neurons at the time of neurotrophin exposure. Since this study shows that three markers of nerve injury and regeneration (GAP-43, Tα1 α-tubulin, and cjun) are all downregulated following NT-3 treatment, it would be of interest to examine whether intrathecal infusion of NT-3 would affect the regeneration process. In experiments involving the immediate intrathecal infusion of NGF, that neurotrophin has also been shown to mitigate injury-induced upregulation of cJUN-ir levels in adult rat L5 DRG neurons (Gold *et al.*, 1993a), but NGF treatment only delayed the onset of the regeneration process for 2 days; it did not alter the maximum rate of regeneration (Gold, 1997).

5.3.5 The Influence of Exogenous NT-3 on Injury-Induced Alterations in SNAP-25 Expression

Here, under normal conditions, all L_5 DRG neurons are shown to express detectable levels of SNAP-25 mRNA, with a subset of large diameter trkC-positive neurons exhibiting the highest SNAP-25 message levels (also see Section 4.2.6). Three weeks following sciatic nerve transection, there is a downregulation in SNAP-25 transcript levels, but a delayed, 7 day intrathecal infusion results in the mitigation of the injury-induced changes in SNAP-25 levels. This upregulation of SNAP-25 expression appears to be primarily restricted to medium and large trkC-expressing neurons. Although there is limited information available delineating the regulation of SNAP-25 in primary sensory neurons, BDNF has been shown to upregulate SNAP-25 levels, as well as those of other proteins associated with vesicle fusion and exocytosis, in embryonic cortical neurons *in vitro* (Takei *et al.*, 1997). The same study (Takei *et al.*, 1997) demonstrated that exogenous BDNF increased stimulation-evoked release of glutamate and increased the number of dense core and clear vesicles in nerve terminals. These data,

together with my findings, suggest that neurotrophins are important to neurotransmitter and neuropeptide release. Also of interest, is a report that prolonged incubation of PC12 cells with NGF resulted in increased SNAP-25 phosphorylation and a concomitant increase in plasma membrane-localized SNAP-25-ir (Kataoka *et al.*, 2000). This suggests that the neurotrophins are not only important in the regulation of SNAP-25 mRNA levels, but also in SNAP-25 activation. SNAP-25 has also been shown to be upregulated by dibutyryl-cAMP in PC12 cells (Sanna *et al.*, 1991). Similarly, potassium chloride induced SNAP-25-ir in hippocampal neurons and PC12 cells, but failed to do so in cerebellar neuron cultures, while arachidonic acid upregulated SNAP-25-ir only in the former cell type (Sepulveda *et al.*, 1998). Conversely, estrogen implants in the pituitary gland of ovariectomized rats resulted in a downregulation of SNAP-25 mRNA levels, although message levels of other SNARE components were not affected (Jacobsson *et al.*, 1998). Taken together, these reports coupled with my findings, indicate that, as for other markers (see Sections 5.3.2, 5.3.4), the regulation of SNAP-25 is complex, involving numerous regulatory molecules, and is also dependent on cell type.

5.3.6 Summary

In primary sensory neurons, delayed intrathecal infusion of NT-3 resulted in a return towards normal, pre-axotomy mRNA levels for all of the phenotypic markers examined here, but only if at least a subset of the neurons expressing the marker also expressed trkC (Table 5.1): NT-3 treatment effected an upregulation in injury-reduced message levels of trkC, p75, NFM, and SNAP-25; with a concomitant downregulation of Tα1 α-tubulin, galanin, NPY, GAP-43, and cjun transcripts. NT-3 infusion did not appear to alter the post-trauma mRNA levels of SOM or VIP, nor did it appear that either of these neuropeptides were coexpressed with trkC. Further, it is of interest to note that although in the case of p75 message levels appeared to be upregulated only in those cells that coexpressed trkC, the influence of NT-3 in regulating post-trauma message levels for more broadly expressed markers (i.e. NFM, Tα1 α-tubulin, cjun) was not limited to the trkC-positive population. These findings may imply that control of the expression for these markers is less specific, more global, than it is for p75. It is also of

Table 5.1 Effect of NT-3 infusion on post-axotomy mRNA levels for selected phenotypic markers in adult rat L_5 DRG neurons.

MARKER	POST-AXOTOMY NT-3 EFFECTED MODULATION
trkC	positive
p75	positive
NFM	positive
Tα1 α-tubulin	negative
galanin	negative
SOM	no apparent qualitative change
NPY	negative
VIP	no apparent qualitative change
GAP-43	negative
cjun	negative
SNAP-25	positive

importance to note that the regulation of the expression of these markers is complex. The presence, or absence, of the neurotrophin may result in altered gene expression; control may be at the level of transcription or be post-transcriptional. For example, since NT-3 effects a downregulation of cjun message levels there may be reduced levels of cJUN, which would result in reduced levels of AP-1 complexes containing that protein, thereby altering the functional capacity of the AP-1 complex in regulating gene transcription (see Sections 4.3.5, 5.3.4). Further, since NGF has been reported to increase the stability of GAP-43 mRNA (Perrone-Bizzozero *et al.*, 1993; Nishizawa, 1994; Tsai *et al.*, 1997), NT-3 might be found to exert similar influences on transcripts for some markers. Moreover, results appear to be dependent on the concentration of the neurotrophin and its receptors, and the rate and mode of its delivery. In addition, it is important to consider that the ability of NT-3 to influence message levels may also be contingent on the presence of other cell types and that other trophic molecules may act synergistically or in opposition to those of the neurotrophin. But, regardless of the mechanism(s) involved, the findings presented here show NT-3 to be an important molecule in maintaining the normal phenotype of at least a subset of mature DRG neurons.

Future work could use the method of data analysis outlined in Section 4.3.7 to determine if NT-3 differentially influenced message levels in selected micropopulations. For example, even though I have shown here that the NT-3 effected upregulation of p75 mRNA levels appears to be limited to the trkC-positive population, because of the strong correlation between p75 and trk expression coupled with the extensive colocalization of trk species, modulation of p75 mRNA levels might be different in trkC/trkA-expressing cells compared to trkC-positive/trkA-negative neurons. In addition, because these data suggest that NT-3 depresses the expression of regeneration-associated genes following injury (GAP-43, cjun, Tα1 α-tubulin), future work could be directed towards determining if this is reflected in a suppression of the ability of these neurons to support an injury response and to successfully regenerate *in vivo*.

6. THE EFFECT OF NT-3 INFUSION ON THE PHENOTYPE OF INTACT DRG NEURONS

6.1 Introduction

NT-3 has been shown to be important in the maintenance of normal neuronal phenotype in at least a subset of adult primary sensory neurons (see Sections 2.2.2.2, 5.2, 5.3), but during some trauma states levels of the trophic factor available to these cells may decrease (see Section 2.2.1.2). Experiments involving the augmentation of endogenous NT-3 supply have been employed to test the efficacy of using this molecule to ultimately treat peripheral neuropathies (Gao *et al.*, 1995; Rodriguez-Pena *et al.*, 1995; Tomlinson *et al.*, 1996, 1997; Helgren *et al.*, 1997; Fernyhough *et al.*, 1998; Mizisin *et al.*, 1998) (see Section 2.2.2.2). Even though application of exogenous neurotrophin may have the desired clinical affect on the injured cells, a number of factors — including the colocalization of trk species in individual neurons (see Sections 2.1.2, 4.2.2.2, 4.3.1); the presence of the pan-neurotrophin-binding receptor, p75, in up to 80% of DRG neurons (see Sections 1.1.2.2, 2.1.3, 4.2.2.3, 4.3.1); and the reported ability of NT-3 to also bind to trkA and trkB (see Section 1.1.2.1.1) — raise the possibility that increasing the concentration of NT-3 above normal levels might alter additional facets of sensory neuron phenotype. Indeed, it has been shown that mice genetically altered to overexpress specific neurotrophins tend to exhibit increased numbers of trk-positive neurons (see Section 2.2.2.1.1), although such models tend to underscore the developmental importance of the manipulated gene and may obscure its role in the adult (see Section 2.2.2.1.4). In this study, I examined whether a 1 week intrathecal infusion of NT-3 modifies the expression of a number of phenotypic markers in otherwise normal adult rat DRG neurons.

6.2 Results

6.2.1 Technical considerations

See Section 4.2.1.

6.2.2 The Effect of Intrathecal Infusion of NT-3 on trkC mRNA Levels

Qualitative assessment of radioautographs of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization to detect trkC mRNA show that a 7 day 600 ng/μl/h intrathecal infusion of NT-3 does not appear to result in alterations in trkC message levels from those seen in non-infused tissue (Figure 6.1).

Scatterplots (e.g. Figure 6.2), constructed from data obtained through quantitative analysis of labeling indices of individual neurons identified in sections of L_5 DRG (7 DRG sections/treatment, average 267 neurons/section), processed for *in situ* hybridization, and subsequent radioautography, to determine perikaryal diameter and to detect trkC mRNA show that under normal conditions trkC message is heterogenously expressed by ~ 40% neurons, with the majority being medium to large in size. Intrathecal infusion of NT-3 does not appear to result in a noticeable change in the relative levels of trkC mRNA expression or in the number of trkC-positive cells. Applying exogenous NT-3 may result in increased perikaryal size, but to be able to say this with scientific accuracy more precise measurements, and a different approach, would be needed (reviewed in: Pover *et al.*, 1993).

Figure 6.3 highlights the trends in alterations in relative levels of trkC mRNA expression following NT-3 infusion for five replicate data sets. Whereas properties inherent to the technique of *in situ* hybridization do not allow for the comparison of silver grain densities between replicates, these bar graphs provide a summary of the data represented in the panel of scatterplots for each replicate set (e.g. Figure 6.2). The median and the 25th and 75th percentile values for trkC mRNA labeling are plotted for each treatment condition (intact, intact plus NT-3). Although there may be small differences between the 75th percentile values of infused and non-infused tissues within each replicate, there is little difference between the medians under these conditions. In

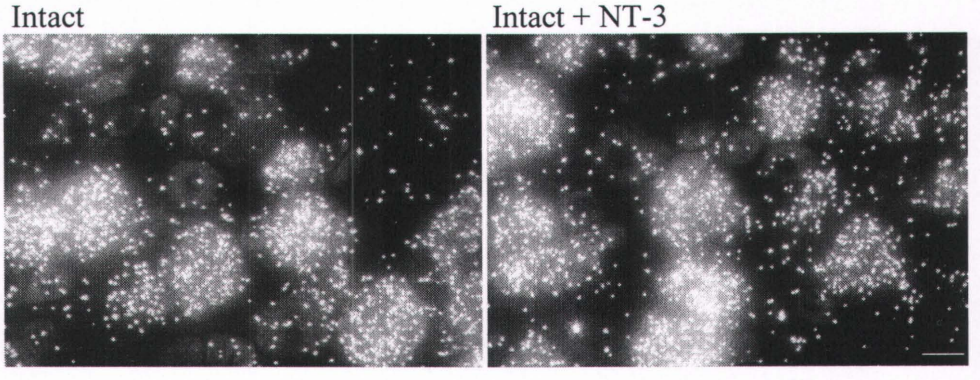

Figure 6.1 Expression of trkC mRNA under normal conditions and following intrathecal infusion of NT-3.

Scanned stained darkfield photomicrographs of 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect trkC mRNA, under normal conditions (intact) and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3 (intact + NT-3). Scale bar = 40 μm.

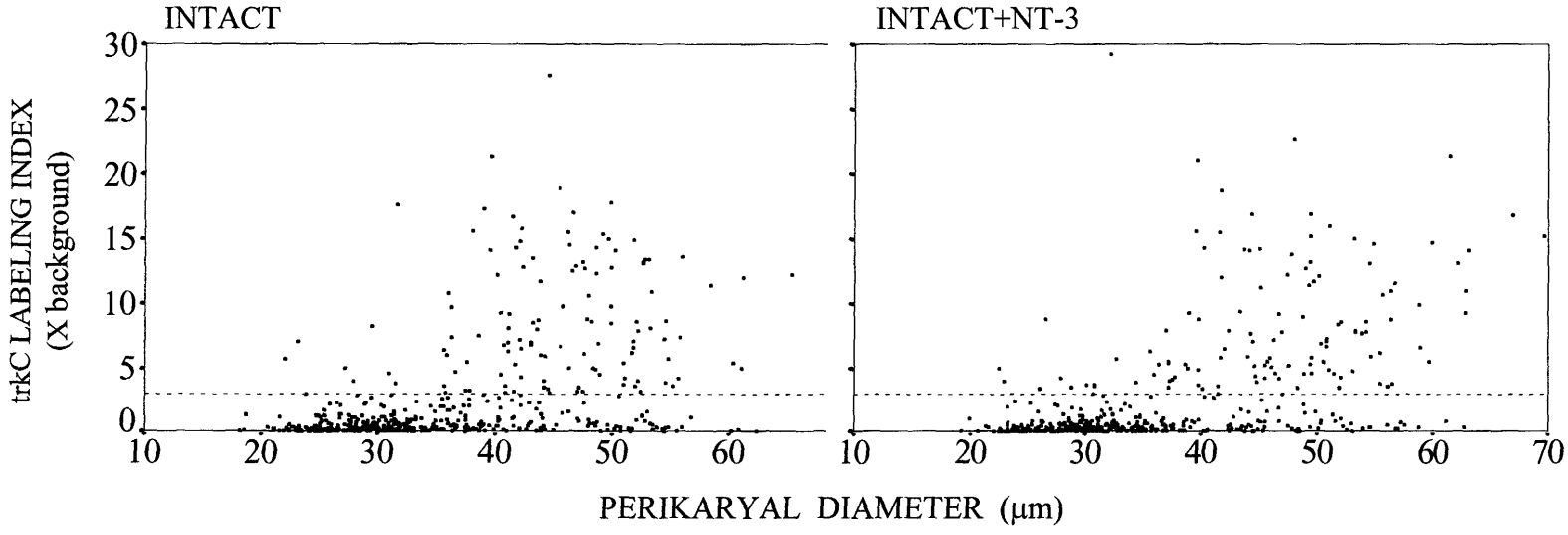

Figure 6.2 Relationship between perikaryal diameter and the relative level of trkC mRNA under normal conditions and with intrathecal infusion of NT-3.

Scatterplots of labeling indices of 492-495 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC mRNA, depict the relationship between perikaryal diameter (x axis) and trkC labeling indices (y axis). Panels show a comparison of data under normal conditions (intact) and intact with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of trkC mRNA. For each plot, neuronal profiles situated above the dashed horizontal line are positive for trkC mRNA.

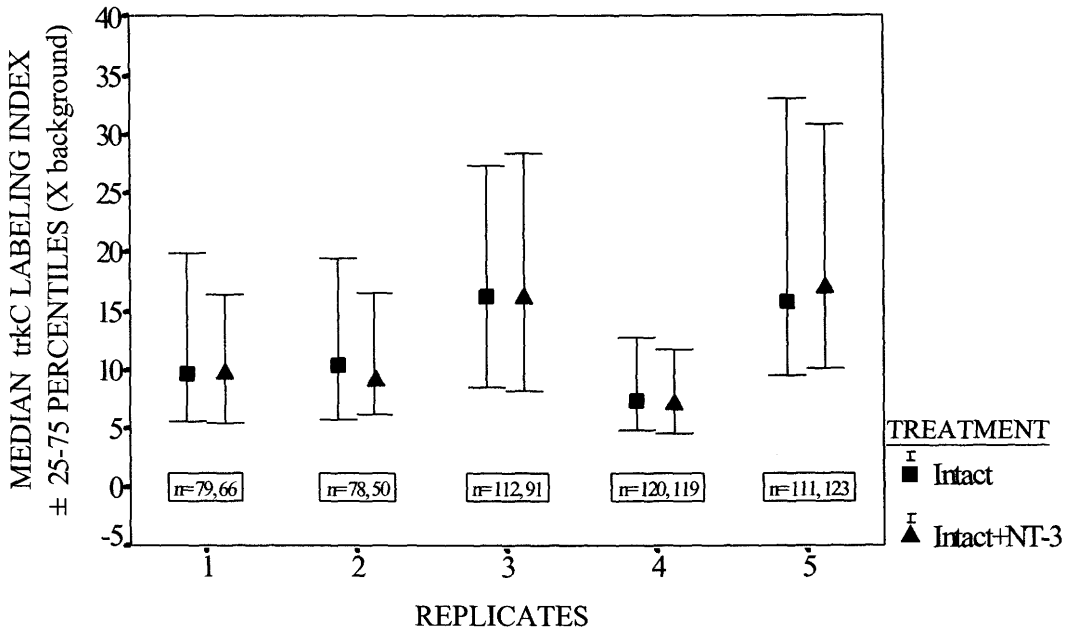

Figure 6.3 The effect of intrathecal infusion of NT-3 on trkC mRNA levels.

Graph depicts the relative differences in trkC mRNA labeling indices between normal conditions (intact) and normal with a 7 day intrathecal infusion of NT-3 (intact + NT-3) in the trkC-positive subpopulation. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, trkC labeling indices for individual neurons were determined from image analysis of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of trkC mRNA. Asterisk indicates significant difference between intact and intact plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-5 (intact, intact + NT-3) are as follows: 228, 226; 200, 168; 262, 240; 492, 495; 252, 226.

all cases, there is no statistically significant difference in trkC labeling indices between normal and NT-3 infused tissues (Mann-Whitney U Test). Data from the five replicates represent analyses of tissues from four different animals; replicates one and two represent analyses of tissue sections from the same animal.

6.2.3 The Effect of NT-3 Infusion on the Phenotype of trkA-Positive Neurons

6.2.3.1 The Effect of Intrathecal Infusion of NT-3 on trkA mRNA Levels

The mRNA for trkC and trkA are abundantly and heterogenously expressed in DRG neurons, and although the trkC-positive subpopulation is predominately medium and large in size and trkA message primarily localized to small and medium diameter neurons, there is a subset of cells in which trkC and trkA mRNA are coexpressed (Figure 6.4). NT-3 infusion does not appear to affect trkC message levels, but seems to result in diminished levels of expression for trkA mRNA, in at least a subpopulation of neurons.

Scatterplots (e.g. Figure 6.5), depicting the relationship between perikaryal diameter and trkA labeling indices of individual neurons (10 DRG sections/ treatment, average 250 neurons/section) under normal conditions and following NT-3 infusion, demonstrate in graph form the reduction in trkA mRNA levels effected by exogenous NT-3. This decrease appears to be most conspicuous in those neurons with higher trkA message levels and does not seem to be restricted to any specific perikaryal size range. Further, NT-3, at least at the concentration used here, does not appear to alter the number of trkA-positive cells compared to the intact state (~ 40% of the total population).

Scatterplots (e.g. Figure 6.6), comparing trkC and trkA mRNA labeling indices for individual neurons (6 DRG sections/treatment/probe, average 264 neurons/section) under normal conditions and following intrathecal infusion of NT-3, show that the NT-3 induced reduction in trkA mRNA expression is most obvious in those cells with higher trkA transcript levels, the trkA-positive/trkC-negative subset.

For the trkA-positive subpopulation (Figure 6.7), the majority of replicates (five of six) show that exogenous NT-3 appears to influence a reduction in median trkA labeling index values. The NT-3 induced diminution of trkA message levels is evident in

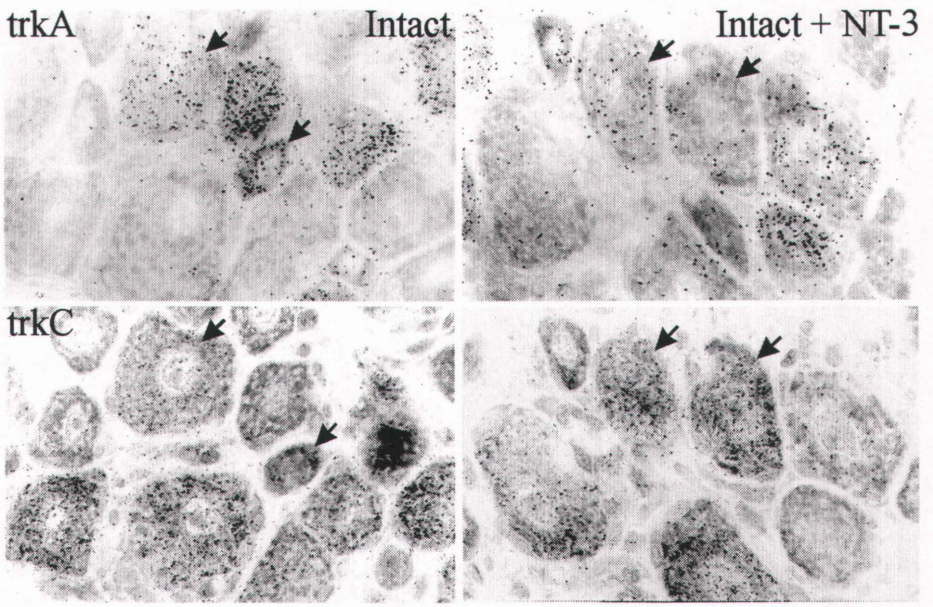

Figure 6.4 Colocalization of trkA and trkC mRNA under normal conditions and following intrathecal infusion of NT-3.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect trkA (upper panel) or trkC (lower panel) mRNA, under normal conditions (intact) and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3 (intact + NT-3). Arrows indicate neurons expressing both markers. Scale bar = 30 μm.

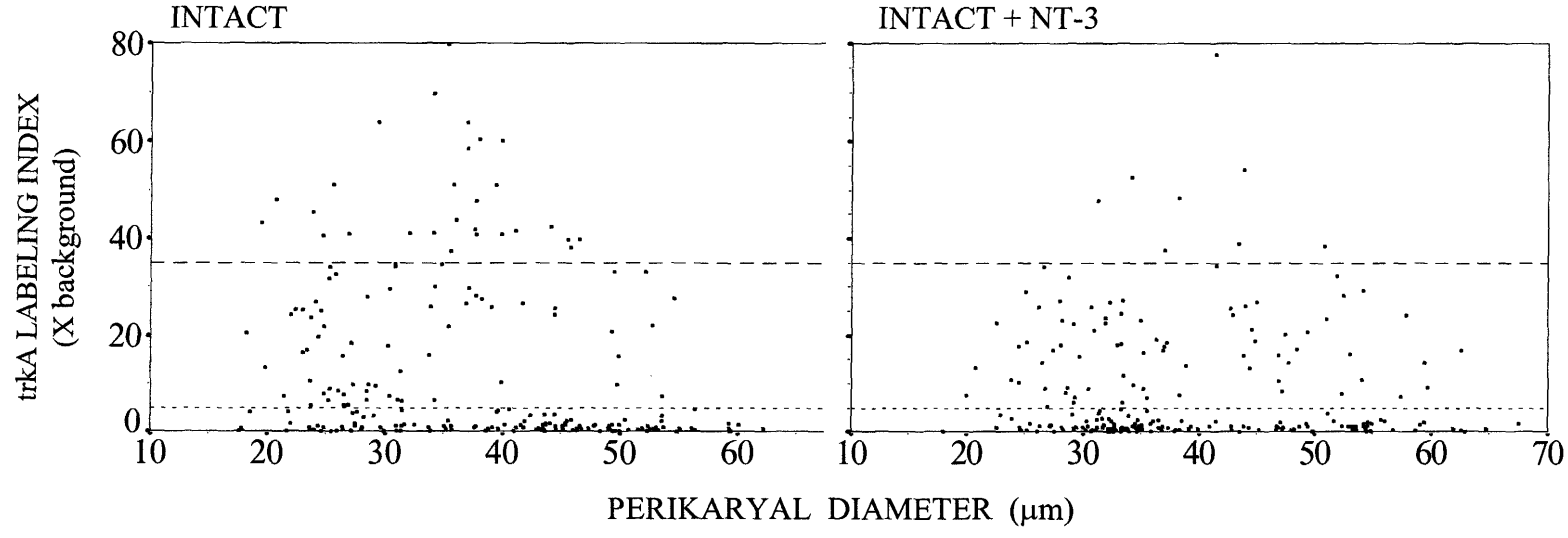

Figure 6.5 Relationship between perikaryal diameter and the relative level of trkA mRNA under the following conditions: normal and normal with intrathecal infusion of NT-3.

Scatterplots of labeling indices of 210-224 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA mRNA, depict the relationship between perikaryal diameter (x axis) and trkA labeling indices (y axis). Panels show a comparison of data under normal conditions (intact) and intact with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of trkA mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for trkA mRNA. The long-dashed lines serve as references to facilitate data interpretation.

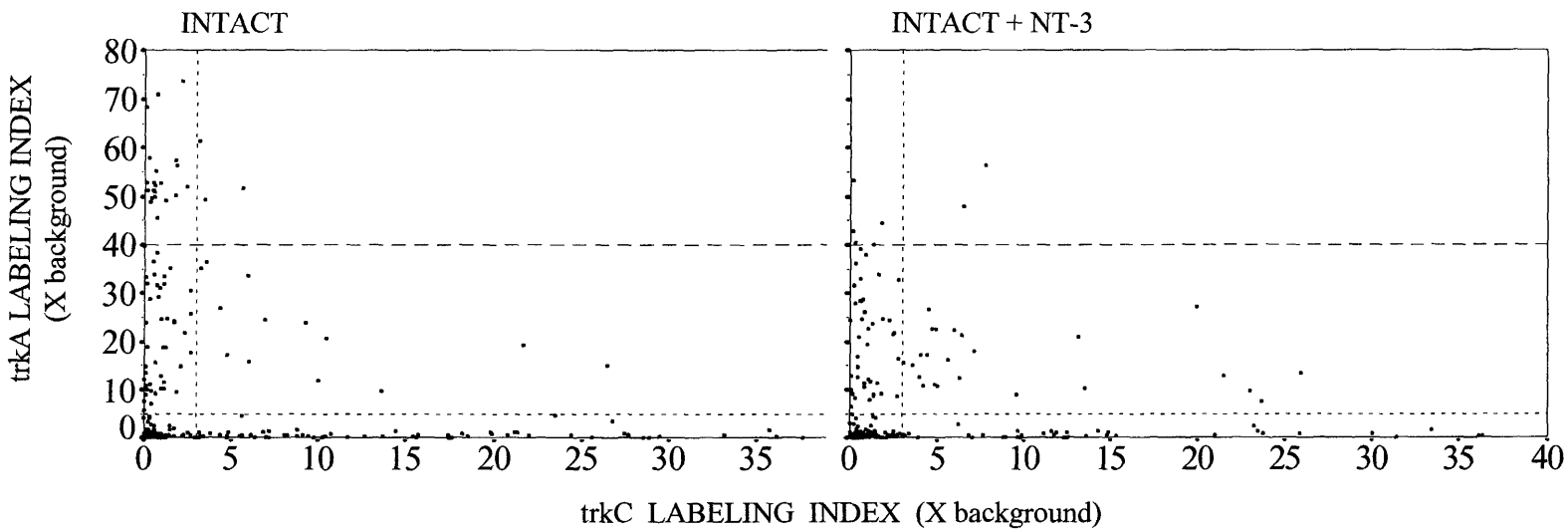

Figure 6.6 Relationship between relative levels of trkC and trkA mRNA under normal conditions and with intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkC and trkA labeling indices for 226-228 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or trkA (y axis) mRNA under normal conditions (intact) and normal with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles situated to the right of the short-dashed vertical line, and above the short-dashed horizontal line, are considered positive for trkC and trkA mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

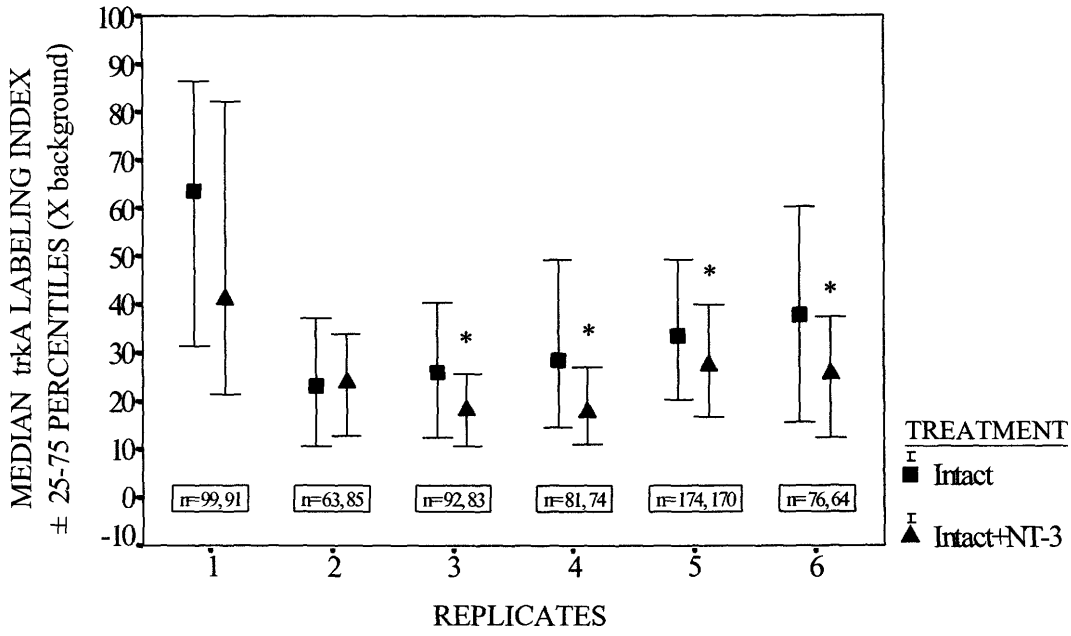

Figure 6.7 The effect of intrathecal infusion of NT-3 on trkA mRNA levels in the subpopulation of neurons expressing trkA.

Graph depicts the relative differences in trkA mRNA labeling indices between normal conditions (intact) and normal with a 7 day intrathecal infusion of NT-3 (intact + NT-3) in the subpopulation of neurons expressing trkA mRNA. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, trkA labeling indices for individual neurons were determined from image analysis of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of trkA mRNA. Asterisk indicates significant difference between intact and intact plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-6 (intact, intact + NT-3) are as follows: 252, 226; 209, 245; 210, 224; 228, 226; 492, 495; 200, 168.

all replicates for the 75th percentile values; the 25th percentile values also decrease (in five of six replicates), but these reductions are generally less pronounced. In four of six cases, the difference in trkA labeling indices between normal and NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.05$). Data from the six replicates represent analyses of tissues from four different animals; replicates three, four, and six represent analyses of tissue sections from the same animal.

6.2.3.2 The Effect of Intrathecal Infusion of NT-3 on the Density of NGF Binding

Analysis of radioautographs processed to detect trkA mRNA and ^{125}I-NGF binding sites, under normal conditions and following a 7 day intrathecal infusion of NT-3 (Figure 6.8), show that neurons with high levels of NGF binding also express trkA mRNA and that exogenous NT-3 results in decreased levels of both markers.

Scatterplots (e.g. Figure 6.9), constructed from data obtained through quantitative analysis of labeling indices of individual neurons identified in DRG sections (3 DRG sections/treatment, average 240 neurons/section) and processed for ^{125}I-NGF binding, depict the relationship between perikaryal diameter and ^{125}I-NGF binding labeling indices. These graphs show that under normal conditions small and medium diameter cells exhibit the highest levels of ^{125}I-NGF binding. Infusion of NT-3 results in decreased ^{125}I-NGF binding labeling index values and this phenomenon does not appear to be limited to any specific neuronal size class.

To distinguish between specific and nonspecific binding, 2560 pM unlabeled NGF was added to the working solution of 40 pM ^{125}I-NGF (Figure 6.10). Image analysis data (2 DRG sections/treatment, average 231 neurons/section) demonstrate that excess unlabeled NGF successfully competes for NGF binding sites, indicating that labeling index values above the "cut off" point recorded following 40 pM ^{125}I-NGF binding experiments represent specific binding of the ligand to NGF receptors.

Scatterplots (e.g. Figure 6.11), depicting the relationship between trkA mRNA and ^{125}I-NGF binding labeling indices for individual neurons (3 DRG

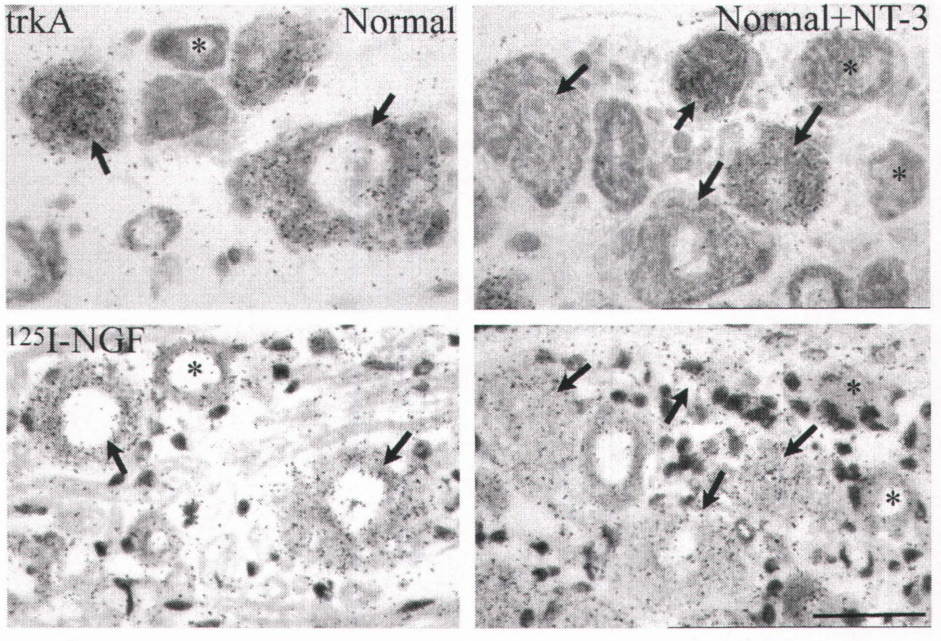

Figure 6.8 Colocalization of trkA mRNA and high-affinity NGF binding sites under normal conditions and following intrathecal infusion of NT-3.

Brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect trkA mRNA (upper panel) and for ^{125}I-NGF binding radioautography (lower panel) under normal conditions and following a 7 day intrathecal infusion of NT-3 (600 ng/μl/h). Arrows indicate neurons expressing trkA that also display high-affinity NGF binding sites, albeit at much lower levels with infusion. Asterisks depict examples of neurons with no discernible trkA mRNA or binding above background levels. Scale bar = 40 μm.

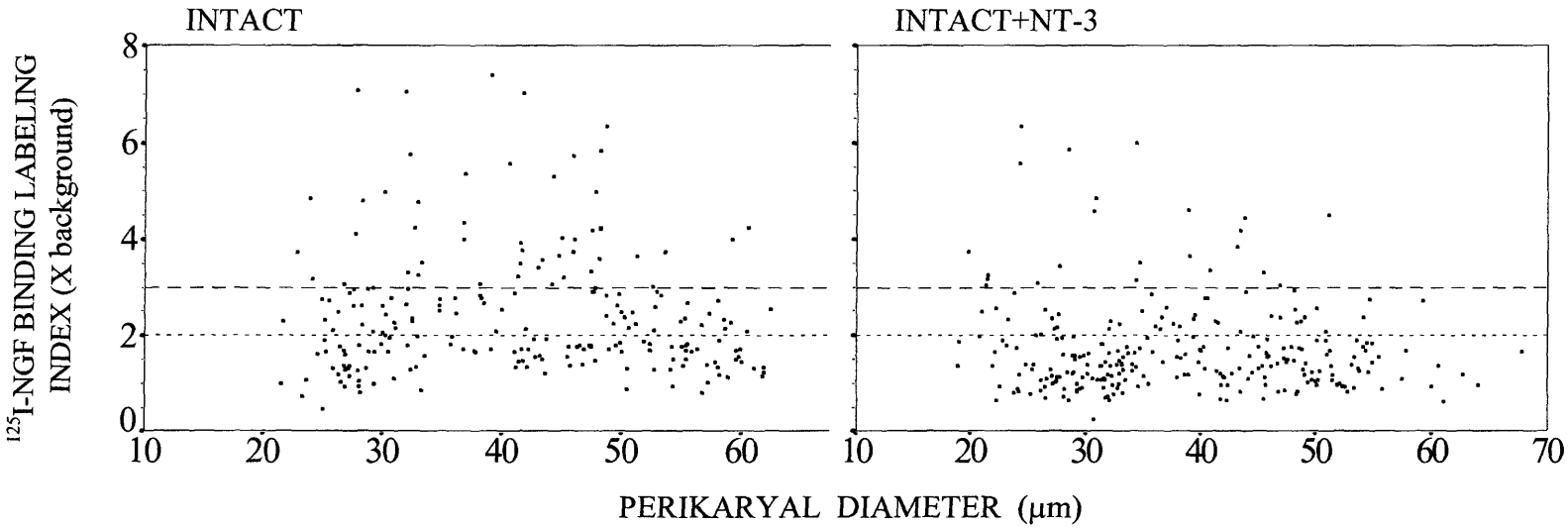

Figure 6.9 Relationship between perikaryal diameter and the relative level of ^{125}I-NGF binding under normal conditions and with intrathecal infusion of NT-3.

Scatterplots of labeling indices of 223-263 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for radioautography to detect ^{125}I-NGF binding depict the relationship between perikaryal diameter (x axis) and ^{125}I-NGF binding labeling indices (y axis). Panels show a comparison of data under normal conditions (intact) and intact with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and is linearly related to the density of high-affinity NGF-binding sites. For each plot, neuronal profiles situated above the short-dashed horizontal line are considered positive for ^{125}I-NGF binding. The long-dashed lines serve as references to facilitate data interpretation.

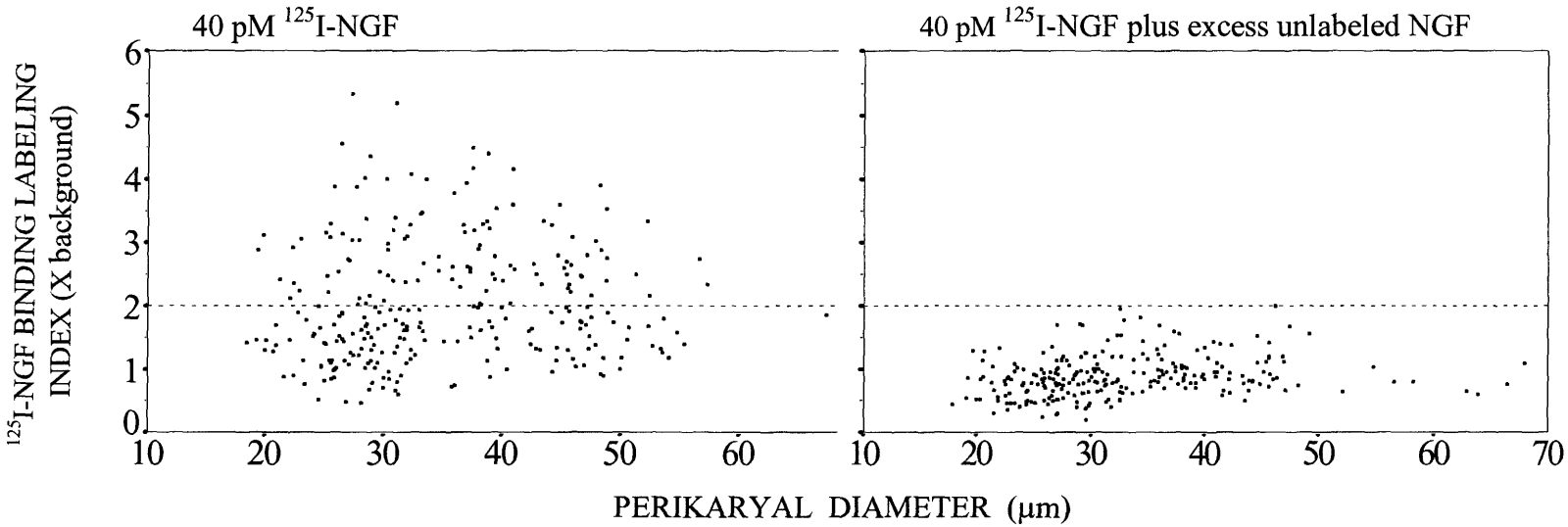

Figure 6.10 Comparison between relative level of ^{125}I-NGF binding in the absence of, and in the presence of, excess unlabeled NGF.

Scatterplots of labeling indices of 250-274 individual neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for radioautography to detect ^{125}I-NGF binding in the absence of, and in the presence of, excess unlabeled NGF. Panels show a comparison of perikaryal diameter (x axis) and labeling index for ^{125}I-NGF (y axis) under normal binding conditions (40 pM ^{125}I-NGF) and 40 pM ^{125}I-NGF with excess, unlabeled NGF (2560 pM). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and is linearly related to the density of high-affinity NGF-binding sites. For each plot, neuronal profiles situated above the dashed horizontal line are positive for ^{125}I-NGF binding.

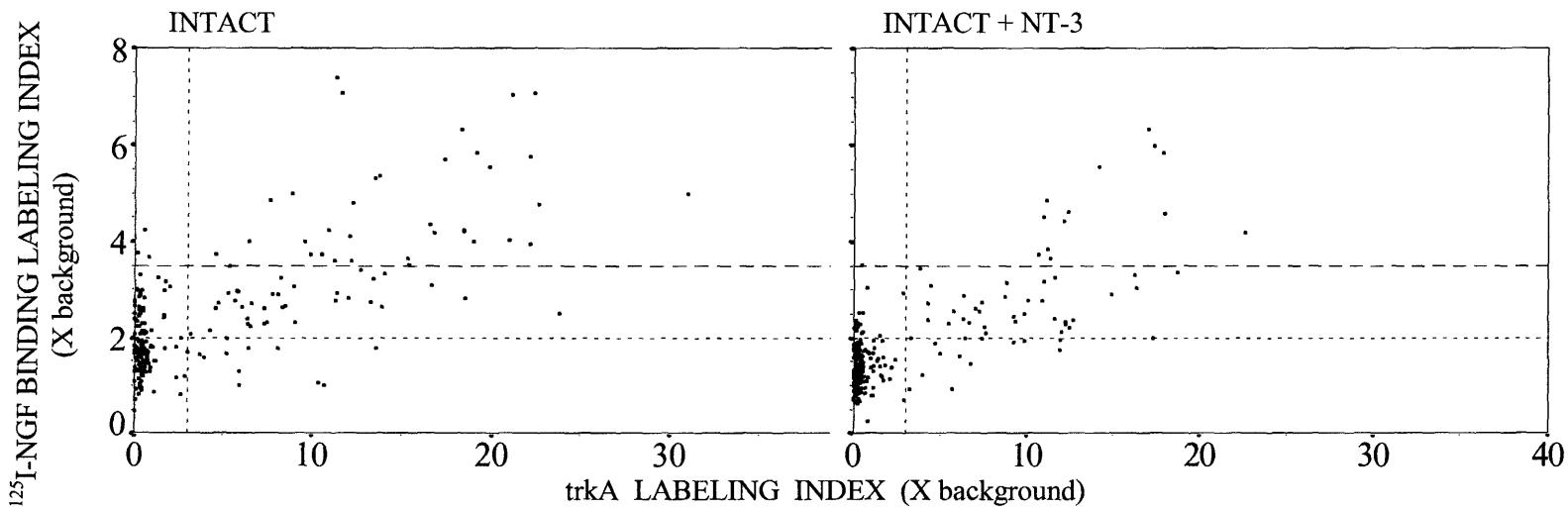

Figure 6.11 Relationship between relative levels of trkA mRNA and ^{125}I-NGF binding under normal conditions and with intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkA and ^{125}I-NGF binding labeling indices for 223-263 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA mRNA (x axis) or ^{125}I-NGF binding (y axis), under normal conditions (intact) and intact with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background), and is linearly related to the density of high-affinity NGF-binding sites or reflects the relative level of trkA mRNA. For each plot, neuronal profiles situated to the right of the short-dashed vertical line, and above the short-dashed horizontal line, are positive for trkA mRNA and ^{125}I-NGF binding, respectively. The long-dashed lines serve as references to facilitate data interpretation.

sections/treatment/marker, average 240 neurons/section), suggest a positive correlation between ^{125}I-NGF binding and trkA mRNA expression. Treatment with NT-3 appears to effect a reduction in both the density of NGF binding sites and trkA message levels.

Figure 6.12 highlights the trend in changes in neuronal ^{125}I-NGF binding labeling indices following NT-3 infusion for three replicate data sets. Although the NT-3 induced decrease in the density of NGF binding sites is evident in all of the replicates, it is most conspicuous when comparing the 75th percentile values between the two experimental conditions. In two of the three cases, the difference in ^{125}I-NGF binding labeling indices between normal and NT-3 infused tissues is statistically significant (Mann-Whitney U Test, $p<0.05$). In addition, it is important to note that there is a poor signal to noise ratio in the aberrant data set. Data from the three replicates represent analyses of tissues from two different animals; replicates one and two represent analyses of tissue sections from the same animal.

6.2.3.3 The Effect of Intrathecal Infusion of NT-3 on SP Expression

Under normal conditions, SP mRNA is detectable in a subpopulation of small- and medium-sized DRG neurons (Figure 6.13). These cells also typically exhibit hybridization signal for trkA, but not trkC. A 7 day intrathecal infusion of NT-3 appears to result in diminished SP message levels.

Scatterplots (e.g. Figure 6.14), illustrating the relationship between perikaryal diameter and SP labeling indices for individual neurons (3 DRG sections/treatment/probe, average 331 neurons/section) under normal conditions and following NT-3 infusion, reflect the qualitative observations. In the intact state, SP mRNA is detectable in ~ 20% of DRG neurons. Although the neuropeptide is heterogenously expressed, the majority of SP-positive cells exhibit high labeling index values and are small in size. NT-3 infusion appears to result in the reduction of the level of SP hybridization signal, although levels of this neuropeptide are still conspicuously high and the proportion of neurons expressing detectable SP mRNA does not seem to be altered.

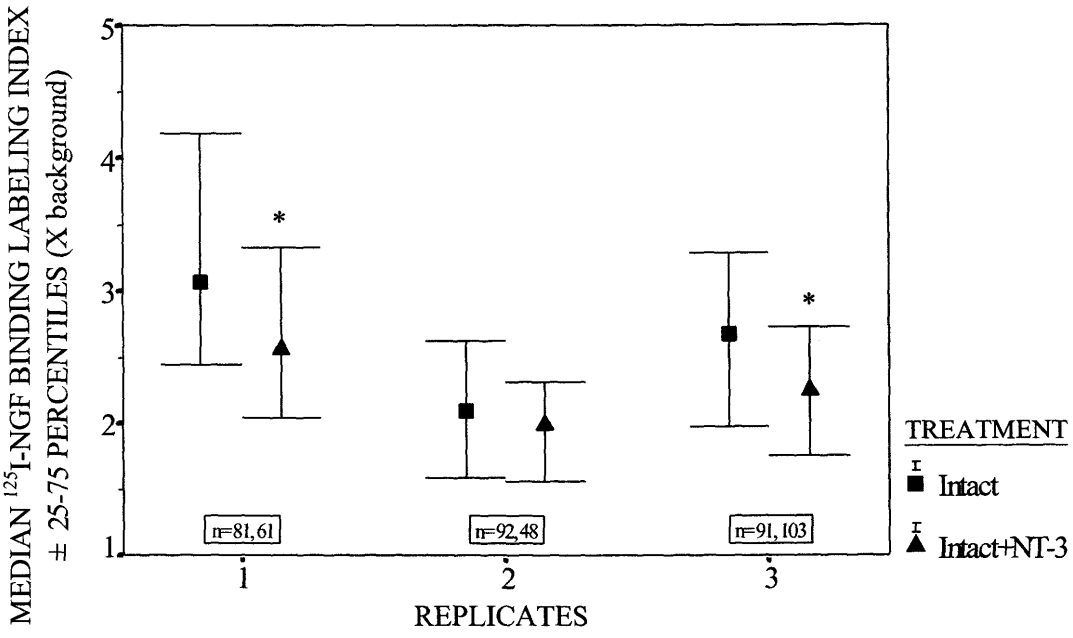

Figure 6.12 The effect of intrathecal infusion of NT-3 on ^{125}I-NGF binding levels in neurons expressing trkA mRNA.

Graph depicts the relative differences in ^{125}I-NGF binding labeling indices between normal conditions (intact) and normal with 7 day intrathecal infusion of NT-3 (intact + NT-3), for the trkA-positive subpopulation. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the three replicates, ^{125}I-NGF binding and trkA labeling indices for individual neurons were determined from image analysis of adjacent 6 μm thick sections of adult rat L_5 DRG processed for ^{125}I-NGF binding or *in situ* hybridization, and subsequent radioautography, to detect NGF binding sites or trkA mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and is linearly related to the density of high-affinity NGF-binding sites. Asterisk indicates significant difference between intact and intact plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-3 (intact, intact + NT-3) are as follows: 223, 263; 214, 200; 274, 268.

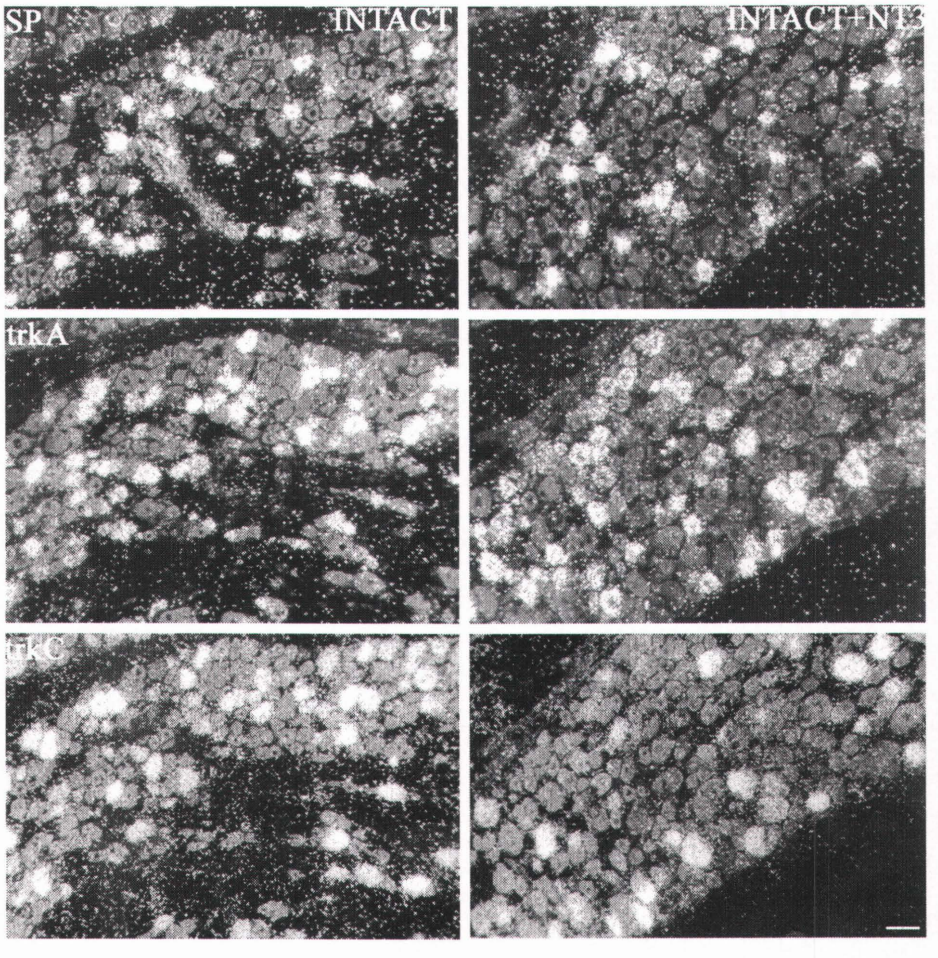

Figure 6.13 Detection of SP, trkA, and trkC mRNA under normal conditions and following intrathecal infusion of NT-3 in serial sections.

Scanned stained darkfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect SP (upper panel), trkA (center panel), and trkC (lower panel) mRNA under normal conditions (intact) and following a 7 day intrathecal infusion of NT-3 (600 ng/μl/h). Scale bar = 60 μm.

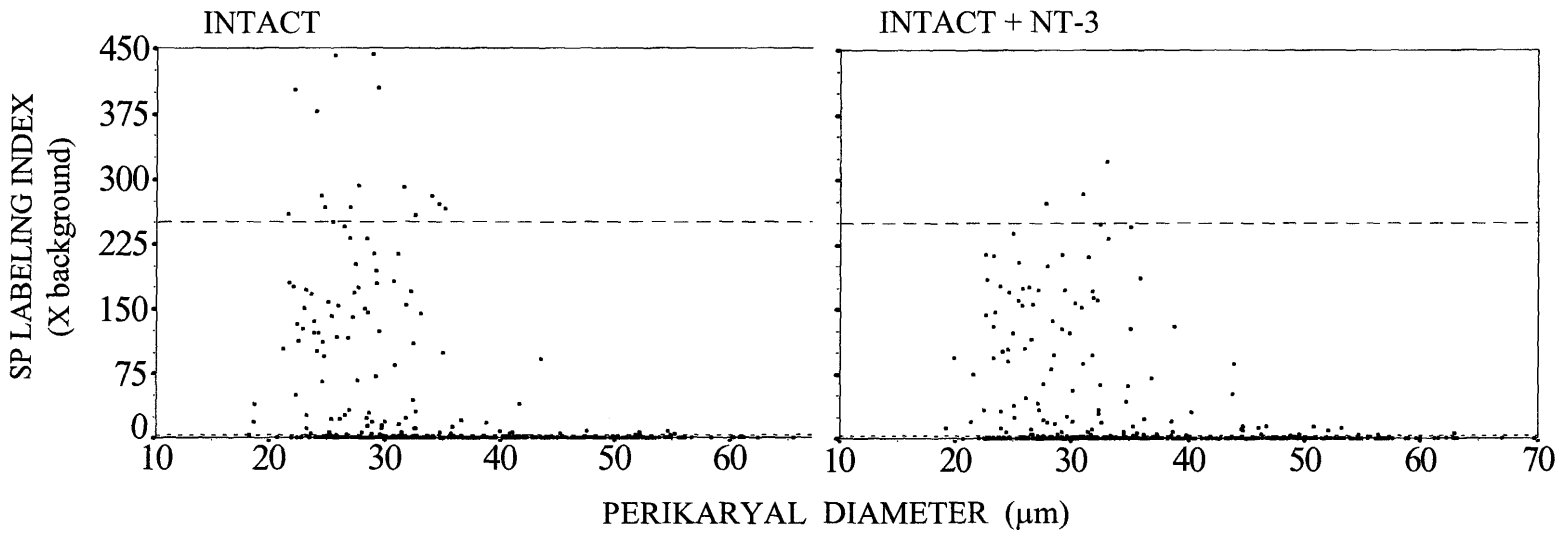

Figure 6.14 Relationship between perikaryal diameter and the relative level of SP mRNA under normal conditions and with intrathecal infusion of NT-3.

Scatterplots of labeling indices of 492-495 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect SP mRNA, depict the relationship between perikaryal diameter (x axis) and SP labeling indices (y axis). Panels show a comparison of data under normal conditions (intact) and intact with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for SP mRNA. The long-dashed lines serve as references to facilitate data interpretation.

Scatterplots (e.g. Figure 6.15), illustrating the relationship between trkC and SP mRNA labeling indices of individual neurons (3 DRG sections/treatment/probe, average 331 neurons/section), indicate that under normal conditions there is a small subpopulation of trkC-positive neurons that coexpress low levels of hybridization signal for both trkC and SP. The majority of SP-expressing cells do not exhibit detectable levels of trkC mRNA, and while these neurons display a range of SP labeling indices, this group includes the subpopulation with the highest levels of SP transcripts. Infusion of NT-3 effects a reduction in SP message, which is most evident in cells that do not also express trkC mRNA.

Scatterplots (e.g. Figure 6.16), delineating the relationship between trkA and SP mRNA labeling indices of individual neurons (2 DRG sections/treatment/probe, average 360 neurons/section) under normal conditions and following infusion of NT-3, show that the majority of SP-expressing neurons also exhibit trkA mRNA. This SP/trkA-positive subpopulation represents neurons with heterogeneous message levels for both markers, although cells with the highest levels of hybridization signal for SP tend to possess moderate levels of trkA signal. SP-positive neurons that do not coexpress detectable trkA mRNA display low to moderate levels of hybridization signal for the neuropeptide. The NT-3 induced lessening of SP mRNA expression appears to be limited to the trkA-positive subpopulation.

Figure 6.17 highlights the trend in changes in SP mRNA expression following NT-3 infusion for three replicate data sets. Although the difference in SP labeling indices between normal and NT-3 infused tissues is statistically significant in only one of the three cases (Mann-Whitney U Test, $p<0.05$), the reduction in SP message levels is clearly evident when comparing the median and 75th percentile values between the two treatments for all of the replicates. Data from the three replicates represent analyses of tissues from two different animals; replicates one and three represent analyses of tissue sections from the same animal.

6.2.4 The Effect of NT-3 Infusion on SNAP-25 Expression

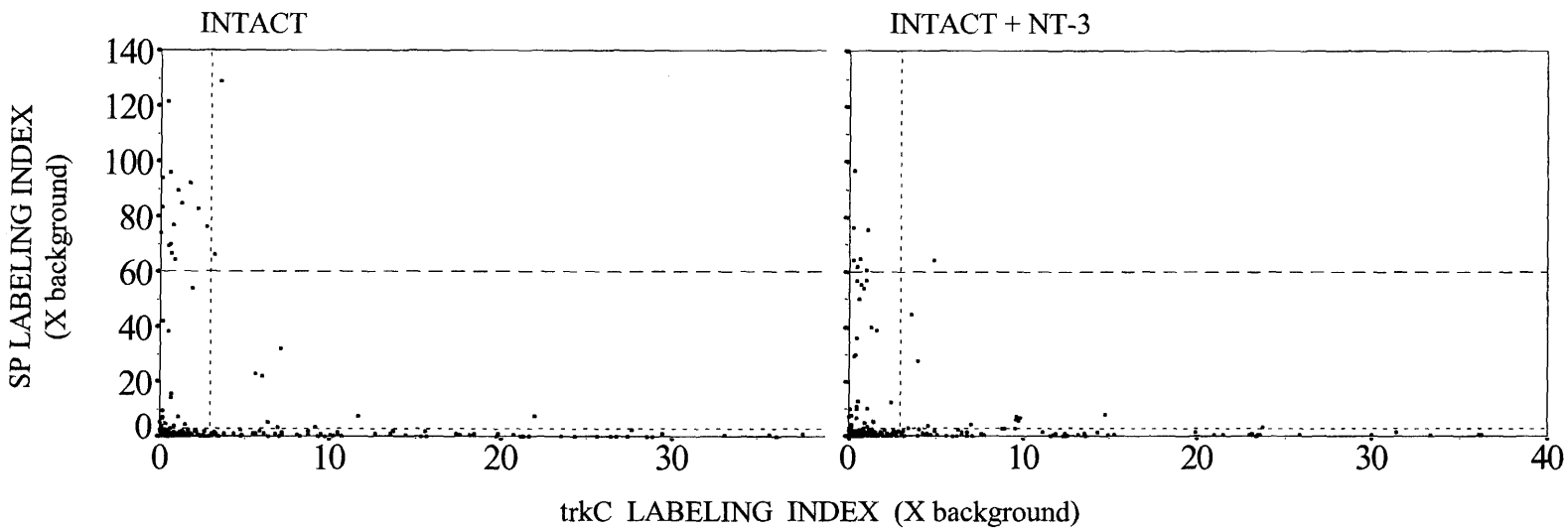

Figure 6.15 Relationship between relative levels of trkC and SP mRNA under normal conditions and normal with intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkC and SP labeling indices for 226-228 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkC (x axis) or SP (y axis) mRNA under normal conditions (intact) and normal with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles situated to the right of the short-dashed vertical line, and above the short-dashed horizontal line, are considered positive for trkC and SP mRNA, respectively. The long-dashed lines serve as references to facilitate data interpretation.

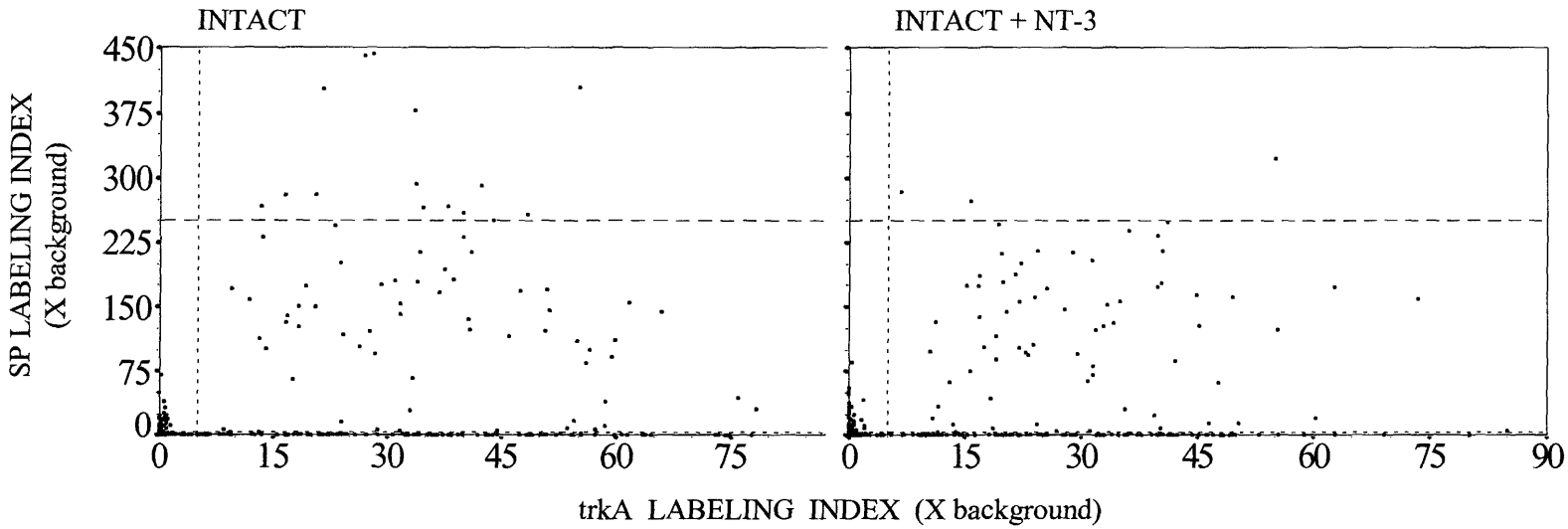

Figure 6.16 Relationship between relative levels of trkA and SP mRNA under normal conditions and normal with intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkA and SP labeling indices for 492-495 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA (x axis) or SP (y axis) mRNA under normal conditions (intact) and normal with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles situated to the right of the short-dashed vertical line, and above the short-dashed horizontal line, are said to be positively labeled for trkA mRNA and SP respectively. The long-dashed lines serve as references to facilitate data interpretation.

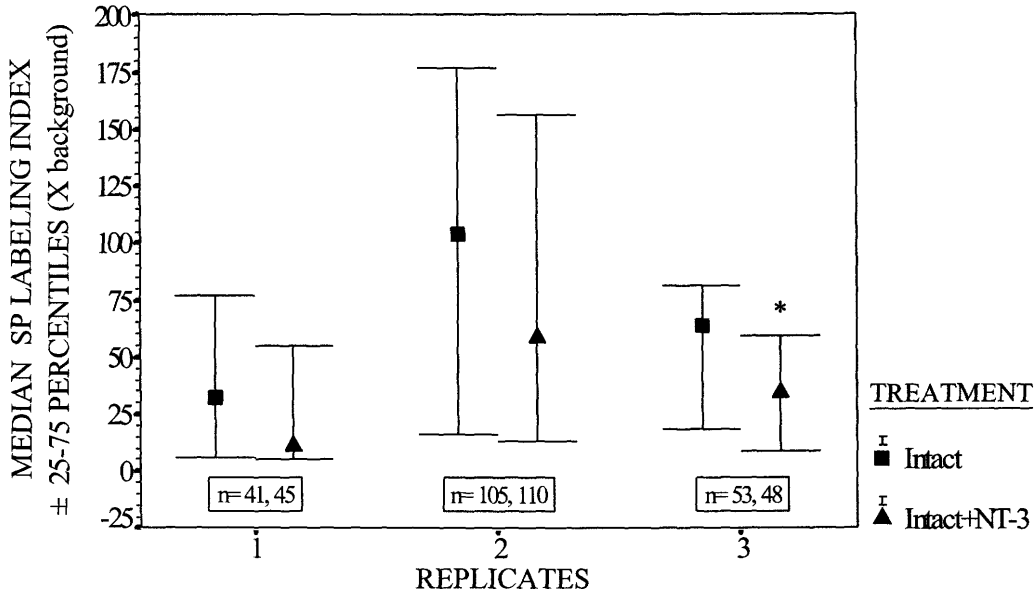

Figure 6.17 The effect of intrathecal infusion of NT-3 on SP mRNA levels.

Graph depicts the relative differences in SP mRNA labeling indices between normal conditions (intact) and normal with a 7 day intrathecal infusion of NT-3 (intact + NT-3) in the subpopulation of DRG neurons expressing SP. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, SP labeling indices for individual neurons were determined from image analysis of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect SP mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of SP mRNA. Asterisk indicates significant difference between intact and intact plus NT-3 infused treatments (Mann-Whitney U Test, $P<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1-3 (intact, intact + NT-3) are as follows: 228, 226; 492, 495; 283, 262.

In the intact state, SNAP-25 mRNA is detectable in most DRG neurons, and therefore, colocalizes to both trkC- and trkA-positive cells (Figure 6.18). Those neurons with the highest levels of SNAP-25 hybridization signal appear to also express trkC, but not trkA, mRNA. A 1 week intrathecal infusion of NT-3 seems to result in a reduction in SNAP-25 hybridization signal in those neurons with the highest levels of the marker.

Scatterplots (e.g. Figure 6.19), illustrating the relationship between perikaryal diameter and SNAP-25 labeling indices for individual neurons (2 DRG sections/treatment/probe, average 200 neurons/section) under normal conditions and following NT-3 infusion underscore qualitative observations, demonstrating the heterogeneous expression of SNAP-25 transcripts and the presence of this marker in the majority of cells. In the intact state, smaller neurons tend to possess lower levels of SNAP-25 mRNA; medium and large diameter perikarya express the highest levels, although some neurons in this size range display more moderate levels of transcripts. A 1 week intrathecal infusion of NT-3 effects a dramatic reduction in SNAP-25 labeling index values. This change is most conspicuous in medium and large diameter neurons, where SNAP-25 labeling indices are now low to moderate in the majority of these cells.

Scatterplots (e.g. Figure 6.20), illustrating the relationship between trkA and SNAP-25 mRNA labeling indices of individual neurons (2 DRG sections/treatment/probe, average 200 neurons/section) under normal conditions and following intrathecal infusion of NT-3, show that cells expressing trkA are among the subset of SNAP-25 positive neurons with low to moderate SNAP-25 labeling indices. Further, in the intact state, virtually all of the neurons with the highest levels of SNAP-25 transcripts do not appear to coexpress trkA mRNA, although there is also a large subset of cells that exhibit low to moderate SNAP-25 labeling indices that lack detectable message for the neurotrophin receptor. Infusion of NT-3 effects a readily observable decrease in SNAP-25 mRNA levels which is most apparent in neurons with the highest message levels of SNAP-25 — the subgroup of cells with trkA labeling index values below background levels.

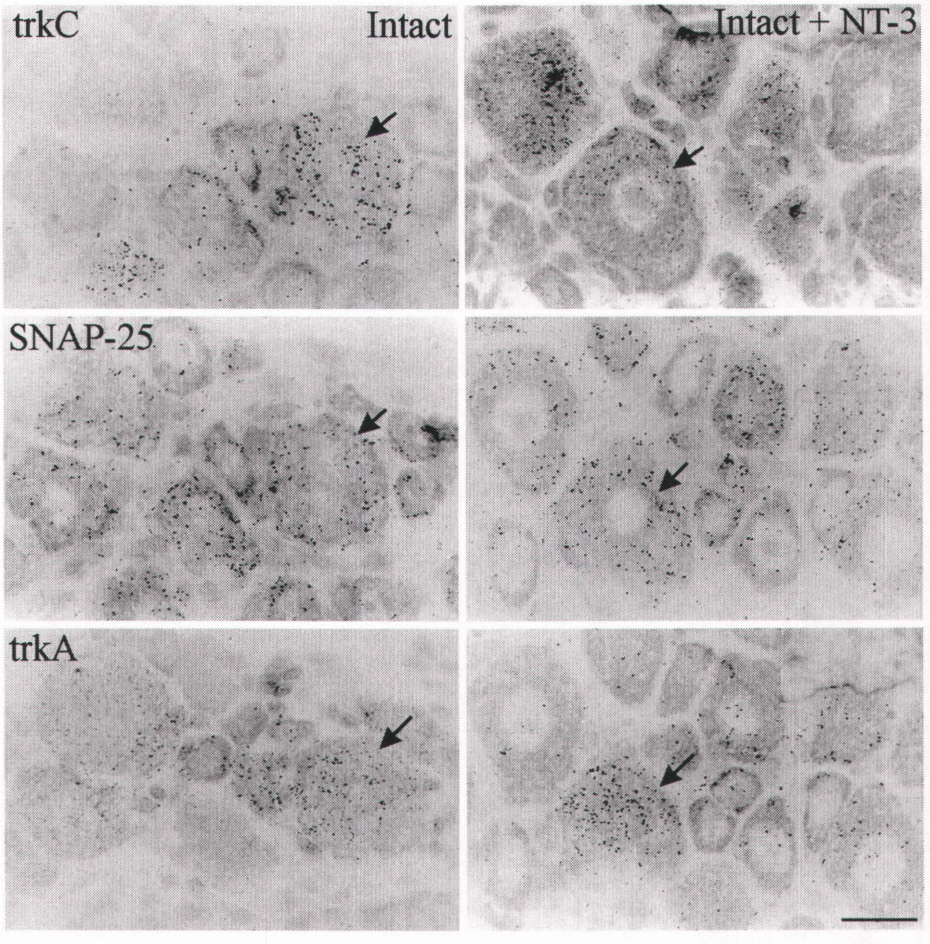

Figure 6.18 Trilocalization of trkC, SNAP-25, and trkA mRNA under normal conditions and following intrathecal infusion of NT-3.

Scanned brightfield photomicrographs of serial 6 μm thick adult rat L_5 DRG sections processed for *in situ* hybridization to detect trkC (upper panel), SNAP-25 (center panel), or trkA (lower panel) mRNA, under normal conditions (intact) and following a 7 day 600 ng/μl/h intrathecal infusion of NT-3 (intact + NT-3). Arrows indicate examples of neurons expressing all three markers. Scale bar = 30 μm.

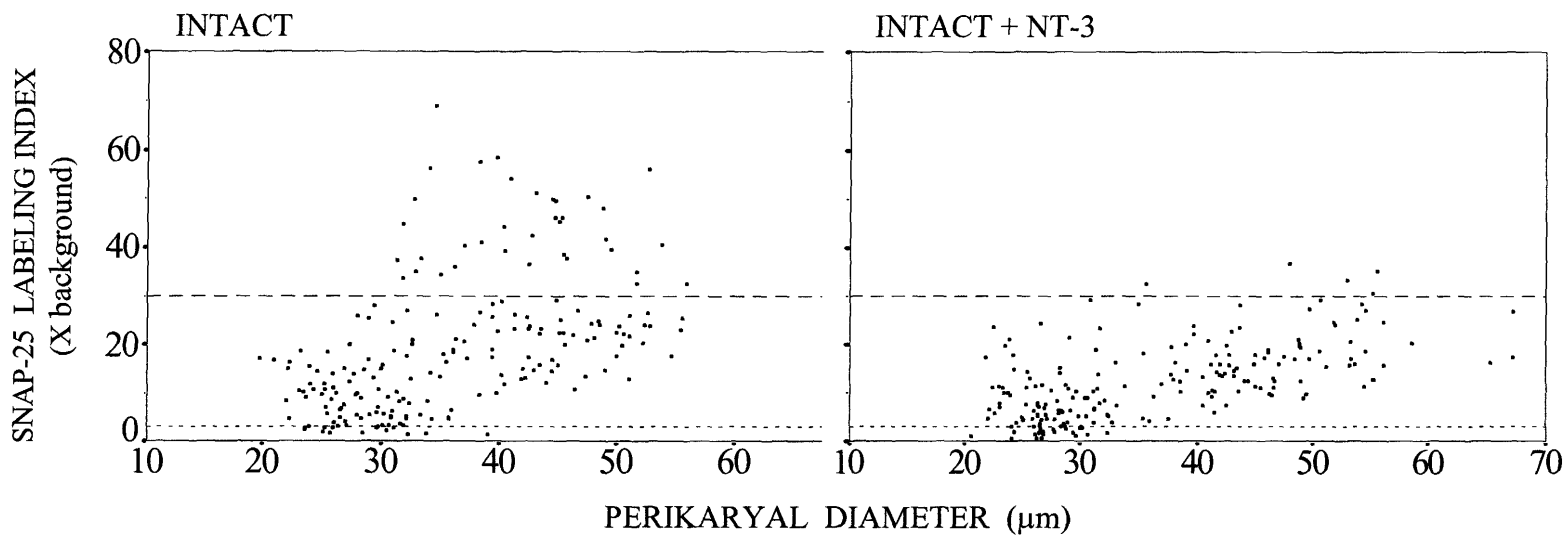

Figure 6.19 Relationship between perikaryal diameter and the relative level of SNAP-25 mRNA under normal conditions and with intrathecal infusion of NT-3.

Scatterplots of labeling indices of 199-200 individual neurons identified in 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect SNAP-25 mRNA, depict the relationship between perikaryal diameter (x axis) and SNAP-25 labeling indices (y axis). Panels show a comparison of data under normal conditions (intact) and intact with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of SNAP-25 mRNA. For each plot, neuronal profiles above the short-dashed horizontal line are considered positive for SNAP-25 mRNA. The long-dashed lines serve as references to facilitate data interpretation.

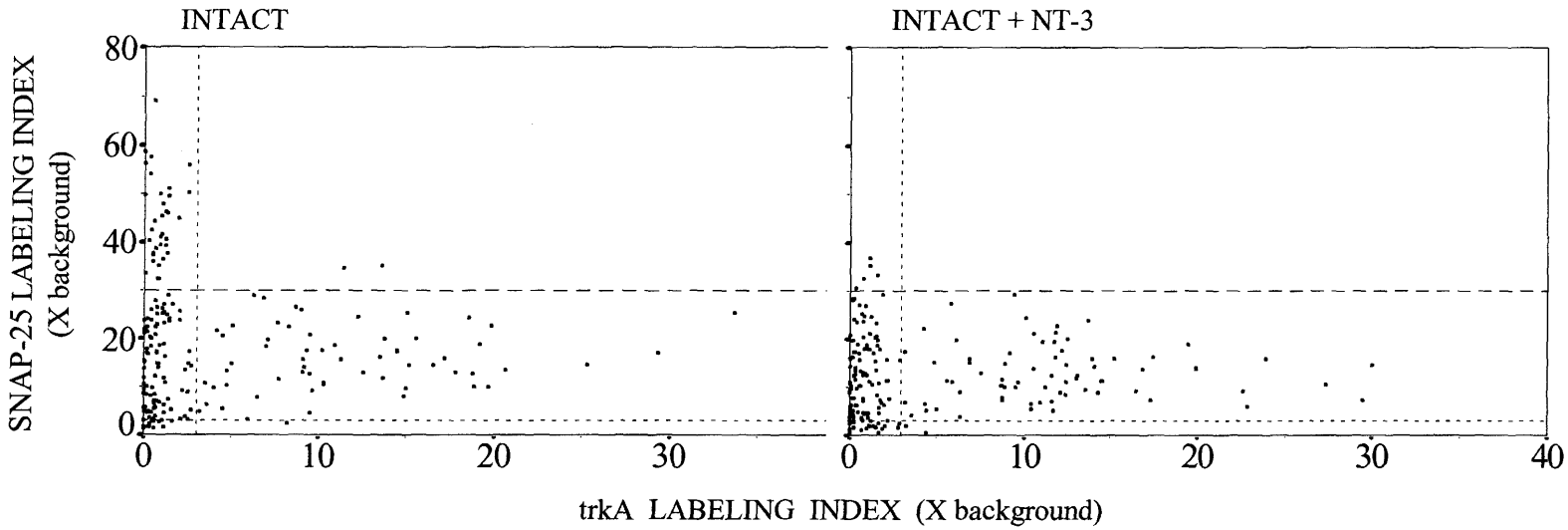

Figure 6.20 Relationship between relative levels of trkA mRNA and SNAP-25 under normal conditions and with intrathecal infusion of NT-3.

Scatterplots demonstrate the relationship between trkA and SNAP-25 labeling indices for 199-200 neurons identified in adjacent 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect trkA mRNA (x axis) or SNAP-25 (y axis) under normal conditions (intact) and intact with a 7 day intrathecal infusion of 600 ng/μl/h NT-3 (intact + NT-3). Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background) and reflects the relative level of indicated mRNA. For each plot, neuronal profiles situated to the right of the short-dashed vertical line and above the short-dashed horizontal line are considered positive for trkA mRNA and SNAP-25, respectively. The long-dashed lines serve as references to facilitate data interpretation.

Figure 6.21 highlights the trends in changes in SNAP-25 mRNA expression following NT-3 infusion for 2 replicate data sets. The difference in SNAP-25 labeling indices between normal and NT-3 infused tissues is statistically significant in both cases (Mann-Whitney U Test, $p<0.05$), with NT-3 effecting a reduction in SNAP-25 labeling indices that is most strongly reflected at the 75th percentile level. Data from the two replicates represent analyses of tissues from two different animals.

6.3 Discussion

This study demonstrates that in otherwise normal animals, two separate populations of mature DRG neurons are affected by NT-3 treatment, both in a seemingly negative manner. Although a 1 week intrathecal infusion of NT-3 does not alter trkC message levels, there is a downregulation of trkA mRNA levels, numbers of high-affinity NGF binding sites, and SP expression. Further, NT-3 effects a reduction in SNAP-25 message levels in those neurons with the highest levels of the marker.

As stated above, a 1 week infusion of NT-3 neither significantly modified trkC mRNA levels nor altered the number of neurons expressing the receptor. This is supported by reports that exogenous NT-3 did not upregulate trkC expression in cultured embryonic or postnatal sympathetic neurons (Belliveau *et al.*, 1997; Wyatt *et al.*, 1999), although one study noted a significant increase in trkC levels in cultured embryonic trigeminal ganglion neurons (Wyatt *et al.*, 1999). Moreover, my findings do not reflect the ability of NT-3 to counteract the injury-reduced levels of trkC expression (see Sections 5.2.2.1, 5.3.1), but rather parallel the influence of NGF on trkA levels in adult primary sensory neurons, where NGF infusion resulted in only a modest increase in receptor message levels in a population that normally expresses low levels of trkA transcripts (Verge *et al.*, 1990a; Tetzlaff *et al.*, 1992; Karchewski *et al.*, submitted). NT-3 treatment also appears to effect an overall increase in perikaryal diameter, although this was not rigorously investigated here. This observation corresponds to the increased fiber diameters observed in transgenic mice engineered to overexpress NT-3 (Albers *et al.*, 1996).

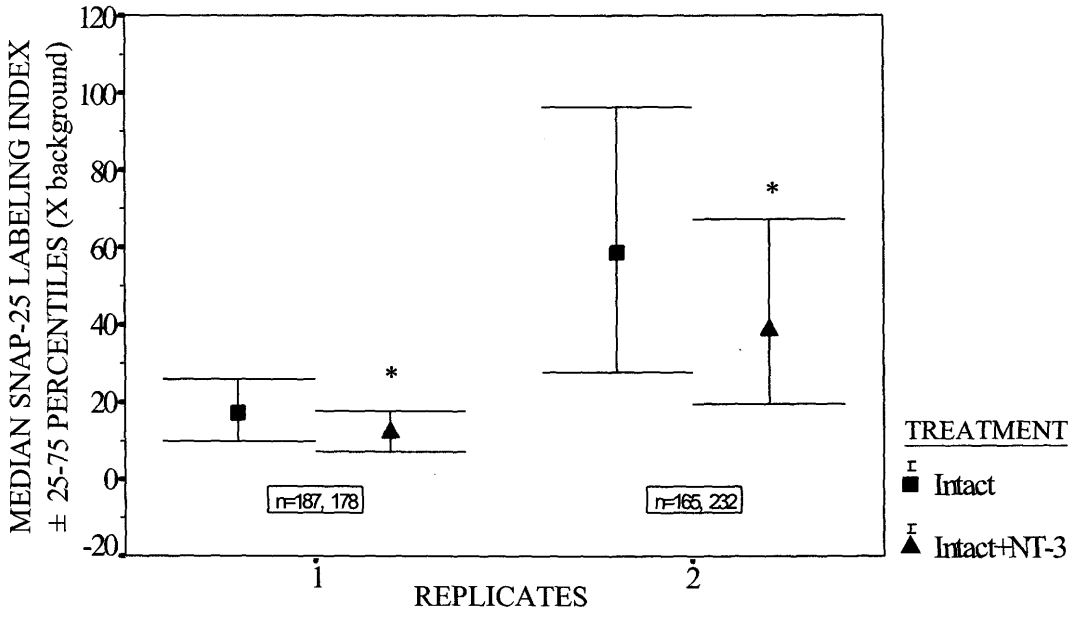

Figure 6.21 The effect of intrathecal infusion of NT-3 on SNAP-25 mRNA levels.

Graph depicts the relative differences in SNAP-25 mRNA labeling indices between normal conditions (intact) and normal with a 7 day intrathecal infusion of NT-3 (intact + NT-3) in the SNAP-25 positive subpopulation. Since the data are not normally distributed, median labeling indices are presented instead of means. The error bars represent the labeling indices of neurons falling between 25% and 75% of the median. In each of the replicates, SNAP-25 labeling indices for individual neurons were determined from image analysis of 6 μm thick sections of adult rat L_5 DRG processed for *in situ* hybridization, and subsequent radioautography, to detect SNAP-25 mRNA. Labeling index refers to the ratio of silver grain density over neuropil devoid of positive hybridization signal (background). Asterisk indicates significant difference between intact and intact plus NT-3 infused treatments (Mann-Whitney U Test, $p<0.05$). Sample sizes (number of neurons) for each treatment are indicated below each replicate. Total number of neurons identified and analyzed for replicates 1 and 2 (intact, intact + NT-3) are as follows: 200, 165; 178, 240.

In sharp contrast to the apparent lack of influence of NT-3 on trkC expression in normal animals, there are significant reductions in trkA message levels, numbers of high-affinity NGF binding sites, and in SP mRNA levels, although the percentages of neurons expressing detectable levels of trkA and SP transcripts are not altered following NT-3 treatment. Interestingly, it appears that treatment of otherwise normal animals with NT-3 appears to place the trkA-positive subpopulation in a NGF-deprived state. The phenomena mimics the injury response in the trkA-positive subpopulation, wherein levels of all three of these markers are depressed with the loss of target-derived NGF (Jessell *et al.*, 1979; Raivich and Kreutzberg, 1987; Verge *et al.*, 1989b, 1990a, 1992a, 1995; Anand *et al.*, 1991; Villar *et al.*, 1991; Noguchi *et al.*, 1994; Ji *et al.*, 1996; Ma and Bisby, 1998b). Further, the NT-3 effected alterations in the phenotype of the trkA-expressing neurons also parallel the effects of experimentally reduced access to endogenous NGF seen with *in vivo* injections of anti-NGF (Ramer *et al.*, 1998; Shadiak *et al.*, 2001). It would seem that NT-3 is acting in a fashion antagonistic to that of NGF in this subset of neurons (Verge *et al.*, 1989a, 1992a, 1995).

That the reduction in trkA mRNA levels effected by NT-3 results in a concomitant loss of high-affinity NGF binding sites is not surprising, because as seen here and reported in the past (Verge *et al.*, 1992a), there is a strong positive correlation between ^{125}I-NGF binding and trkA expression. In addition, the concurrent downregulation of SP expression is also not unexpected, given the reduction in trkA message levels, since the neuropeptide is a predominant marker of trkA-positive DRG neurons (Verge *et al.*, 1989a) (see Section 4.2.4.2) and its expression has been shown to be regulated in a positive manner by NGF (Lindsay *et al.*, 1989; Verge *et al.*, 1995; Ji *et al.*, 1996). It is also of interest to note that in experiments that exposed chicken chorioallantoic membrane to a continual supply of NT-3, the once predominantly unmyelinated nociceptive fibers attained physiological characteristics associated with low threshold mechanoreceptors. This suggests that excess NT-3 might not only alter the phenotype of trkA neurons, but change the physiology of this subset, such that developing nociceptors become more functionally similar to mechanoreceptors (Lewin, 1996).

Although trkA and trkC transcripts colocalize in some adult rat L_4/L_5 DRG neurons (see Sections 2.1.2, 4.2.2.2, 4.3.2), it does not appear that this is the subpopulation most notably influenced by the infusion of NT-3, since the reduction in trkA expression is most conspicuous in those neurons with the highest labeling indices for that receptor, the trkA-positive/trkC-negative subset (see Sections 4.2.2.2, 4.3.2). Similarly, although SP and trkC colocalize in some neurons in the intact state (see Section 4.2.4.2), the reduction in SP expression is evidenced in those cells with the highest levels of SP, the trkA-positive/trkC-negative subpopulation. These observations suggest that the influence of NT-3 in depressing aspects of the phenotype of trkA-positive neurons does not necessarily occur through the activation of trkC, but potentially through the neurotrophin binding to other receptors, including trkA or $trkA_{ei6,}$ (see Section 1.1.2.1.1), where it could potentially activate a signal transduction pathway different from that of NGF (see Section 1.1.2.1.2). Alternatively, NT-3 might effect these changes through an interaction with p75 (see Section 1.1.2.2). For example, BDNF, *via* p75, has been shown to affect a serine phosphorylation on trkA that results in a dampening of the NGF signaling response (MacPhee and Barker, 1997) and to lower the affinity state of NGF/trkA binding (Ross *et al.*, 1998). That NT-3 has been shown to alter the binding site of p75 in a manner similar to that of BDNF (Shamovsky *et al.*, 1999), suggests that NT-3 has the potential to behave like BDNF in altering NGF/trkA binding.

Since trkA neurons are known to function in nociception (reviewed in: Lewin and Mendell, 1993; Woolf, 1996, McMahon *et al.*, 1997; Millan, 1999; Stucky, 2001), then the ability of NT-3 to downregulate aspects of their phenotype might be of benefit in offsetting neuropathic pain states. In support of this, our laboratory has recently shown that two other markers of this nociceptive population, PACAP and BDNF, are also influenced by exogenous NT-3. The neuropeptide, PACAP (see Section 2.2.2.2), is believed to play a role in nociception (Moller *et al.*, 1993; Mulder *et al.*, 1994; Zhang *et al.*, 1996; reviewed in: Mulder *et al.*, 1999; Vaudry *et al.*, 2000). In normal DRG neurons virtually all PACAP-positive cells coexpress trkA, and NT-3 infusion effected a dramatic downregulation in the levels of the neuropeptide (Jongsma Wallin *et al.*, 2001).

Even though the NT-3 induced depression of PACAP signal levels appeared to occur in both neurons that expressed trkC and in those that lacked detectable trkC transcripts, it was most evident in the subset of trkA-positive/trkC-negative cells, which normally express the highest levels of the neuropeptide (Jongsma Wallin *et al.*, 2001). BDNF has been reported to play a role in central sensitization and in inflammation-associated pain (Kerr *et al.*, 1999; Mannion *et al.*, 1999), and unlike NT-3 or NGF, it is constituitively expressed in mature DRG neurons (Ernfors *et al.*, 1990b; Wetmore *et al.*, 1991; Wetmore and Olson, 1995; Apfel *et al.*, 1996; Kashiba *et al.*, 1997b; Michael *et al.*, 1997, 1999). In the intact state, it is primarily localized to trkA neurons (Apfel *et al.*, 1996; Kashiba *et al.*, 1997b; Michael *et al.*, 1997, 1999), although some trkC-positive cells also express the neurotrophin (Michael *et al.*, 1997, 1999). In addition, under normal conditions NGF has been shown to stimulate both BDNF expression (Cho *et al.*, 1997a, 1997b) and its release (Michael *et al.*, 1997). In contrast, but consistent with the findings reported here, we have shown that a 1 week intrathecal infusion of NT-3 resulted in decreased BDNF mRNA and protein levels, in trkC-positive neurons and in those that lack detectable levels of the receptor (Karchewski *et al.*, submitted). Moreover, if trkB is dependent on the presence of its ligand to maintain normal levels of its expression in DRG neurons, as are other neurotrophin receptors (Johnson *et al.*, 1987; Verge *et al.*, 1989b, 1992a; Ernfors *et al.*, 1993; Funakoshi *et al.*, 1993; Sebert and Shooter, 1993; Zhou *et al.*, 1996; Kashiba *et al.*, 1998), these data raise the possibility that NT-3 treatment may also result in reduced trkB levels. Further, a 7 day intrathecal infusion of NT-3 in adult rats prevented much of the increased thermal hyperalgesia seen after chronic constriction injury, but it did not appear to alter post-injury sensitivity to mechanical stimulation (Wilson and Verge, 2001). But, there are seemingly conflicting reports. Another study reported that a 14 day intrathecal infusion of NT-3 (200 ng/d) significantly reduced the threshold to mechanical stimuli in otherwise normal rats, indicating that NT-3 in this case appeared to act in an algesic, not analgesic, fashion (White, 1998). The apparent discrepancies between these data and my observations may be due to the differing concentrations of NT-3 used in each study, between 200 ng/day for 14 days and 600 ng/μl/hour for 7 days. The lesser concentration of NT-3 may have

failed to reduce trkA expression and failed to place these neurons in an NGF-deprived state. The author of that study (White, 1998) attributed the increased levels of sensitivity to mechanical stimuli to the aberrant Aβ fiber sprouting into lamina II of the spinal cord dorsal horn caused by a similar concentration of NT-3 (White, 1997b, 1998), mimicking an injury-induced response seen in these fibers (see Section 2.2.2.2). Interestingly, intrathecal infusion of GDNF has been shown to prevent this sprouting (Bennett *et al.*, 1998). Thus, this, coupled with the report that post-axotomy the majority of large diameter neurons express GFRα1, a receptor for GDNF (Bennett *et al.*, 2000b), and that the sprouting is from Aβ, not C fibers, suggests that the population affected by the infusion of NT-3 in the White study (1998) express trkC, not trkA. In spite of these conflicting findings, the ability of NT-3 to depress the phenotype of trkA-positive DRG neurons may be clinically beneficial in conditions when levels of NGF are abnormally high, as in chronic inflammation (reviewed in: McMahon, 1996); any correlation between NT-3 treatment and the dampening of nociception needs further study. It would also be of interest to determine whether the NT-3 effected reduction in trkA mRNA levels alters the ability of that receptor to interact with p75, since the ratio of p75 to trkA appears to be important to trkA signal transduction (see Section 1.1.2.2.2).

SNAP-25, is an important protein in vesicle-membrane fusion and exocytosis (see Section 4.3.6). Under normal conditions, transcripts are detectable in virtually all mature DRG neurons, with a subset of trkC-expressing cells exhibiting the highest labeling indices for SNAP-25 (see Section 4.2.6). Infusion of NT-3 effects a downregulation of SNAP-25 levels, and this reduction is most evident in those trkC-positive neurons with the highest levels of SNAP-25. Interestingly, NT-3 treatment does not appear to significantly alter SNAP-25 mRNA levels in trkA-expressing cells (see Figure 6.20). This is unexpected, given the downregulation of trkA, SP, BDNF, and PACAP in predominantly in trkA neurons. It is also interesting because when NT-3 is provided to peripherally axotomized DRG neurons it tends to effect a return towards the normal, intact phenotype in trkC-positive cells (see Chapter 5). Further, it is important to note that the reduction in SNAP-25 mRNA levels apparently occurs without a concomitant loss of trkC expression. These findings could reflect a similar dose-

dependent effect (see Section 5.3.1), as evidenced in an *in vitro* study detailing the ability of NT-3 to regulate GAP-43 and Tα1 α-tubulin (Mohiuddin *et al.*, 1995). That study found that lower concentrations of NT-3 effected an upregulation in message levels for both markers, but that the use of higher concentrations of the neurotrophin resulted in levels of expression only slightly higher than those of untreated cultures (Mohiuddin *et al.*, 1995). Given the importance of SNAP-25 to synaptic functioning, including neurotransmitter release (O'Conner *et al.*, 1997; reviewed in: Mochida, 2000), and given the necessitated high firing rate of putatively trkC-positive rapidly adapting mechanoreceptors (Carr *et al.*, 1989a) (see Section 2.2.2.2), it would be interesting to learn whether the reduction in SNAP-25 message levels is translated into a reduced ability of these neurons to function.

Together, the ability of exogenous NT-3 to effect a dampening of the phenotype of trkA-expressing neurons and a downregulation of SNAP-25 mRNA levels, suggest that if NT-3 is to be used therapeutically (Gao *et al.*, 1995; Rodriguez-Pena *et al.*, 1995; Tomlinson *et al.*, 1996, 1997; Helgren *et al.*, 1997; Fernyhough *et al.*, 1998; Mizisin *et al.*, 1998) then these factors should be considered, since not all of the NT-3 induced changes might be desirable; whether or not these observations are important beyond their experimental or therapeutic use is unknown.

7. CONCLUSIONS

The objective of this study was to gain a better understanding of the role of NT-3 in mature primary sensory neurons. Taken together, my data establish evidence for the existence of phenotypically unique micropopulations within the subset of trkC-positive neurons and suggests that NT-3 can act in both a positive and negative manner to modulate aspects of neuronal phenotype.

In order to attempt to determine the role of NT-3 in sensory neurons it was necessary to characterize the phenotype of the putative NT-3 responsive population, the trkC-positive subset, which represents ~ 40% of DRG neurons. My findings (see Chapter 4, Table 4.1) indicate that, whether under normal or axotomized conditions, beyond the presence of trkC transcripts, no single marker, including trkA, p75, NFM, Tα1 α-tubulin, SP, α-CGRP, galanin, NPY, GAP-43, cjun, and SNAP-25, can be used to define the subgroup of primary sensory neurons responsive to NT-3. Rather, the trkC-positive subset is made up of phenotypically unique micropopulations, each expressing varying levels of the markers, in variable combinations, which may reflect neuronal populations with differing sensory functions.

To determine the function of NT-3 in these cells, the neurons were peripherally axotomized to deprive them of a source of target-derived neurotrophin and then NT-3 was reintroduced *via* intrathecal infusion. Injury resulted in the upregulation of some markers and a downregulation of others. If a marker colocalized with trkC then expression levels of the marker returned towards normal values, at least within a subset of those cells post-infusion (see Chapter 5, Table 5.1). These findings suggest that NT-3 is important in maintaining these neurons in their normal state. But, conversely, when normal animals were treated with exogenous NT-3 phenotypic markers within two

separate subpopulations of neurons appeared to negatively influenced (see Chapter 6). The biological importance of this observation is not known, but it may be important in determining the clinical relevance of the neurotrophin.

Although there is little literature (see Section 2.2.2.2) available describing the role of NT-3 in adult primary sensory neurons, a comparison between it and my findings, indicates that the influence of the neurotrophin over the expression of phenotypic markers may be dependent on the concentration of the neurotrophin and on the mode and rate of its delivery, and of the level of receptor expression (see Sections 5.3.1, 5.3.2). In addition, it also appears to be dependent on specific cell type, whether other types are present, and whether the experiment is conducted *in vitro* or *in vivo* (see Sections 5.3.2, 5.3.3, 5.3.4). Moreover, delineating the role of NT-3 is even more complex given its promiscuous nature — that of potentially binding to receptors other than trkC (see Sections 1.1.2.1.1, 1.1.2.2).

Although the means by which NT-3 can modulate neuronal phenotype were not examined here, it seems that this too may be dependent on complex interactions between it and other neurotrophic molecules, or between the neurotrophin and potential receptor signal transduction pathways. For example, activation of trkA can evoke at least five different signal transduction cascades, with unique, overlapping, or cooperating second messenger components (see Section 1.1.2.1.2). Moreover, there may be cooperation or positive/negative regulation of phenotype through a combination of neurotrophic molecules. NGF and NT-3 have been shown to influence BDNF expression in DRG neurons, in a positive and negative manner, respectively (Cho *et al.*, 1997a; Karchewski *et al.*, submitted), and BDNF has been shown to regulate NT-3 levels in cerebellar granule neurons (Leingärtner *et al.*, 1994). In addition, while NT-3 (see Section 5.2.4.1), NGF (Verge *et al.*, 1995; Corness *et al.*, 1998; Ramer *et al.*, 1998), and aFGF (Ji *et al.*, 1996) act to negatively regulate galanin levels, the injury-induced cytokines, IL-6 and LIF (Curtis *et al.*, 1994; Murphy *et al.*, 1995, 1999a, 1999b; Kurek *et al.*, 1996a, 1996b; Sun and Zigmond, 1996; Arruda *et al.*, 1998), appear to be responsible for its upregulation after nerve injury (Corness *et al.*, 1998; Ramer *et al.*, 1998; Thompson *et al.*, 1998b; Kerekes *et al.*, 1999; Murphy *et al.*, 1999b). As a further example of the

potential interconnections between regulatory systems, the ability of NT-3 to depress cjun mRNA levels (see Sections 5.2.5.2, 5.3.4) may influence the level of cJUN available to participate as a component of AP-1 complexes (see Section 4.3.5), which can bind to a site on the GAP-43 gene (Eggen *et al.*, 1994; de Groen *et al.*, 1995), potentially regulating its transcription. This seeming complexity of interactions at the various levels of regulation may be necessary to allow for fine tuning of the cell's response to the trophic factor.

With the emerging potential of using the neurotrophins as therapeutic molecules (Eide *et al.*, 1993; Barinaga, 1994; Schätzl, 1995), it is important to note that the data presented here should have relevance in humans, since human trks exhibit a high degree of similarity in gene sequence and tissue expression to their murine counterparts (Shelton *et al.*, 1995). Even though NT-3 has been shown to be effective in counteracting aspects of experimentally-induced large fiber neuropathies (see Section 2.2.2.2), the report here that NT-3 may also negatively regulate SNAP-25 expression and the phenotype of trkA-positive neurons must be taken into account when considering the efficacy of its use in clinical situations. Moreover, NT-3 may not only influence primary sensory neurons, it has also been shown to be important in motorneuron survival and regeneration (Li *et al.*, 1994; Grill *et al.*, 1997; Sterne *et al.*, 1997b; Simon *et al.*, 2000), as well as in restoring injured muscle fibers, the target of motorneurons (Sterne *et al.*, 1997b). Further, the presence of the neurotrophin in many different cell types, including kidney, spleen, and liver suggests that NT-3 may have an additional role outside of the nervous system (Zhou and Rush, 1993).

In conclusion, the data presented in this work indicate that multiple subsets of mature DRG neurons have the potential to be responsive to NT-3. In addition, NT-3 has been shown to be effective in counteracting many of the injury-induced changes in many primary sensory neurons, not all of which express trkC. This suggests a role for the neurotrophin in the maintenance of normal neuronal phenotype. Conversely, infusion of NT-3 in normal animals resulted in a negative regulation of phenotype in two different subpopulations, a finding that needs to be taken into account when considering therapeutic applications for the neurotrophin.

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APPENDIX

0.01 M Sodium Acetate Buffer

0.2 M Stock Solutions

0.2 M Sodium Acetate

Under RNase-free conditions, dissolve 16.6 g sodium acetate in 1 l distilled water.

0.2 M Acetic Acid

Under RNase-free conditions, add 12 ml glacial acetic acid to 988 ml distilled water.

0.01 M Working Solution

Under RNase-free conditions, add 16.5 ml 0.2 M sodium acetate stock and 3 ml 0.2 M acetic acid stock to 180 ml distilled water. Adjust pH of working solution to pH 4-4.5 (for toluidine blue) or pH 5.2 (for DTT) with additional 0.2 M acetic acid.

Diethyl-pyrocarbonate (DEPC) Treated Water and Glassware

0.1% DEPC Treated Water

Under RNase-free conditions and in a fume hood, add 1.0 ml DEPC (Sigma) to 999 ml distilled water. Leave solution for 12 hours at 37 °C. Autoclave the solution to deactivate DEPC. Also, all glassware and pipette tips that were exposed to DEPC must be autoclaved.

0.1% DEPC Treated Glassware

Prepare DEPC solution as described above. Completely submerge glassware to be treated in the fluid, leave for at least 12 hours, and then deactivate the DEPC by autoclaving all glassware and pipette tips exposed to DEPC.

5 M Dithiothreitol (DTT)

Under RNase-free conditions, dissolve 15.45 g DTT (Ultrapure; United States Biochemical, Cleveland, OH) in 20 ml 0.01 M sodium acetate buffer, pH 5.2. Filter sterilize with a 0.45 µm pore nylon syringe filter (Nalge Nunc International Corp.), dispense into 1 ml aliquots, and store at -20 °C. Return used aliquots to the freezer for reuse. Do not autoclave DTT or solutions containing DTT.

2% Formaldehyde with 2% Glutaraldehyde in 0.1 M PBS, pH 7.4

In a fume hood and under RNase-free conditions, heat 200 ml 0.1 M DPBS to 70 °C. With stirring, add 5 g paraformaldehyde. Clear solution with several drops of 1 N sodium hydroxide. Cool solution to room temperature, adjust pH to 7.4, and filter. Add 10 ml 50% glutaraldehyde and bring final volume to 250 ml with 0.1 M DPBS. Fixative was made within 24 hours of use and was stored at 4 °C until needed.

Hybridization Cocktail Stock

Under RNase-free conditions and in a fume hood, add, with stirring, 50 ml of deionized formamide to 20 ml of 20X SSC. Continue stirring, while adding 1 ml 100X Denhardt's solution, 10 ml 0.2 M sodium phosphate buffer (pH 7.0), and 10 g dextran sulfate (Sigma). After all ingredients are dissolved, add 5 ml 20% sarcosyl (N-lauroylsarcosine; Sigma). Avoid foaming the solution. Store in 5 ml aliquots at -20 °C. Return used aliquots to the freezer for reuse.

Deionized Formamide

Under RNase-free conditions, deionize the required volume by stirring gently for 1 hour with 1 g of analytical grade mixed-bed ion exchange resin (20-50 mesh, Bio-Rad Laboratories, Hercules, CA) for 10 ml of formamide (Fisher Scientific, Fair Lawn, NJ). Dispense into 5-15 ml aliquots and store at -20 °C. Return used aliquots to the freezer for reuse.

100X Denhardt's Solution

Under RNase-free conditions, add 5 g ficoll (Sigma), 5 g polyvinal-pyrolidone (Sigma), and 5 g BSA (Fraction V; Sigma) to 200 ml distilled water. Bring final volume to 250 ml, dispense into 2.5 ml aliquots, and store at -20 °C.

0.2 M Phosphate Buffer, pH 7.0

Add, with stirring, 11.2 g sodium phosphate dibasic and 2.74 g sodium phosphate monobasic to 400 ml distilled water. Adjust pH of solution to 7.0 and bring final volume to 500 ml.

Lactoperoxidase, 0.05 mg/µl in 0.1 M PB

Prepare solution for ^{125}I-NGF iodination and binding within 24 hours of use and under RNase-free conditions. Add 100 µl cold 0.1 M PB to the shipping vial containing 5 mg lyophilized lactoperoxidase (Sigma). Once the lactoperoxidase is in solution transfer it to a 1.5 ml microcentrifuge tube to allow for ease in aliquoting; place the tube and 10 µl aliquots (0.5 mg lactoperoxidase) on ice while working. Store aliquots at -70 °C.

Lana's Fixative

In a fume hood, heat 800 ml of 0.1 M PB (pH 7.4) to 60-70 °C and add, with mixing, 20 g paraformaldehyde. Clear solution with several drops of 1 N sodium hydroxide. Cool solution to room temperature, adjust pH to 7.4, and filter. Add 15 ml saturated picric acid and bring final volume to 1 l with 0.1 M PB. Fixative was made within 24 hours of use and was stored at 4 °C until needed.

0.1 M Phosphate Buffer (PB), pH 7.4

sodium phosphate dibasic $2H_2O$	7.03 g
sodium phosphate monobasic H_2O	1.58 g

With stirring, add the above to 400 ml distilled water. Adjust pH of solution to 7.4 and bring final volume to 500 ml. If to be used for ^{125}I-NGF iodination and binding, prepare within 24 hours of use and under RNase-free conditions.

0.1 M Phosphate Buffered Saline (PBS), pH 7.4

Prepare solution for ^{125}I-NGF iodination and binding within 24 hours of use and under RNase-free conditions. Add the following ingredients in the order listed to 1.5 l distilled water; allow each to dissolve completely before adding the next.

sodium phosphate dibasic $2H_2O$	2.67 g
sodium chloride	16 g
potassium chloride	0.4 g
potassium dihydrogen orthophosphate	0.4 g

Adjust pH to 7.4 and bring final volume to 2 l.

Salmon Sperm DNA (Denatured)

Under RNase-free conditions, add 1 g salmon sperm DNA to 100 ml DEPC treated water, seal container, and let stir at room temperature for 16-18 hours. Dispense into 1 ml aliquots in 1.5 ml microcentrifuge tubes. To disrupt the DNA, sonicate at setting 5 for three times, 15 seconds each (Tissue Tearor model 3985-370; Biospec Products, Inc., Bartlesville, OK). Store sonicated aliquots at -20 °C. Return used aliquots to the freezer for reuse.

0.05 M Sodium Acetate, pH 4.0

Prepare solution for ^{125}I-NGF iodination and binding within 24 hours of use and under RNase-free conditions. Add 0.025 g sodium acetate to 40 ml distilled water. Adjust pH of solution to 4.0 and bring final volume to 50 ml.

SSC Buffer

To prepare 20X SSC, dissolve 175.3 g sodium chloride in 800 ml distilled water under RNase-free conditions. With stirring, add 88.2 g sodium citrate. Adjust pH of solution to pH 7.0 and bring final volume to 1 l. Autoclave and store at room temperature. Dilute to 1X SSC as needed with additional distilled water.

20% TCA Solution

100% TCA Stock Solution

Prepare TCA stock solution immediately prior to use by adding 20 g TCA to 20 ml distilled water. The preparation of a concentrated stock solution was necessary since a directly prepared 20% solution yielded inconsistent results, possibly due to the hygroscopic nature of the TCA crystals.

20% TCA Working Solution

Dilute TCA stock solution to 20% with distilled water.

0.5% Toluidine Blue, pH 4-4.5

Suspend 0.5 g toluidine blue in 100 ml 0.01 M sodium acetate buffer, pH 4-4.5. If tissue is difficult to stain add 0.1% sodium borate to the above solution.

0.05 M Tris-Hydrochloride (Tris- HCl), pH 7.6

Prepare solutions for ^{125}I-NGF iodination and binding within 24 hours of use and under RNase-free conditions. Do not use DEPC treated water to prepare the solution. Add 38.5 ml 0.1 N hydrochloric acid to 50 ml 0.1 M Tris. Adjust pH to 7.6 and bring final volume to 100 ml with distilled water.

0.1 M Tris

Dissolve 1.21 g Tris base in 100 ml distilled water.

0.1 N Hydrochloric Acid

Add 415 µl of concentrated hydrochloric acid to 50 ml distilled water.

0.05 M Tris-HCl, pH 9.0

Under RNase-free conditions, add 7.0 ml 0.1 N hydrochloric acid to 50 ml 0.1 M Tris. Adjust pH to 9.0 and bring final volume to 100 ml with distilled water. Do not use DEPC treated water to prepare the solution.

0.1 M Tris-HCl, pH 8.0

Under RNase-free conditions, prepare 250 ml each of 0.1 M Tris base and 0.1 M Tris-HCl. Add one to the other to reach pH 8.0, autoclave, and store at 4 °C. Do not use DEPC treated water to prepare the solution. To prepare 0.1 M Tris base, add 3.029 g Tris base to 250 ml distilled water. To prepare 0.1 M Tris-HCl, add 3.94 g Tris-HCl to 250 ml distilled water.