THE ROLE OF NEUROTENSIN IN PREVENTING NEURONAL DEATH FOLLOWING PERIPHERAL NERVE INJURY

By

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I. Acknowledgements

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IV. Abstract

Traumatic nerve injury frequently leads to loss of function due to neuronal degeneration and death. The molecular mechanisms involved in neuronal death are poorly understood, but are thought to parallel apoptosis, or programmed cell death. Several molecules have recently been identified as key players in the induction of neuronal death. Sortilin, a recently discovered receptor expressed in neuronal tissues, has been shown to interact with the p75 neurotrophin receptor ($p75^{NTR}$) in the induction of apoptosis upon binding unprocessed nerve growth factor, also known as proNGF (Nykjaer et al., 2004). Sortilin also functions as a receptor for the peptide neurotransmitter neurotensin. Neurotensin occupies the sortilin receptor and thereby prevents the proNGF-induced formation of the sortilin-$p75^{NTR}$ receptor complex. Therefore, proNGF-activation of the $p75^{NTR}$ apoptotic signaling cascade is thought to be prevented by neurotensin. We sought to determine if the absence of neurotensin *in vivo* would result in significantly greater neuronal death following peripheral nerve injury. To test this hypothesis, we assessed neuronal survival in L4/5 dorsal root ganglia (DRG) seven days following sciatic nerve transection amongst neurotensin null-mutant ($NT^{-/-}$), heterozygous ($NT^{+/-}$), and wild-type ($NT^{+/+}$) mice (Dr. Paul Dobner, University of Massachusetts Medical Center). In response to sciatic nerve transection, $NT^{-/-}$ mice experience the most dramatic neuronal loss (53%), while $NT^{+/-}$ and $NT^{+/+}$ mice also suffer a significant neuronal loss of 38% and 26%, respectively. Unexpectedly, exogenous delivery of either 10 µg or 100 µg of neurotensin (7 intrathecal injections total/ 24-hour cycles) did not prevent

neuronal death amongst $NT^{-/-}$ mice after nerve injury. However, $NT^{+/+}$ mice which received either 10 µg or 100 µg of neurotensin had 61% and 67% more neurons than control mice after injury, respectively. These differential effects produced amongst null-mutant and wild-type mice raise important questions of how neurotensin mediates pro-survival effects. Overall, these data suggest an important and novel role for neurotensin in promoting survival amongst sensory neurons after peripheral nerve injury.

V. Introduction

1. Peripheral Nerve Injury

Following peripheral nerve injury, a complex and finely regulated series of events ensues in order to remove damaged tissue and commence the process of regeneration. However, a nerve's response to injury is not localized to the site of injury but also includes changes within the cell body. For successful regeneration and full functional recovery, neurons must survive the injury. Days to weeks after the initial peripheral nerve injury, apoptosis occurs throughout sensory neurons, causing the cells to self-destruct. This neuronal death is a major factor responsible for poor sensory functional outcome after peripheral nerve trauma.

Following a traumatic nerve injury, many pathophysiological events take place within the cell body including morphological and metabolic changes. These changes begin to occur within several hours after the initial injury. The cell body, nucleus and nucleolus all swell in size in a process called chromatolysis. The function of the metabolic machinery in the cell transforms from production of neurotransmitters to upregulation of genes needed for neuronal survival. This includes increased production of ribonucleic acid, protein components, and lipids. There are also many changes occurring at the site of the injury. Wallerian degeneration occurs after peripheral nerve injury. This process starts immediately after injury and involves the breakdown of Schwann cells distal to the injury (Fig. i). Subsequently, there is a migration of Schwann cells and

macrophages to the injured axon. Macrophages digest the debris and degenerating tissue. It may take up to 3 months for complete clearing of the debris.

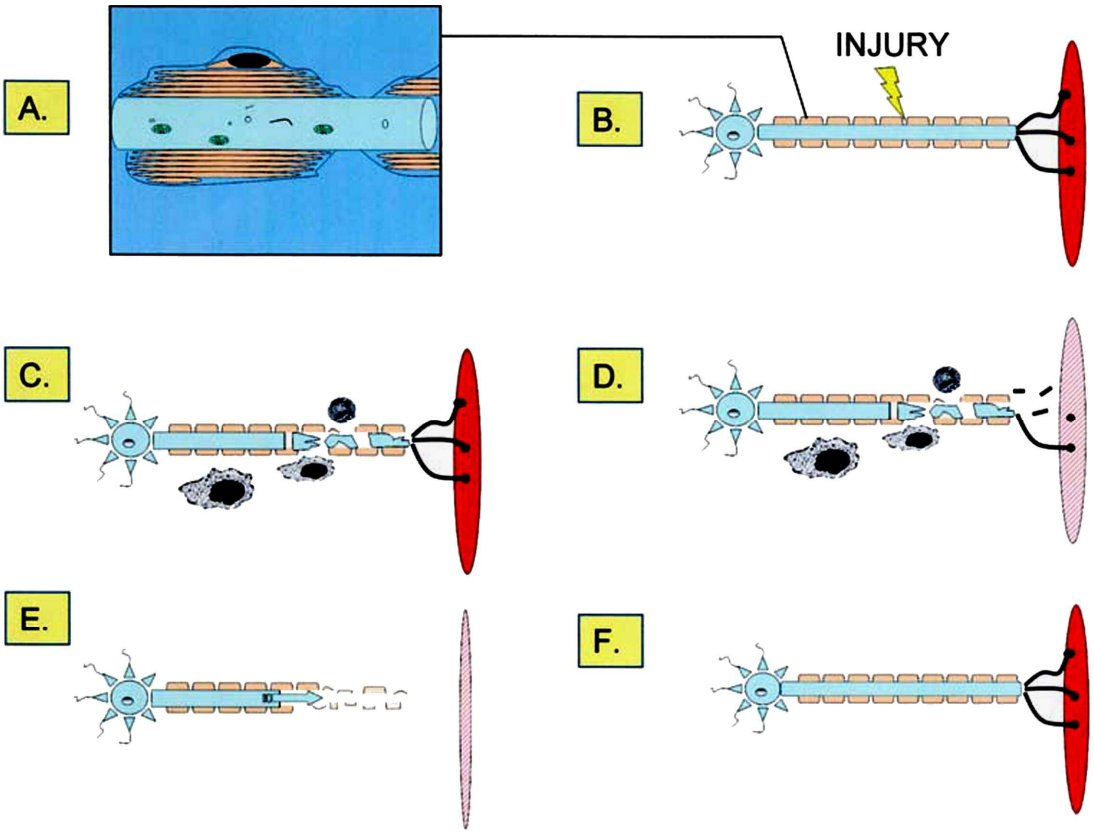


Figure i: Wallerian Degeneration of the Peripheral Nerve. In the uninjured (A) and injured form (B), Schwann cells align the length of the axon and form multiple layers of myelin. The gaps between the Schwann cells are nodes of Ranvier. After traumatic nerve injury (C), the nerve begins to degenerate causing a breakdown of the axon and surrounding myelin. Phagocytic macrophages interact with Schwann cells to remove injured tissue. Thus, connection with the target muscle is lost (D), leading to muscle atrophy. Once the degenerative events are complete (E), all that remains is a column of collapsed Schwann cells

known as bands of Büngner. An advancing fingerlike growth cone begins to sprout using the Schwann cells as guides. After reinnervation (F), the newly connected axon matures and the preinjury cytoarchitecture and function are restored. (Burnett and Zager et al., 2004).

In recent years, treatment of peripheral nerve injuries has centered on surgical repair of nerve interruption. However, this attempt has produced disappointing results by offering little improvement in adequate cutaneous innervation and quality of sensation.

Survival of the neuron after injury results in restoration of nerve continuity, regeneration of axons and reinnervation of the sensory receptors in the periphery. Therefore, maintaining the cell body is critical to ensuring a desirable outcome following nerve injury. Thus, it is clear that to improve functional outcome after peripheral nerve injury the focus needs to be redirected to the molecular mechanisms involved in neuronal death and survival. The knowledge of these mechanisms will be crucial for the development of new clinically relevant therapeutic strategies aimed at the treatment of disorders involving neuronal loss.

The studies presented here address the neurobiology involved in neuronal loss and propose a mechanism targeted at preventing cell death amongst sensory neurons following peripheral nerve injury, thereby avoiding the unsatisfactory results in functional outcome often associated with traumatic nerve damage.

2. ProNGF

Mature nerve growth factor (NGF) is generated from a precursor that contains a signal peptide at the N-terminus followed by a pro-region which is trailed by sequences of contiguous basic amino acids (Figure ii).

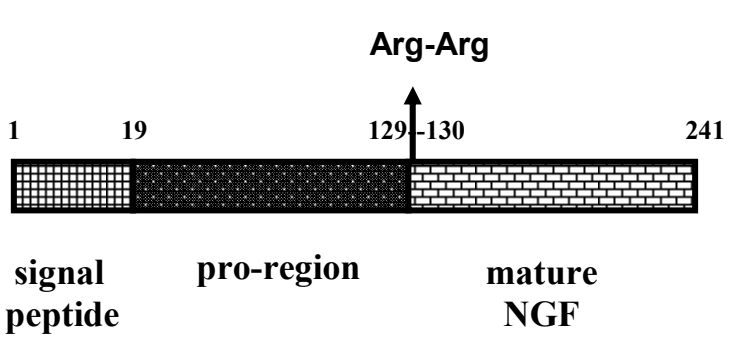


Figure ii: The ProNGF Peptide. ProNGF is a 241 amino acid molecule. It includes a signal peptide at the N-terminus, followed by the pro-domain which precedes mature NGF. There is a monobasic amino acid sequence which separates the pro-domain from its mature form at amino acids 129 and 130. These adjoining basic amino acid sequences serve as cleavage sites for production of mature NGF. Intracellular cleavage of proNGF occurs in the trans golgi network (TGN). Candidates for intracellular cleavage of proNGF include the proprotein convertases furin, PACE4, and PC5 (Seidah et al., 1996). Intracellular cleavage of proNGF takes place after the following pairs of basic amino acids: Arg-Ser-(Lys/Arg)-Arg↓, with the arrow representing the site of cleavage. Matrix metalloproteinase 7 (MMP-7) and plasmin have been identified as extracellular proteases also capable of cleaving proNGF (Lee et al., 2001).

Until recently, it was generally accepted that proneurotrophins were inactive precursors to their mature counterparts. However, recent studies have shown that proneurotrophins, including proNGF, are able to escape intracellular cleavage for secretion into the extracellular environment (Lee et al., 2001). In effect, proneurotrophins account for a significant proportion (40-60%) of total neurotrophins released extracellularly (Farhadi et al., 2000; Heymach et al., 1996; and Mowla et al., 1999). ProNGF has been identified as the predominant form of NGF detectable in many tissues (Table i).

Table i: ProNGF is the Predominant Form of NGF in Many Different Tissues

Tissue	ProNGF detectable	NGF detectable	Source
Rat DRG	√	Barely	Reinshagen et al., 2000
Mouse/rat/human brain	√		Fahnestock et al., 2001
Rat spermatids	√		Chen et al., 1997
Human prostatic stromal cells	√		Delsite and Djakiew, 1999
Ovine (sheep) skin	√		Yardley et al., 2000

Several proNGF forms ranging from 27-200 kDa have been identified (Edwards et al., 1988, Reinshagen et al., 2000 and Saboori et al., 1986). The proNGF protein produces various high-molecular-weight forms due to the existence of several potential targets for cleavage, glycosylation, sulphation, and

acetylation (Earl et al., 2006). Thus, varying forms of proNGF are present from one tissue to another.

Recently, proNGF has been implicated in many disease states. The existence of several proNGF forms are significantly increased in the frontal cortex of brains affected by Alzheimer's disease (AD) (Fahnestock et al., 2001). Furthermore, the proNGF purified from the brains of AD patients has been shown to be effective in inducing apoptosis via the $p75^{NTR}$ (Pedraza et al., 2005). Likewise, proNGF levels were found to be increased in inflamed colon extracts from patients with Crohn's disease (Yiangou et al., 2002).

ProNGF has been reported to interact with $p75^{NTR}$ and to selectively induce $p75^{NTR}$ – dependent apoptosis in neurons (Beattie et al., 2002). Inhibition of sortilin in neurons which express $p75^{NTR}$ prevents cell death (Nykjaer et al., 2004). Likewise, DRG of $p75^{NTR}$ null-mutant mice are resistant to injury-induced neuronal death (Sorensen et al., 2003). Therefore, proNGF mediated apoptosis requires signaling via both sortilin and $p75^{NTR}$.

ProNGF displays at least fivefold greater affinity for $p75^{NTR}$ than mature NGF and only neglible binding to the tyrosine kinase receptor A (*trk* A) (Ibanez et al., 2002). Therefore, while proNGF is a high-affinity ligand for the apoptosis-inducing receptor 75^{NTR}, mature NGF is the preferred ligand for the survival-promoting receptor *trk* A. In turn, proNGF will induce apoptosis upon activating sortilin-$p75^{NTR}$ while mature NGF will bind the *trk* A-$p75^{NTR}$ receptor complex and mediate cell survival. *trk* A mediates survival by receptor dimerization and autophosphorylation on Tyr-490 and Tyr-785. This results in activation of two

independent pathways: ras-Extracellular signal-Regulated Kinasas 1/2 (ERK 1/2) and Phosphatidylinositol-3 Kinase (PI_3K)/AKT (protein kinase B) pathways. The Ras-ERK 1/2 pathway regulates the expression of survival – promoting genes through transcription factors such as c-fos and CREB binding protein (CBP). The PI_3K pathway regulates neuronal survival by modulating the phosphorylation of members of the pro-apoptotic bcl-2 family (Alberghina and Colangelo, 2006). Thus, neuronal fate can be determined by the proportions of mature and processed NGF forms available to cells as well as differential receptor expression (Figure iii).

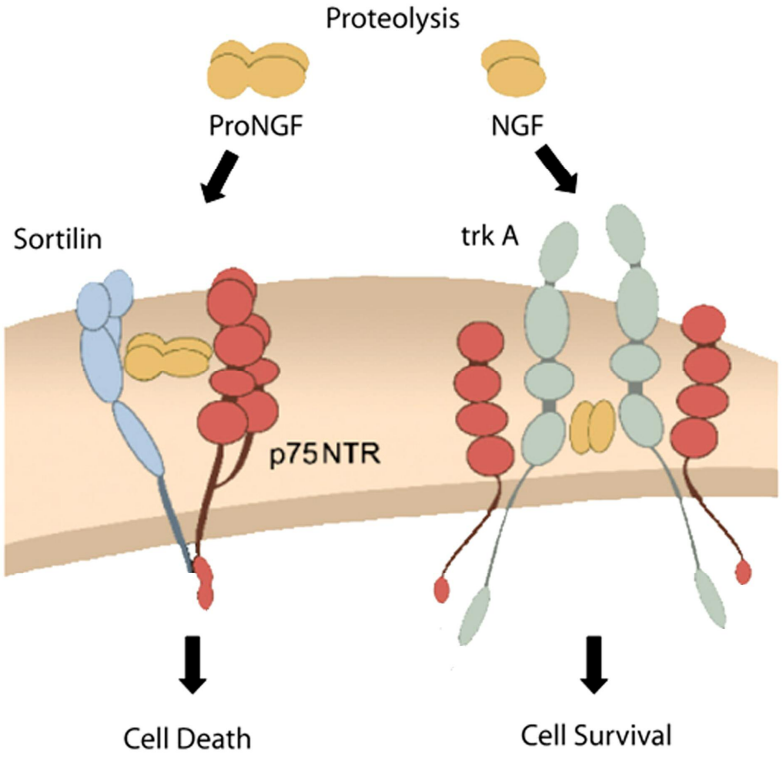


Figure iii: Determination of Cell Fate by Differential NGF Forms. Protolytically unprocessed proNGF will bind to the sortilin-$p75^{NTR}$ receptor complex and induce $p75^{NTR}$ mediated-apoptosis while processed, mature NGF

will bind to the trkA-p75^{NTR} complex and promote cell survival. (Nykjaer et al., 2004).

3. p75^{NTR}

The p75^{NTR} is a member of the tumor necrosis factor receptor (TNFR) superfamily (Chao, 2003). Members of this family share a common cysteine motif which is repeated two to six times within their ligand binding domain. p75^{NTR} also contains a homologous cytoplasmic region to the “death domain” of TNFR family members TNF-1 and Fas (Chapman, 1995), and a juxtamembrane death domain named “chopper”, which has been found to be necessary to initiate neuronal death (Coulson et al., 2000) (Figure iv).

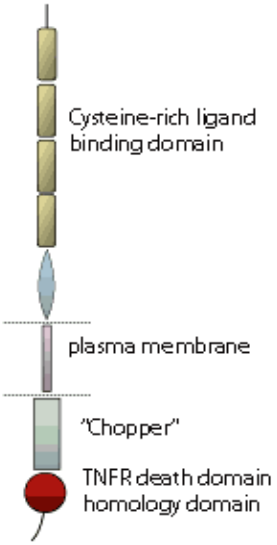


Figure iv: The p75 Neurotrophin Receptor. Regions of p75^{NTR} include the extracellular domain necessary to bind ligands, the lipid anchor which attaches p75^{NTR} to the plasma membrane, the region nicknamed “Chopper” which initiates neuronal death, and a region homologous to the “death domain” of the TNF receptors Fas and TNF-1.

p75^{NTR} signaling results in diverse neuronal responses. These different roles for p75^{NTR} are determined by the extracellular ligand and interactions with other receptors. For instance, the presence of the sortilin receptor modulates the activity of p75^{NTR}. Specifically, the absence or presence of sortilin determines

whether or not $p75^{NTR}$ functions as a death receptor (Kaplan and Miller, 2004). On the other hand, $p75^{NTR}$ promotes cell survival by cooperating with *trk* receptors to increase the number of high-affinity binding sites for mature neurotrophins (Esposito et al., 2001).

$p75^{NTR}$, also referred to as the low-affinity neurotrophin receptor, binds each neurotrophin (nerve growth factor [NGF], brain derived neurotrophic factor [BDNF], neurtrophin-3 [NT-3], and neurotrophin-4 [NT-4]), but with less affinity than their specific tyrosine kinase receptor (*trk* A, *trk* B, *trk* C, and *trk* B, respectively). $p75^{NTR}$ can act as an accessory receptor for the *trk* receptors and increase *trk* signaling (Battleman et al., 1993; Hantzopoulos et al., 1994; and Verdi et al., 1994). Thus, trophic signals are relayed to cells via simultaneous binding to *trk* receptors and $p75^{NTR}$. Paradoxically, $p75^{NTR}$ is also capable of mediating cell death. Upon binding of proNGF, $p75^{NTR}$ induces apoptosis in sympathetic neurons, vascular smooth muscle cells, and oligodendrocytes (Nykjaer et al., 2004 and Beattie et al., 2002). DRG neurons in $p75^{NTR}$ null-mutant mice have been shown to be less vulnerable to cell death in response to nerve injury than wild-type mice, suggesting a possible mechanism by which $p75^{NTR}$ triggers an apoptotic response following nerve injury (Sorensen et al., 2003).

The exact molecular mechanisms by which $p75^{NTR}$ induces apoptosis are not known, but they are thought to involve the activation of Jun N-terminal kinase (JNK). Further downstream events include phosphorylation of c-jun, activation of both p53 and the “BH3-domain only” family members Bad and Bim, mitochondrial

release of cytochrome c and activation of caspases 9, 6, and 3 (Gentry et al., 2004; Bhakar et al., 2003; Okuno et al., 2004; Barker 2004; and Becker et al., 2004) (Figure v).

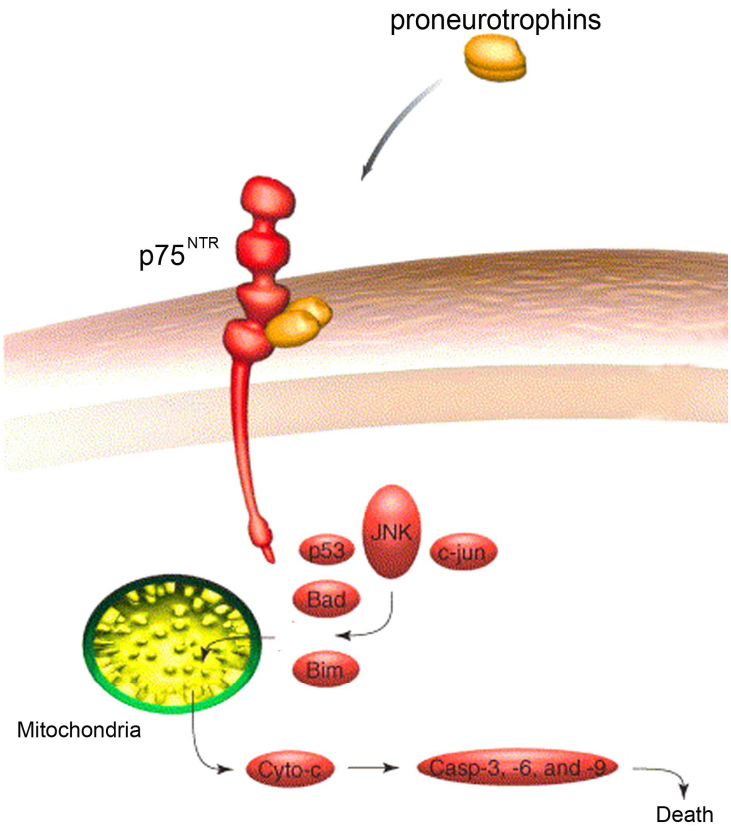


Figure v: p75^{NTR} Induces Apoptosis Upon Binding Proneurotrophins. Binding of pro-neurotrophins to p75^{NTR} results in activation of JNK which indirectly induces phosphorylation of c-jun and activation of p53, Bad, and Bim to cause leakage of cytochrome c from the mitochondria which results in activation of caspases (Adapted from Nykjaer et al., 2005)

p75^{NTR} is expressed in almost all DRG neurons that express *trk* A or *trk* B, but is co-expressed in only 50% of *trk* C-expressing neurons. Interestingly, p75^{NTR} expression is absent in the small, unmyelinated, non-peptidergic population of DRG neurons (Wright and Snider, 1995). As p75^{NTR} is required for proNGF-induced sortilin-mediated apoptosis, its pattern-specific expression may provide clues into the vulnerability of particular neuronal populations to undergo apoptosis.

4. Sortilin

Sortilin, the third and most recently discovered receptor for neurotensin, binds neurotensin with very high affinity (binding affinity = 0.3 nM) (Mazella et al., 1998). High sortilin expression has been detected in the mammalian brain, spinal cord, skeletal muscle, and testis (Mazella, 2001). The 100 kDa receptor was first isolated from human brain by receptor-associated-protein (RAP) affinity chromatography in 1997 (Petersen et al., 1997). It is produced as an inactive precursor which is converted to its mature, 95 kDa active form upon furin-mediated cleavage of the 5 kDa propeptide domain in the trans-Golgi-network (TGN) (Nielsen et al., 1999). It is a typical type-1 membrane receptor, consisting of a large extracellular domain, a single transmembrane segment, and a short, C-terminal cytoplasmic tail (Petersen et al., 1997). Both the extracellular and cytoplasmic domains of sortilin bear a distinct resemblance to well known sorting receptors. The vacuolar protein sorting receptor 10 (Vps10p) is responsible for sorting the enzyme carboxypeptidase Y (CPY) to the vacuolar compartment of yeast. Sortilin shares homology to Vps10p in the alignment of ten conserved cysteine consensus regions (10CC) in its extracellular domain (Hampe et al., 2001). The cation-independent mannose-6-phosphate receptor (CI-M6P) is responsible for transporting newly synthesized enzymes from the Golgi complex to lysosomes in mammalian cells. The C-terminal segment of sortilin's cytoplasmic tail is homologous to an acidic, di-leucine motif (HDDSDEDLL) in the cytoplasmic tail of the cation-independent mannose-6 phosphate receptor (CI-M6P) (Figure vi).

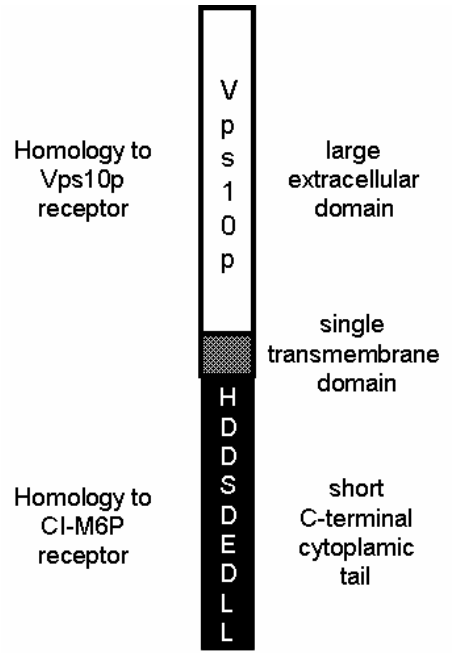


Figure vi: The Sortilin Receptor. The sortilin receptor is a typical type I membrane receptor with a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. The extracellular domain shares homology to the vacuolar protein sorting receptor 10 (Vps10p). Likewise, it also shares homology within its cytoplasmic tail to the cation-independent mannose-6-phosphate receptor (CI-M6P).

Based on these structural features, sortilin was first proposed to function in trafficking lysosomal proteins from the TGN to the lysosome. Sortilin has been identified as the sorting receptor which targets the sphingolipid activator protein (SAP) prosaposin from the TGN to the lysosomal compartment of mammalian cells (Lefrancois et al., 2003). It has recently been shown that efficient sorting of brain derived neurotrophic factor (BDNF) to the regulated secretory pathway in neurons is dependent on interaction between the pro-domain of proBDNF and sortilin (Chen et al., 2005). Furthermore, sortilin was found to co-localize with BDNF in secretory vesicles, confirming that sortilin is properly localized in the secretory granule compartment as BDNF is delivered to the plasma cell membrane for secretion.

Previous reports have indicated that sortilin predominates within intracellular compartments. Approximately 90% of sortilin receptors are found

within the Golgi apparatus and other intracellular compartments while only ~10% of sortilin receptors are expressed on the plasma cell membrane (Nielsen et al., 1999; Morris et al., 1998). In the presence of neurotensin, the sortilin receptor is translocated from intracellular compartments to the plasma cell membrane. Specifically, the neurotensin-induced internal sequestration of NTR1, one of two G protein-coupled receptors for neurotensin expressed on the cell surface, serves as the signal which triggers the appearance of the sortilin receptors on the cell surface of neurons (Chabry et al., 1993). The mechanism by which this occurs is not currently understood. Neurotensin is efficiently internalized after binding to the sortilin receptor, and despite this internalization, the amount of the sortilin receptor present on the cell surface is maintained thus, suggesting that neurotensin binding to the sortilin receptor may induce activation of a signaling pathway which leads to the translocation of sortilin to the plasma membrane from intracellular compartments (Navarro et al., 2001). Sortilin has also been shown to co-localize within intracellular vesicles which contain the glucose transporter GLUT4 in adipocytes. In response to insulin, the sortilin receptor levels on the plasma membrane are increased by 1.7 fold in rat adipocytes (Morris et al., 1998).

Sortilin has been shown to bind and mediate rapid endocytosis of the pro-domain of proNGF (Westergaard et al., 2004). Sortilin will bind proNGF with an affinity in the low nanomolar range (~5 nm). However, the affinity of sortilin for proNGF is increased ~20 fold in cells which co-express $p75^{NTR}$ (binding affinity = ~0.25 nM) (Nykjaer et al., 2005). Thus, a tertiary complex is formed with proNGF

acting as a crosslinker with its pro-domain binding to the sortilin receptor and the mature NGF-domain binding to the $p75^{NTR}$ receptor. Upon binding to the sortilin-$p75^{NTR}$ receptor complex, proNGF induces apoptosis in vascular smooth muscle cells and neurons (Nykjaer et al., 2004). Studies have shown that sympathetic neurons are rescued from proNGF-induced apoptosis in the presence of the sortilin antagonist neurotensin. Furthermore, $p75^{NTR}$ null-mutant mice that express endogenous sortilin are insensitive to the pro-apoptotic effects of proNGF (Nykjaer et al., 2005). Therefore, simultaneous binding of proNGF to both the sortilin and the $p75^{NTR}$ receptor is crucial for the induction of apoptosis. These data are suggestive of a role for sortilin as a key determinant in the regulation of neuronal apoptosis.

5. Neurotensin

Neurotensin is a 13 amino acid peptide neurotransmitter (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) which was originally isolated from bovine hypothalamic extracts in 1973 (Carraway and Leeman, 1973). Since its discovery, neurotensin expression has been well characterized within the gastrointestinal tract and the central nervous system. In the rat, ~85% of neurotensin immunoreactivity is found in the gut and ~10% within the brain (Eysselein, 1984). Neurotensin expression is distributed throughout the central nervous system, with highest levels in the hypothalamus, amygdala and nucleus accumbens (Vincent et al., 1999). In the brain, neurotensin induces potent hypothermic and analgesic effects. Within the gastrointestinal tract, neurotensin immunoreactivity is found at highest levels within the small intestine, specifically the mucosa of the ileum (Eysselein, 1984). Neurotensin is released into the circulation after food ingestion. Its digestive functions include stimulation of pancreatic secretions, inhibition of gastric sections and motility, and stimulation of colonic motility (Ferris 1985).

Neurotensin is synthesized as part of a 170-amino acid precursor which starts with a 22-residue signal peptide. The precursor also contains neuromedin N (NMN), a six amino acid structurally related analogue of neurotensin (Dobner et al., 1987). NMN shares many properties with neurotensin but its effects are less potent and it is more easily degraded than neurotensin. Agents that increase neurotensin synthesis also increase NMN synthesis. Both neurotensin and NMN are located within the carboxy terminus (C-terminus) of the precursor.

A dibasic site (Lysine [Lys] - Arginine [Arg] residues) separates NMN and neurotensin. Two other Lys-Arg sequences flank the amino terminus (N-terminus) of NMN and the C-terminus of neurotensin (Figure vii). Another monobasic sequence (Arg-Arg) precedes an NMN-like sequence and is present within the central region of the precursor**.** These dibasic amino acid sequences provide signals for cleavage by members of the proprotein convertase family (PCs). In tissues which express the neurotensin/NMN precursor (pro-NT/NMN), the three C-terminal di-basic sites (Lys-Arg) are cleaved, whereas the monobasic site (Arg-Arg) upstream of the NMN-like sequence is not processed (Rovère et al., 1996). Likewise, an Arg-Arg sequence that occurs within neurotensin is not cleaved and therefore must somehow be protected from cleavage or not be recognized by the processing enzyme. Recent evidence indicates that pro-NT/NMN is differentially processed in brain versus intestinal tissues. Within the mammalian brain, PC5A and PC2 may act jointly to process pro-NT/NMN. PC5A, which is particularly efficient at cleaving the two most C-terminal Lys-Arg sequences of pro-NT/NMN, may be acting as early as the Golgi apparatus to release neurotensin; whereas PC2, which is considerably more active than PC5A in cleaving the third C-terminal dibasic site, may be predominantly involved further distally along the secretory pathway to release NMN (Villeneuve et al., 2000). Therefore, in the brain, cleavage of pro-NT/NMN gives rise to equimolecular amounts of neurotensin and NMN. In the gut, the precursor is preferentially cleaved at the two most C-terminal pairs of dibasic residues, giving rise to comparable amounts of neurotensin and a large biologically active peptide

beginning after the signal peptide and ending with the NMN sequence (Carraway et al., 1992). PC1 has been identified as the enzyme required for cleavage of pro-NT/NMN in the mammalian gut (Villeneuve et al., 2002 and Rehfeld et al., 2003).

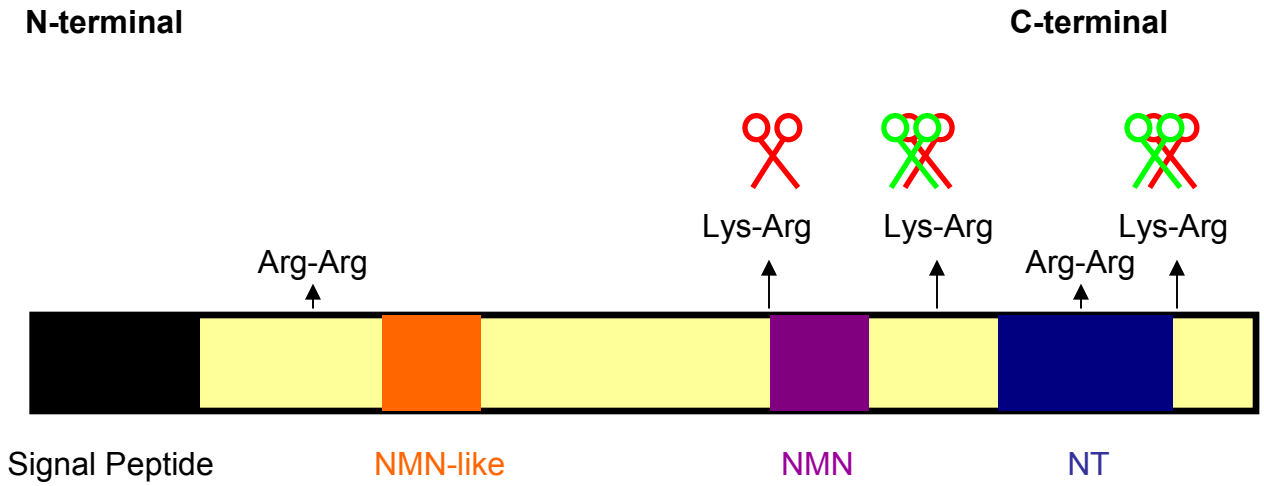


Figure vii: The NT/NMN Precursor. A dibasic sequence (Lys-Arg) flanks the N-terminus of NMN and the C-terminus of neurotensin. Another dibasic sequence separates NMN and neurotensin. The Arg-Arg sequences within neurotensin and preceding the NMN-like sequence are somehow protected from cleavage. Sequences cleaved in the mouse brain are indicated by red scissors, while sites cleaved in the mouse intestine are represented by green scissors.

Neurotensin's effects are mediated by signaling membrane receptors. Two such receptors were cloned in recent years. Neurotensin receptor 1 (NTR1) and neurotensin receptor 2 (NTR2) are both members of the family of G protein-coupled receptors (Chalon et al., 1996). NTR1 has a high affinity for neurotensin

(k_d [binding affinity] = 0.1-0.3 nM) whereas NTR2 displays a lower affinity for neurotensin (binding affinity = 3-5 nM). NTR1 expression is found within the brain and intestine, while NTR2 expression is localized mostly in the brain (Mazella et al., 1996). Additionally, NTR1 is found within the ~23% of the neurons in the rat L4 and L5 DRG (L4/5). These neurons are of a small, non-peptidergic population. Synthesis of NTR1 is decreased in response to nerve injury in the DRG (Zhang et al., 1995). The signaling pathway for NTR1 is well documented, including the release of calcium following inositol triphosphate (IP3) stimulation in response to coupling of NTR1 to phospholipase C (PLC), and activation of mitogen-activated protein (MAP) kinases via the protein kinase C (PKC). NTR3 receptor/gp95 sortilin (sortilin) was recently identified as the third receptor for neurotensin. A non-G protein-coupled receptor, sortilin is structurally different from NTR1 and NTR2 and may therefore play a unique role in the processing of neurotensin.

In uninjured rat L4/5 DRG, only a few neurons express neurotensin immunoreactivity. In the rat, a marked increase in neurotensin (message and peptide) is observed in many large-sized neurons in L4/5 DRG in response to sciatic nerve axotomy (Zhang et al., 1996). Similarly, neurotensin expression has been shown to increase following sciatic nerve axotomy in mice (Tanabe et al., 2003). This increase in neurotensin expression might be a consequence of the general rule that after injury neurons downregulate compounds related to chemical transmission and upregulate those important for repair or survival (Barron, 1984) thus, suggesting an important role in regulating survival.

6. Goal of the Study

Functional outcome following peripheral nerve injury remains disappointingly poor despite highly precise microsurgical repair techniques. Prevention of neuronal death after peripheral nerve injury is vital to regaining quality of sensation and cutaneous innervation density (Wiberg et al., 2003). Focusing on the neurobiology of injury-induced neuronal loss will result in the creation of new, successful therapeutic interventions that preclude these anatomical and functional deficits. The main goal of this study is to better understand the role of neurotensin in preventing neuronal death after injury. Previous studies have demonstrated the ability of neurotensin to impair the induction of proNGF-induced cell death by up to 90% in cultured neurons (Nykjaer et al., 2004). Here, we investigate the role of neurotensin in hindering injury-induced neuronal death *in vivo*. A novel neuroprotective role for neurotensin offers important clinical significance in designing new treatments aimed at preventing sensory deficits associated with nerve injury while simultaneously providing insight into the etiology of neurodegenerative diseases such as Alzheimer's disease. Expanding our understanding of the molecular mechanisms involved in neuronal loss will precede an increase in the depth of knowledge surrounding the pathology of neurodegeneration and improved clinical strategies concentrated on preventing the functional deficits and cognitive impairments associated with neuronal loss.

VI. Chapter One: proNGF, sortilin, and $p75^{NTR}$: Potential Mediators of Injury-Induced Apoptosis in Primary Sensory Neurons of the Mouse DRG. Arnett M.G., Ryals J.M., Wright D.E. (2007).

A. Abstract

The nerve growth factor precursor (proNGF) may function as a death-inducing ligand that mediates its apoptotic effects via $p75^{NTR}$. ProNGF-induced apoptosis is postulated to be dependent upon membrane expression of the sortilin receptor, which interacts with $p75^{NTR}$ to promote a high affinity-binding site for proNGF. Here, we explore the expression of proNGF, sortilin and $p75^{NTR}$ in the mouse dorsal root ganglion (DRG) to understand the potential for this trimeric signaling complex to function in injury-induced neuronal death of DRG neurons. Our results reveal the expression of all three components within the DRG and that a subpopulation of neurons coexpresses sortilin and $p75^{NTR}$. Following nerve transection, the expression of these peptides appears insensitive to injury; however, the majority of small $p75^{NTR}$-sortilin coexpressing neurons are lost 25 days after nerve transection. These results propose proNGF-induced, $p75^{NTR}$-sortilin mediated neuronal death as a critical modulator of nerve-injury induced death in the DRG.

B. Introduction

Traumatic nerve injury frequently leads to loss of function due to neuronal degeneration and death. The molecular mechanisms involved in injury-induced neuronal death are poorly understood but are thought to involve apoptosis, or programmed cell death (Ekstrom, 1995). Recently, several molecules have been identified as key players in the induction of neuronal death. Sortilin, a type I membrane receptor expressed in neuronal tissues involved in membrane trafficking, has been shown to interact with the p75 neurotrophin receptor ($p75^{NTR}$) in the induction of apoptosis upon binding unprocessed nerve growth factor, also known as proNGF (Nykjaer et al., 2004).

Neurotrophins are growth factors released by target tissues that can regulate cell survival and death signaling. Neurotrophins, such as nerve growth factor (NGF), are synthesized as precursors known as proneurotrophins. These pro-forms are susceptible to intracellular as well as extracellular cleavage by numerous proteolytic enzymes including furin, plasmin and MMP (Lee et al., 2001). Proneurotrophins have long been thought to be inactive precursors to their mature counterparts, but recent evidence has identified proNGF as the predominant form of nerve growth factor in many tissues, recognizing it as a potentially biologically important molecule (Lee et al., 2001; Yardley et al., 2000; Fahnestock et al., 2001; Pedraza et al., 2005; and Reinshagen et al., 2000). Neurotrophins are capable of mediating profound diverse biological effects depending on processing of the pro-forms and their interaction with two cell surface receptors, $p75^{NTR}$ and the *trk* family of tyrosine kinase receptors. For

example, mature NGF preferentially binds to *trk* A and promotes cell survival. However, recent reports have demonstrated that proneurotrophins, such as proNGF, selectively bind to $p75^{NTR}$ and activate its pro-apoptotic signaling cascade (Nykjaer et al., 2004). The formation of a signaling complex in which the sortilin receptor interacts with $p75^{NTR}$ is required for proNGF-mediated neuronal death to occur (Nykjaer et al., 2004). ProNGF simultaneously binds sortilin (via the pro-domain) and $p75^{NTR}$ (via the mature domain) and thereby acts as a crosslinker, creating a tertiary complex that results in the activation of neuronal apoptosis.

Evidence identifying proNGF as a physiological ligand for $p75^{NTR}$ under pathological conditions continues to build. Recent reports demonstrate that proNGF expression is induced after brain injury and causes death of adult corticospinal neurons upon binding $p75^{NTR}$ *in vivo* (Harrington et al., 2004). Likewise, proNGF mediates cell death of oligodendrocytes following spinal cord injury *in vivo* (Beattie et al., 2002) and has been suggested to be responsible for the neurodegeneration found in the brains of Alzheimer's disease patients (Pedraza et al., 2005).

In this report, we investigate whether members of the proNGF-$p75^{NTR}$-sortilin signaling complex could potentially be responsible for the injury-induced apoptosis of primary sensory neurons in the dorsal root ganglia (DRG). The results presented here demonstrate the following: (1) members of this signaling complex are present in the DRG; (2) their expression is insensitive to the effects of injury; (3) there is a population of DRG neurons that coexpresses both

receptors, and further, (4) that this unique subpopulation of neurons is particularly vulnerable to injury-induced neuronal death as more than 59% of small-sized sortilin-$p75^{NTR}$ coexpressing neurons are lost 25 days following sciatic nerve transection. These data propose this signaling complex as an apoptotic-inducing mechanism amongst sensory neurons following injury.

C. Results

Sortilin is present in the mouse DRG and its expression is not responsive to injury

Immunocytochemistry was performed to identify DRG neurons that express sortilin in conjunction with the neuronal marker NeuN. NeuN is a neuronal protein that is observed in the nucleus of sensory neurons (Mullen et al., 1992). 73.8% +/- 4.82 of L4/5 DRG neurons express the sortilin receptor (Fig. 1C). A diffuse cytoplasmic distribution of sortilin is visible in neurons which express the receptor (Fig. 1B). These data are consistent with previous studies that localize over 90% of sortilin expression to intracellular vesicles including the golgi apparatus and endo- and lysosomal compartments (Petersen et al., 1997 and Mazella et al., 1998). The studies carried out here are the first to identify the presence of the sortilin receptor in primary sensory DRG neurons of the mouse.

Next, we evaluated the responsiveness of sortilin expression to injury. L4/5 DRG were examined for sortilin expression three days following sciatic nerve transection. At this time point, injury results in little neuronal death but effects the expression of many neuropeptides (Shi et al., 2001 and McKay Hart

et al., 2002). This allows us to determine injury-induced effects upon sortilin expression while excluding the possibility of significant cell loss. 72.8% +/- 3.50 of neurons were positive for sortilin expression after injury (Fig. 2C). An unpaired t-test determined that there was no significant difference in the number sortilin-expressing neurons between injured and uninjured groups (P = 0.3573). To substantiate this result, we used Western blot analysis to quantify sortilin peptide expression. Using the same antisera, we were able to detect a band of 95 kDa, which is consistent with the molecular weight of sortilin (Petersen et al., 1997) (Fig. 3). Peptide expression was quantified by analyzing band intensity and no significant difference in the number of neurons expressing sortilin was observed between injured and uninjured groups (P = 0.8643). These data support our previous finding that sortilin expression is not altered in response to injury in DRG neurons.

Sortilin is expressed in several neuronal subpopulations within the lumbar DRG

Next, we sought to determine if sortilin expression is confined to a specific neuronal subpopulation within the DRG. DRG neurons can be divided into 3 broad subcategories based on expression of neuronal markers. The first class consists of neurons that do not express the classic neuropeptides generally associated with pain transmission; however, they will bind the isolectin IB4, which is specific for small, non-peptidergic sensory neurons (Kashiba et al., 2001). The second subpopulation consists of neurons that produce calcitonin gene-related peptide (CGRP), which exclusively identifies the small- and medium-sized

peptidergic sensory neurons (Silverman et al., 1988). Finally, there is a class of neurons that can be identified using the neuronal marker N52, which is specific for large, myelinated neurons. Uninjured DRG neurons that express both the sortilin receptor and a specific neuronal marker were counted. Immunocytochemistry revealed 13.6% +/- 1.16 of sortilin-positive neurons express IB4. Alternatively, 31.7% +/- 3.69 of IB4-positive neurons express the sortilin receptor (Fig. 4C). Approximately 20.6% +/- 1.47 of sortilin-expressing neurons coexpress CGRP, while 44.1% +/- 3.10 of CGRP-positive neurons express sortilin (Fig. 5C). Finally, approximately 18.2% +/- 2.57 of neurons that express sortilin also coexpress N52. On the contrary, 52.8% +/- 4.95 of N52-expressing neurons display sortilin coexpression (Fig. 6C). In conclusion, sortilin expression is found within the small non-peptidergic neurons, the small-medium sized peptidergic neurons, and finally the large, myelinated neurons. These data indicate that sortilin expression is not confined strictly to one primary sensory neuronal population, but rather it is found within all classes of DRG neurons as categorized here.

$p75^{NTR}$ is present in the DRG and its expression is not responsive to injury

DRG neurons expressing $p75^{NTR}$ were identified using a primary polyclonal antibody in combination with the pan-neuronal marker NeuN. Using immunocytochemistry, we determined that 66.1% +/- 2.83 of DRG neurons from uninjured mice express $p75^{NTR}$ (Fig. 7C). According to the literature, $p75^{NTR}$ expression is upregulated in response to injury, throughout various disease

states, and during development (King et al., 2000; Syroid et al., 2000; and Crockett et al., 2000). However, these studies were carried out in the central nervous system. We were curious to determine if injury alters $p75^{NTR}$ expression in the peripheral nervous system. To address this question, we evaluated the DRG from mice that underwent sciatic nerve transection. Three days following injury, 67.2% +/- 4.43 of neurons express $p75^{NTR}$ (Fig. 8C). An unpaired t-test confirmed no significant difference in the average number of neurons expressing $p75^{NTR}$ between groups (P = 0.8248). To confirm these results, we employed Western blot analysis to re-examine the effects of injury upon $p75^{NTR}$ expression. Application of the primary polyclonal antibody resulted in the presence of a ~75 kDa band along with bands of approximately ~55, ~135, and ~155 kDa in size (Fig. 9). These multiple bands were present in samples taken from both injured and uninjured mice. Band intensity was analyzed to detect any alteration in peptide expression. An unpaired t-test did not detect any significant difference in $p75^{NTR}$ expression between groups (P = 0.7836), confirming our immunocytochemical results.

proNGF is the predominant form of NGF present in the mouse DRG

Next, we sought to determine if the pro-form of nerve growth factor is present in the mouse DRG and if its expression is responsive to injury. To date, this was the first assessment of proNGF expression in the mouse DRG. Western blot analysis using a polyclonal antibody designed to detect the pro-form of proNGF detects bands of approximately ~35, ~40, ~44, and ~87 kDa in size (Fig.

10A). Each band is present in all of the treatment groups (no injury and sciatic nerve crush or transection). The ~ 35 kDa band closely resembles the molecular weight of the unglycosylated NGF precursor, as detected by Yardley and colleagues (Yardley et al., 2000). The ~40, ~44, and ~87 kDa bands most likely represent post-translationally modified forms of proNGF (Yiangou et al., 2002 and Lobos et al., 2005). To address the effects of injury upon proNGF expression, band intensity was analyzed between treatment groups. ANOVA did not reveal a significant effect of group on band intensity, indicating that proNGF expression is not altered in mouse sensory neurons in response to peripheral nerve injury (P = 0.9990). Next, we inquired if the bands detected by our proNGF antibody truly represented peptides of proNGF nature. To address this question, we implemented the use of a polyclonal antibody aimed at identifying the mature domain of NGF. First, using a commercial source of recombinant NGF peptide, we ensured that this particular antibody would indeed detect mature NGF (Fig. 10B). Similarly, the same bands of ~35, ~40, ~44, and ~87 kDa were again detected in each treatment group. On the contrary, a band of ~91 kDa was also present which was not detectable with the use of proNGF antisera, which suggest the band represented at ~91 kDa to not be of true NGF origin (Fig. 10C). Interestingly, we were not able to distinguish a band for mature NGF (~13 kDa), signifying that mature NGF is not present in detectable quantities under injured nor uninjured conditions in the mouse DRG. Again, band intensity was analyzed and no significant difference in peptide expression was detected between groups using one way ANOVA (P = 0.9868).

Identification of a unique population of DRG neurons that coexpress sortilin and p75^{NTR}

Next, we examined if there exists within the DRG a unique population of neurons that coexpress the sortilin and p75^{NTR} receptors, making them particularly vulnerable to proNGF-induced neuronal death. To examine this matter, we used immunocytochemistry to identify which neurons were positive for both sortilin and p75^{NTR} expression. First, the total number of neurons expressing either the sortilin or the p75^{NTR} receptor was determined separately. Next, we assessed the percentage of sortilin-positive neurons coexpressing p75^{NTR} and vice versa, the number of p75^{NTR}-positive neurons coexpressing sortilin in uninjured animals. Analysis revealed 52.5% +/- 3.77 of sortilin-positive neurons express p75^{NTR}. Alternatively, 50.7% +/- 3.84 of p75^{NTR}-positive neurons coexpress the sortilin receptor (Fig. 11C). Three days following sciatic nerve transection, 54.5% +/- 2.82 of sortilin-positive neurons continue to coexpress p75^{NTR} and 49.9% +/- 0.83 of p75^{NTR}-positive neurons maintain sortilin expression (Fig. 12C). These data suggest that there is no significant change in the number of neurons coexpressing sortilin and p75^{NTR} after injury.

To determine if this neuronal subpopulation was vulnerable to death following injury, we extended the time-frame at which neuronal death was assessed and examined the number of sortilin-p75^{NTR} coexpressing neurons twenty-five days following sciatic nerve transection. We observed a 59.4% loss of sortilin-p75^{NTR} coexpressing neurons with 803 neurons counted as sortilin-

p75^{NTR}-positive in uninjured animals and only 326 sortilin-p75^{NTR}-positive neurons present in mice 25 days following sciatic nerve axotomy.

Small – medium sized, sortilin-p75^{NTR} coexpressing DRG neurons are vulnerable to injury-induced neuronal death

After establishing that a large percentage of sortilin-p75^{NTR} coexpressing neurons are lost due to the effects of injury, we sought to define which particular class of neurons among the sortilin-p75^{NTR} coexpressing neuronal population is particularly susceptible to injury-induced neuronal death. To identify this population, we measured the size of sortilin-p75^{NTR} coexpressing neurons present in uninjured mice but absent 25 days following sciatic nerve axotomy. These data indicate that small neurons (0-200 μm^2) comprise 58.03% of the total number of neurons in uninjured mice. However, neurons of an equivalent area were completely absent in the injured group. Likewise, medium-sized neurons (200-400 μm^2) compose 22.79% of the uninjured population while only 1.84% of neurons measured were found of the same area within the injured group. Finally, 19.18% of the total neuronal population can be classified as large (<400 μm^2) in uninjured mice while this size class accounts for the majority of the total neuronal population (98.16%) in injured mice. These results are represented in a histogram-based model by a large shift to the right, indicating that the percentage of small and medium-sized neurons is drastically reduced in injured mice while the large-sized neuronal population is maintained (Fig. 13). These data suggest that among sortilin-p75^{NTR} coexpressing DRG neurons, the small and medium-

sized neuronal populations are especially vulnerable to injury-induced neuronal death.

D. Figures

Figure 1A-2C: Immunohistochemcial Analysis of Sortilin Expression in Injured and Uninjured DRG of C57BL/6 Mice. Immunohistochemistry performed on L4/5 DRG of C57BL/6 uninjured (1 A-C) and injured (2 A-C) (three days after sciatic nerve transection) mice. The neuronal marker NeuN (red) (1A and 2A) identifies neurons that express sortilin (green) (1B and 2B). 73.8% ± 4.82 of neurons express sortilin in uninjured mice (1C) while 72.8% ± 3.50 of neurons express the sortilin receptor in injured mice (2C). Filled arrows identify neurons that express the sortilin receptor. Open arrows indicate neurons that are negative for sortilin expression (1C and 2C).

Figure 1A – 2C: Immunohistochemical Analysis of Sortilin Expression in Injured and Uninjured DRG of C57BL/6 Mice.

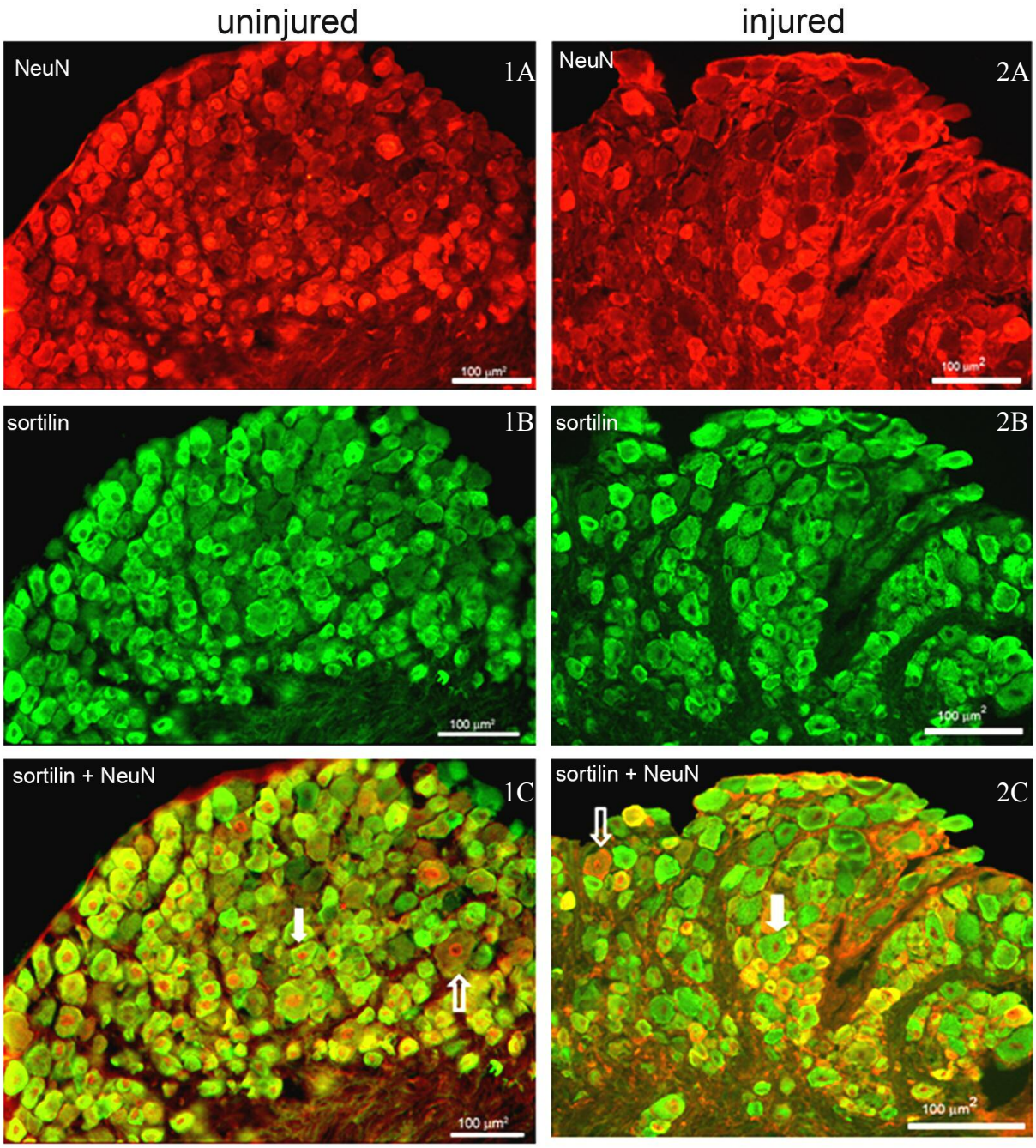


Figure 3: Western Blot Analysis of Sortilin Expression in Injured and Uninjured DRG of C57BL/6 Mice. Immunoblotting analysis performed using L4/5 DRG three days following no injury (lane 1) or sciatic nerve transection (lane 2). A band present at ~95 kDa represents the sortilin receptor. All protein loading was normalized to cyclophilin A (18 kDa). An unpaired t-test performed on band intensity revealed no significant difference in sortilin expression between uninjured and injured groups (P = 0.8643).

Figure 3: Western Blot Analysis of Sortilin Expression in Injured and Uninjured DRG of C57BL/6 Mice.

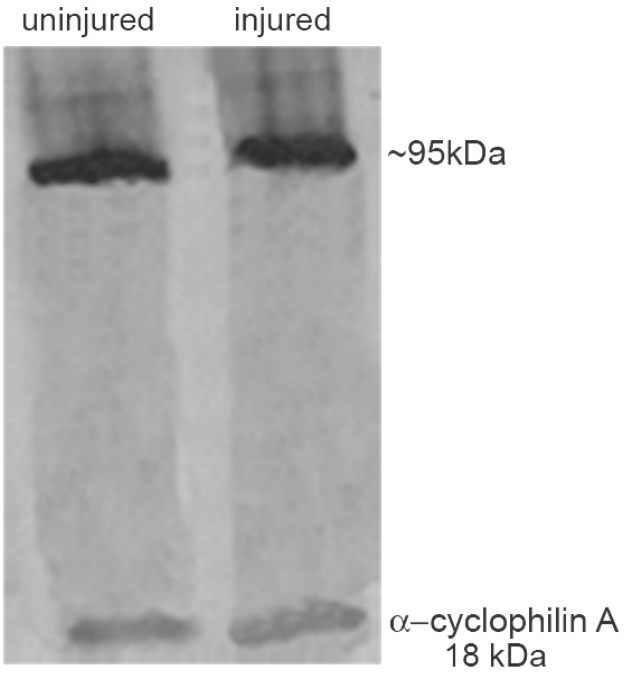


Figure 4A-6C: Sortilin Expression in the CGRP, IB4, and N52 Neuronal Subpopulations of the C57BL/6 DRG. Immunohistochemistry analysis performed on L4/5 DRG from C57Bl/6 uninjured mice using a primary polyclonal sortilin (red) antibody (4B, 5B, and 6B) in conjunction with the neuronal marker IB4 (green) (4A), which identifies the small, non-peptidergic sensory neuronal population, the neuronal marker CGRP (red) (5A), which identifies the small-medium sized, peptidergic sensory neuronal population, and the neuronal marker N52 (green) (6A), which identifies the large, myelinated sensory neuronal population. 13.6% ± 1.16 of sortilin-positive neurons express IB4 and 31.7% ± 3.69 of IB4-positive neurons express the sortilin receptor (4C). 20.6% ± 1.47 of sortilin-positive neurons express CGRP while 44.1% ± 3.10 of CGRP-positive neurons express sortilin (5C) and finally, an average of 18.2% ± 2.57 of sortilin-positive neurons express N52 and 52.8% ± 4.95 of N52-positive neurons express sortilin (6C).

Figure 4A-6C: Sortilin Expression in the CGRP, IB4, and N52 Neuronal Subpopulations in the C57BL/6 DRG.

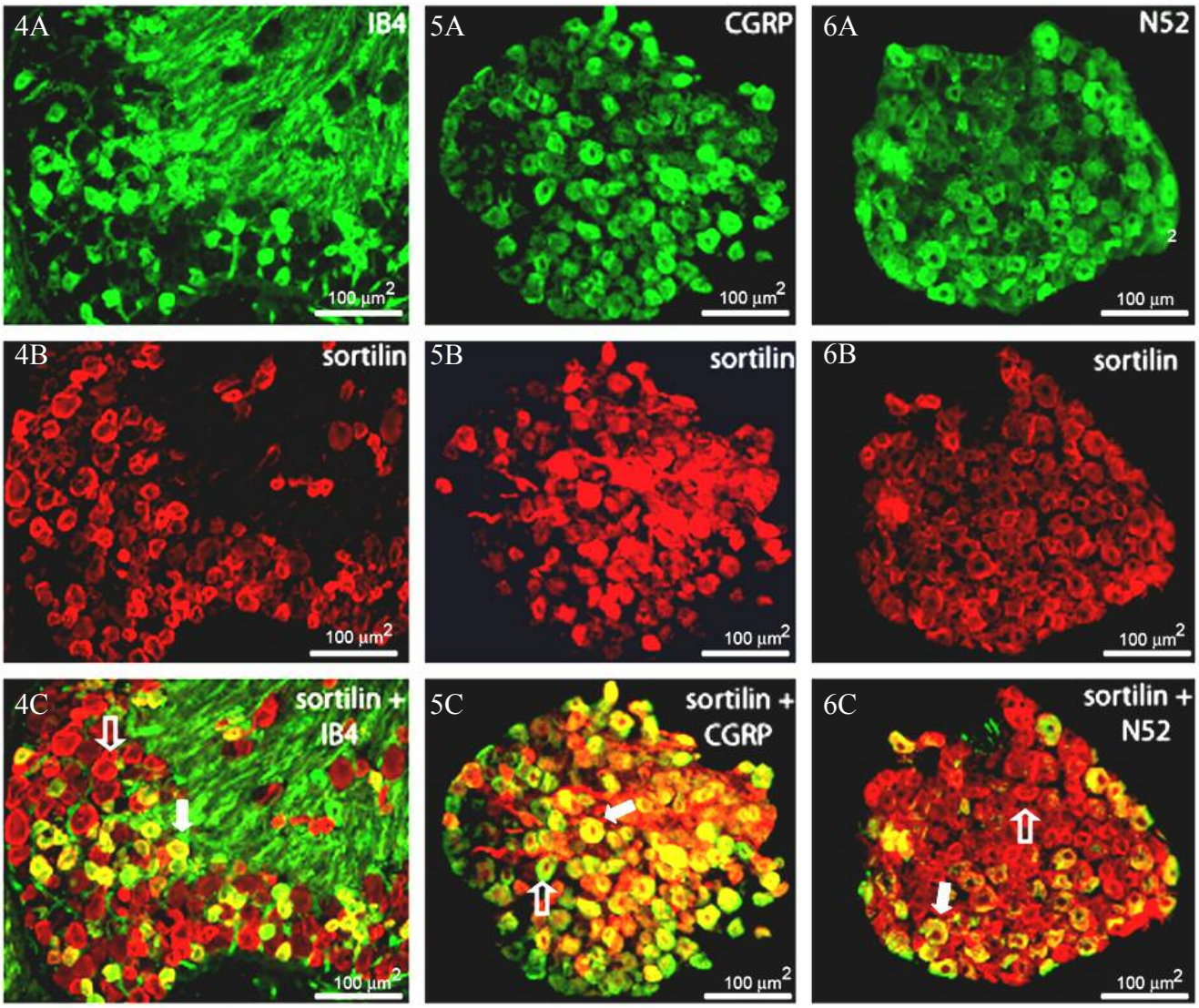


Figure 7A-8C: Immunohistochemical Analysis of $p75^{NTR}$ Expression in the C57BL/6 Mouse DRG. Immunohistochemistry performed on L4/5 DRG of C57Bl/6 uninjured (7 A-C) and injured (8 A-C) (three days after sciatic nerve transection) mice. The neuronal marker NeuN (red) (7A and 8A) identifies neurons that express $p75^{NTR}$ (green) (7B and 8B). 66.1% ± 2.83 of neurons express $p75^{NTR}$ in uninjured mice (7C). 67.2% ± 4.43 of neurons express $p75^{NTR}$ (8C) in injured mice. Filled arrows identify neurons that express $p75^{NTR}$. Neurons that do not express $p75^{NTR}$ are identified by open arrows.

Figure 7A-8C: Immunohistochemical Analysis of p75^{NTR} Expression in the C57BL/6 Mouse DRG.

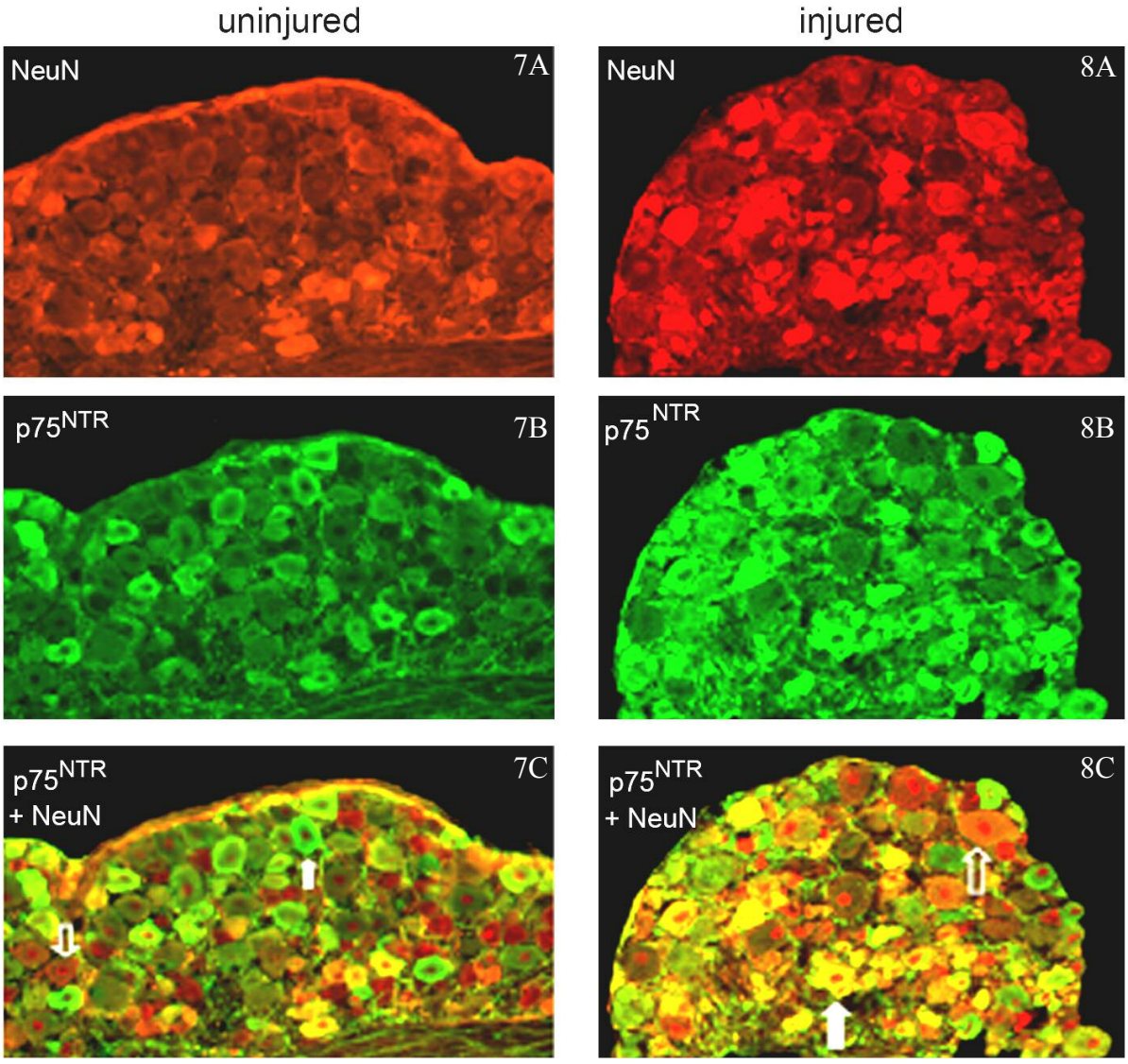


Figure 9: Western Blot Analysis of $p75^{NTR}$ Expression in the C57BL/6 Mouse DRG. Immunoblotting analysis performed using L4/5 DRG three days following no injury (lane 1) or sciatic nerve transection (lane 2). A band present at ~75 kDa represents the full-length $p75^{NTR}$ receptor while other bands represent the ecto-domain (55 kDa) and possible post-translationally modified forms of $p75^{NTR}$ (135 and 155 kDa). All protein loading was normalized to cyclophilin A (18 kDa). An unpaired t-test performed on band intensity revealed no significant difference in $p75^{NTR}$ expression between uninjured and injured groups ($P = 0.7836$).

Figure 9: Western Blot Analysis of $p75^{NTR}$ Expression in the C57BL/6 Mouse DRG.

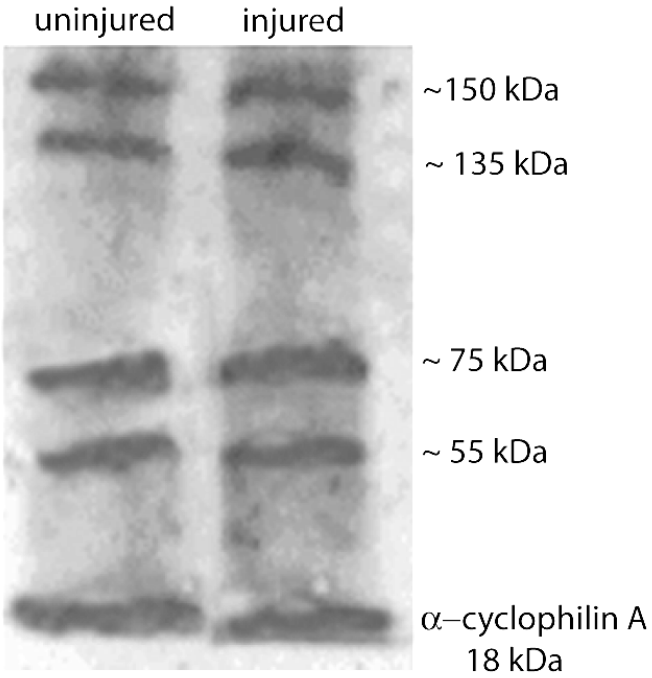


Figure 10 A-C: Western Blot Analysis of proNGF Expression in the C57Bl/6 Mouse DRG.

10A: Immunoblotting analysis performed using L4/5 DRG from the following groups of mice: uninjured (lane 1), three day post-sciatic nerve crush (lane 2), or transection (lane 3). A polyclonal antibody specific for the pro-domain of proNGF detected multiple bands present at ~35, ~40, ~44, and ~87 kDa. The unglycosylated NGF precursor was identified as the ~35 kDa band. Post-translationally modified forms of proNGF are represented as bands of ~40, ~44, and ~87 kDa. Statistical analysis using ANOVA revealed no significant difference in band intensity amongst groups (P = 0.9990). All protein loading was normalized to cyclophilin A (18 kDa).

10B: Recombinant mouse NGF (20 ng/lane) was loaded as a positive control to test the specificity of the anti-mature NGF antibody.

10C: Samples were taken from the following groups of mice: uninjured (lane 1), three day sciatic nerve crush (land 2), or transection (lane 3). A primary polyclonal antibody against the mature domain of NGF detected bands of ~35, ~40, ~44, and ~87 kDa represent post-translationally modified forms of proNGF. A band of ~91 kDa, which was not detectable with the proNGF antibody, was determined to correspond with a peptide of non-NGF origin. Mature NGF (~13 kDa) was not present in detectable concentrations. No significant change in peptide expression was detected by ANOVA between treatment groups (P = 0.9868). All protein loading was normalized to cyclophilin A (18 kDa).

Figure 10 A-C: Western Blot Analysis of proNGF Expression in the C57Bl/6 Mouse DRG.

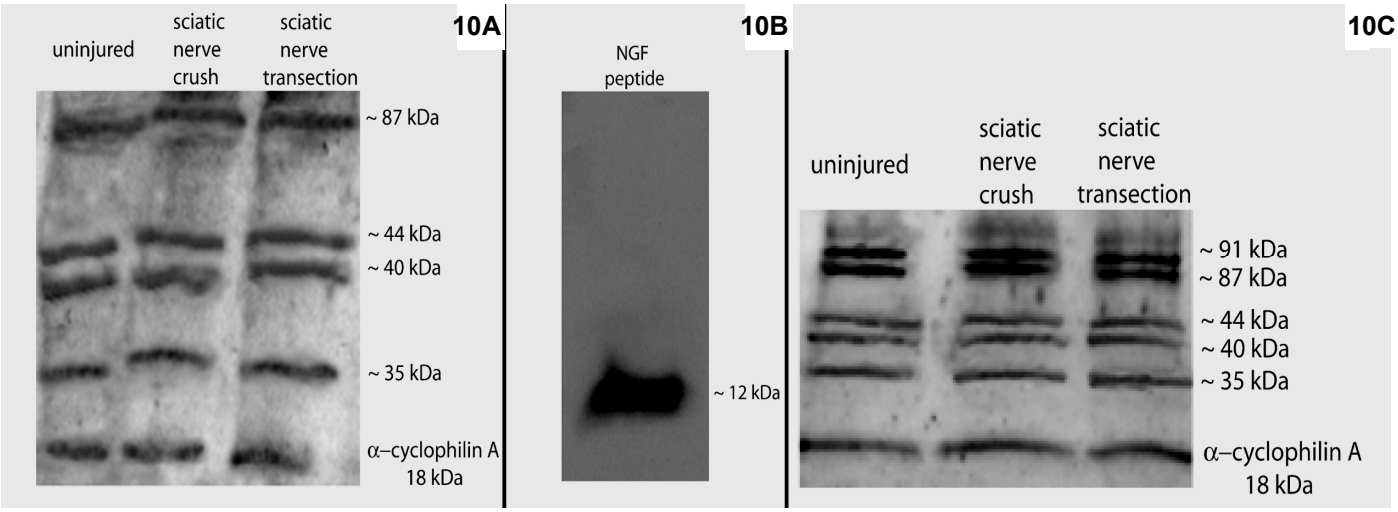


Figure 11A-12C: Sortilin and $p75^{NTR}$ Coexpression in the C57BL/6 Mouse DRG. Immunohistochemistry was performed on L4/5 DRG of C57BL/6 uninjured (11 A-C) and injured (12 A-C) (three days after sciatic nerve transection) mice using primary antibodies to detect neurons which express both $p75^{NTR}$ (green) (11A and 12A) and sortilin (red) (11B and 12B). On average, 52.5% ± 3.77 of sortilin-positive neurons express $p75^{NTR}$ in uninjured mice (11C). Alternatively, 50.7% ± 3.84 of $p75^{NTR}$-positive neurons coexpress the sortilin receptor (11C). In injured mice, 54.5% ± 2.82 of sortilin-positive neurons express $p75^{NTR}$ and 49.98% ± 0.83 of $p75^{NTR}$-positive neurons express sortilin (12C). Filled arrows identify neurons which coexpress both $p75^{NTR}$ and sortilin. Neurons that do not coexpress $p75^{NTR}$ and sortilin are identified with open arrows (11C and 12C).

Figure 11A-12C: Sortilin and p75^{NTR} Coexpression in the C57BL/6 Mouse DRG.

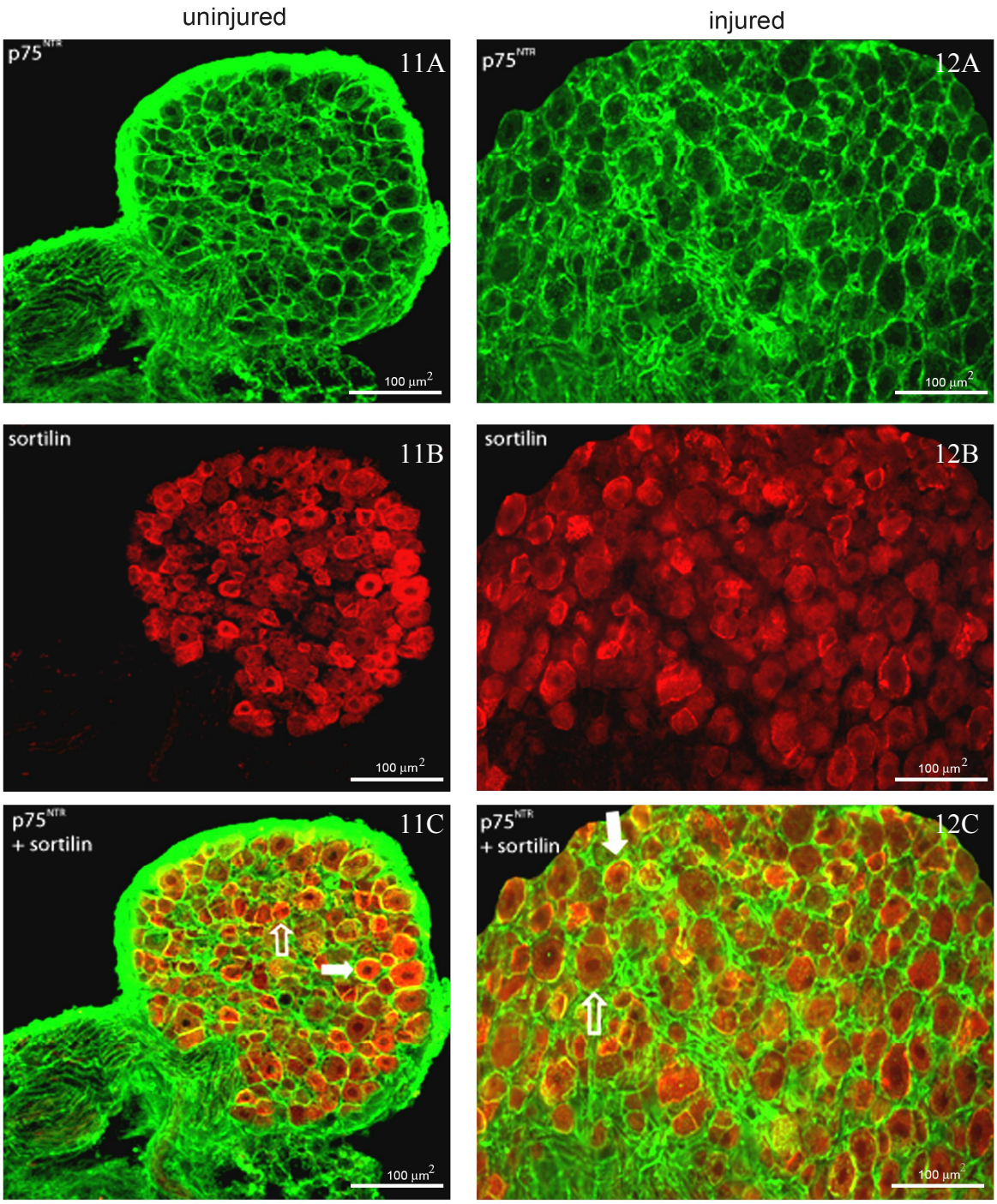
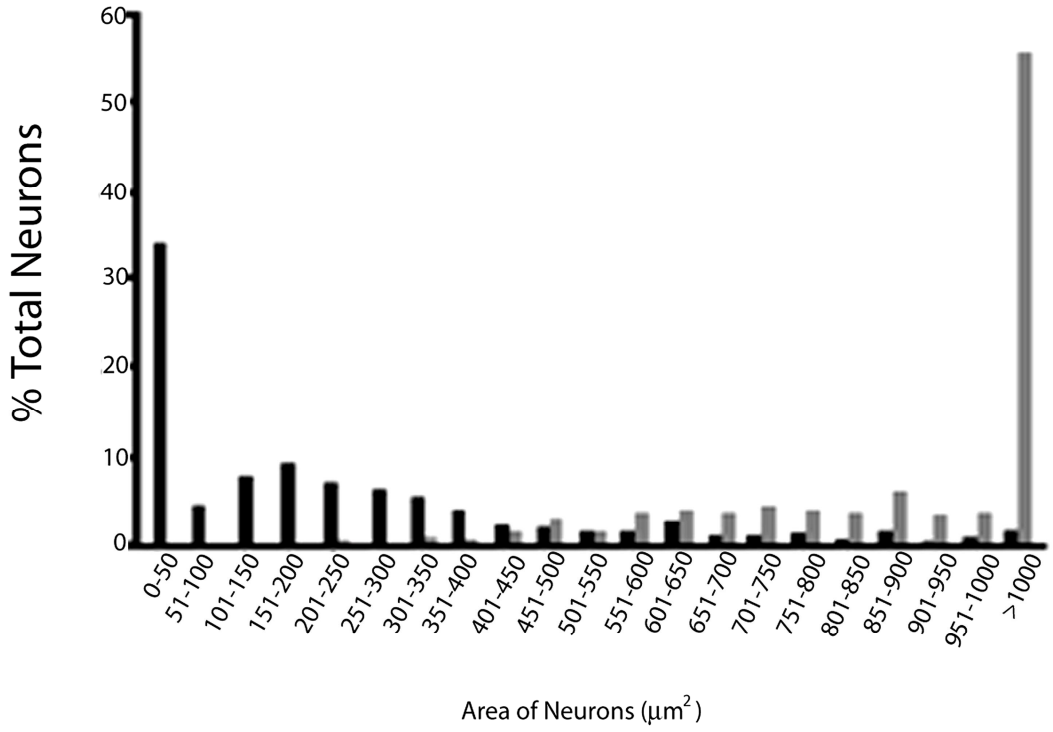


Figure 13: Size-Based Histogram of Injured and Uninjured DRG. Size-frequency histogram illustrating the distribution of neuronal area (μm^2) of L4/5 DRG neurons in mice with no injury (black bars) and mice that underwent sciatic nerve transection (grey bars) twenty-five days prior. Small neurons (0-200 μm^2) comprise 58.03% of the total number of neurons in uninjured mice. This same neuronal population is completely absent in the injured group. Medium-sized neurons (200-400 μm^2) comprise 22.79% of the neurons in uninjured mice but only 1.84% in injured mice.

Figure 13: Size-Based Histogram of Injured and Uninjured DRG.



E. Discussion

Peripheral nerve injuries are very common, with several hundred thousand cases occurring each year within the United States alone (Wiberg et al., 2003). Neurodegeneration occurs as a result of traumatic nerve injury. To date, the mechanisms underlying this pronounced neuron loss remain to be revealed. It is clear that a better understanding of the molecular mechanisms involved in injury-induced neuronal death is critical to the creation of new therapeutic strategies aimed at preventing the sensory deficits that result from peripheral nerve injuries.

The purpose of this study was to identify a possible mechanism for the neuronal death that occurs amongst the primary sensory DRG neurons following peripheral nerve injury. Recently, several key players have been implicated in the induction of neuronal death; including the sortilin and $p75^{NTR}$ receptors and the death-inducing ligand, proNGF. Sortilin and $p75^{NTR}$ cooperate to promote a high-affinity binding site for proNGF (Nykjaer et al., 2004). These three individual components thereby interact to create a trimeric signaling complex that is capable of inducing apoptosis.

Our first goal was to confirm the existence of these pro-apoptotic components in the DRG in order to determine if these participants might indeed be responsible for the injury-induced neurodegeneration observed here.

proNGF

Neurotrophins, such as nerve growth factor (NGF), are growth factors that regulate neuronal survival and death signaling (Hempstead 2006). They are

synthesized as precursors, known as proneurotrophins, which can be cleaved intracellularly for release as mature ligands. Proneurotrophins, such as proNGF, have long been thought to be inactive precursors to their mature counterparts. However, recent publications show that proneurotrophins are detectable in the extracellular environment, indicating that they may have biologically important roles (Lee et al., 2001). In fact, proNGF is the dominant form of NGF present in many tissues (Chen et al., 1997; Harrington et al., 2004; Yardley et al., 2000; Fahnestock et al., 2001; Pedraza et al., 2005; Fahnestock et al., 2004, Seidah et al., 1996 and Reinshagen et al., 2000). Recently, it has been reported that proNGF induces apoptosis in cells coexpressing sortilin and $p75^{NTR}$ *in vivo,* identifying proNGF as a pathological ligand (Pedraza et al., 2005; Harrington et al., 2004; Beattie et al., 2002; and Volosin et al., 2006). We sought to determine if proNGF was present in the mouse DRG and might therefore behave as a pro-apoptotic ligand. In our study, we were able to detect several different molecular weight forms of proNGF. The multiple isoforms represent the numerous cleavage and glycosylation sites available, rendering proNGF susceptible to post-translational modification. All of the isoforms were present in each of the treatment groups; no injury, and sciatic nerve crush or transection. Furthermore, none of the isoforms displayed any modification in response to injury. Interestingly, we were not able to detect the presence of mature NGF (~13 kDa). Taken together, these data suggest that proNGF is present as the predominant form of nerve growth factor in the mouse DRG and furthermore, that proNGF expression is not modified following nerve injury, in contrast to other

neurotrophins such as brain derived neurotrophic factor (BDNF) (Karchewski et al., 2002).

sortilin

Sortilin, a member of the Vps10p family of receptors, cooperates with $p75^{NTR}$ to promote high-affinity binding of proNGF. It is a required component for proNGF-induced, $p75^{NTR}$-mediated apoptosis, identifying it as a key determinant in the induction of post-traumatic nerve injury-induced apoptosis (Nykjaer et al., 2004). Sortilin expression is widely distributed throughout the central nervous system (Sarret et al., 2003) as well in several peripheral cell types including adipocytes (Morris et al., 1998), skeletal muscle, placenta, heart, thyroid, and testis (Peterson et al., 1997). We sought to determine if the sortilin receptor was expressed in sensory neuronal tissues, a novel approach to determining if these primary sensory neurons are prone to neuronal apoptosis via the sortilin-$p75^{NTR}$-proNGF complex. Our data indicate that the majority of DRG neurons express sortilin (~73%). Furthermore, 72.8% of neurons continue to express sortilin three days after injury. Since there is no significant neuronal loss in L5 mouse DRG three days following sciatic nerve axotomy (Shi et al., 2001), we can assume that sortilin peptide expression is not altered in response to injury.

Next, we aimed to investigate which DRG subpopulations express sortilin. DRG neurons can be divided into three broad subcategories based on soma size, peptide production, neurotrophic support, and expression of neuronal markers. The first neuronal subpopulation can be classified as the non-

peptidergic group. These neurons do not produce the classic neuropeptides generally associated with pain transmission; however, they will bind the isolectin IB4 and are therefore commonly referred to as the IB4-positive subpopulation. Here, we report an average of 13.6% of sortilin-positive neurons express the neuronal marker IB4. Alternatively, 31.7% of IB4-positive neurons express the sortilin receptor. The second category consists of small-medium sized peptidergic neurons which produce calcitonin gene related peptide (CGRP). On average, 20.6% of sortilin-positive neurons express CGRP while 44.1% of CGRP-positive neurons express sortilin. Finally, there is a class of neurons which consists of predominantly large fiber proprioceptive neurons that can be identified using the neuronal marker N52. 18.2% of sortilin-positive neurons express N52 while 52.8% of N52-positive neurons express sortilin. Although sortilin expression is found in all subpopulations of DRG neurons, it is unclear why sortilin expression is not found within all neurons of the DRG. One explanation is that sortilin might define a target specific pattern of expression. For example, neurons which project to muscle and skin might express sortilin, but not neurons navigating to viscera. These questions can be addressed in the future using retrograde labeling studies to help determine if sortilin expression is target dependent.

p75^{NTR}

p75^{NTR} is a member of the tumor necrosis factor (TNF) receptor superfamily (Roux and Barker, 2002). To date, there has been no enzymatic

activity associated with $p75^{NTR}$, but its associations with several cytoplasmic interactors, such as NRIF, TRAF6, and NRAGE, allow it to induce apoptosis via a cytoplasmic signaling cascade (Kenchappa et al., 2006). The exact molecular events involved in activation of the $p75^{NTR}$ apoptotic pathway are not completely understood but are thought to involve the activation of c-Jun N-terminal kinase (JNK). Further downstream events include phosphorylation of c-jun, and activation of p53 as well as the "BH3-domain only" family members Bad and Bid, which results in mitochondrial release of cytochrome c and the consequent activation of caspases (Nykjaer et al., 2005). $p75^{NTR}$ is a requirement for proNGF-induced neuronal death to occur. This study demonstrates the presence of $p75^{NTR}$ in a majority of DRG neurons (66.1%), localizing the third and final component of the death-inducing complex discussed here to the DRG. Furthermore, the data presented here establish that $p75^{NTR}$ expression is found in 67.2% of neurons following injury, indicating the $p75^{NTR}$ expression is not altered following peripheral nerve injury in the DRG. This result was surprising as previous studies have described dramatic alterations in $p75^{NTR}$ expression after injury (Byers et al., 1992, Arendt et al., 1995, Vaudano et al., 1998, Syroid et al., 2000, King et al., 2000, Xie et al., 2003). One possible explanation for these inconsistencies is the time-frame at which $p75^{NTR}$ expression was assessed after injury. Although the expression of many peptides is modified three days after injury, the discrepancies could potentially be attributed to the designated stage of examination in this study.

We found four distinct forms of the $p75^{NTR}$ receptor by western blot analysis. The band of ~ 75 kDa represents the full-length receptor. The band of ~ 55 kDa represents the intracellular domain (ICD), which is cleaved by γ-secretase in response to binding proapoptotic ligands in neurons (Kenchappa et al., 2006 and Humpert et al., 2007). Higher molecular weight bands of ~155 and ~135 kDa potentially represent a $p75^{NTR}$ dimer and accompanying post-translational modifications, respectively.

Previous studies have shown that $p75^{NTR}$ expression is confined to specific subclasses of neurons within the DRG. $p75^{NTR}$ is coexpressed in almost all of the neurons that express the t*rk* A or t*rk* B receptor (CGRP-positive neuronal subpopulation) while only approximately 50% of the t*rk* C-expressing neurons (N52-positive neuronal subpopulation) are positive for $p75^{NTR}$ expression. Interestingly, $p75^{NTR}$ expression is not found in the small, non-peptidergic sensory neurons (IB4-positive neuronal subpopulation), theoretically rendering this class of neurons resistant to proNGF-induced neuronal death (Wright and Snider 1995). Results presented here indicate that small sortilin-$p75^{NTR}$ coexpressing neurons (<200 μm^2) are lost 25 days after injury. Reportedly, small non-peptidergic neurons do not express $p75^{NTR}$. Thus, suggesting the small peptidergic DRG neurons as the population of neurons which are lost after peripheral nerve injury.

sortilin-p75^{NTR} coexpressing neurons

Here we define a unique DRG neuronal subpopulation coexpressing sortilin and p75^{NTR}, making this distinct neuronal class particularly vulnerable to proNGF-induced neuronal death. Previous studies have established a 54% loss of DRG neurons amongst mice 28 days following sciatic nerve transection (Shi et al., 2001). We report in this study that neither p75^{NTR} nor sortilin peptide expression is altered three days following sciatic nerve transection. Therefore, we are able to attribute a reduction in p75^{NTR} and sortilin immunoreactivity to a loss of neurons due to injury-induced apoptosis. Our results determine that approximately 59% of sortilin-p75^{NTR} coexpressing DRG neurons are lost twenty-five days following sciatic nerve axotomy. Taken together, these data suggest that the injury-induced neuronal loss reported from others to be amongst the sortilin-p75^{NTR} coexpression subpopulation. Thus, these data represent a possible mechanism for mediating neuronal apoptosis amongst peripheral neurons.

Conclusion

In conclusion, we have shown that proNGF, sortilin, and p75^{NTR} are present in mouse DRG neurons, identifying them as potential mediators of neuronal death. Furthermore, we have demonstrated that the number of sortilin-p75^{NTR} coexpressing neurons is reduced dramatically in response to injury. These data suggest a pathological role for proNGF in mediating apoptosis of sensory neurons following peripheral nerve injury *in vivo*. By identifying a likely

mechanism for cell death in a particularly vulnerable population, we can now focus on potential therapeutic strategies aimed at preventing the death of these neurons thereby preventing loss of sensory function.

F. Experimental Methods

Materials

Phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, aprotinin, antipain, sodium fluoride, sodium orthovanadate, ethylenediaminetetraacetic acid disodium dihydrate and all other reagent grade chemicals were purchased from Sigma (St. Louis, MO). Normal donkey/goat sera, as well as porcine gelatin, were obtained from Jackson Immunoresearch (West Grove, PA). 12.5% Tris-HCl gels and 0.4 µm nitrocellulose membranes were purchased from Bio Rad (Hercules, CA).

The anti-sortilin rabbit polyclonal antibody, specific for the extracellular (luminal) domain of sortilin, was generously donated by Dr. Claus Munck Petersen, University of Aarhus, Denmark. The polyclonal antibody specific to the extracellular domain of the $p75^{NTR}$ was a gift from Dr. Louis Reichardt, Howard Hughes Medical Center. The anti-nerve growth factor beta and the anti-pro-NGF polyclonal antibodies were purchased from Chemicon International (Temecula, CA) and Sigma, respectively. Primary antibodies for the neuronal markers CGRP, N52, and IB4 were purchased from Chemicon, Sigma and Molecular Probes (Eugnene, OR), respectively. The secondary antibodies, Alexa Fluro

488/555 and horseradish peroxidase conjugated affinity purified secondary antibody, were purchased from Molecular Probes and Chemicon, respectively.

Animal Model

The experiments were carried out on six-week old, male C57BL/6 mice (Charles River, Wilmington, MA) and were conducted according to the guidelines from the National Institutes of Health and the University of Kansas Medical Center Animal Care and Use Protocol. The animals were housed in 12/12-h light/dark cycle and allowed free access to food and water. Anesthesia was induced by an intraperitoneal injection of a 1.25% avertin solution (2.5 g of 2,2,2-tribromoethyl alcohol and 5 ml tert-amyl alcohol [Sigma] dissolved in 200 ml deionized water, 0.2 ml/10 g body weight). Sciatic nerves were bilaterally exposed at the mid-thigh level (between 18 and 22 mm distal to the DRG) and transected or not injured. Following transection, a 5 mm portion of the nerve was resected to prevent regeneration. The sciatic nerves of mice in the uninjured group were viewed anatomically without further intrusion. Nerves of the crush and transected groups received either mild pressure applied with forceps for approximately five seconds or were completely severed, respectively. The surgical incision was closed by applying Nexaband® liquid tissue adhesive (Webster Veterinary Supply, Raleigh, NC). Mice were allowed to survive for three days following surgical treatment of the sciatic nerves for western blot or immunocytochemical analysis. For neuronal survival assessment, L4/5 DRG were bilaterally removed twenty-five days following sciatic nerve transection.

Western blot Analysis

L4/5 dorsal root ganglia (DRG) (n = four mice per treatment group) were pooled together for optimal protein concentration. Tissue was placed in liquid nitrogen upon dissection and homogenized using a PRO250 homogenizer (PRO Scientific, Monroe, CT) in 500 µL ice cold RIPA buffer (1% triton, 1% deoxycholic acid, 0.1% SDS, 15 M NaCl, 50 mM Tris pH 7.5, 1% NP-40, and 1 mM PMSF) for 30 seconds. Samples were then centrifuged for 30 minutes at 15,000 rpm at +4ºC. Total protein concentration was determined using the Bradford Assay method (Bradford, 1976). Samples were denatured for 10 minutes at 100ºC in bromophenol blue. Protein samples (50 µg/lane) were separated by SDS-page on 12.5% gels for the detection of mature NGF, proNGF, sortilin, and $p75^{NTR}$. Recombinant mouse NGF (R&D Systems, 20ng/lane) was loaded as a positive control to test the specificity of the anti-mature NGF antibody. All protein loading was normalized to cyclophilin A (1:1,000). Primary antibodies were diluted as follows: anti-sortilin (1:4,000), anti-$p75^{NTR}$ (1:2,000), anti-mature NGF (1:2,000), and anti-proNGF (1:2,000). Membranes were incubated in the secondary antibody (HRP-conjugated affinity-purified secondary antibody; 1:20,000) for one hour at +4ºC. The proteins were detected using an enhanced chemilluminescence reagent kit (Pierce, Rockford, IL) and imaged using a ChemiDoc™ XRS Imager (Bio Rad). Relative band density was analyzed with Quantity One software (Bio Rad) by applying the following equation:

$$\text{Band Intensity} = \frac{\text{density (the total intensity of pixels enclosed in the volume box)}}{\text{intensity (the area of the volume box [mm}^2\text{])}}$$

Immunocytochemistry

Sortilin and $p75^{NTR}$ expression was assessed in L4/5 DRG obtained from mice with bilaterally uninjured or transected sciatic nerves (n = 10/treatment group). Mice were deeply anesthetized with avertin (0.2 ml/10 g body weight) and transcardially perfused with ice cold saline followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (PBS) (pH 7.4). L4/5 DRG were dissected and allowed to post-fix in the same solution for one hour. Following post-fixation, the tissue was cryoprotected using 30% sucrose. 12 µm sections were cut using a cryostat maintained at -20ºC and mounted onto Superfrost Plus microscope slides (Fisher, Chicago, IL). Immunocytochemistry was performed using previously published protocols (Christianson et al., 2003). Briefly, sections were blocked for 1 hour in 1.5% normal goat/donkey serum, 0.5% porcine gelatin, and 0.2% Triton X-100 in Superblock buffer (pH 7.4, Pierce, Rockford, IL). Sections were incubated overnight with primary antisera to sortilin (1:5,000) or $p75^{NTR}$ (1:2,000) at +4ºC under humidified conditions. The primary antibody was removed by two washes in PBS-triton (0.5%) and sections were then incubated with secondary antibodies (Alexa Fluro 488 and 555; 1:2,000) for one hour at +4ºC. Fluorescently labeled sections were rinsed and coverslipped with PBS and viewed using a Nikon E800 microscope attached to a Magnafire digital camera.

Sortilin expression within DRG neuronal subpopulations

Sortilin expression in DRG neuronal subpopulations was defined by removing L4/5 DRG bilaterally from uninjured C57Bl/6 mice (n = 5). A polyclonal primary sortilin antibody (1:5,000) was applied to 14 μm sections in conjunction with the individual neuronal markers CRGP (1:500), IB4 (1:20), and N52 (1:400). Immunocytochemistry was performed as described above.

sortilin-p75^{NTR}-coexpressing neurons

To identify DRG neurons that coexpress sortilin and p75^{NTR}, L4/5 DRG from C57BL/6 mice (n = 5) were removed and perfused three days following no injury or sciatic nerve transection. Primary polyclonal sortilin and p75^{NTR} antibodies were used together on 14 μm DRG sections. The number of neurons coexpressing both receptors was counted in random, non-sequential sections of DRG. Approximately ten sections per DRG per animal were counted to arrive at an average number of neurons coexpressing both receptors.

Analysis of Neuronal Survival

The number of neurons in L4/5 DRG coexpressing the sortilin and p75^{NTR} receptors was assessed in C57BL/6 mice (n = 7/treatment group) that were perfused 25 days following no injury or bilateral sciatic nerve transection. To identify a subpopulation of neurons vulnerable to sortilin-p75^{NTR}-mediated proNGF-induced neuronal death, immunocytochemistry was performed on DRG sections using primary antisera specific for the sortilin and p75^{NTR} receptors. The

number of neurons coexpressing sortilin and $p75^{NTR}$ were counted in both injured and uninjured groups of mice. The area of neurons was measured, in square microns (μm^2), using NIH Image J software. Neurons were categorized into different size-based groups based on their area as follows: small neurons (0-200 μm^2), medium neurons (200-400 μm^2), and large neurons (<400 μm^2). The number of neurons in each group was counted and regarded as a percentage of the total number of neurons within the overall treatment group (no injury or 25 days post-sciatic nerve transection).

Statistical Analysis

Statistical analysis was performed using either ANOVA or an unpaired t-test to determine a significant difference between treatment groups. Statistical significance was set at $P < 0.05$. The variations in number of neurons are given as mean +/- S.E.M.

VII. Chapter Two: Neurotensin Prevents Injury-Induced Neuronal Death of Sensory Neurons. Arnett M.G., Ryals J.M., and Wright D.E. (2007).

A. Abstract

Traumatic nerve injury frequently leads to loss of function due to neuronal degeneration and death. The molecular mechanisms involved in neuronal death are poorly understood, but are thought to parallel apoptosis, or programmed cell death. Several molecules have recently been identified as key players in the induction of neuronal death. Sortilin, a recently discovered receptor expressed in neuronal tissues, has been shown to interact with the p75 neurotrophin receptor ($p75^{NTR}$) in the induction of apoptosis upon binding unprocessed nerve growth factor, also known as proNGF (Nykjaer et al., 2004). Sortilin also functions as a receptor for the peptide neurotransmitter neurotensin. Neurotensin occupies the sortilin receptor and thereby prevents the proNGF-induced formation of the sortilin-$p75^{NTR}$ receptor complex. Therefore, proNGF-activation of the $p75^{NTR}$ apoptotic signaling cascade can be prevented by neurotensin. We sought to determine if the absence of neurotensin *in vivo* would result in significantly greater neuronal death following peripheral nerve injury. To test this hypothesis, we assessed neuronal survival in the L4/5 dorsal root ganglia (DRG) seven days following sciatic nerve axotomy amongst neurotensin null-mutant ($NT^{-/-}$) heterozygous ($NT^{+/-}$), or wild-type ($NT^{+/+}$) mice. We found that in response to peripheral nerve injury, $NT^{-/-}$ mice experienced the most dramatic neuronal loss (53%, $P = 0.0015$), while $NT^{+/-}$ and $NT^{+/+}$ mice also suffered a significant loss of

39% (*P* = 0.0348) and 27% (*P* = 0.0043), respectively as compared to uninjured mice. Unexpectedly, intrathecal delivery of neurotensin (low [10 µg] and high [100 µg] doses) to $NT^{-/-}$ mice did not result in a significant increase in the number of L4/5 DRG neurons after injury as compared to control mice receiving artificial cerebrospinal fluid [aCSF]). However, $NT^{+/+}$ mice receiving 10 µg neurotensin daily, had an average of 61% more neurons after injury as compared to uninjured mice (*P* = 0.0069). Furthermore, $NT^{+/+}$ mice given 100 µg neurotensin daily had 67% more neurons as compared to uninjured mice (*P* = 0.0023). The differential effects of neurotensin upon $NT^{-/-}$ and $NT^{+/+}$ mice may offer important insight into how neurotensin mediates survival. Taken together, these data suggest a neuroprotective role for neurotensin *in vivo* in the prevention of neuronal death following injury.

B. Introduction

Peripheral nerve injury can result in a significant loss of sensory neurons due to programmed cell death, or apoptosis (Groves et al., 1997). A better understanding of the molecular mechanisms involved in this neuronal loss is critical to the creation of novel therapeutic strategies that target the loss of neurons and the subsequent loss of motor function or active range of motion, weakness in the affected body part, and/or pain ranging from a tingling sensation to extreme, burning pain that often accompany a nerve injury (Polston et al., 2004). The particular key players involved in the induction of injury-induced cell death have remained elusive until recently when proNGF, the unprocessed nerve growth factor (NGF) precursor, was implicated as a death inducing ligand that acts via a sortilin-$p75^{NTR}$ receptor complex to mediate neuronal death through activation of the $p75^{NTR}$ apoptotic cascade (Nykjaer et al., 2004 and Teng et al., 2005). Little attention has previously been focused on the physiological effects of proneurotrophins, mainly due to the fact that they contain a typical cluster of basic amino acids immediately preceding the mature domain making them vulnerable targets for cleavage by widely distributed proteases such as furin, plasmin and matrix metalloproteinases (Barde et al., 2004). However, recent findings demonstrate that proneurotrophins can escape intracellular cleavage and account for a majority of the total neurotrophin present extracellularly in many tissues (Lee et al., 2001).

proNGF binds with high affinity to $p75^{NTR}$ but not to *trk* A, the tyrosine kinase receptor responsible for mediating the trophic actions of mature NGF, thus

leading to cell death via activation of the apoptotic cascade associated with $p75^{NTR}$ (Lee et al., 2001). Mature NGF, however, preferentially binds and activates both receptors simultaneously resulting in cell survival by superseding the $p75^{NTR}$-mediated apoptotic signal. Therefore, cell fate depends largely on the operative ligand available as well as receptor expression.

In addition to binding proNGF, sortilin also serves to bind the tridecapeptide neurotensin. Neurotensin was first isolated from bovine hypothalamic extracts in 1976. It has received little attention outside of its role in the central nervous system where it functions as an antipsychotic and potent analgesic, and in the gastrointestinal tract where it aids in lipid digestion and stimulates colonic motility. Recently, a new role has been defined for neurotensin as a sortilin receptor antagonist (Folkers et al., 1976; Cáceda et al., 2006; Buhler et al., 2005; Hellström and Rosell, 1981 and DeGolier et al., 1999). Thus, neurotensin may play an important role in preventing cell death by interfering with the binding of proNGF to the sortilin-$p75^{NTR}$ receptor complex. In fact, recent findings indicate that an excessive amount of neurotensin is able to impair the induction of cell death in cultured neurons transfected with sortilin and $p75^{NTR}$ by more than 90% (Nykjaer et al., 2004). In this report, we investigate whether neurotensin is able to prevent the injury-induced neuronal death that occurs amongst sensory neurons *in vivo*.

These findings may contribute to treatment of disorders involving neuronal loss by providing a therapeutic approach aimed at improving the functional outcomes of peripheral nerve injuries.

C. Results

Stereological studies

Total number of neurons in uninjured DRG.

Using profile counts we found 5774.68 ± 939.18 neurons in L4/5 DRG of $NT^{+/+}$ adult mice (n = 7), versus 4186.27 ± 658.58 neurons in $NT^{+/-}$ adult mice (n = 7) and 4461.09 ± 405.16 neurons in $NT^{-/-}$ adult mice (n = 8) (Fig. 14). These values demonstrate a significant difference in the average number of L4/5 DRG neurons between $NT^{+/+}$ and $NT^{+/-}$ mice (27.5% decrease, P = 0.0381) However, there was no significant difference in neuronal number between $NT^{+/+}$ and $NT^{-/-}$ mice (22.7% decrease, P = 0.0743) or $NT^{+/-}$ and $NT^{-/-}$ mice (6% increase, P = 0.7031).

Effect of axotomy on DRG neuron numbers.

There was a significant loss of neurons after axotomy (Table 1). Profile counts revealed an average of 4236.64 ± 208.57 neurons in $NT^{+/+}$ L4/5 DRG seven days following sciatic nerve axotomy. This results in a 26.63% (P = 0.0443) loss of neurons as compared to uninjured $NT^{+/+}$ mice. $NT^{+/-}$ mice had an average of 2568.32 ± 137.80 neurons following injury, resulting in a 38.65% (P = 0.0348) loss as compared to uninjured $NT^{+/-}$ mice, and finally, there was an average of 2089.75 ± 316.12 neurons in $NT^{-/-}$ mice after injury, resulting in a 53.15% (P = 0.0015) loss in comparison to uninjured $NT^{-/-}$ mice.

Effect of neurotensin on neuron numbers following axotomy.

$NT^{-/-}$ mice

Intrathecal delivery of neurotensin was not able to prevent a significant loss of L4/5 DRG neurons in $NT^{-/-}$ mice after injury (Fig. 15). Control $NT^{-/-}$ mice receiving intrathecal injections of artificial cerebrospinal fluid (aCSF) have 3975 ± 1351.23 (n = 4) neurons in L4/5 DRG seven days following sciatic nerve axotomy. $NT^{-/-}$ mice receiving 10 µg daily injections of neurotensin after injury have 2846.08 ± 71.07 L4/5 DRG neurons after injury and 3348 ± 2264.686 neurons when receiving 100 µg neurotensin (Fig. 15). One-way ANOVA *(P* = 0.7428) revealed no significant effect of group on total number of neurons.

$NT^{+/+}$ mice

Control $NT^{+/+}$ mice receiving daily intrathecal injections of aCSF after sciatic nerve axotomy have an average of 1808.48 ± 732.72 neurons in L4/5 DRG. $NT^{+/+}$ mice receiving 10 µg daily injections of neurotensin after injury have an average of 4639.63 ± 1528.27 neurons (Fig. 16). Finally, $NT^{+/+}$ mice given 100 µg neurotensin daily after injury have an average of 5529.28 ± 792.39 in L4/5 DRG.

D. Figures

Figure 14: Mean Total Number of Neurons in Uninjured and Injured $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ Mice. Mean total number of L4/5 DRG neurons from $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ mice assessed seven days following no injury or sciatic nerve transection. Statistical differences in total mean neuronal number between groups are reported as *P* values obtained from a one-way ANOVA followed by Fisher's protected least significant difference test (FPLSD), ($P < 0.05$). There are an average of 5774.68 ± 939.18 neurons in L4/5 DRGs of $NT^{+/+}$ adult mice (n = 7), 4186.27 ± 658.58 neurons in $NT^{+/-}$ adult mice (n = 7) and 4461.09 ± 405.16 neurons in $NT^{-/-}$ adult mice (n = 8). These values demonstrate a significant difference in the average number of L4/5 DRG neurons between $NT^{+/+}$ and $NT^{+/-}$ mice (27.5% decrease, $P = 0.0381$) However, there was no significant difference in neuronal number between $NT^{+/+}$ and $NT^{-/-}$ mice (22.7% decrease, $P = 0.0743$) or $NT^{+/-}$ and $NT^{-/-}$ mice (6% increase, $P = 0.7031$). Seven days after sciatic nerve transection, there are 4236.64 ± 208.57 neurons in $NT^{+/+}$ L4/5 DRG. This results in a 26.63% ($P = 0.0443$) loss of neurons as compared to uninjured $NT^{+/+}$ mice. $NT^{+/-}$ mice have an average of 2568.32 ± 137.80 neurons following injury, resulting in a 38.65% ($P = 0.0348$) loss as compared to uninjured $NT^{+/-}$ mice, and finally, there were an average of 2089.75 ± 316.12 neurons in $NT^{-/-}$ mice after injury, resulting in a 53.15% ($P = 0.0015$) loss in comparison to uninjured $NT^{-/-}$ mice.

Figure 14: Mean Total Number of Neurons in Uninjured and Injured $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ Mice.

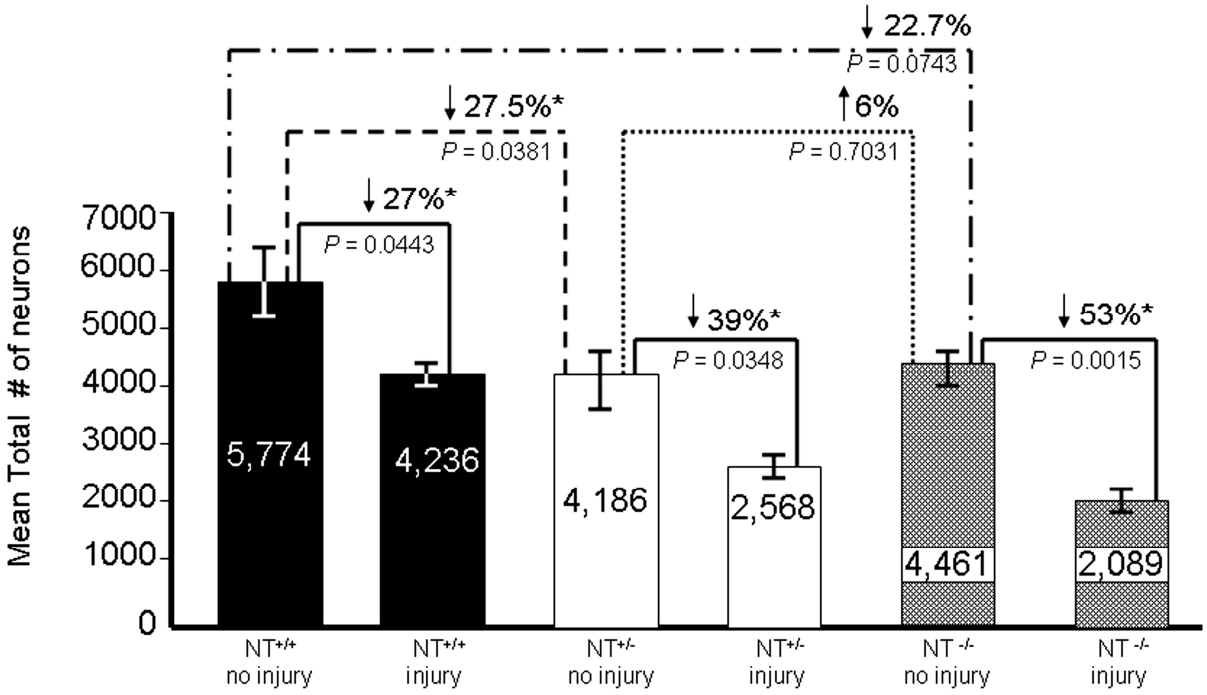


Table 1: Percent Neuronal Loss Amongst $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ Mice After Injury. Summary of the total mean neuronal number (± S.E.M.) in $NT^{+/+}$, $NT^{+/-}$, or $NT^{-/-}$ L4/5 DRG seven days following no injury or sciatic nerve axotomy as reported in Figure 14. Average neuronal loss is reported within genotypes as a percentage difference between uninjured and injured groups.

Table 1: Percent Neuronal Loss Amongst $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ Mice After Injury.

	Total mean neuron number		
genotype	uninjured sciatic nerve (left L4/5 DRG)	transected sciatic nerve (right L4/5 DRG)	% loss of neurons
$NT^{+/+}$	5774.68 ± 939.18	4236.64 ± 208.57	26.63 %
$NT^{+/-}$	4186.27 ± 658.58	2568.32 ± 137.80	38.65 %
$NT^{-/-}$	4461.09 ± 405.16	2089.75 ± 316.12	53.15 %

Table 2: Groups of $NT^{-/-}$ Mice Receiving Intrathecal Injections. The sample size, dosage, and volume of injections are listed for each group.

Table 2: Groups of $NT^{-/-}$ Mice Receiving Intrathecal Injections.

Group	Genotype	Intrathecal Injections	Volume
(control) group 1(N = 7)	$NT^{-/-}$	aCSF	50 µL
group 2 (N = 7)	$NT^{-/-}$	10 µg neurotensin	50 µL
group 3 (N = 7)	$NT^{-/-}$	100 µg neurotensin	50 µL

Table 3: Groups of $NT^{+/+}$ Mice Receiving Intrathecal Injections. The sample size, dosage, and volume of injections are listed for each group.

Table 3: Groups of $NT^{+/+}$ Mice Receiving Intrathecal Injections.

Group	Genotype	Intrathecal Injections	Volume
(control) group 1(N = 7)	$NT^{+/+}$	aCSF	50 μL
group 2 (N = 7)	$NT^{+/+}$	10 μg neurotensin	50 μL
group 3 (N = 7)	$NT^{+/+}$	100 μg neurotensin	50 μL

Table 4: Timeline of Injury, Intrathecal Injections, and Sacrifice. A summary of the schedule for $NT^{-/-}$ and $NT^{+/+}$ mice (tables 2 and 3) to receive sciatic nerve injury and intrathecal injections. The time of sacrifice relative to intrathecal injections is also listed.

Table 4: Timeline of Injury, Intrathecal Injections, and Sacrifice.

	group 1, 2, & 3	group 1, 2, & 3	group 1, 2, & 3	group 1, 2, & 3	group 1, 2, & 3	group 1, 2, & 3	group 1, 2, & 3
	day 1	2	3	4	5	6	7
bi-lateral sciatic nerve transection	x						
intrathecal injections	x	x	x	x	x	x	x
sacrifice							x

Figure 15: Effect of Neurotensin on Total Mean Neuronal Number in $NT^{-/-}$ Mice. The mean number of neurons in $NT^{-/-}$ mice receiving aCSF (control), 10 µg neurotensin (10 µg), and 100 µg neurotensin (100 µg) seven days following sciatic nerve axotomy. There are an average of 3975.08 ± 1351.23 neurons in the control group, 2846.08 ± 71.08 neurons in mice receiving 10 µg neurotensin, and 3348.0 ± 2264.69 neurons in mice given 100 µg neurotensin after injury. One-way ANOVA found no significant difference of neurotensin on group (P = 0.7428).

Figure 15: Effect of Neurotensin on Total Mean Neuronal Number in NT$^{-/-}$ Mice.

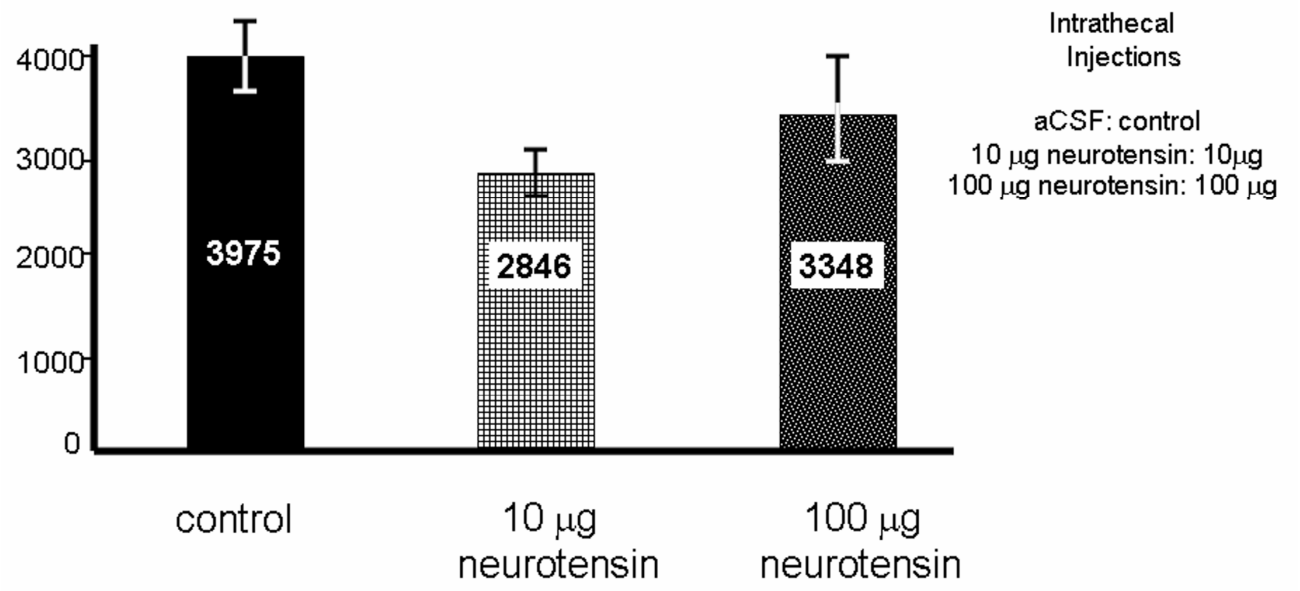


Figure 16: Effect of Neurotensin on Total Mean Neuronal Number in $NT^{+/+}$ Mice. The mean number of neurons in $NT^{+/+}$ mice receiving aCSF (control), 10 µg neurotensin (10 µg), and 100 µg neurotensin (100 µg) seven days following sciatic nerve axotomy. There are an average of 1808.48 ± 732.72 neurons in the control group, 4639.63 ± 1528.27 neurons in mice receiving 10 µg neurotensin, and 5529.28 ± 792.39 neurons in mice receiving 100 µg neurotensin after injury. Mice receiving 10 µg of neurotensin have 61% more neurons than control mice after injury (P = 0.0069). Mice receiving 100 µg of neurotensin have 67% more neurons than control mice after injury (P = 0.0023). There is no significant difference in the mean total number of neurons between the 10 µg and 100 µg group (P = 0.3250).

Figure 16: Effect of Neurotensin on Total Mean Neuronal Number in $NT^{+/+}$ Mice.

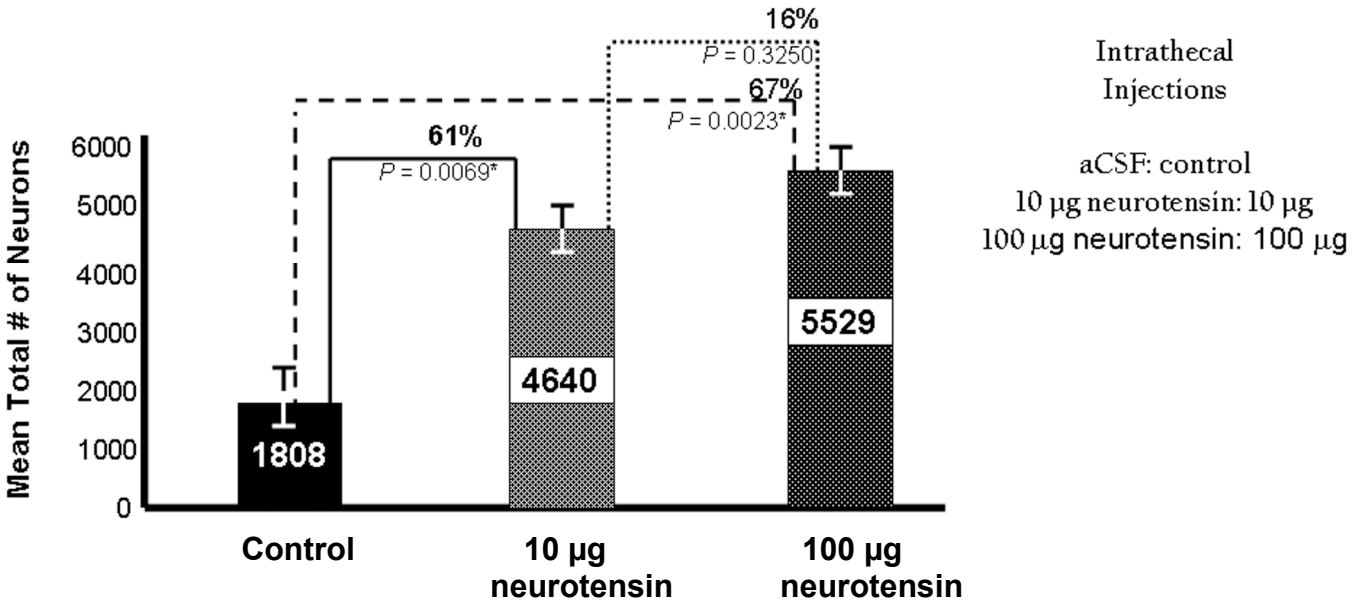


Figure 17: Thionin-Stained DRG from $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ Mice. Photomicrographs (20x) of thionin-stained L4/5 DRG sections from the following groups of mice: $NT^{+/+}$, uninjured sciatic nerve (A); $NT^{+/+}$, transected sciatic nerve (B); $NT^{+/-}$, uninjured sciatic nerve (C); $NT^{+/-}$, transected sciatic nerve (D); $NT^{-/-}$, uninjured sciatic nerve (E); and $NT^{-/-}$, transected sciatic nerve (F). All DRG were removed and processed seven days following treatment of the sciatic nerve. Scale bar = 100 μm.

Figure 17: Thionin-Stained DRG from $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ Mice.

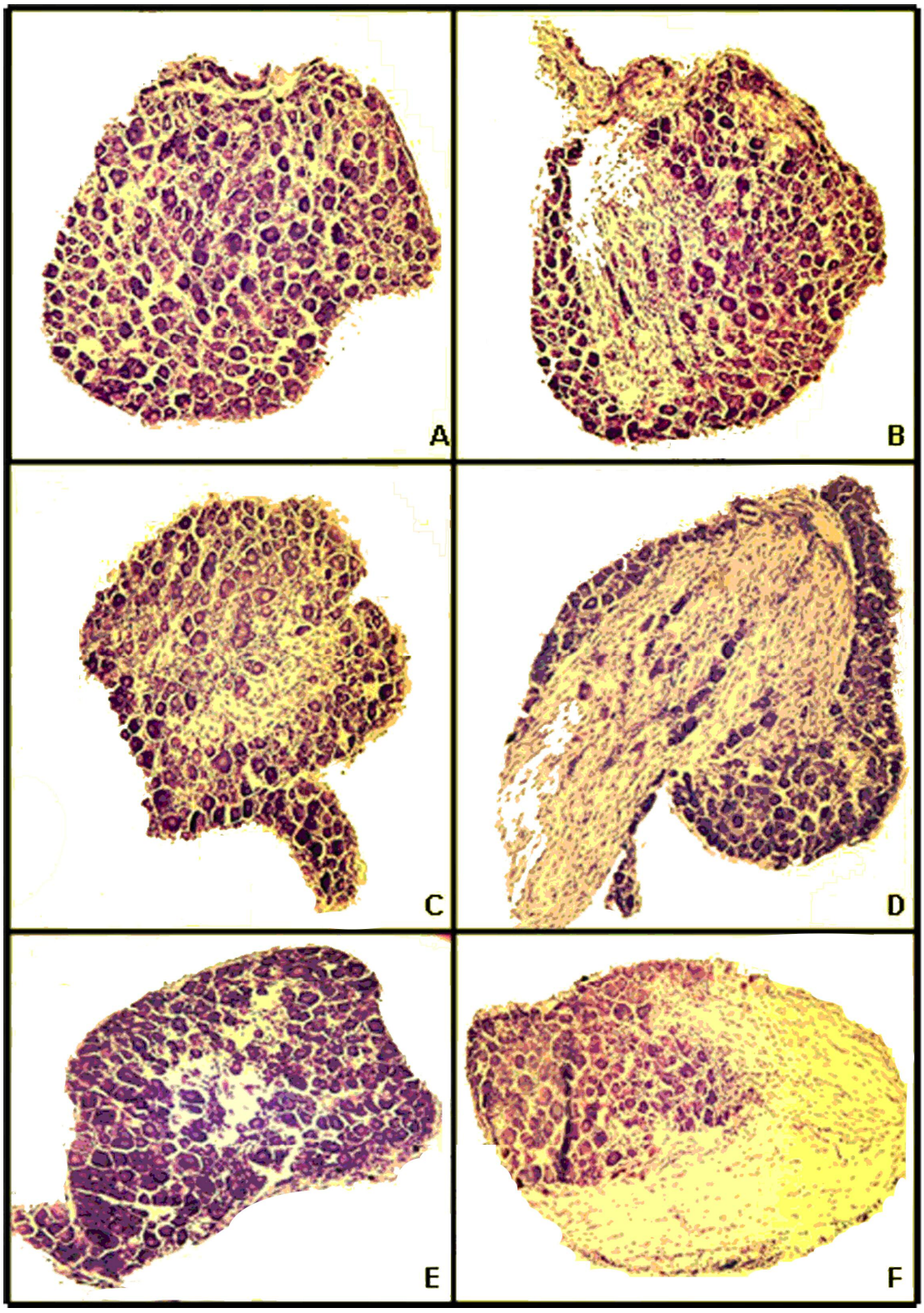
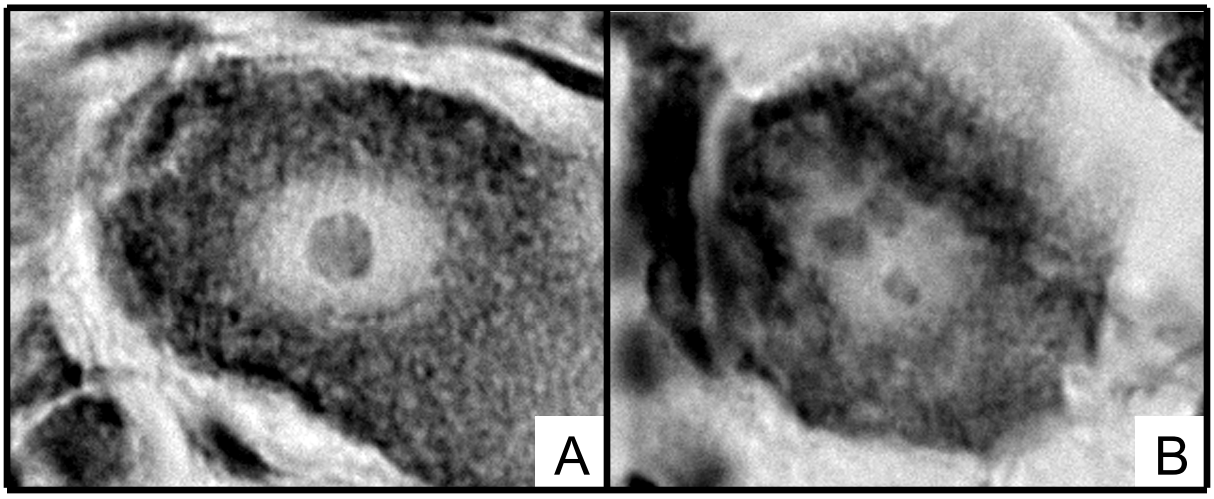


Figure 18: Healthy and Apoptotic Sensory Neurons. Examples of healthy (A) and apoptotic (B) neurons stained with the nuclear stain thionin. The healthy neuron (A) contains a centrally located nucleolus, a lightly stained nucleus, and a uniform, dense cytoplasm. The neuron undergoing apoptosis (B) is smaller than the healthy neuron, displays an irregular shape and contains a few darkly stained granules within the nucleus referred to as apoptotic bodies.

Figure 18: Healthy and Apoptotic Sensory Neurons.



E. Discussion

Neurotrophins are most commonly secreted as the pro-form, known as proneurotrophins (Bruno and Cuello, 2006; Fahnestock et al., 2004; Lou et al., 2005; Lee et al., 2001; and Seidah et al., 1996). The proneurotrophin proNGF is able to promote the formation of a high-affinity signaling complex between the sortilin and $p75^{NTR}$ receptors and induce apoptosis (Nykjaer et al., 2004). Neurotensin expression is dramatically upregulated in sensory neurons following sciatic nerve injury (Tanabe et al., 2003 and Zhang et al., 1996). Thus, indicating a possible pro-survival role for neurotensin.

The studies presented here reveal a marked difference in the number of DRG neurons found amongst uninjured $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ mice. On average, there are 1,588.41, or 27.50%, fewer neurons in the $NT^{+/-}$ DRG as compared to the $NT^{+/+}$ DRG. However, there was no parallel loss of neurons found between $NT^{+/-}$ and $NT^{-/-}$ mice, (+ 8%) or any further significant loss observed between $NT^{+/+}$ and $NT^{-/-}$ mice (21% loss), suggesting that the deletion of one neurotensin gene is sufficient for maximum neuronal loss and no further loss occurs with the deletion of the final copy of the gene. These data suggest that the signaling complex discussed here may play an important role in neuronal survival/death signaling during development. The results of this study are unaffected by these variations as comparisons of injury-induced neuronal loss are calculated within the same genotypic group.

Here we report an almost 27% neuronal loss in $NT^{+/+}$ L4/5 DRG seven days following sciatic nerve axotomy. These data are consistent with previous findings

(Shi et al., 2001) which report a 24% or 54% loss of L5 DRG neurons at seven or twenty-eight days following sciatic nerve axotomy in adult C57BL/6 mice, respectively. We have previously demonstrated that there is a 59% loss of sortilin-p75^{NTR} co-expressing L4/5 DRG neurons in the adult C57BL/6 mouse twenty-five days after sciatic nerve axotomy (Arnett et al., 2007). Taken together, these data suggest a death-inducing role for pro-NGF *in vivo* amongst sensory neurons following injury as the majority of neurons lost express both receptors (sortilin and p75^{NTR}) required for efficient binding of proNGF and the subsequent cell death that ensues (Nykjaer et al., 2004).

Additional data which suggests a pro-survival role for neurotensin is an increasing neuronal loss with deletion of the neurotensin gene. For example, here we report an 27% loss (P = 0.0443) of neurons in mice with two functional copies of the neurotensin gene (NT$^{+/+}$), a further decrease in total neuron number (39%, P = 0.0348) in mice with only one functional copy of the neurotensin gene (NT$^{+/-}$), and finally the most significant neuronal loss (54%, P = 0.0015) amongst mice which are null-mutant for the neurotensin gene (NT$^{-/-}$).

Here, we demonstrate NT$^{+/+}$ mice receiving intrathecal injections of either 10 µg or 100 µg of neurotensin daily after injury have significantly more neurons as compared to control mice. However, we did not see such an effect in NT$^{-/-}$ mice. One possible explanation in the differential effects of neurotensin amongst NT$^{+/+}$ and NT$^{-/-}$ mice could be the location of the sortilin receptor. Previous studies have shown that neurotensin internalization via one of two G protein-coupled receptors for neurotensin results in translocation of sortilin from intracellular

compartments to the plasma cell membrane. Once on the membrane, it can interact with neurotensin and promote cell survival (Chabry et al., 1993). Because $NT^{-/-}$ mice do not produce neurotensin, there is no signal for sortilin to be inserted onto the cell membrane, therefore making it inaccessible to neurotensin that is delivered into the extracellular environment by intrathecal injections. Another possible interpretation of these data can be explained by the fact that in the trans-golgi-network (TGN), sortilin's 5 kDa pro-peptide domain is cleaved rendering the 95 kDa sortilin receptor active. Once cleaved, the pro-peptide competes with neurotensin and proNGF for binding to active sortilin. In the absence of neurotensin, as in $NT^{-/-}$ mice, either the pro-peptide or proNGF would be uninhibited from binding to the sortilin receptor while still residing in the TGN, making sortilin once again inaccessible to bind to neurotensin on the cell membrane.

Previous studies have shown that neurotensin is able to prevent cultured neurons expressing both sortilin and $p75^{NTR}$ from undergoing apoptosis by competing with proNGF for binding to sortilin (Nykjaer et al., 2004 and Volsin et al., 2006). The goal of this study was to investigate the capability of neurotensin to prevent injury-induced death of sensory neurons *in vivo*. By interfering with proNGF binding to sortilin, neurotensin may provide a therapeutic approach in the treatment of disorders involving neuronal loss.

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F. Experimental Methods

Experimental animals

$NT^{-/-}$ were generously donated by Dr. Paul Dobner, University of Massachusetts Medical School, Worcester, MA. These mice were created using gene-targeting methods to disrupt the gene encoding neurotensin and the related hexapeptide neuromedin N (Dobner et al., 2001). $NT^{+/-}$ mice are mated to produce $NT^{+/+}$, $NT^{+/-}$, and $NT^{-/-}$ offspring. Polymerase chain reaction is performed on tail DNA from pups to determine the genotype of the offspring. Three specific primers are used to amplify regions of the NT gene fragment:

5'-CCCAGTCACGACGTTGTAAAACGAC-3'

5'-CATCCCTCACAGTTCACTCACTTTG-3"

5'-CCTGGATTCATTTACCTGAGTAGCA-3'

The reaction conditions are as follows: 94° for 2 minutes, and 35 cycles of: 94° for 30 seconds, 57° for 30 seconds, and 68° for 30 seconds. The PCR products are electrophoresed through a 1.5% agarose gel at 100V for 35 minutes. $NT^{+/+}$ mice are identified by a band of 270 bp, $NT^{+/-}$ mice are identified by bands of 270 bp and 188 bp, and $NT^{-/-}$ are identified by a band of 188 bp. Mice are housed and bred in the Laboratory Animal Resource facility at the University of Kansas Medical Center. All studies are performed in accordance with the guidelines specified by the National Institutes of Health laboratory animal care and use protocols. All mice used in this study are between six and eight weeks of age.

Sciatic nerve axotomy

Mice are deeply anesthetized with an intraperitoneal injection (200 µL/10 g body weight) of avertin (2.5 g 2,2,2 tribomoethanol [Sigma, St. Louis, MO] dissolved in 5 ml *tert*-Amyl alcohol [Sigma] and 200 ml distilled water). Sciatic nerves are either unilaterally or bilaterally transected at the mid-thigh level (between 18 and 22 mm distal to the DRG) and a 5mm segment of the nerve is resected to prevent regeneration. In the case of unilateral nerve axotomy, the right sciatic nerve is axotomized and the left sciatic nerve serves as the sham control. The sham nerve is viewed surgically for anatomical purposes but undergoes no further intrusion. Surgical wounds are closed by applying Nexaband® liquid tissue adhesive (Webster Veterinary Supply, Raleigh, NC). Mice are allowed to survive for 7 days following surgery.

Intrathecal Injections

Mice were randomly assigned into a low-dose (10 µg neurotensin) or high-dose (100 µg neurotensin) treatment group. Synthetic neurotensin was purchased from EMD Biosciences (San Diego, CA). Control mice received intrathecal injections of artificial cerebrospinal fluid (aCSF). aCSF was prepared to closely mimic endogenous CSF to ensure physiological compatibility. Briefly, the following solutions are combined in a 1:1 ratio. Solution 1: 8.66 g NaCl, 0.224 g KCl, 0.206 g $CaCl_2 \cdot 2H_2O$, and 0.163 g $MgCl_2 \cdot 6H_2O$ dissolved in 500 ml sterile water. Solution 2: 0.214 g $Na_2HPO_4 \cdot 7H_2O$ and 0.027 g $NaH_2PO_4 \cdot H_2O$ also dissolved in 500 ml sterile water.

Intrathecal injections were performed under avertin anesthesia via freehand lumbar puncture between L4/5 DRG. Mice received the first intrathecal injection concomitantly with sciatic nerve axotomy. Injections continued on a daily basis, 24-hour cycles, and were maintained for the next 6 days for a total of seven injections (Tables 2 and 3). Animals showing neurological deficits were excluded from the study. Mice were sacrificed approximately six hours following the last injection and DRG were removed and processed according to the tissue preparation protocol below.

Tissue preparation

Mice are anesthetized and transcardially perfused with 50 ml of ice-cold 0.1 M phosphate buffer, pH 7.4 (PBS) followed by 4% paraformaldehyde in PBS. Following dissection, L4/5 DRG are post-fixed in the same solution for one hour. DRG are rinsed in PBS overnight and stored in 70% ETOH at +4ºC until further processing. Ganglia are embedded in paraffin and cut into 10 µm sections. Sections are stained in thionin (0.5 g thionin [Sigma] dissolved in 100 ml distilled water and 0.1 ml acetic acid [Sigma]) and air dried for 4-5 days.

Profile counts

Neuronal profiles are counted in a one-in-five series of sections through the nucleolus using a 40x dry objective. On average, ten sections per ganglia are examined using a Nicon E800 microscope attached to a Magnafire digital camera. Neuronal profiles must meet specific criteria to be counted as healthy, non-apoptotic cells, including: (i) the presence of a stained cytoplasm

surrounding (ii) a clear nucleus with (iii) an identifiable clump of nucleolar material (Fig. 18). Total neuron number is calculated by multiplying profile counts by five.

Correction factors

The Abercrombie correction factor (Abercrombie, 1946) is applied to total neuronal counts to correct for overestimating neuronal number based on the size of the nucleolus relative to section thickness:

True number = (total neuron number)[T/(T+h)]

where T is section thickness and h is the average height of the nucleolus.

Section thickness

Section thickness is estimated by focusing up and down within the z plane of a representative tissue section using the 100x oil immersion lens of a Nicon E800 microscope. Three random neurons are measured per section and represent average thickness for that individual section. Total DRG thickness corresponds to an average of all section thicknesses per DRG (in microns).

Nucleoli height

The diameter of three neuronal nucleoli per section is obtained using NIH Image J software. In neurons with two nucleoli, h is measured between the centers of the two nucleoli. Section height represents the average of these

measurements. Total DRG height symbolizes the average of all section heights per DRG (in microns).

Statistics

Comparison between the number of L4/5 DRG neurons amongst experimental groups was made using a one-way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference test (PLSD). The level of significance was set to $P < 0.05$. The variations in number of neurons are given as mean ± S.E.M.

VIII. Chapter Three: Neurotensin Expression is Dramatically Upregulated in Sensory Neurons Following Peripheral Nerve Injury. Arnett M.G., Ryals J.M., and Wright D.E. (2007).

A. Abstract

It has been previously reported that several changes occur in sensory neurons following peripheral nerve injury. Among these changes are loss of neurons and changes in neuropeptide expression. In an attempt to prevent sensory deficits, there is an increase in the expression of survival promoting genes following injury. To investigate the upregulation of potential survival-promoting mRNAs, we examined changes in gene expression within the C57BL/6 dorsal root ganglia (DRG) three days following sciatic nerve injury. Here, we focus our attention on one gene in particular, the endogenous tridecapeptide neurotensin (NT), which underwent a dramatic increase in expression after injury. In the current study, we report a 3.78 fold-increase in NT expression as detected by microarray analysis (n = 3) and an 11.48 fold-increase as demonstrated by real-time RT-PCR (reverse transcriptase polymerase chain reaction) (n = 3) three days following sciatic nerve crush. NT expression appears to be directly correlated to severity of injury as real-time RT-PCR (n = 3) analysis reveals a 28.44 fold-increase in NT gene expression three days after sciatic nerve axotomy. This marked increase in expression is suggestive of an important role in regulating survival amongst sensory neurons following peripheral nerve injury.

B. Introduction

Traumatic nerve injury often leads to loss of function due to neuronal loss. Identification of pro-survival peptides will assist in the discovery of new therapeutic strategies aimed at preventing these sensory deficits. Neurotensin (NT), an endogenous tridecapeptide neurotransmitter (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH), is widely distributed throughout the central and peripheral nervous system (Carraway and Leeman, 1973). Since its initial isolation from bovine hypothalamic extracts in 1973, the research surrounding NT has focused mainly on its effects in the gastrointestinal (GI) tract, where it induces contraction of the ileum and relaxation of the duodenum (Folkers et al., 1976) and in the central nervous system, where it induces potent hypothermic and analgesic effects. NT mediates these effects via two closely related G protein-coupled receptors (GPCR), NTR1 and NTR2 (Nemeroff et al., 1992). Sortilin, a type-I membrane non-GPCR, has recently been identified as the third NT receptor (Petersen et al., 1997). Sortilin interacts with the p75 neurotrophin receptor, $p75^{NTR}$, to promote a high-affinity binding site for the pro-apoptotic ligand proNGF. By acting as a sortilin antagonist, NT is able to interfere with proNGF binding and prevent neuronal death amongst cultured neurons by up to 90% (Nykjaer et al., 2004). Whether or not NT is able to prevent neuronal death *in vivo* is still unclear, but its ability to disrupt pro-apoptotic signaling interactions is suggestive of a survival-promoting role. Here, we investigate NT expression amongst sensory neurons in response to peripheral nerve injury as another indicator of a pro-survival role for NT.

C. Results

Microarray Analysis

Many genes were significantly upregulated (>1.5 fold) following sciatic nerve crush in the mouse DRG (Table 5). Among the genes are galanin, preproneuropeptide Y, activating transcription factor 3, and neurotensin. Data generated from microarray analysis (n = 3) demonstrate a 3.78 fold-increase in NT gene expression, as compared to NT expression in uninjured mice (Table 3 and Fig. 19).

Real-time RT-PCR (reverse transcriptase polymerase chain reaction)

Real-time RT-PCR experiments were performed to confirm the increase in NT expression as detected by microarray analysis. The average threshold cycle (ΔCT) for the uninjured group was 25.95 ± 0.98, 23.20 ± 0.22 for the crush DRG group, and 22.78 ± 0.33 for the axotomized DRG group for the samples with NT primers and 20.05 ± 0.12, 20.82 ± 0.13, and 21.71 ± 0.29 for the uninjured, DRG crush, and DRG axotomized samples using the GAPDH primers. This data is converted into fold-change of gene expression (see Experimental Methods). NT expression is upregulated 11.48 fold three days after sciatic nerve crush, in comparison to uninjured mice (Fig. 21) (n = 3). NT expression was upregulated 28.44 fold following a complete severing of the sciatic nerve as compared to NT expression in uninjured mice (Fig. 21) (n = 3).

D. Figures

Table 5: Genes Significantly Upregulated in the Mouse DRG After Sciatic Nerve Crush. Fold-Change in 28 genes as determined by microarray analysis performed on L4/5 DRG three days following sciatic nerve crush. Genes with a significant increase in fold-change (>1.5 fold) are listed in descending order. The accession number along with the common name is included for each gene.

Table 5: Genes Significantly Upregulated in the Mouse DRG After Sciatic Nerve Crush.

Gene Name	Common Name	Genbank Acc. Number	Fold Change
small proline-rich protein 1A	Sprr1a	NM_009264.1	37.6
activating transcription factor 3	ATF3	BC019946.1	15.61
leucine-rich alpha-2-glycoprotein		NM_029796.1	12.47
preproneuropeptide Y	NPY	NM_023456.1	10.09
stathmin-like 4	Stmn4	NM_019675.1	8.7
galanin	Gal	NM_010253.1	7.83
tissue inhibitor of metalloproteinase	TIMP	BC008107.1	5.59
growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	NM_007836.1	5.57
chondroadherin	Chad	NM_007689.1	5.08
annexin A10	Anxa10	NM_011922.1	5.02
follistatin	Fst	NM_008046.1	5.0
growth arrest and DNA-damage-inducible 45 gamma	Gaddd4g	AK007410.1	4.78
gastrin-releasing peptide		BC024515.1	4.6
serine proteinase inhibitor, clade B, member 1a	Serpinb1a	AB030426	4.37
kinesin-like 4		BB251322	4.07
xanthine dehydrogenase	Xdh	AV286265	3.97
syndecan 1		BC010560.1	3.9
endothelial protein C receptor,	Procr	NM_011171.1	3.85
SRY-box containing gene 11	Sox11	BG072739	3.84
neurotensin	NTS	NM_024435.1	3.78
IL2-inducible T-cell kinase	Itk	NM_010583.1	3.77
complement component 1, q subcomponent	C1qb	BB111335	3.75
matrix metalloproteinase 16	Mmp16	BB041237	3.66
serpinb1		AF426024.1	3.62
Eph receptor A3	Epha3	BB292785	3.49
adipocyte complement related protein of 30 kDa	Acrp30	NM_009605.1	3.37

Figure 19: Microarray Analysis of Neurotensin Expression Following Injury. Neurotensin gene expression is upregulated 3.78 fold in L4/5 DRG neurons of C57Bl/6 mice three days following sciatic nerve crush.

Figure 19: Microarray Analysis of Neurotensin Expression Following Injury.

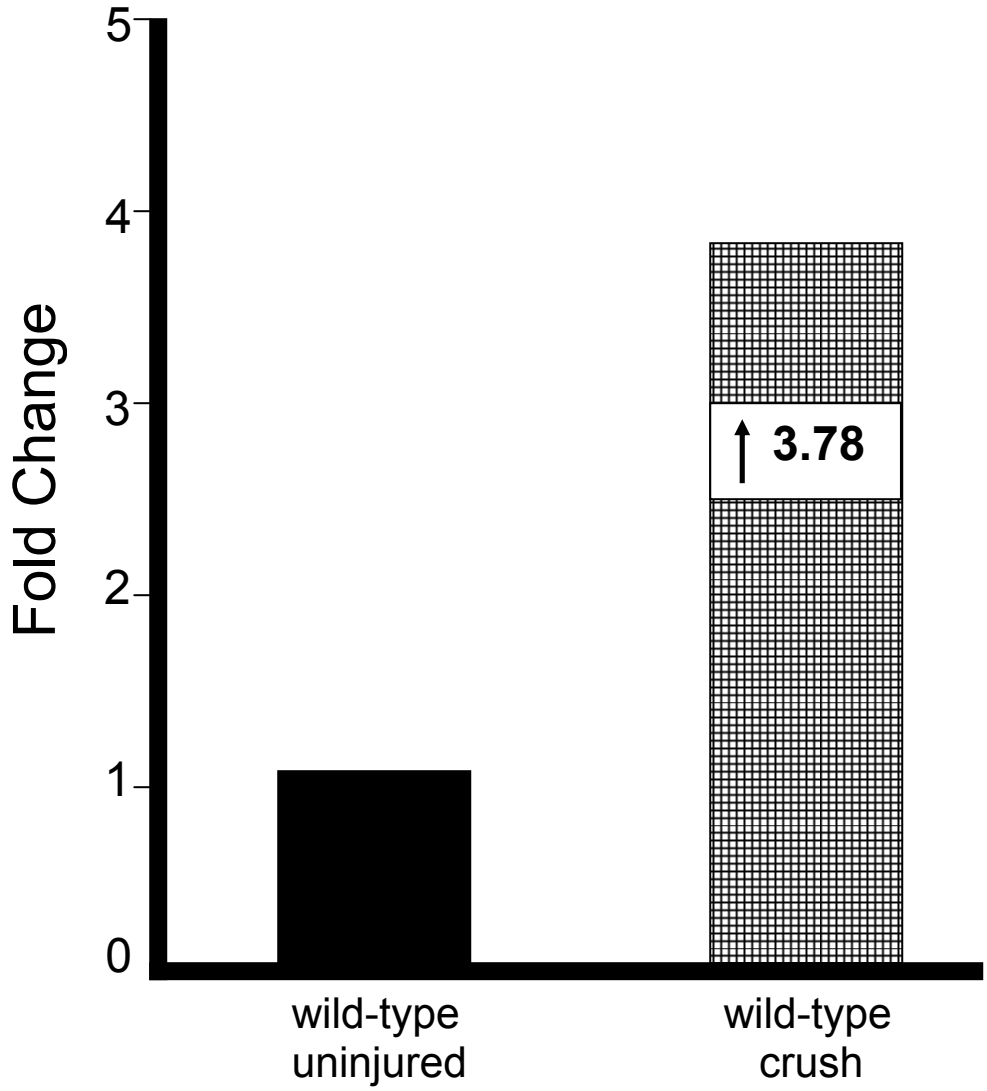


Figure 20. Real-Time RT-PCR Average Threshold Cycle. Differences in expression between sham and experimental groups are detected when compared to expression levels of the house-keeping gene glucose 6 phosphate dehydrogenase (GAPDH). Each treatment group consisted of three samples and each sample was assessed in triplicate. The mean triplicate data determine the average threshold cycle (C_t) for samples using NT primers and GAPDH primers, separately.

Figure 20. Real-Time RT-PCR Average Threshold Cycle.

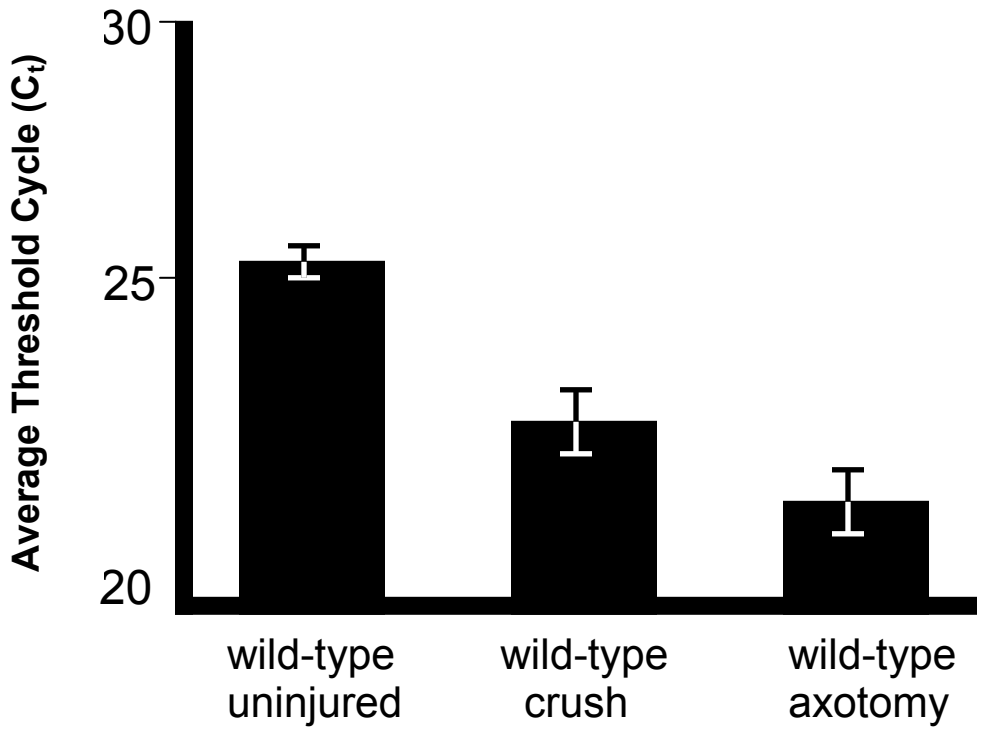
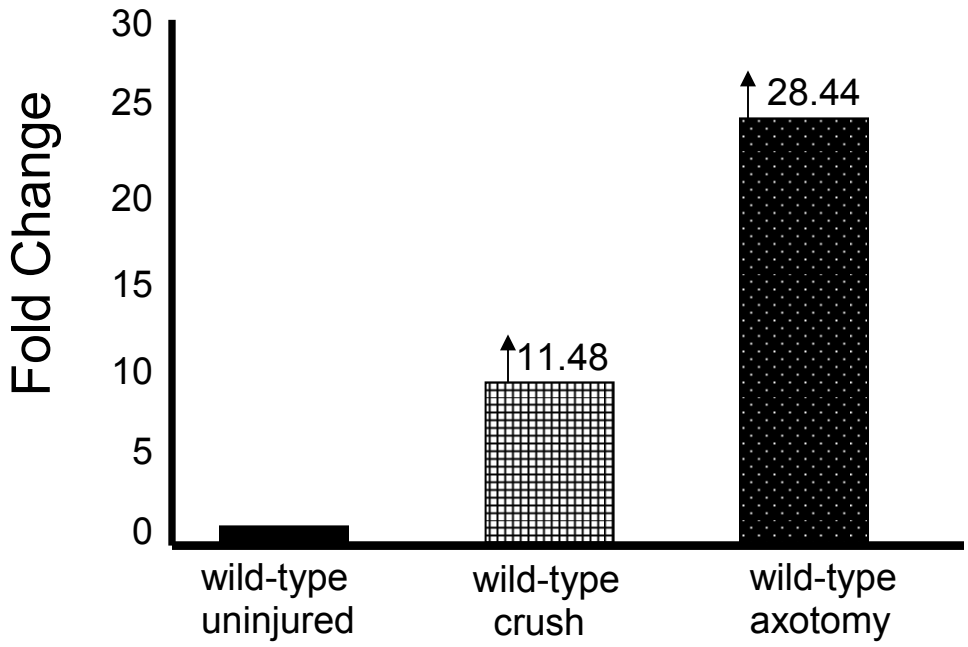


Figure 21: Fold Change in Neurotensin Gene Expression Following Sciatic Nerve Crush or Axotomy. Fold change in neurotensin gene expression in L4/5 DRG of C57BL/6 mice three days following sciatic nerve crush or axotomy. The C_t (Fig. 19) is mathematically translated into fold-change in gene expression by applying the following formula:

$2^{|\Delta CT_{uninjured} - \Delta CT_{injured}|}$.

There is an 11.48 fold-increase in neurotensin gene expression three days following sciatic nerve crush. An even more robust increase in neurotensin expression is observed following sciatic nerve axotomy (28.44 fold-increase).

Figure 21: Fold Change in Neurotensin Gene Expression Following Sciatic Nerve Crush or Axotomy.



E. Discussion

Over the past 30 thirty years since its first isolation from bovine hypothalamic extracts, little focus has been put on the role of neurotensin outside of its classic effects in the central and peripheral nervous system. In the central nervous system it provides analgesic properties and functions as an anti-psychotic (Seta et al., 2001 and Dobner et al., 2001) while it stimulates colonic motility and potentiates lipid digestion in the gastrointestinal tract (Evers et al., 1990). Recently, a new role has been proposed for neurotensin as a sortilin receptor antagonist. Sortilin, a type I membrane receptor expressed throughout the central and peripheral nervous system, (Arnett et al., 2007 and Petersen et al., 1997), has been shown to interact with the p75 neurotrophin receptor ($p75^{NTR}$) and promote apoptosis upon binding proneurotrophins. This cell death can be inhibited up to 90% by NT (Nykjaer et al., 2004).

More than half of the sensory neurons in the DRG undergo cell death approximately one month following peripheral nerve injury (Shi et al., 2001). In order to prevent this neuronal loss, an increase in pro-survival peptides must be observed (Tanabe et al., 2003). To identify potential pro-survival peptides, we analyzed the DRG following sciatic nerve crush using microarray analysis. We found a dramatic increase in NT expression amongst sensory neurons in response to peripheral nerve injury. The ability of NT to disrupt interactions between pro-apoptotic molecules coupled with its marked increase in expression after injury is suggestive of a pro-survival role.

Cell death becomes more extensive with increasing severity of injury, therefore; the expression of neuroprotective peptides should concomitantly increase with the degree of injury. We investigated the effect of sciatic crush and axotomy on NT expression in sensory neurons and found that there is a much greater increase in gene expression due to a complete transection of the nerve rather than a simple crush injury.

NT expression appears to be regulated differently among species in response to injury. Others have reported a 25.2 fold-increase in NT expression seven days following sciatic nerve axotomy in the adult C57BL/6 mouse DRG, similar to what we report in this study (Tanabe et al., 2003). However, rats do not experience the same degree of upregulation in NT expression following injury. Approximately 1.4% of L4/5 DRG neurons are NT mRNA-positive in uninjured rats. Seven days following sciatic nerve axotomy, 7.7% of L4/5 DRG neurons are reported as expressing NT mRNA, an approximate increase of only 6.3% (Zhang et al., 1996). Mice may therefore represent a unique animal model that experiences a distinct marked increase in neurotensin expression after injury.

If neurotensin functions as a pro-survival peptide, mice should experience less neuronal death after injury than rats, as they undergo a greater increase in NT expression. However, this is not the case as mice suffer a 24% loss of DRG neurons at 7 days and 54% at 28 days following sciatic nerve axotomy (Shi et al., 2001) while rats experience only a 15% loss of neurons at seven days and reach a maximum neuronal loss of 35% two months following sciatic nerve axotomy

(McKay et al., 2002). One possible explanation for these results is the proximity of the nerve injury in reference to the cell bodies within the DRG. For example, in mice, an injury to the mid-thigh region of the sciatic nerve results in trauma to a majority of the smaller peripheral nerve branches traveling in parallel along the larger sciatic nerve, thus, resulting in an injury to a majority of the peripheral axons within the sciatic nerve. In rats, many of the smaller peripheral nerves have already branched off to reach their somatic targets and are no longer affected by a mid-thigh sciatic nerve injury, therefore resulting in fewer peripheral nerves being affected by the injury.

In the current study, we investigate the effect of injury on NT gene expression. A role for preventing neuronal death *in vitro* has recently been suggested for NT. Here, we report further evidence of a marked increase in NT expression in response to nerve injury. Taken together, these data suggest a neuroprotective role for NT in preventing neuronal death *in vivo* amongst sensory neurons following injury.

F. Experimental Methods

Experimental Animals

All experiments in the current study were performed in six-week old, male C57BL/6 mice (Charles River, Wilmington, MA) and were conducted according to the guidelines from the National Institutes of Health and the University of Kansas Medical Center Animal Care and Use Protocol.

Surgical Procedures

Anesthesia was induced by an intraperitoneal injection of a 1.25% avertin solution (2.5 g of 2,2,2-tribromoethyl alcohol and 5 ml tert-amyl alcohol [Sigma] dissolved in 200 ml deionized water, 0.2 ml/10 g body weight). Sciatic nerves were bilaterally exposed at the mid-thigh level (between 18 and 22 mm distal to the DRG) and transected or not injured. Following transection, a 5 mm portion of the nerve was resected to prevent regeneration. The sciatic nerves of mice in the uninjured group were viewed anatomically without further intrusion. Nerves of the crush and transected groups received either mild pressure applied with forceps for approximately five seconds or were completely severed, respectively. The surgical incision was closed by applying Nexaband® liquid tissue adhesive (Webster Veterinary Supply, Raleigh, NC).

Tissue Harvesting / RNA Isolation

Mice were sacrificed three days following treatment of sciatic nerves. L4/5 DRG were removed and immediately stored in liquid nitrogen. Tissue was homogenized for approximately 30 seconds using a PRO250 homogenizer (PRO Scientific, Monroe, CN). Total RNA was harvested from L4/5 DRG using 1 mg Trizol® (Sigma-Aldrich, St. Louis, MO) per 50-100 mg tissue and 1 mg/ml mussel glycogen (Sigma-Aldrich). The RNA yield and the ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280} ratio) were measured with the SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA).

Microarray Analysis

Total RNA from control, crush, and axotomized DRG samples were subjected to microarray analysis using the Affymetrix 430A gene chip which uses 45,000 probe sets to analyze the expression level of over 39,000 transcripts and variants from over 34,000 well characterized mouse genes. Each treatment group consisted of three samples and each sample was assessed in triplicate. The microarray gene expression analysis was performed by the Biotechnology Support Facility at the University of Kansas Medical Center, Kansas City, Kansas.

Real-Time RT-PCR

L4/5 DRG are dissected out and immediately stored in liquid nitrogen. Total RNA is extracted using Trizol reagent (Invitrogen, Carlsbad, CA). 1.25 µL of RNA is reverse transcribed into cDNA at 55º for 10 minutes using reverse transcriptase and 5X iScript reaction mix (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA). Following degradation of the remaining mRNA, the transcription product is amplified through 45 cycles using two oligonucleotide primers specific for NT:

NT forward primer: 5'-GCTAGCTCCTTCAGTGTCTG-3'

NT reverse primer: 5'-CTGAAAGCCAGGAGAGTCAG-3'

The PCR product is quantitatively measured using SYBR Green Supermix (iQ SYBER® Green Supermix, Bio-Rad). Differences in expression between sham and experimental groups are detected when compared to expression levels of

the house-keeping gene glucose 6 phosphate dehydrogenase (GAPDH). Each treatment group consisted of three samples and each sample was assessed in triplicate. The triplicate data are averaged to determine the average threshold cycle (C_t) for samples using NT primers and GAPDH primers, separately. The C_t can be mathematically translated into fold-change in gene expression by applying the following formula:

$2^{|\Delta CT_{uninjured} - \Delta CT_{injured}|}$.

IX. Chapter Four:

Conclusions

A. Injury-Induced Neuronal Death in Sensory Neurons

Peripheral nerve injury induces a significant loss of sensory neurons (Shi et al., 2001). This neuronal loss occurs through a series of pre-programmed events known as apoptosis, or cell suicide (Oliveira, 2000). Preventing neuronal death after injury is vital to maintaining adequate cutaneous innervation and quality of sensation which, once lost, result in poor functional outcomes. Despite recent advances in microsurgical techniques and proven neuroprotective therapies, the outcome of peripheral nerve injuries remains disappointing. Therefore, we must now focus on acquiring a better understanding of the neurobiology that surrounds nerve injury to develop clinically significant strategies aimed at preventing these sensory deficits. The first task targeted at precluding neurodegeneration lies in identifying the molecular mechanisms involved in mediating injury-induced apoptosis in neurons. Long thought to be biologically inactive precursors, proneurotrophins, or unprocessed neurotrophins, have been shown to bind with high-affinity to $p75^{NTR}$. The apoptotic cascade associated with $p75^{NTR}$ is activated upon interaction with proNGF and sortilin, a type I membrane receptor that aids in efficient binding between proNGF to $p75^{NTR}$. We sought to determine if sortilin and $p75^{NTR}$ were expressed amongst sensory neurons, thereby rendering them susceptible to proNGF-induced apoptosis. The data presented here report that on average, 66.1% ± 2.83 and 73.8% ± 4.82 of L4/5 DRG neurons in the C57BL/6 mouse express $p75^{NTR}$ and sortilin, respectively. There

is no significant difference in the number of neurons expressing either receptor after injury with 67.2% ± 4.43 and 72.8% ± 3.50 neurons expressing $p75^{NTR}$ and sortilin three days following sciatic nerve axotomy. Because both receptors are required to efficiently bind proNGF and induce apoptosis, we next investigated if there is a population of DRG neurons that express both receptors. Approximately 52.5% ± 3.77 neurons express both sortilin and $p75^{NTR}$. Again, we found no significant difference in the number of neurons expressing both receptors after injury with 54.5% ± 2.82 neurons expressing $p75^{NTR}$ and sortilin three days following sciatic nerve axotomy. If proNGF is behaving as a death-inducing ligand amongst sensory neurons, there should be a concomitant loss of sortilin-$p75^{NTR}$ bearing neurons with injury. In the studies presented here, we report a 58% loss of sortilin-$p75^{NTR}$ coexpressing neurons twenty-five days following sciatic nerve axotomy, suggesting that proNGF is inducing apoptosis amongst sortilin-$p75^{NTR}$ coexpressing neurons after injury. Others report a total neuron loss of 54% in the C57BL/6 mouse DRG twenty-eight days following sciatic nerve axotomy (Shi et al., 2001). The data reported here indicate this total loss of neurons to be amongst the sortilin-$p75^{NTR}$ coexpressing neurons, further suggesting that proNGF induces apoptosis after injury. Overall, these data increase the body of knowledge surrounding neurodegeneration by proposing a mechanism for inducing and mediating cell death amongst sensory neurons in response to injury.

IX. Conclusions; cont.

B. Pro-Survival Role for Neurotensin

Neurotensin, a tridecapeptide neurotransmitter originally isolated from bovine hypothalamic extracts in 1973, is expressed throughout the peripheral as well as central nervous systems. It is most noted for exerting potent analgesic and hypothermic effects in the central nervous system (Clineschmidt et al., 1979; Yaksh et al., 1982 and Spampinato et al., 1988). Peripherally, it acts as an endocrine peptide that functions to stimulate pancreatic and biliary secretions, inhibit gastric secretions and motility, and induce colonic motility (Kitabgi and Nemeroff, 1992). Neurotensin is synthesized as part of a precursor molecule that also contains neuromedin N, a six amino acid neurotensin-like peptide. The active neurotensin peptide is released from its precursor upon cleavage by pro-protein convertases. Neurotensin mediates its effects in the central and peripheral nervous systems via two G protein-coupled receptors known as NTR1 and NTR2. A third receptor for neurotensin, NTR3 or sortilin, was recently cloned (Petersen et al., 1997). Approximately 90% of sortilin resides intracellularly where it functions to sort proteins to their appropriate cellular compartment (Mazella et al., 2001). To date, there has been no signaling cascade associated with sortilin; however, it is able to promote apoptosis by interacting with $p75^{NTR}$ to efficiently bind the pro-apoptotic ligand proNGF. Therefore, sortilin serves as a molecular switch, determining cell fate depending on its ligand of choice. By acting as a sortilin antagonist, neurotensin is able to bind to the sortilin receptor and prevent association with proNGF. In cultured

neurons, neurotensin has been shown to inhibit the induction of proNGF-induced apoptosis by up to 90% (Nykjaer et al., 2004). However, whether or not sortilin functions as a true neurotensin receptor *in vivo* capable of mediating pro-survival effects, was not yet established. In this study, we investigate the role of neurotensin in preventing neuronal death *in vivo*.

The first data suggestive of a neuroprotective role for neurotensin *in vivo* was an observance of an increase in its expression after injury. Microarray analysis performed on L4/5 DRG three days following sciatic nerve crush demonstrate a 3.78 fold-increase in neurotensin gene expression. Real-time RT-PCR results confirm these data by revealing an 11.48 fold-increase in neurotensin gene expression after sciatic nerve crush and a even more robust increase of 28.44 fold-increase following sciatic nerve axotomy.

Data presented here report an injury-induced neuronal loss of 27% (P = 0.0443) amongst $NT^{+/+}$ mice, 39% amongst $NT^{+/-}$ mice (P = 0.0348) and the most dramatic neuronal loss of 53% amongst $NT^{-/-}$ mice (P = 0.0015). These data support an increasing neuronal loss concomitant with deletion of the neurotensin gene. Unexpectedly, exogenous delivery of neurotensin to $NT^{-/-}$ mice did not prevent neuronal loss after injury. However, $NT^{+/+}$ mice that received intrathecal injections of neurotensin after injury had significantly more neurons as compared to mice receiving artificial cerebrospinal fluid (aCSF) after injury. These data may reflect the significance of how neurotensin mediates its pro-survival effects. For example, previous studies have shown that sortilin is translocated from

intracellular compartments to the plasma cell membrane upon the internalization of neurotensin via one of two G protein-couple receptors, NTR1 or NTR2.

Once on the membrane, it can interact with neurotensin and promote cell survival (Chabry et al., 1993). In the absence of neurotensin, there is no signal for expression of sortilin on the cell membrane in $NT^{-/-}$ mice, rendering it inaccessible to neurotensin. An alternative interpretation of these data is that in the trans-golgi-network (TGN), the 5 kDa pro-peptide domain located within the N'-terminal of sortilin is cleaved transforming the 100 kDa peptide into a fully-active 95 kDa receptor. Upon cleavage, the pro-peptide competes for binding to active sortilin with proNGF and neurotensin in the TGN. In $NT^{-/-}$ mice, neither the pro-peptide nor proNGF would be inhibited from binding to the sortilin receptor, rendering sortilin once again inaccessible to neurotensin and thereby unable to prevent cell death. Overall, the data presented here support an important and novel role for neurotensin in preventing neuronal death amongst sensory neurons following injury.

IX. Conclusions; cont.

C. Clinical Significance

Peripheral nerve injury is common and often results in significant disability to patients and costs to society. Although damaged peripheral nerve fibers can and often do regenerate in the PNS, recovery of function is often incomplete due to a variety of biological factors. First, neurons must survive the injury. Days to weeks after the initial peripheral nerve injury, apoptosis occurs throughout sensory neurons, causing the cells to self-destruct. This neuronal death is a major factor responsible for poor sensory functional outcome after peripheral nerve trauma. Although genes have been identified that appear to regulate apoptosis, researchers still don't know enough to be able to specify the exact biochemical events that cause a cell to switch it on - or turn it off. It would therefore be of great clinical benefit to develop neuroprotective strategies to combat apoptotic cell death. The results produced from these studies offer clinical significance two-fold. First, we have identified a receptor complex that mediates cell death *in vivo*. This novel finding provides a new target system for therapeutic intervention. Furthermore, we designate a neuroprotective role for neurotensin. By modulating these key cellular structures, therapeutic strategies can be aimed at controlling the molecular responses of neurons and their target structures following injury in order to improve functional outcome.

X. References

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