A Role for the p75 Neurotrophin Receptor in Axonal Degeneration and Apoptosis Induced by Oxidative Stress

By

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6-hydroxydopamine	6-OHDA
Alzheimer's Disease	AD
Apoptotic protease activating factor 1	Apaf-1
Arabinocytidine	Ara-C
Ankyrin repeat-rich membrane spanning	ARMS
Brain-derived Neurotrophic Factor	BDNF
Central Nervous System	CNS
4,6-diamidino-2-phenylindole	DAPI
Degeneration Index	DI
Dimethyl sulfoxide	DMSO
2,4-dinitrophenylhydrazine	DNPH
Dorsal Root Ganglia	DRG
Extracellular signal-related kinase	ERK
GNU image-manipulation program	GIMP
G-protein-coupled inwardly rectifying potassium	GIRK
Hypoxia-inducible factor-1α	HIF1α
4-hydroxy-2-nonenal	HNE
Intracellular Domain	ICD
IκB kinase	IKK
Interleukin-12	IL-12
Interleukin-1ß	IL-1ß
Immunoreactivity	ir
c-jun N-terminal Kinase	JNK

Melanoma-associated antigen	MAGE
Malondialdehyde	MDA
Matrix Metalloproteinase-7	MMP-7
Mitochondrial permeability transition	MPT
Nuclear factor- κB	NF-κB
$p75^{NTR}$-associated death executioner	NADE
Norepinephrine transporter	NET
Nerve Growth Factor	NGF
Neurotrophin receptor-interacting MAGE homolog	NRAGE
Neurotrophin Receptor Homolog 2	NRH2
Neurotrophin Receptor-Interacting Factor	NRIF
Neurotrophin-3	NT3
Neurotrophin-4	NT4
p75 Neurotrophin Receptor	$p75^{NTR}$
Antibody specific for extracellular domain of $p75^{NTR}$	α-p75-ECD
p75 Neurotrophin Receptor – c-terminal fragment	$p75^{NTR}$-CTF
p75 Neurotrophin Receptor – Intracellular Domain	$p75^{NTR}$-ICD
Parkinson's Disease	PD
Phosphatidylinositol-3-kinase	PI3K
Phosphatidylinositol 4,5-bisphosphate	PIP_2
Phospholipase C-γ	PLC-γ
Phosphatase and tensin homolog deleted on chromosome 10	PTEN
Regulated intramembrane proteolysis	RIP
Receptor-interacting protein 2	RIP2
Rho kinase	ROCK

Reactive oxygen species	ROS
Short form of $p75^{NTR}$	s-$p75^{NTR}$
Superoxide dismutase	SOD
Tyrosine hydroxylase	TH
Tissue inhibitor of matrix metalloproteinase-1	TIMP-1
Tumor necrosis factor	TNF
Tumor necrosis factor-α	TNFα
Tumor necrosis factor receptor	TNFR
TNF receptor associated factor	TRAF
Tropomyosin-related kinase A	TrkA
Tropomyosin-related kinase B	TrkB
Tropomyosin-related kinase C	TrkC
Tissue plasminogen activator	tPA
Terminal deoxynucleotidyl transferase dUTP nick end labeling	TUNEL

CHAPTER I

INTRODUCTION

Neurotrophins and Neurotrophin Receptors

Neurotrophins are diffusible growth factors secreted from various tissues innervated by the nervous system, as well as from neurons and glia. While these factors were originally characterized for their ability to promote the survival and outgrowth of developing neurons to ensure proper innervation of target tissues, neurotrophins are now known to mediate a wide range of developmental and non-developmental biological functions. The founding member of the neurotrophin family, Nerve Growth Factor (NGF), was first discovered after a series of ablation and transplantation studies by Rita Levi-Montalcini and Viktor Hamburger led to the search for a diffusible factor responsible for the survival of sensory and sympathetic neurons innervating peripheral tissues (1,2). Stanley Cohen, in collaboration with Levi-Montalcini, was eventually able to purify NGF from snake venom (3), and later, from mouse salivary gland. Injection of antibody specific for NGF was found to cause death of sympathetic neurons, thus demonstrating the importance of the factor for sympathetic neuron survival *in vivo* (4). Observations that NGF promoted the survival of some, but not all sensory neurons ultimately paved the way for the search for other neurotrophic factors (5). Brain-Derived Neurotrophic Factor (BDNF), which was purified from pig brain, was initially discovered to be a trophic factor for placode-derived sensory neurons (6) and eventually identified as an NGF gene family member (7). Later, using polymerase chain reaction primers designed to amplify conserved transcript regions of NGF and BDNF, two additional NGF gene family

members were discovered: neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (8-11). After the initial cloning of NT-4 in *Xenopus*, the equivalent human cDNA was considered suitably different to be a separate gene, and thus it was termed neurotrophin-5 (12). Subsequently, however, it was realized that the two are homologous genes, and thus this neurotrophin is often termed neurotrophin-4/5 (13).

The discovery of Nerve growth factor (NGF) as the factor released by peripheral targets to promote survival and differentiation of sympathetic neurons quickly led to the hunt for the receptor involved in mediating its actions. Early studies characterizing radiolabeled NGF binding to peripheral neurons revealed that NGF bound its receptors in a complex manner that likely involved multiple sites (14,15). Subsequently, two distinct NGF binding sites, one with high affinity and one with low affinity, were demonstrated in sensory neurons (16). Cross-linking studies confirmed two receptor components in sympathetic neurons (17) and PC12 cells (18,19) of approximately 140-200 kD and 70-100 kD, with the lower molecular weight species being most abundant. These binding studies set the stage for the expression cloning of the NGF receptor by Chao and colleagues (20) and, independently, by the Shooter lab (21). Both groups identified the cDNA for the lower molecular weight species; it was termed the p75 receptor and proved to contain the low affinity binding site. Eventually, the proto-oncogene Tropomyosin-related kinase A, or TrkA, was recognized as another NGF receptor accounting for the higher molecular weight component (22,23), and a complex of both p75 and TrkA was shown to comprise the high affinity binding site (24). Following screening of cDNA libraries, two additional Trk receptors, TrkB and TrkC, have since been discovered (25). Currently, we know that each of the four members of the neurotrophin family preferentially

bind to particular Trk receptors to exert a variety of biological functions. NGF selectively associates with TrkA, while BDNF and NT-4 both preferentially bind to TrkB, and NT-3 associates with TrkC. Depending upon the cellular context, these interactions can activate well-studied phosphatidylinositol-3-kinase (PI3K)/Akt, Ras/extracellular signal–regulated kinase (ERK), and phospholipase C- γ (PLC- γ) signaling pathways, as well as other signaling cascades, and thereby regulate a diversity of trophic functions, including promotion of cell survival, neurite outgrowth, cell differentiation, synapse formation, and long-term potentiation (26,27). In contrast to the Trk receptors, the signaling mechanisms employed by the p75 receptor have remained much more enigmatic. All four neurotrophins can associate with p75, and, though more poorly understood, these interactions can also have substantial effects on neuronal survival and differentiation, neurite outgrowth, and synaptic plasticity (28). Of these functions for the receptor, over two decades of research has indicated that the effects of p75 signaling on cell survival can be especially profound (28). The remainder of this chapter will focus on the role of the p75 receptor in regulating cell survival, reviewing its established signaling mechanisms and the functional effects of such signaling on neurodevelopment and cellular responses to injury.

The p75 Neurotrophin Receptor

Because the p75 receptor has no catalytic domain, while Trk receptors are tyrosine kinases with potent signaling capability, for several years the p75 receptor was thought to simply function as a binding partner for TrkA, perhaps acting to increase the local concentration of NGF to facilitate activation of TrkA (29). However, there were a number of observations that piqued the interest of researchers in the field, causing them to further

explore the role of p75; for example, the receptor is expressed widely in the developing nervous system, with expression in peripheral neurons, within the spinal cord, and throughout the brain (30). It is expressed by many neuronal cell types, as well as neural stem cells, some astrocytes, oligodendrocyte precursors, Schwann cells and olfactory ensheathing glia (31). Several non-neural tissues also express the receptor during some stage of development, such as kidney and muscle (30,32). In contrast, the Trk receptors exhibit a much more restricted expression pattern. In addition, the p75 receptor is strongly upregulated in many neurons and glial cells following injury, suggesting that it has a functional role in such conditions (discussed below). Finally, there are portions of the intracellular domain, where signaling would initiate, that are highly conserved across species, from chicken to human (33,34). These findings prompted further study of the p75 receptor, and in the 25 years since its initial cloning, it has been shown to regulate an amazing array of cellular responses, including cell survival, cell cycle, neurite outgrowth, synaptic function, and myelination (28).

Structure

After the cloning of p75 it was quickly recognized that it not only bound NGF, but also Brain-derived neurotrophic factor (BDNF) (35), Neurotrophin-3 (NT-3) (36) and Neurotrophin-4 (NT-4) (37), with similar affinity, although with somewhat different kinetics (36). The ability of the receptor to bind all neurotrophins led to its designation as the p75 neurotrophin receptor ($p75^{NTR}$), as opposed to the p75 NGF receptor. The $p75^{NTR}$ interacts with the neurotrophins through the four cysteine-rich domains in its extracellular domain (38). The initial X-ray crystallography structural analysis of the extracellular domain of $p75^{NTR}$ bound to NGF indicated that the receptor monomer binds NGF in an

asymmetrical fashion, resulting in a 1:2 ratio (39). However, considerable biochemical data have since indicated that $p75^{NTR}$ associates with neurotrophins in a 2:2 ratio. Binding analyses using cross-linkers to attach neurotrophins to the receptor indicated a dimer of $p75^{NTR}$ bound to a neurotrophin dimer (40). Further crystallographic analyses support a 2:2 complex between neurotrophins and $p75^{NTR}$ (41,42) and it has been suggested that the 1:2 asymmetrical binding may represent an intermediate in the formation of the 2:2 complex (42).

At least a fraction of $p75^{NTR}$ has been shown to pre-exist as a disulfide-linked dimer (40,43), and a highly conserved cysteine (257) in the transmembrane domain responsible for linking the monomers was recently identified, although non-covalent dimerization still occurred even when cysteine 257 was mutated (44). Further analysis revealed that a conserved $AxxxG^{266}$ sequence in the transmembrane region, which is often found in self-associating transmembrane proteins, is required for the formation of dimers. Through their studies, the authors elucidated an interesting aspect of the receptor's structural dynamics that provided a mechanism by which $p75^{NTR}$ transduces its signal upon ligand binding: the disulfide in the transmembrane domain acts as a pivot point, such that when the extracellular domain clamps down on a neurotrophin, the intracellular domains separate. The parting of the dimerized intracellular portions of the receptor facilitates binding of signaling molecules necessary for $p75^{NTR}$-mediated cell death (Vilar et al., 2009).

The intracellular domain (ICD) of $p75^{NTR}$ contains a region similar to the Tumor necrosis factor receptor (TNFR) and the Fas antigen (45-47). Since TNFR and Fas mediate apoptotic signals, this portion of their ICD was termed the "death domain." The

3-D structure of $p75^{NTR}$'s death domain was determined by NMR and was similar to the structure of the Fas death domain, although there were a few differences. In particular, the death domain of Fas and TNFR self-assemble, while that of $p75^{NTR}$ does not (48). This result is in agreement with the ability of TNFR and Fas to signal by recruiting other death domain-containing proteins while the intracellular interactors of $p75^{NTR}$ so far identified do not contain a death domain.

In addition to the full length form of $p75^{NTR}$, a splice variant was reported lacking exon III, which encodes the cysteine-rich domains 2, 3 and 4 that are required for neurotrophin binding (49). The original $p75^{NTR}$ knockout mouse was created by deleting exon III (50); thus the short form of $p75^{NTR}$ (s-$p75^{NTR}$) could still be detected in these mice, in principle. The existence of s-$p75^{NTR}$, however, remains rather controversial, and its function is not known. Nevertheless, an alternative mutant mouse was created lacking exon IV, such that both splice isoforms of $p75^{NTR}$ are deleted (49). These mice exhibit a number of neurological and vascular defects similar to the exon III knockout mice, but with a more severe phenotype. However, understanding the phenotype of the exon IV mutants is complicated by the fact that the targeting strategy created a cryptic truncated protein encoding an extracellular stalk with the entire transmembrane and intracellular domains of the receptor (51). Since expression of the intracellular domain of the receptor can initiate signaling independent of ligand (52), some phenotypic characteristics of this mouse may be due to the expression of this fragment. Clearly, results from using either of these genetically altered mice need to be interpreted with caution, and further study is needed to understand the role of s-$p75^{NTR}$.

Apoptotic Signaling

Although p75^{NTR} was first discovered for its ability to bind NGF, which promotes neuronal survival, the most investigated function of the receptor is, ironically, its ability to induce programmed cell death. One of the earliest indications of this function was revealed in a study by Bredesen's group demonstrating that ectopic expression of p75^{NTR} in an immortalized neural cell line increased apoptosis after serum withdrawal (53). These results proved challenging to reproduce in primary cells with the endogenous receptor; however, the groups of Barde and Chao found that activation of endogenous p75^{NTR} by NGF could induce apoptosis in early retinal neurons in the chick (54) and oligodendrocytes in rat (55), respectively. The ability of p75^{NTR} to induce programmed cell death in response to ligand binding has now been observed in a wide variety of neuronal and non-neuronal cell types, including sympathetic (56-58), motor (59), and hippocampal neurons (60); photoreceptor cells (61); oligodendrocytes (55); Schwann cells (62,63); and other cells (64-66). These *in vitro* studies together with the analysis of *p75^{NTR}-/-* mice have established this receptor as a critical regulator of developmental apoptosis, promoting the naturally occurring elimination of neurons within the developing basal forebrain (67), trigeminal ganglia (68), retina (54), superior cervical ganglion (57), and spinal cord(69). This developmental role of p75^{NTR} has been particularly well characterized in sympathetic neurons. These neurons express TrkA and p75^{NTR}, which together mediate a survival signal in response to NGF (discussed below); however, Miller and colleagues demonstrated that selective activation of p75^{NTR} by BDNF led to apoptosis (57). Furthermore, deletion of the receptor resulted in an increase in the number of neurons during the development of the superior cervical ganglia, suggesting that p75^{NTR}

mediates normal developmental death in this population. Ginty's group later demonstrated that these neurons produce BDNF in response to NGF and suggested a model in which neurons receiving robust trophic support through NGF-induced activation of TrkA produce BDNF, thereby promoting $p75^{NTR}$-dependent death of neighboring neurons receiving insufficient NGF signal (70). Their computer simulations based on this model quite accurately predicted the normal developmental kinetics of cell death in the superior cervical ganglia.

Activation of the Mitochondrial Cascade

Over the past decade, significant progress has been made in understanding the cellular mechanisms through which $p75^{NTR}$ promotes apoptosis, although many facets of the receptor's signaling remain enigmatic. Members of the TNF receptor superfamily can activate two pathways that regulate cell survival. Through their death domain, they recruit other death domain-containing adaptors, such as TRADD and FADD, leading to caspase-8 activation and induction of a terminal caspase cascade (71). Despite attempts to detect activation of caspase-8 (72,73), no evidence supports $p75^{NTR}$ utilizing this pathway, which agrees with the structural divergence of $p75^{NTR}$'s death domain from that of TNFR and Fas. The second pathway initiated by many members of the TNF receptor family involves stimulation of the stress-activated kinase c-Jun N-terminal kinase (JNK) and of the transcription factor NF-κB (71). JNK activation causes cell death by inducing phosphorylation of the transcription factor c-Jun and the tumor suppressors p53 and p73 (74), resulting in transcriptional up-regulation of an array of pro-apoptotic genes, including Bax (75), PUMA (76), Bak (77), and Caspase-6 (78), among others (79). In addition, JNK directly phosphorylates several Bcl-2 family proteins, causing inhibition of pro-survival

members such as Bcl-2 (80) and activation of pro-death members such as Bim (81) and Bad (82). These events ultimately lead to the release of cytochrome c from mitochondria and caspase-dependent apoptosis (77).

An accumulation of evidence has indicated that $p75^{NTR}$-induced apoptosis occurs via this mitochondrial cascade. Activation of JNK in response to ligand binding to endogenous $p75^{NTR}$ has been demonstrated in oligodendocytes (55), sympathetic neurons (57) and hippocampal neurons (83), and inhibition of the kinase prevented the induction of apoptosis (83-87). Overexpressing $p75^{NTR}$ in cortical neurons also resulted in activation of JNK (88). In mammals, there are 3 genes encoding the JNK family, JNK1-3. While JNK1 and JNK2 are ubiquitously expressed, JNK3 is selectively expressed in the nervous system and heart (89-91) and has been suggested to be the primary isoform mediating neuronal death in response to a variety of ligands and insults (74). Of these three JNK isoforms, JNK3 was selectively activated following ligand binding to $p75^{NTR}$ in oligodendrocytes (85) and sympathetic neurons (87), and gene deletion of JNK3 prevented receptor-mediated apoptosis both *in vitro* and *in vivo* (87,92).

Further support for $p75^{NTR}$ activating a JNK-p53 apoptotic pathway comes from the fact that cell death mediated by the receptor is associated with upregulation of p53 (93,94). Induction of apoptosis by $p75^{NTR}$ has also been linked to phosphorylation of Bim (95) and Bad (88), cytochrome c release (88), and cleavage of procaspase-3, -6, -7, or -9 (88,96). Curiously, however, the receptor does not require c-Jun for killing sympathetic neurons (97).

Cytosolic Factors Linking p75^{NTR} to JNK

Like many other receptors of the Tumor Necrosis Factor (TNF) receptor superfamily, p75^{NTR} promotes downstream signaling via association with a number of cytosolic interactors (Fig. 1). One group of p75^{NTR} interactors that contributes to activation of JNK is the family of TNF receptor associated factors (TRAFs). TRAF family proteins are distinguished by a conserved C-terminal domain that is responsible for their oligomerization and interactions with the cytoplasmic domains of TNF receptor family members (98). With the exception of TRAF1, all TRAF family members also feature an N-terminal domain-containing RING and zinc finger structures that are critical for their signaling function. The RING finger domain in the TRAFs acts as an E3 ubiquitin ligase, but instead of targeting proteins for proteasomal degradation, the TRAFs form a ubiquitin chain through Lysine 63 linkages, which serve as protein-protein interaction motifs (99,100). TRAF1-6 have been reported to associate with p75^{NTR}, with TRAF2, 4 and 6 shown to modulate p75^{NTR}-induced cell death via interactions with the ICD of the receptor (101,102). However, the role of TRAF6 in p75^{NTR} signaling has been the most thoroughly studied. TRAF6 associates with p75^{NTR} in a ligand-dependent manner (102) and mediates signaling from the receptor to both JNK and NF-κB (86,102). Sympathetic neurons from *traf6-/-* mice fail to activate JNK in response to BDNF binding to p75^{NTR} and fail to undergo apoptosis (86). Furthermore, there is reduced developmental cell death in the superior cervical ganglia in *traf6-/-* mice relative to the wild type, indicating that TRAF6 is essential for p75^{NTR}-mediated apoptotic signaling *in vivo*.

TRAF6 also associates with the neurotrophin receptor-interacting factor (NRIF) to promote JNK activation (94,103). NRIF is a zinc-finger protein that was first identified in

a yeast 2-hybrid screen for proteins interacting with the ICD of $p75^{NTR}$ (104). NRIF and TRAF6 can directly interact, and overexpression of NRIF together with TRAF6 enhanced TRAF6-mediated JNK activation (105). Furthermore, BDNF-induced JNK activation and cell death were significantly attenuated in *nrif-/-* sympathetic neurons (94). Gene deletion revealed that NRIF was required for developmental apoptosis in the retina (104), which is a $p75^{NTR}$-dependent process (54). Thus, interaction of NRIF with TRAF6 and $p75^{NTR}$ appears to be critical for $p75^{NTR}$-mediated JNK activation and apoptosis. However, expression of NRIF alone in mouse embryonic fibroblasts was not sufficient to activate the kinase, although it did induce cell death (94). Exactly how NRIF contributes to the activation of JNK is not clear, but it may facilitate oligomerization of TRAF6, which is necessary for it to mediate its biological actions (106).

Another intracellular binding partner of $p75^{NTR}$ that is linked to JNK activation is the Neurotrophin receptor-interacting MAGE homolog, NRAGE (also known as Maged1 and dlxin)(107). NRAGE contains a melanoma-associated antigen (MAGE) domain, which is a region of homology defining the MAGE family of proteins. The function of the MAGE proteins is poorly understood, but many have been implicated in the regulation of cell cycle and apoptosis (108). Ectopic expression of NRAGE along with $p75^{NTR}$ in a sympathetic precursor cell line enabled NGF-dependent cell death, thereby implicating this interactor in the apoptotic pathway activated by $p75^{NTR}$ (109). Overexpression of NRAGE in PC12 cells led to potent activation of JNK, release of cytochrome c from mitochondria, and the induction of caspases -3, -6, and -9, ultimately resulting in cell death (107). These results suggested that NRAGE could be involved in $p75^{NTR}$-mediated stimulation of JNK. Corroborating evidence came from analysis of *nrage-/-* mice: $p75^{NTR}$-

induced JNK activation in *nrage-/-* sympathetic neurons was significantly reduced compared to wild type neurons (110). Furthermore, the null animals have an increased number of neurons in their superior cervical ganglia, like *p75^{NTR}-/-* mice, and sympathetic neurons isolated from *nrage-/-* mice were resistant to p75^{NTR}-mediated apoptosis (110). These results suggest a function for NRAGE as an adaptor protein, linking the receptor to JNK activation and apoptosis. Whether NRAGE, TRAF6, and NRIF form a complex or function independently to regulate the kinase remains an open question; however, they may function at different stages of the cascade to affect the kinetics of JNK activity (discussed below). It should be noted that sequestering the anti-apoptotic factor XIAP (111,112) and promoting degradation of the anti-apoptotic transcription factor Che1 (113) have also been suggested as mechanisms through which NRAGE affects cell survival, though these interactions have not been studied in the context of p75^{NTR} signaling.

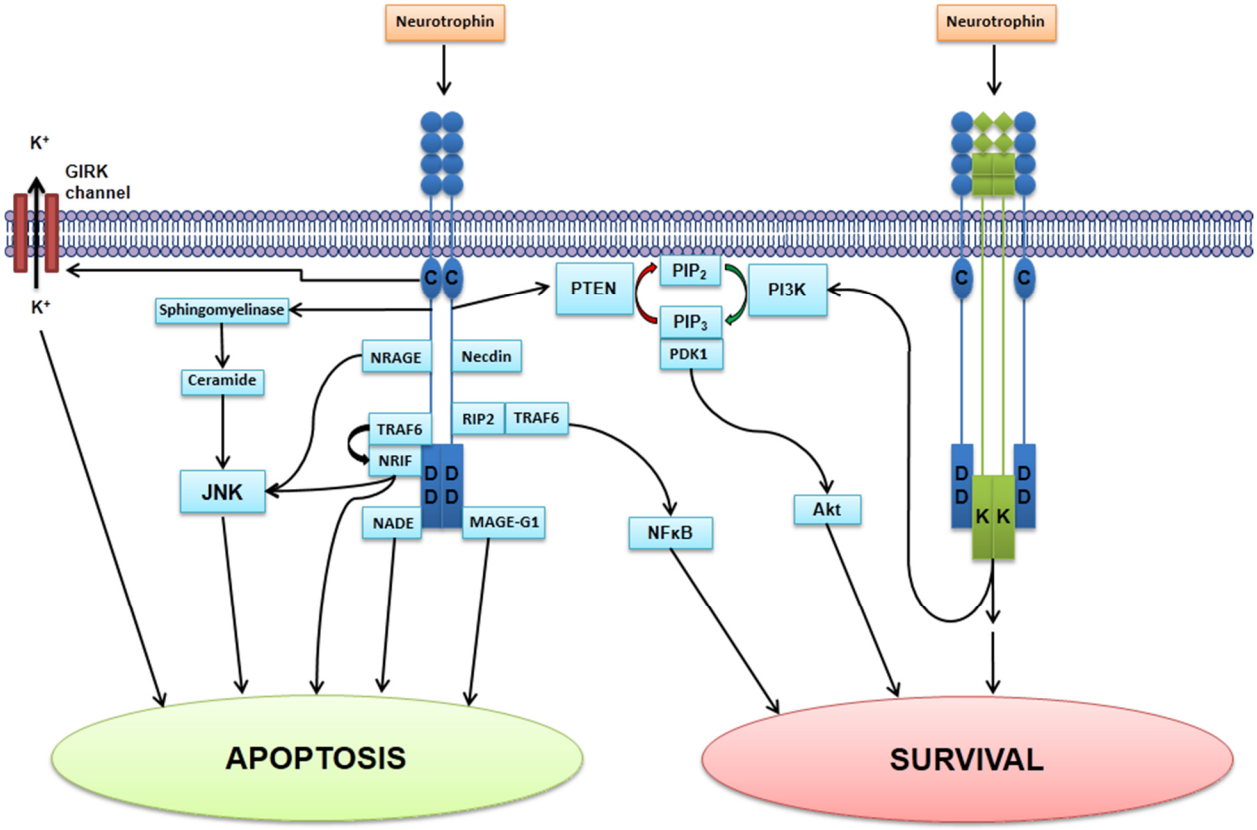

Figure 1: Signaling pathways mediated by p75^{NTR} that regulate cell survival and apoptosis. In response to neurotrophin binding, p75^{NTR} promotes JNK activation via interactions with NRAGE, TRAF6, and NRIF, thus leading to apoptosis. Activation of JNK by p75^{NTR} also occurs through induction of sphingomyelinases. The chopper domain of p75^{NTR} promotes apoptosis by facilitating depletion of internal K^+ through GIRK channels. Other cytosolic interactors contribute to p75^{NTR}-mediated cell death, including NADE, MAGE-G1, and Necdin. In response to pro-neurotrophins, p75^{NTR} inhibits Trk-mediated survival signaling via induction of PTEN and the resultant inhibition of PI3K-Akt survival signaling. Promotion of cell survival by p75^{NTR} is facilitated by its interactions with Trk receptors which enhance Trk-mediated PI3K-Akt survival signaling, as well as other Trk-mediated survival pathways. P75^{NTR} may also promote survival via activation of NFκB, possibly through associations between RIP2 and TRAF6 (abbreviations: DD, p75^{NTR} death domain; C, p75^{NTR} chopper domain; K, Trk receptor tyrosine kinase domain).

Another mechanism through which p75^{NTR} has been suggested to regulate JNK involves production of the lipid signaling molecule ceramide (Fig. 1). When the field was searching for evidence of signaling by p75^{NTR}, a NGF-mediated increase in ceramide levels through activation of neutral sphingomyelinase in T9 glioma cells was one of the first signals detected (114). Multiple reports have since confirmed the ability of p75^{NTR} to stimulate ceramide production in other cell types, including in oligodendrocytes (55), hippocampal neurons (115), Schwann cells (116), and mesencephalic neurons (117). One known downstream effect of elevated ceramide is activation of JNK (118), and thus ceramide may couple p75^{NTR} to JNK phosphorylation. Indeed, in cultured hippocampal neurons activation of p75^{NTR} resulted in upregulation of ceramide, stimulation of JNK, and cell death (115). Furthermore, inhibition of sphingomyelinase in these neurons prevented ceramide accumulation, JNK activation, and the induction of apoptosis. However, increasing ceramide levels does not always result in cell death. In fact, p75^{NTR}-mediated ceramide production has also been linked to promotion of cell survival (119,120). Understanding this lipid signaling pathway is complicated by the fact that ceramide is a central intermediate in sphingolipid metabolism and can have a variety of effects depending on the specific fatty acid chain attached and its cellular concentration and localization (121). Further studies are needed to elucidate the mechanisms by which p75^{NTR} activates sphingomyelinase and to reveal how ceramide elicits its effects in various cellular contexts.

Other Factors Involved in p75^{NTR}-Mediated Apoptosis

Apart from TRAF6, NRIF, and NRAGE, several other cytosolic proteins have been shown to associate with p75^{NTR} and suggested to regulate its apoptotic signaling. For

example, $p75^{NTR}$ associated cell death executor (NADE), a novel protein isolated in a two-hybrid screening for proteins binding to the ICD of the receptor, was reported to associate with endogenous $p75^{NTR}$ in PC12 cells (122). Overexpression of NADE together with $p75^{NTR}$ in HEK 293 cells induced apoptosis, (122) and expression of a fragment of NADE lacking the region identified as necessary for promoting apoptosis blocked receptor-mediated cell death in oligodendrocytes (123). Currently, though, how NADE contributes to $p75^{NTR}$-mediated apoptotic signaling is unknown. In addition, MAGE-G1, MAGE-H1 and the MAGE-related protein, Necdin, have also been shown to interact with $p75^{NTR}$ (124,125). Both Necdin and MAGE-G1 associate with E2F1, a transcription factor that is important for G1/S transition in the cell cycle and that can induce apoptosis in postmitotic cells (126). When the ICD of $p75^{NTR}$ was overexpressed in a neuroblastoma cell line, Necdin and MAGE-G1 bound to the receptor ICD, thereby releasing E2F1 and triggering apoptosis (125,127). Additional studies are needed to determine whether Necdin and MAGE-G1 regulate ligand-mediated cell death in primary cells. The $p75^{NTR}$ has also been reported to promote apoptosis through upregulation of the sugar binding protein Galectin-1 (128). Embryonic stem (ES) cells were engineered to express $p75^{NTR}$ when they are induced to differentiate into neurons. Expression of $p75^{NTR}$ was found to induce neurite degeneration which correlated with expression of Galectin-1. Expression of Galectin-1 was also found to promote neurite degeneration and death of the ES cells, as well as cortical neurons (128). Furthermore, mice lacking Galectin-1 were resistant to neuronal apoptosis caused by pilocarpine-induced seizures (129), which was demonstrated to be a $p75^{NTR}$-dependent process (60,130). The mechanisms by which this lectin causes cell death remain to be determined.

Regulated Intramembrane Proteolysis of p75^{NTR}

In a manner similar to Notch and Amyloid precursor protein (APP), p75^{NTR} undergoes regulated intramembrane proteolysis (RIP). Proteolysis of p75^{NTR} was first described as a response to phorbol esters in HEK293 cells transfected with the receptor (131,132). The extracellular region of p75^{NTR} is first cleaved by the metalloproteinase TNFα-Converting Enzyme (TACE, also known as ADAM17), thereby producing a 24 kD membrane-bound C-terminal fragment (p75^{NTR}-CTF) (133). This cleavage event appears to be quite promiscuous in terms of the amino acid sequence; however, deletion analysis revealed that at least 15 residues extracellular to the transmembrane domain are required (134). Following release of the soluble ectodomain, the p75^{NTR}-CTF is then further cleaved within its transmembrane region by the γ-secretase complex, thereby releasing the 19 kD intracellular domain of the receptor (p75^{NTR}-ICD). Similar to proteolysis by TACE, cleavage by γ-secretase is quite permissive for various amino acids; nevertheless, there must be some sequence specificity for both enzymes since substituting the transmembrane domain of Fas for that of p75^{NTR} blocked cutting by γ-secretase, and replacing the 15 juxtamembrane sequence with the Fas sequence blocked p75^{NTR} proteolysis by TACE (134). The order of the two cleavage reactions is also invariant, with TACE acting on the receptor prior to γ-secretase. This was determined by studies in which cleavage of p75^{NTR} by γ-secretase was prevented by TACE inhibition, but inhibition of γ-secretase did not affect TACE activity, thus indicating that release of the extracellular domain is required for further proteolysis of the receptor within the transmembrane domain (87,134). Since the initial finding of RIP of p75^{NTR} in response to phorbol esters, a number of reports have demonstrated that proteolysis of p75^{NTR} occurs through a ligand-dependent mechanism; for example, treatment of sympathetic neurons with BDNF

(87,135), Schwann cells with NGF (136) and cerebellar neurons with Myelin associated glycoprotein (137) resulted in RIP. It is unclear, however, whether ligand-activated $p75^{NTR}$ always results in RIP.

One functional role of $p75^{NTR}$ cleavage, like for many γ-secretase substrates, is to facilitate signaling to the nucleus. Release of the $p75^{NTR}$-ICD may facilitate nuclear translocation of associated factors such as NRIF. Although NRIF was shown to be required for $p75^{NTR}$-mediated apoptotic signaling based on analyses of *nrif-/-* mice (94,104), exactly how it contributed to the cell death was not clear. NRIF contains a classic C2H2 zinc-finger motif (104), which are typically found among DNA binding transcription factors (138), suggesting that in addition to facilitating JNK activation, NRIF could bind DNA and regulate transcription. The recognition of $p75^{NTR}$ proteolysis by γ-secretase revealed a possible mechanism by which NRIF could be translocated from the surface-bound ICD of the receptor to the nucleus. Indeed, it was demonstrated that BDNF-induced cleavage of the receptor in sympathetic neurons facilitated nuclear localization of NRIF and, subsequently, apoptosis (135). Blocking receptor cleavage prevented both localization of NRIF to the nucleus and cell death. A similar signaling cascade has been detected in hippocampal neurons, where neuronal death due to pilocarpine-induced seizures was associated with $p75^{NTR}$ proteolysis and NRIF nuclear translocation. Moreover, the number of apoptotic neurons after seizure was significantly reduced in *$p75^{NTR}$-/-* (139) and in *nrif-/-* mice (60).

The mechanism of NRIF nuclear translocation also depends on TRAF6-mediated ubiquitylation. TRAF6 was shown to ubiquitylate NRIF following ligand binding to $p75^{NTR}$, and blocking this event by mutating the ubiquitin-attachment site of NRIF prevented its

nuclear translocation and inhibited $p75^{NTR}$-mediated apoptosis (140). The ubiquitylation of NRIF required $p75^{NTR}$ cleavage (135), suggesting that receptor proteolysis facilitates an interaction between NRIF and TRAF6, enabling ubiquitylation of NRIF, which is needed for it to enter the nucleus, and oligomerization of TRAF6, which promotes the activation of JNK (Fig. 2.).

The cleavage of $p75^{NTR}$ and the activation of JNK were recently shown to occur through interdependent pathways. In sympathetic neurons, JNK activation was required for ligand-induced proteolysis of the receptor by both TACE and γ-secretase (87), as blocking JNK activity or deleting JNK3 prevented receptor cleavage by both proteases. The activation of JNK facilitated the transcriptional upregulation of TACE and, through an unknown mechanism, stimulated both TACE and γ-secretase, thereby inducing $p75^{NTR}$ processing. Interestingly, the release of the receptor's ICD, along with NRIF and TRAF6, was necessary for prolonged JNK stimulation by the receptor. Expression of a non-cleavable mutant $p75^{NTR}$ prevented JNK activation at 24 hours, yet the kinase was still activated for the first hour after ligand binding (87). Hence, there appears to be a biphasic activation of JNK by $p75^{NTR}$, with an early signal, perhaps initiated through NRAGE, inducing proteolytic processing of the receptor, which allows NRIF and TRAF6 to promote long-term stimulation of the kinase as well as nuclear signaling, ultimately resulting in cell death (Fig. 2).

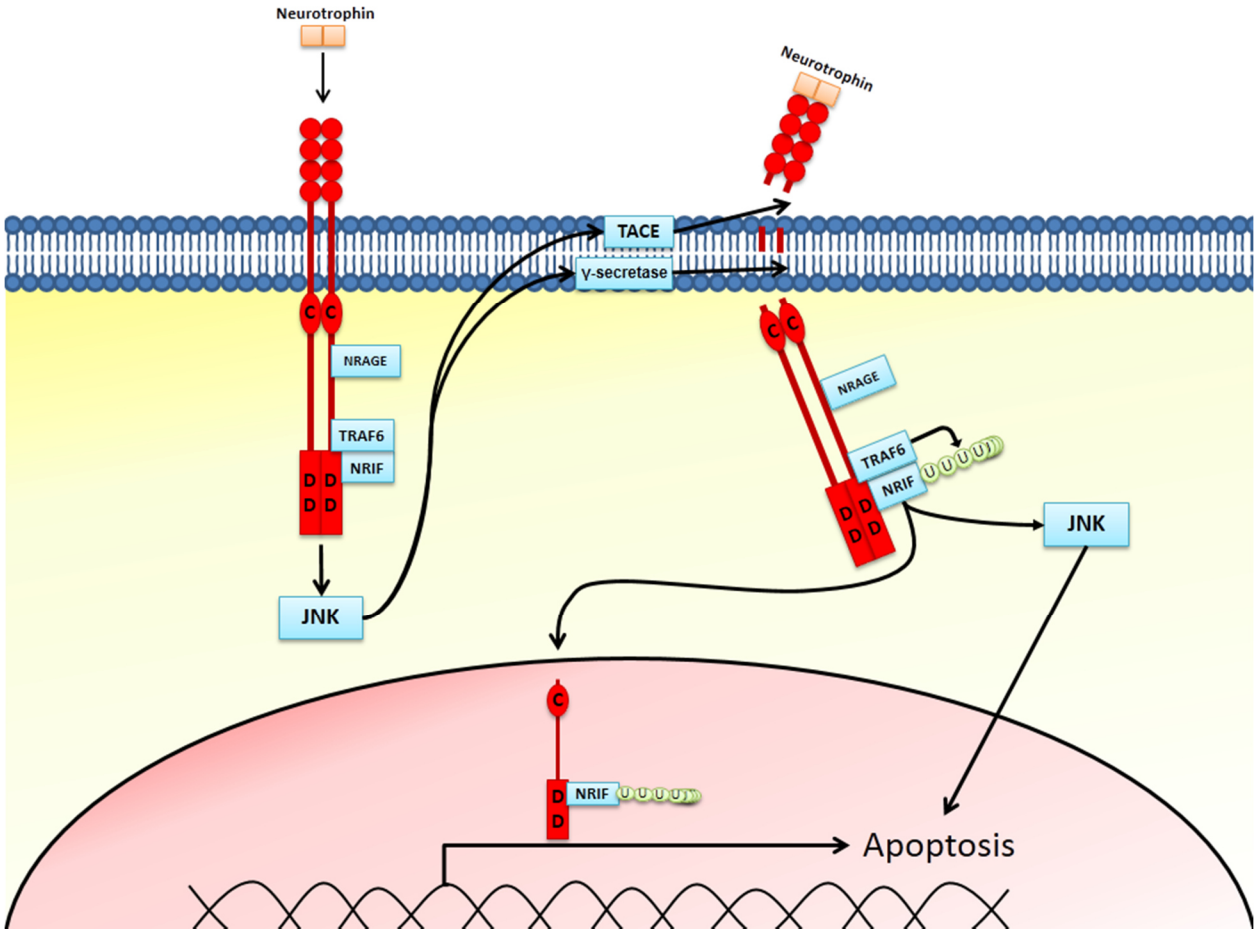

Figure 2: Cell death signaling initiated by regulated intramembrane proteolysis (RIP) of p75^{NTR}. Stimulation of p75^{NTR} by neurotrophins promotes an early phase of JNK activation, occurring within 30 min of ligand binding. Through a mechanism currently unknown, JNK induces sequential proteolytic cleavage of p75^{NTR} by TACE and γ-secretase. Release of the p75^{NTR} intracellular domain promotes TRAF6-dependent ubiquitylation and nuclear translocation of NRIF, as well as persistent JNK activation, ultimately leading to induction of programmed cell death (abbreviations: DD, death domain; C, chopper domain; U, ubiquitin).

In contrast to the evidence that proteolytic processing of $p75^{NTR}$ induces apoptosis by releasing the $p75^{NTR}$-ICD, in certain cellular contexts programmed cell death may be activated by the $p75^{NTR}$-CTF alone. Coulson et al. found that overexpression of the $p75^{NTR}$-CTF was sufficient to promote the apoptosis of dorsal root ganglion (DRG) neurons and that the death domain was not necessary (34,141). This function of the $p75^{NTR}$-CTF required a 29 amino acid sequence in the cytoplasmic juxtamembrane region of the receptor termed the “chopper domain” (34). Coulson and colleagues demonstrated that ectopic expression of membrane-associated fragments of $p75^{NTR}$ containing the chopper domain promoted apoptosis by inducing a Rac-dependent increase in phosphatidylinositol 4,5-bisphosphate (PIP_2). In turn, PIP_2 stimulated G-protein-coupled inwardly rectifying potassium (GIRK) channels, causing a depletion of internal potassium that ultimately activated an apoptotic protease activating factor 1 (APAF-1)-dependent cell death pathway (142-144). It should be cautioned, however, that these studies relied on overexpression of the CTF; thus further studies are needed to determine how the various fragments of the receptor regulate cell death under different physiological conditions.

Proneurotrophins and Sortilin

The initial discovery that $p75^{NTR}$ can induce programmed cell death was somewhat puzzling, as *in vitro* studies indicated that relatively high concentrations of neurotrophins were needed to induce apoptosis, and in certain cell types, cross-reactivity of neurotrophins with Trk receptors could potentially promote an opposing, pro-survival signal. An answer was found, at least in part, by Hempstead’s group, who discovered that precursor forms of neurotrophins are biologically active, selective ligands for $p75^{NTR}$. Like

most secreted proteins, neurotrophins are initially synthesized as larger precursors, which are enzymatically cleaved to generate the mature form of the protein (145,146). Proneurotrophins have an amino-terminal pro-domain that assists in their proper folding and dimerization (147-149). The pro-domain can be proteolytically removed by furin and pro-protein convertases in the endoplasmic reticulum and Golgi apparatus (150). Alternatively, the cleavage of the pro-domain can also be mediated by plasmin and matrix metalloproteases following secretion of the proneurotrophin into the extracellular milieu (151). While it was originally thought that mature neurotrophins are the only physiologically active ligands for $p75^{NTR}$, it is now well-established that endogenous proneurotrophins can be secreted to function as potent activators of $p75^{NTR}$ signaling (56,151-154).

Proneurotrophins do not activate Trk receptors (151,155) and have been demonstrated to induce significant $p75^{NTR}$ mediated cell death at sub-nanomolar concentrations (151). Thus, proteolytic processing determines the functional fate of nascent neurotrophins, with uncleaved forms selectively triggering $p75^{NTR}$-mediated cell death and mature forms activating either $p75^{NTR}$ or Trk receptors, depending upon the cellular context. Proneurotrophins induce programmed cell death by binding to a high affinity protein complex containing $p75^{NTR}$ and its co-receptor Sortilin, a member of the Vps10p-domain receptor family (56,156). Mammalian members of the Vps10p family, which consists of Sortilin, SorLA, and SorCS-1, -2, and -3, are type I transmembrane receptors with multifunctional roles that include the modulation of protein sorting and trafficking, as well regulation of signal transduction (157). Proneurotrophins bind to Sortilin via their pro-domain and to $p75^{NTR}$ by their mature domain, thus facilitating the association

of these two receptors to initiate programmed cell death (56,156,158). Following initial reports that Sortilin mediates neurotrophin-induced cell death *in vitro* (56,156), studies have indicated that Sortilin is required for developmental $p75^{NTR}$-mediated cell death *in vivo*. For example, mice lacking Sortilin have a reduction in the developmental apoptosis of retinal ganglion cells that is indistinguishable from that of $p75^{NTR}$-deficient mice (159). However, Sortilin may not be required for all $p75^{NTR}$-mediated cell death, as these mice did not have defects in the apoptosis of sympathetic neurons during the developmental time period in which $p75^{NTR}$-mediated death is known to occur (159). Loss of Sortilin did, however, impair age-related degeneration of these neurons, suggesting that proneurotrophins may not have been involved in the early development of the sympathetic neurons, but do have a role in their loss during aging.

Apoptotic Role of $p75^{NTR}$ in Pathology

In addition to its critical role during neurodevelopment, $p75^{NTR}$ is a stress-activated receptor that stimulates the death of cells within injured tissue. Though the receptor is downregulated in most regions of the nervous system after early postnatal development, re-expression of $p75^{NTR}$ occurs in response to many forms of cellular damage. For example, increases in $p75^{NTR}$ expression have been reported following neuronal axotomy (153,160-163), mechanical damage (152,164,165), elevated intraocular pressure (166), seizures (60,130), and focal ischemia (167). Beyond measuring increases in expression of the receptor, multiple studies have more definitively demonstrated that $p75^{NTR}$ signaling is responsible for injury-induced cell death *in vivo*. In one such study, unilateral administration of kainic acid to the basal forebrain resulted in re-expression of $p75^{NTR}$ in the degenerating cholinergic neurons, which correlated with their apoptosis.

Administration of a function-blocking $p75^{NTR}$ antibody prevented this cell death, thereby indicating that $p75^{NTR}$ signaling contributes to excitotoxin-induced death of basal forebrain neurons (168). Similarly, expression of $p75^{NTR}$ was induced and associated with programmed cell death caused by axotomy of corticospinal neurons, and antibodies to $p75^{NTR}$ prevented this apoptosis (160). Although these two studies indicated that $p75^{NTR}$ promotes neuronal death after injury, whether proneurotrophins contribute to the death caused by these injuries was not known. In a later report, injury to the spinal cord was found to induce production of proNGF and to stimulate $p75^{NTR}$-dependent apoptosis of spinal cord oligodendrocytes (152). ProNGF extracted from the injured region elicited apoptosis of cultured oligodendrocytes expressing $p75^{NTR}$, but not of *$p75^{NTR}$-/-* oligodendrocytes. Thus, this work suggested that proNGF functions to promote the elimination of damaged cells by activating $p75^{NTR}$ after spinal cord injury (152). A subsequent study by Yoon and colleagues demonstrated that axotomy of corticospinal neurons also resulted in apoptosis of the neurons through a proNGF–$p75^{NTR}$-dependent mechanism (Harrington et al., 2004). Following lesion of the internal capsule, proNGF was detected in cerebral spinal fluid, indicating that proNGF is produced and secreted *in vivo* after brain injury. In the cortex of lesioned animals, an interaction between proNGF and $p75^{NTR}$ was detected *in vivo*, and disruption of this interaction by infusion of an antibody specific for proNGF prevented the apoptosis caused by the injury (153). These experiments provided the first conclusive evidence that proNGF is a pathophysiological ligand that induces apoptosis in response to neuronal damage. Since then, a growing body of evidence has linked proneurotrophins to cell death induced by various types of injury. For example, hippocampal seizures stimulated the upregulation and secretion of

proNGF *in vivo*, and antibodies specific for proNGF prevented seizure-induced apoptosis of neurons within the dentate gyrus (60). In another study, increases in proNGF, along with $p75^{NTR}$ and Sortilin, were reported in the retina after exposure of albino mice to intense light, and blockade of Sortilin with the pro-domain of proNGF attenuated light-induced retinal cell death (169). The ability of proneurotrophins to induce cell death in response to cellular damage is likely not specific to proNGF, as proBDNF has also been demonstrated to promote apoptosis in a number of cell culture models (56,170,171), and upregulation of proBDNF has been detected in *in vivo* injury models, such as in an animal model of cochlear damage (172). ProBDNF has also been implicated in apoptosis occurring due to neuronal axotomy, as infusion of a proBDNF antibody prevented the death of sensory neurons induced by lesion of the sciatic nerve *in vivo* (170).

While the signaling mechanisms responsible for the induction of $p75^{NTR}$ and the proneurotrophins after injury are not well understood, several studies have provided some clues. One possibility is that cellular damage prevents the proteolytic processing of neurotrophins, thus increasing the release of death-inducing proneurotrophins. A recent study by Friedman and colleagues has revealed that following kainic acid-induced seizures, the proneurotrophin-processing enzyme matrix metalloproteinase-7 (MMP-7) and its inhibitor tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) were regulated in a manner that would hinder cleavage of proneurotrophins and lead to increased release of proNGF (173). Decreased MMP-7 production has also been observed in samples from human patients and animal models with diabetic retinopathy (174). These findings suggest that regulation of proteolytic processing of proneurotrophins is one mechanism by which the levels of these factors are modulated, though a greater understanding of the

pathways regulating their release in the unprocessed versus the mature form is needed. Along with increases in the levels of proneurotrophins, upregulation of $p75^{NTR}$ after injury may occur due to inflammatory signals released in response to tissue damage. The inflammatory cytokines interleukin-12 (IL-12), tumor necrosis factor alpha (TNFα), and interleukin-1ß have been demonstrated to increase $p75^{NTR}$ expression in a variety of *in vitro* systems, such as in cultured hippocampal neurons (175), natural killer cells (176), or astrocytes (175). Interestingly, a recent report indicated that trauma-induced upregulation of $p75^{NTR}$ could also result from calcium influx. Within axotomized hippocampal neurons, cellular responses to GABA change from hyperpolarizing to depolarizing, leading to increased intracellular calcium and the subsequent activation of Rho kinase (ROCK). The activation of ROCK resulted in upregulation of $p75^{NTR}$, ultimately leading to neuronal death (177). Thus, multiple signals may contribute to trauma-induced upregulation of the receptor. However, the mechanisms by which these signals increase $p75^{NTR}$ transcription are still poorly understood. The ubiquitous transcription factor Sp1 has been linked to $p75^{NTR}$ basal expression (178) and upregulation following hypo-osmotic stress (179,180), but whether this factor is involved in other forms of injury is not known. It should also be added that long-term treatment of SH-SY5Y cell lines with IGF1 resulted in a significant upregulation of $p75^{NTR}$ levels (181), suggesting that a factor that responds to IGF1 signaling may also be involved.

In addition to regulating cell death following injury, $p75^{NTR}$ signaling has been suggested to contribute to neurodegeneration caused by a number of diseases. Among these disorders, the link between $p75^{NTR}$ and Alzheimer's disease (AD) has been most studied. Besides Purkinje neurons in the cerebellum, $p75^{NTR}$ is expressed at high levels

in cholinergic neurons of the adult basal forebrain, a population of neurons that undergoes severe degeneration early in the progression of AD pathology. Additionally, several *in vitro* studies have indicated that amyloid beta 1-42 (Aß), the main component of plaques commonly found within brains of AD patients, is a pro-apoptotic ligand for $p75^{NTR}$ (182-184). These findings have led to the hypothesis that activation of $p75^{NTR}$ by Aß contributes to neurodegeneration caused by AD. This idea has remained controversial, however, due to other reports indicating that expression of $p75^{NTR}$ is protective against Aß induced toxicity (185,186). Nonetheless, a role for $p75^{NTR}$ in Aß induced neurotoxicity was recently strengthened by an *in vivo* finding that deletion of $p75^{NTR}$ prevented the degeneration of cholinergic basal forebrain neurons *in vivo* following Aß injection into the hippocampus (187). Furthermore, when *$p75^{NTR}$-/-* mice were crossed with the Thy1-$hAPP^{Lond/Swe}$ mouse model of AD, the degeneration of hippocampal and forebrain cholinergic fibers was dramatically rescued (188). Just as for the *in vitro* studies, however, these *in vivo* studies were also challenged by a recent study by Wang et al, which indicated that $p75^{NTR}$ signaling induces production of Aß, since deletion of the *$p75^{NTR}$* gene in the APPswe/PS1dE mouse model of AD resulted in decreased production of Aß within cortical neurons (189). Despite some differences, these findings together suggest that $p75^{NTR}$ signaling by Aß peptides contributes to overall AD pathology. Apart from Aß-induced apoptosis, studies have also implicated proNGF in AD pathology. Increased expression of proNGF has been detected in human brains affected by AD (190,191), and proNGF isolated from these brain samples induced $p75^{NTR}$-mediated death of cultured sympathetic neurons (190,192). Thus, in addition to activation of $p75^{NTR}$ by Aß, enhanced production of proNGF may contribute to neurodegeneration within the AD brain. While

these studies provide multiple links between $p75^{NTR}$ signaling and AD induced-neurodegeneration, collective evidence suggests that the degeneration of neurons in AD occurs near the end-stages of the disease (193). Hence, understanding whether $p75^{NTR}$ plays a critical role in the onset and early progression of AD remains essential.

While the majority of studies related to $p75^{NTR}$ and neurodegenerative disease have focused on the contributions of the receptor to AD, it is perhaps not surprising that $p75^{NTR}$ has been linked to a number of other disorders. For example, $p75^{NTR}$ may contribute to degeneration of motor neurons during the progression of amyotrophic lateral sclerosis (ALS). Though $p75^{NTR}$ is downregulated in motor neurons of the spinal cord during the perinatal period, re-expression of the receptor was detected in spinal motorneurons of an ALS mouse model (194,195), as well as in spinal cord samples from human patients with ALS (195,196). Furthermore, the receptor was implicated in ALS-associated motoneuron death by a study in which knockdown of $p75^{NTR}$ delayed locomotor impairment and mortality in the SOD1G93A mouse model of ALS (197). However, when the SOD1G93A mice were crossed with the *$p75^{NTR}$-/-* mice, prolonged survival was only detected in the female mice, and this improvement did not correlate with increased motorneuron survival, but with reduced astrocytosis (198). Nevertheless, the SOD mutation represents a very small fraction of ALS patients, and thus further study into the role of the receptor in this disease is warranted.

Degeneration of dopaminergic neurons in Parkinson's disease (PD) could also involve $p75^{NTR}$. A study by Simon and colleagues demonstrated that loss of the *Engrailed* transcription factors results in increased expression of $p75^{NTR}$ in the ventral midbrain (199). This finding has implications for Parkinson's disease because mice deficient in

Engrailed-1 and Engrailed-2 exhibit progressive loss of mesencephalic dopaminergic neurons and have PD-like motor deficiencies (199). Importantly, knocking down $p75^{NTR}$ or addition of a receptor-blocking antibody prevented the apoptosis of mesencephalic dopaminergic neurons in cultures from the *engrailed-1/2* double-knockout mice. Upregulation of $p75^{NTR}$ in dopaminergic nigro-striatal neurons has also been reported following kainic-acid treatment (200). However, direct evidence for $p75^{NTR}$ expression in nigral dopaminergic neurons in PD and causal evidence linking expression of $p75^{NTR}$ to PD-associated nigral neurodegeneration *in vivo* is still missing.

In addition to these neurodegenerative conditions, evidence continues to grow implicating $p75^{NTR}$ in the pathology of other neurological diseases. For example, $p75^{NTR}$ has been suggested as having a role in spongiform encephalomyelopathy (201), diabetes-related impairment of neovascularization (202), and psoriasis (203), among others. The abundance of links between $p75^{NTR}$ and such a variety of diseases indicates that the receptor may function in a broader sense as a stress-induced apoptotic signal that is activated by a mechanism common to all of these pathological conditions. Thus, further elucidation of the mechanisms by which $p75^{NTR}$ is upregulated and activated during these pathological conditions and of the contributions of the receptor to the resulting neurodegeneration may be of critical therapeutic importance.

Promotion of Cell Survival

Because $p75^{NTR}$ was originally identified as a receptor for NGF, early studies investigated the ability of the receptor to promote a trophic signal. Despite the abundance of current evidence demonstrating a role for the receptor in stimulating neuronal

apoptosis, many of the early investigations found that $p75^{NTR}$ can also have the opposite function: promoting cell survival. One of the first indications of this role came from analysis of *$p75^{NTR}$-/-* mice, which revealed a significant loss of sensory innervation of limbs (50). Subsequently, the number of neurons in the dorsal root ganglia (DRG) was reported to be reduced by 50-75% in the knockout mice (204). Although the DRG is a very heterogeneous population of neurons, a decrease in virtually all types of neurons was detected, based on morphological criteria (205,206) or expression of various markers (207). Since then, numerous reports have suggested that $p75^{NTR}$ promotes survival in a wide variety of cell types. For example, neurotrophin-dependent activation of $p75^{NTR}$ has been demonstrated to inhibit the death of neuroblastoma cells (208), of hippocampal neurons treated with NMDA (209), and of both sensory neurons (210) and cortical subplate neurons deprived of trophic support (119). Also, despite the aforementioned role of $p75^{NTR}$ in promoting apoptosis of damaged cells, for certain cell types the receptor may play a protective role after injury. For example, $p75^{NTR}$-/- mice have increased death of primary auditory neurons following acoustic trauma (211). Though some reports have indicated that the receptor can independently promote a survival signal in response to neurotrophins (discussed later in this chapter), the majority of studies have suggested that pro-survival signaling by $p75^{NTR}$ occurs through cooperation with members of the Trk family of receptors.

$P75^{NTR}$ Forms a High Affinity Complex with the Trks

Shortly after TrkA was identified as a receptor for NGF, Chao and colleagues demonstrated that $p75^{NTR}$ interacts with TrkA to form a high affinity binding complex (24).

While TrkA alone was found to bind NGF with sub-nanomolar affinity, co-expression with $p75^{NTR}$ was discovered to increase this interaction by 100-fold (24,212). Thus, $p75^{NTR}$ can augment Trk-mediated survival by increasing its interaction with neurotrophins. Given that neurotrophins are typically present in limiting amounts in the target tissues, the presence of high-affinity receptors is an obvious advantage. The requirement for $p75^{NTR}$ in forming the high-affinity complex was initially offered as an explanation for the sensory neuron loss in the animals lacking the receptor. Indeed, neurotrophin dose-response curves revealed that higher doses of NGF were needed to promote survival of sensory and sympathetic neurons from *$p75^{NTR}$-/-* mice (50,213). However, a critical element unanswered by this interpretation of the data relates to the fact that, unlike the loss of neurons in the DRG, *$p75^{NTR}$-/-* mice actually have excess sympathetic neurons (70,214,215). As discussed earlier, $p75^{NTR}$ also contributes to normal, developmental apoptosis of sympathetic neurons, which could explain the increased neuronal number in the knockout mice. However, why the receptor functions differently in sensory neurons has yet to be resolved.

Remarkably, in addition to enhancing the affinity of the complex for neurotrophins, Trk – $p75^{NTR}$ interactions also regulate the neurotrophin-selectivity of the tyrosine kinase receptor. For example, in the absence of $p75^{NTR}$, TrkA can respond to both NT3 and NGF; however, the Trk – $p75^{NTR}$ complex is highly selective for NGF (216,217). Ginty and colleagues demonstrated that, during the development of sympathetic neurons, intermediate targets such as blood vessels produce NT3 and promote axon growth, but not survival, through TrkA signaling. However, as the neurons innervate their NGF-secreting targets, $p75^{NTR}$ is upregulated, causing TrkA to become selective for NGF over

NT3. The NGF–Trk–p75^{NTR} complex is then retrogradely transported to promote survival (218). Hence, p75^{NTR} can function as a switch factor, allowing differential TrkA responses. Similar selectivity has been observed with TrkB ligands; co-expression of p75^{NTR} with TrkB increased its selectivity for BDNF over NT3 and NT4 (219).

Beyond regulating the affinity and selectivity of Trks for neurotrophins, p75^{NTR} also potentiates Trk-mediated survival signaling. Prevention of neurotrophin binding to p75^{NTR} attenuated TrkA signaling in several *in vitro* systems (220-223). While the mechanism by which p75^{NTR} enhances Trk signaling remains poorly understood, Barker and colleagues demonstrated that co-expression of p75^{NTR} with TrkA attenuated TrkA ubiquitylation and delayed the NGF-dependent internalization and degradation of the tyrosine kinase receptor (224). Therefore, one mechanism utilized by p75^{NTR} to augment Trk-mediated survival signaling is prolonging cell surface expression of the Trk receptor.

p75^{NTR} and Trk Receptors: Mechanisms of Association

Although functional interaction between p75^{NTR} and Trk receptors is clear, the molecular details are not fully understood. Surprisingly, the transmembrane and intracellular domains of p75^{NTR}, but not the neurotrophin-binding portion of the extracellular domain, are required for the high-affinity complex (225). Furthermore, structure analysis by X-ray crystallography and complementation assays (using fragments of beta-galactosidase), indicated that complexes of each receptor bind NGF independently and that there is no direct interaction between p75^{NTR} and TrkA (226). The structural analysis disagrees with many early cross-linking experiments (discussed above) and co-immunoprecipitation studies in HEK293 cells (e.g. (219)) that indicate the

presence of a complex of both receptors. Clearly, further study is required to resolve the nature of the high affinity complex.

Perhaps the key to identifying circumstances in which the receptor contributes to survival or death lies within understanding the mechanisms of Trk-p75^{NTR} interactions. While the exact stoichiometry of the high-affinity Trk-p75^{NTR} complex has yet to be resolved, two molecular factors have been identified which may play important roles in governing signaling between these two receptors. Ankyrin repeat-rich membrane spanning (ARMS/Kidins220) is a large transmembrane protein that can physically associate with both TrkA and p75^{NTR} to form a ternary complex (227,228). ARMS is tyrosine phosphorylated following neurotrophin treatment and is expressed in many of the neuronal populations which receive neurotrophin stimulation (227). These data suggest that ARMS may serve as a link between p75^{NTR} and Trk receptors. However, expression of ARMS was discovered to decrease association of TrkA with p75^{NTR} (228), and the functional role of the protein has remained poorly understood. Recently, though, a study revealed that loss of ARMS *in vivo* results in apoptosis of sensory neurons (229), a phenotype similar to that observed in p75$^{NTR-/-}$ animals (205,229), thus further highlighting the potential importance of this interactor in p75^{NTR}-mediated regulation of cell survival.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is another factor which mediates the balance between Trk and p75^{NTR} signaling. In basal forebrain neurons receiving trophic support through BDNF-TrkB signaling, activation of p75^{NTR} by ProNGF results in apoptosis, despite TrkB phosphorylation. Friedman and colleagues demonstrated that this p75^{NTR}-induced cell death requires PTEN, which functions to suppress Trk-mediated phosphoinositide-3 kinase (PI3K) signaling (230). These

experiments indicate that PTEN is a critical determinant of survival outcome in neurons receiving neurotrophin stimulation. More studies are needed, however, to better understand this function of PTEN and how it may be differentially regulated in other cell types.

Interestingly, signaling by Trk receptors can also modulate proteolytic cleavage of $p75^{NTR}$. In PC12 cells and cerebellar granular neurons, cleavage of $p75^{NTR}$ is induced by NGF and BDNF, respectively. Surprisingly, this proteolysis was found to require Trk receptor activation, rather than occupancy of $p75^{NTR}$ with ligand (231). Trk receptor activation was discovered to promote phosphorylation of TACE, leading to cleavage of $p75^{NTR}$, which was ultimately necessary for potentiation of neurotrophin-induced survival signaling (232). It is also important to note that a $p75^{NTR}$ homolog, Neurotrophin Receptor Homolog 2 (NRH2) was recently identified (233). Like $p75^{NTR}$, NRH2 can also undergo cleavage by TACE and γ-secretase (233), associate with Sortilin (234) and form a high-affinity NGF receptor with TrkA (235). NRH2 is co-expressed with $p75^{NTR}$ in multiple neuronal subtypes. Thus, understanding how NRH2 and $p75^{NTR}$ function together with their co-receptors is necessary to interpret the phenotype of *$p75^{NTR}$-/-* mice.

Downstream Pro-survival Signals Induced by $p75^{NTR}$

A few downstream pro-survival signals activated by $p75^{NTR}$ have been identified, though detailed mechanisms have mostly remained elusive. Multiple studies have indicated that $p75^{NTR}$ activates nuclear factor kappaB (NFκB), a pro-survival transcription factor that is also induced by other members of the TNF receptor family (236). NFκB is best characterized for its role in the immune system, where it is activated by many

cytokine and Toll-like receptors, leading to upregulation of other cytokines and pro-survival genes (237). NFκB exists as a dimer, held in the cytosol through binding to its inhibitor IκB. The transcription factor is activated through phosphorylation of IκB by the IκB kinase (IKK) complex, leading to proteasomal degradation of the inhibitor and release of the NFκB dimer to translocate into the nucleus (237). The activation of NFκB by $p75^{NTR}$ was first reported in Schwann cells (238) and has since been demonstrated in a variety of cell types, including Schwannoma cells (239), primary Schwann cells (63), trigeminal neurons (240), and hippocampal neurons (241). How induction of $p75^{NTR}$ results in NFκB activation and enhanced cell survival in some circumstances, while leading to apoptosis in other cellular contexts, is not fully understood. However, as discussed earlier, neurotrophin binding to $p75^{NTR}$ can recruit members of the TRAF family, which activate the IKK complex (99,100). Specifically, TRAF6 was shown to mediate activation of NFκB, as Schwann cells from *traf6-/-* mice did not respond to $p75^{NTR}$ activation (242). Since TRAF6 promotes both NFκB and JNK activation, it was recognized as a potential nodal point for determining survival vs apoptotic signaling. How TRAF6 selectively promotes one pathway over the other remains to be fully elucidated; however, the finding that the adaptor protein Receptor-interacting protein 2 (RIP2) directly associates with the death domain of $p75^{NTR}$ provided an important clue. Chao and colleagues demonstrated that expression of RIP2 in Schwann cells conferred NGF-dependent activation of NFκB through interaction with TRAF6. Expression of RIP2 in these cells also reduced JNK activation and the subsequent apoptosis (63). Thus, RIP2 expression may serve as the key toggle, switching TRAF6 signaling to NFκB from JNK.

Promotion of cell survival by $p75^{NTR}$ may also occur through activation of the serine/threonine kinase Akt, an upstream regulator of NFκB, since expression of the receptor in PC12nnr5 cells caused PI3K-dependent activation of Akt. Expression of $p75^{NTR}$ in these studies was also demonstrated to reduce apoptosis induced by staurosporine or by serum starvation (243). Interestingly, some reports have suggested that survival signals induced by $p75^{NTR}$ may occur independently of Trk signaling. Indeed, activation of Akt in PC12nn5 cells was found to be Trk-independent (243), and $p75^{NTR}$-mediated induction of NFκB in trigeminal neurons was blocked by an anti-$p75^{NTR}$ antibody (240).

It is also notable that the $p75^{NTR}$ was recently shown to regulate the stability of hypoxia inducible factor-1α (HIF1α), a transcription factor induced by oxidative stress that controls the expression of a wide variety of genes involved in protection from reactive oxygen species and, importantly, promoting cell survival (244). Le Moan et al reported that the ICD of the receptor can bind the E3 ubiquitin ligase Siah2, which targets HIF1α for degradation (245). The interaction between $p75^{NTR}$-ICD and Siah2 lead to upregulation of HIF1α and increased expression of vascular endothelial growth factor, which promoted angiogenesis after retinal hypoxia. While the authors did not address a potential role in regulating survival, given that many target genes of HIF1α are pro-survival, it would be interesting to determine whether this pathway has a role in promoting neuronal survival in response to activation of the receptor.

Altogether, these studies demonstrate that the factors modulating $p75^{NTR}$ signaling on cell survival are highly intricate, with the overall outcome on cell survival being determined by interactions between $p75^{NTR}$ and Trk receptors, upstream regulation of

those interactions by factors like ARMS and PTEN, and independent or Trk-facilitated activation of downstream signals such as Akt and NFκB.

Determinants of Survival Outcome after p75^{NTR} Activation

Because of the ability of p75^{NTR} to augment Trk-mediated survival signaling and ligand selectivity, the overall effect of p75^{NTR} on cell survival is quite variable by cell type and highly dependent upon the presence or absence of Trk receptor co-expression. In general, though with exception, simultaneous activation of both Trk receptors and p75^{NTR} by mature neurotrophins leads to cell survival. However, induction of p75^{NTR} by mature neurotrophins in the absence of Trk-receptor activation more often promotes cell death. For example, NGF treatment of sympathetic neurons, which express both p75^{NTR} and TrkA, promotes neuronal survival. Stimulation of these neurons with BDNF, however, results in apoptosis, since these neurons do not express TrkB (57). Additionally, activation of p75^{NTR} by proneurotrophins can result in cell death despite simultaneous activation of Trk receptors by mature neurotrophins. This has been demonstrated in cultured basal forebrain neurons, in which treatment with ProNGF elicited cell death despite the activation of TrkB receptors due to pretreatment with BDNF(64). The proliferative state of the cell also may influence the effects of p75^{NTR} signaling, as the majority of demonstrations of p75^{NTR}-mediated cell death have involved studies with post-mitotic neurons, while studies of p75^{NTR} in proliferative cells have revealed more variable survival outcomes (143).

Summary

In summary, $p75^{NTR}$ is versatile receptor with a variety of complex signaling mechanisms to regulate cell survival. The ultimate effect of the receptor is governed by the cleavage state of the neurotrophin ligand, associations between $p75^{NTR}$ and co-receptors of the Trk or Vps10p-domain family, regulated proteolysis of $p75^{NTR}$ itself, and interactions between the receptor with a multitude of cytosolic signaling factors. These events are necessary not only for proper modulation of cell survival during the development of the nervous system, but also for cellular responses to injurious or pathological conditions.

Oxidative Stress

Sources of Reactive Oxygen Species

Oxidative stress is a deleterious condition that results from a state of imbalance between excessive formation of reactive oxygen species (ROS) and limited production or activity of cellular antioxidants. The term ROS is a collective descriptor that includes a variety of free radicals, as well as non-radical derivatives of molecular oxygen (246). Superoxide anion ($O_2^{-}\bullet$), the product of a one-electron reduction of molecular oxygen, is a precursor to most types of ROS (247). While $O_2^{-}\bullet$ itself is not a strong oxidant, dismutation of $O_2^{-}\bullet$ can produce hydrogen peroxide (H_2O_2), which can easily traverse cell membranes to reach numerous compartments of the cell. In the presence of $O_2^{-}\bullet$ or reduced transition metals such as iron (Fe^{2+}), H_2O_2 can be cleaved to generate the highly reactive hydroxyl radical (248). Additionally $O_2^{-}\bullet$ can combine with other radical species such as nitric oxide (NO•) to produce peroxynitrite, another highly reactive oxidant (249). There are numerous cellular sources of $O_2^{-}\bullet$. For most cells, mitochondria are the

greatest contributor of $O_2^{-}\bullet$, since the electron transport chain consists of numerous redox centers which may leak electrons to reduce molecular oxygen. Indeed, $O_2^{-}\bullet$ is a natural byproduct of oxidative phosphorylation, since an estimated 1-2% of molecular oxygen consumed during cellular respiration is converted to $O_2^{-}\bullet$ (247). Additionally, however, $O_2^{-}\bullet$ and other ROS can be produced as an intermediate in enzymatic reactions. For example, proteolytic conversion of xanthine dehydrogenase to xanthine oxidase produces both $O_2^{-}\bullet$ and H_2O_2. NADPH oxidases and cytochrome P_{450}-dependent oxygenases also produce $O_2^{-}\bullet$ (250), and H_2O_2 is a byproduct of oxidative deamination catalyzed by monoamine oxidase (247).

Antioxidants

With so many different sources of regular ROS production, cells must employ a variety of defense mechanisms to prevent toxic ROS accumulation. A family of metalloenzymes called superoxide dismutases (SOD) serve as a primary defense against $O_2^{-}\bullet$ accumulation by catalyzing the conversion of $O_2^{-}\bullet$ to H_2O_2. A specific form of SOD containing manganese in its active-site catalyzes the dismutation of $O_2^{-}\bullet$ within the matrix of mitochondria, while active-site copper and zinc SODs carry out this role within the mitochondrial intermembrane space and within other compartments of the cell (246). Because SODs produce H_2O_2, mammalian cells must also defend against accumulation of peroxides. Within the brain, peroxiredoxins, a family of peroxidases which can reduce H_2O_2, peroxynitrite, and other peroxides, may represent the most important source of H_2O_2 removal due to their high abundance in all sub-cellular organelles and within the cytosol (246,251). However, glutathione peroxidases, which couple electron donation

from glutathione with reduction of H_2O_2, are also major contributors of H_2O_2 removal (250). Catalases are also major H_2O_2-detoxifying enzymes found within peroxisomes and in mitochondria of cardiomyocytes; however, the brain expresses relatively low levels of catalases, and thus other enzymes are likely greater contributors of H_2O_2 removal within the CNS (247,250). Apart from these enzymes, many other proteins and molecular factors aid in the defense against ROS. For example, several components of the electron transport chain, such as cytochrome c, can be reduced by $O_2^{-}\bullet$, thus driving the synthesis of ATP (250,252). Additionally, the brain is enriched with many ROS scavengers, including glutathione, ascorbate (vitamin C), and α-tocopherol, one of four tocopherols which comprise the vitamin E family (246).

Oxidative Stress-induced Apoptotic Signaling

Because of the abundant antioxidant capabilities of most cells, at low levels, ROS are fairly innocuous, and small fluctuations in ROS are even essential for healthy cell signaling events. However, when antioxidants are depleted or when ROS accumulate to uncontrollable levels, ROS exert a wide variety of disruptive effects, ultimately leading to the cell's demise. For example, ROS can promote release of cytochrome c from mitochondria by disrupting its attachment to cardiolipin, an anionic phospholipid which functions to anchor cytochrome c to the inner mitochondrial membrane (247). Once released, cytochrome c can interact with Apaf-1 and, in the presence of ATP, polymerize to form the "apoptosome", a pro-death complex which triggers a cascade of proteolytic events by inducing cleavage of caspase-9, which then cleaves caspase-3, leading to activation of numerous other proteases and DNAses (250). Oxidants can also modify

components of the mitochondrial permeability transition (MPT) pore, a channel-forming protein complex located at contact sites between the inner and outer mitochondrial membranes, thereby causing loss of mitochondrial membrane potential, impaired mitochondrial respiration, and further release of cytochrome c to the cytosol (253,254). Jun N-terminal Kinases (JNKs) are also significant pro-apoptotic kinases activated during oxidative stress (255). JNK signaling can promote programmed cell death through multiple pathways, including direct phosphorylation of BH3-only members of the Bcl2 family Bim, Bmf, and Bad, as well as through nuclear translocation and phosphorylation of c-Jun, a member of the AP-1 transcription factor complex which induces expression of numerous pro-apoptotic genes such as TNFα, Fas-L, and Bak (74). In addition to these distinct signaling events, ROS oxidize bioactive molecules such as proteins and nucleic acids to cause a wide array of cellular disruptions, including protein misfolding, increased proteolytic degradation, impairment of enzymatic activities, abnormal accumulation of protein aggregates, defects in the ubiquitin-proteosome system, single- and double-stranded DNA breaks, and crosslinking of DNA with other molecules (246,247).

Lipid Peroxidation

Poly-unsaturated fatty acids within lipid bilayers are extremely vulnerable to oxidation. In a process termed lipid peroxidation, unstable ROS attack poly-unsaturated fatty acids such as arachidonic acid or linoleic acid within lipid membranes, resulting in the production of more stable, yet still actively damaging molecules. 4-Hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and acrolein are all major aldehyde end-products of lipid peroxidation capable of cellular damage by forming adducts with nucleophiles such as

proteins and DNA. Among these, acrolein is most reactive, though the compound has received less attention due to its relatively low abundance among lipid peroxidation products (256). MDA is widely used as a biomarker of lipid peroxidation, and increased levels of MDA-adducts are associated with several neurodegenerative conditions (257,258). Currently, however, HNE has displayed the highest biological activity and thus is the most intensively studied lipid peroxidation product in association with neurodegeneration (256). HNE is a highly reactive α,ß-unsaturated aldehyde with three reactive components: a C1=O carbonyl group, a C2=C3 double bond, and a C4-OH group. Through Michael adduction or formation of Schiff bases, HNE can react with a wide range of cellular macromolecules (259). Through interactions with histidine, lysine, or cysteine residues, HNE can bond with and alter the function of numerous proteins. Additionally, HNE can react with amino groups of lipids or with DNA, primarily through interactions with the deoxyguanosine (259,260). Through these versatile interactions, HNE can function as a regulator of signal transduction, enzyme modulation, and gene expression, ultimately effecting cellular functions such as growth and differentiation (260). However, high concentrations of HNE are correlated with numerous neurodegenerative disorders associated with redox imbalance, including Alzheimer's disease, macular degeneration, cardiovascular disorders, metabolic syndrome, and Parkinson's disease (257,259). In addition to these studies, HNE has been demonstrated to promote neuronal programmed cell death through a variety of molecular mechanisms, and thus is widely regarded as a key mediator of neuronal apoptosis induced by oxidative stress (259-262). For example, HNE can directly bind and activate JNK, and the compound has been demonstrated to induce JNK-dependent apoptosis in a variety of

cells, including PC12 cells, sympathetic neurons, and Jurkat cells (263-265). Additionally, the lipid peroxidation product has been found to induce apoptosis through a variety of other mechanisms: HNE can stimulate the extrinsic apoptotic pathway by promoting production of FasL, activate intrinsic cell death signaling through p53, impair the peptidase activity of the 20S proteasome, promote protein aggregate accumulation, and induce ER stress (259). Lastly, HNE can disrupt mitochondrial function or deplete cellular antioxidants such as glutathione, leading to further production of ROS (266-269).

Dissertation Goals: Investigating an Hypothesis about Oxidative stress and p75^{NTR}

While the evidence linking p75^{NTR} to neuronal apoptosis after cellular injury is clear, how signaling by the receptor is initiated by such a variety of conditions is not fully understood. Upregulation and release of proneurotrophins may be the key mechanism of receptor induction after certain injuries; however, expression of the intracellular domain of p75^{NTR} is reportedly sufficient for induction of apoptosis (270,271), thus allowing for the possibility that p75^{NTR} signaling is stimulated by ligand-independent mechanisms which lead to cleavage of the receptor. Our lab recently demonstrated that activation of JNK is sufficient to induce cleavage of p75^{NTR} (87), thus revealing the possibility that cellular injuries may induce JNK, thereby triggering ligand-independent apoptotic signaling through p75^{NTR}. Of the many types of cellular injury known to activate JNK, oxidative stress has been particularly well-established as a condition associated with activation of the kinase (264,272,273). Additionally, p75^{NTR} has been implicated as a contributing factor to numerous neurodegenerative conditions associated with oxidative stress (274-279). Therefore, my dissertation research, which is described in the following two

chapters, investigated whether oxidative stress promotes apoptosis through activation of $p75^{NTR}$. The findings enhance our understanding of both the mechanisms by which $p75^{NTR}$ signaling can be induced as well as the potential roles of this receptor in neurodegenerative disease.

CHAPTER II

A ROLE FOR p75^{NTR} IN NEURODEGENERATION INDUCED BY OXIDATIVE STRESS

Introduction

Studies of *p75^{NTR}*-/- animals have revealed that the receptor is critical for naturally occurring developmental apoptosis within the retina, superior cervical ganglia, spinal cord, and basal forebrain (54,57,67,69). In addition to promoting cellular apoptosis, more recent findings have demonstrated that p75^{NTR} also causes breakdown of mislocalized axons, as the receptor has been found to promote degeneration of aberrantly sprouting septal cholinergic axons, thereby preventing the fibers from growing into myelinated tracts (280), as well as to mediate developmental pruning of sympathetic axons projecting to the iris (281). Hence, p75^{NTR} functions as a regulator of neurodevelopment, ensuring the removal of unsuitable neurons or neuronal projections. Apart from these roles, however, numerous studies have indicated that p75^{NTR} also promotes neuronal apoptosis in response to cellular injuries or pathological conditions. The receptor is necessary for programmed cell death caused by seizures (60,168), corticospinal axotomy (153,160), and spinal cord injury (152). Additionally, p75^{NTR} has been linked to neurodegeneration occurring in models of Alzheimer's disease (282), amyotrophic lateral sclerosis (197), and ischemia (167,283-285). Thus, p75^{NTR} appears to function as a stress-activated receptor which promotes degeneration in response to neuronal injury.

Of the numerous pathological conditions in which p75^{NTR} signaling has been implicated, nearly all are associated with oxidative stress (274-279). In this study we used sympathetic neurons exposed to HNE, a lipid peroxidation product naturally generated

during oxidative stress, to model oxidative stress-induced neurite degeneration and apoptosis, evaluating whether $p75^{NTR}$ has a functional role. Treatment of sympathetic neurons with HNE caused neurite degeneration and neuronal apoptosis; however, these neurodegenerative effects were markedly attenuated in neurons lacking *$p75^{NTR}$*. These results indicate a novel role for $p75^{NTR}$ in promoting axonal degeneration and neuronal apoptosis in response to oxidative stress.

Experimental Procedures

Sympathetic Neuron Culture

All experiments with animals were approved by the Animal Care and Use Committee at Vanderbilt University. Superior cervical ganglia were dissected from postnatal day 5/6 Sprague Dawley rats, C57BL/6J mice, or C57BL/6J *$p75^{NTR(exonIII)-/-}$* mice and dissociated with 0.08% trypsin (Worthington) and 0.3% collagenase (Sigma). Dissociated cells were then plated at a density of 5000-7000 neurons per 0.7 mm^2 on 8-well chamber slides (Thermo Scientific) or cell culture plates coated with poly-D-lysine (MP Biomedicals) and laminin (Invitrogen). All neurons were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco), 40 ng/ml Nerve growth factor (Harlan), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). To inhibit the proliferation of non-neuronal cells, the neurons were treated with 5-10 µM cytosine arabinofuranoside [(Ara-C), Sigma] 24 hours after plating. Following 3 days of exposure, Ara-C was removed for 24 hours, and the neurons were then treated with the indicated concentrations of HNE or different pharmacological reagents. During pilot studies, we observed increased toxicity of HNE at lower cell densities, as has been reported for 6-hydroxydopamine (6-OHDA) (286), and

therefore for all experiments neuron plating densities and Ara-C exposures were equivalent across all experimental conditions.

HNE Treatment

HNE was produced as previously described (287) as well as obtained from Calbiochem, and its concentration was determined by measuring optical density at 224 nm and using an extinction coefficient of 13,750/M. HNE is a highly reactive lipid aldehyde, and some variability in toxicity was observed between different batches of the compound. Every effort was therefore made to limit exposure of the HNE to oxygen, including its storage at -80° C under inert gas.

Western Blot Analyses

After treating rat sympathetic neurons as indicated with HNE, the neurons were lysed in NP-40 lysis buffer [25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1% NP-40, 10% glycerol,] supplemented with Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche) and PhosStop-Phosphatase Inhibitor Cocktail Tablet (Roche). Cell lysates were subjected to western blot analysis using antibodies to cleaved Caspase-3 (1:500, Cell Signaling, #9664S) and Tubulin (1:1000, Calbiochem, #CP06). For analysis of norepinephrine transporter (NET), superior cervical ganglia from adult C57BL6/J or C57BL/6J *p75*$^{NTR(exonIII)-/-}$ mice were collected and lysed in NP-40 lysis buffer. Lysates were then subjected to western blot analysis using the rabbit polyclonal NET antibody 43411 (generously provided by Randy Blakely, Vanderbilt University).

Quantification of Neurite Degeneration

Analyses of neurite degeneration were performed as previously described (288-290), with slight modification. Following the indicated treatments, sympathetic neurons in 8-well chamber slides were fixed with 4% paraformaldehyde and visualized via a 20x optical lens on a Leica inverted fluorescence microscope. Phase-contrast images of five fields of view per well were captured with 16 ms exposure by a Nikon DXM1200C digital camera. To ensure accurate measurement of neurites, images were captured from blindly-selected regions with well-separated axon tracts. Using an automated method of image analysis, the fragmentation of the neurites was then measured. Levels of neurite degeneration are reported as a degeneration index (DI), which is the ratio of fragmented neurite area over total neurite area. To process images for DI calculation, the auto-level function of the software GNU-image Manipulation Program (GIMP) was first used to adjust image gray levels to objectively provide uniform background intensity to all of the images. ImageJ software was then utilized to binarize the image and to remove all cell bodies, rendering an image composed of black neurites on white background. While healthy neurites appear continuous, degenerating neurites have a disrupted, particulate structure due to blebbing and fragmentation. To measure the area of fragments from degenerating neurites, the Particle Analyzer algorithm of ImageJ was applied to identify regions of fragmentation based on size (20-10,000 pixels) and circularity (0.2 – 1.0). The total area of these detected neurite fragments was then divided by the total black, neurite area to determine the DI. In agreement with other studies (288), a DI of 0.2 or greater accurately indicated neurite degeneration, while a DI of 1.0 would theoretically represent neurites that have completely degenerated into fragmented particles.

Quantification of Neuronal Death

Following the indicated treatments, sympathetic neurons in 8-well chamber slides were fixed with 4% paraformaldehyde and immunostained with neuron-specific anti-TUJ1 primary antibody (Neuronal Class III β-Tubulin, 1:500, Covance, #MMS-435P) and Alexa Fluor® 488 secondary antibody (1:1000, Life Technologies, #A11001). Slides were then mounted using Vectashield with 4,6-diamidino-2-phenylindole (Vector Labs), and the neurons were blindly scored as apoptotic or non-apoptotic based on the appearance of the nucleus, apoptotic nuclei being condensed or fragmented. At least 75 TUJ1-positive neurons were counted per condition in all experiments.

Measurement of Protein Carbonylation

Detection of protein carbonylation was performed using the Oxyblot kit (Millipore) by following the manufacturer's instructions. Briefly, cell lysates were treated with 2,4-dinitrophenylhydrazine (DNPH) to derivatize protein side chain carbonyl groups to 2,4-dinitrophenylhydrazone (DNP-hydrazone). Separately, as a negative control, aliquots of all cell lysates were treated with solution lacking DNPH. Protein samples were then separated by polyacrylamide gel electrophoresis and analyzed by Western blot using an anti-DNP antibody.

In vivo Assessment of 6-OHDA-induced Axonal Degeneration

Adult, age-matched *p75*$^{NTR(exonIII)}$ -/- or +/+ mice were administered 100mg/kg 6-OHDA**-**hydrobromide (Sigma, freshly prepared in Phosphate Buffered Saline, pH 7.3 supplemented with 0.02% ascorbate) or vehicle solution by intraperitoneal injection once daily for two days. Animals were sacrificed one week later, and the spleens were collected

for determination of norepinephrine concentrations or used for immunohistochemical localization of tyrosine hydroxylase (TH)-immunoreactive (-ir) axons. For the latter studies, animals were transcardially perfused with heparized saline, followed by 4% paraformaldehyde. The spleens were then collected, post-fixed, cryosectioned at 12 µm, and collected onto slides. Noradrenergic axons were detected by immunofluorescent localization of TH-ir using a mouse anti-TH antibody (1:750; Abcam, Cambridge, MA). Splenic norepinephrine concentrations were determined by high performance liquid chromatography (HPLC) with electrochemical detection, following our previously described method (291).

In vivo Assessment of 6-OHDA-induced Apoptosis

Adult, age-matched *p75*$^{NTR(exonIII)}$ -/- or +/+ mice were administered 100mg/kg 6-OHDA-hydrobromide (Sigma, freshly prepared in Phosphate Buffered Saline, pH 7.3 supplemented with 0.02% ascorbate) or vehicle solution by intraperitoneal injection once daily for two days. One week later, the animals were transcardially perfused with heparized saline, followed by 4% paraformaldehyde. Superior cervical ganglia were then collected, cryosectioned, and processed for detection of apoptosis using the ApopTag Plus *In Situ* Apoptosis Fluorescein Detection Kit (Millipore, S7111), per manufacturer's recommendations. The sections were then mounted in ProLong® Gold Antifade Mountant with DAPI (Molecular Probes, P36931) and visualized with a Zeiss Axioskop 2 epifluorescent microscope. As a positive control, cultured HeLa cells were treated with 1 µM staurosporine for 20 hours before fixation with 4% paraformaldehyde, followed by similar staining and assessment for apoptosis.

Results

P75^{NTR} is Required for HNE-induced Neuronal Apoptosis

Previous studies have demonstrated that sympathetic neurons exposed to the naturally produced oxidant HNE undergo caspase-dependent programmed cell death (264). To examine the effect of HNE on neuronal survival in our culture system, rat sympathetic neurons were treated with a range of concentrations of HNE and scored for apoptosis based on nuclear morphology. HNE dose-dependently induced death of sympathetic neurons (Fig. 3*a*). We confirmed that the cell death induced by HNE was apoptotic, indicated by a marked increase in the levels of cleaved caspase-3 (Fig. 3*b*). HNE is known to promote apoptosis through its ability to form protein adducts and modify cell signaling (259). Additionally, HNE can propagate oxidative stress through mitochondrial impairment or depletion of antioxidants (266-269). Because amino acid side chains are abundant targets of oxidation by reactive oxygen species and lipid-derived α,ß-unsaturated aldehydes, increased protein carbonylation is commonly used as a biomarker of oxidative stress (292). Treatment of sympathetic neurons with HNE caused a rapid increase in protein carbonylation, observed within 30 minutes of HNE treatment (Fig. 3*c*). These results indicate that exposure of sympathetic neurons to HNE models oxidative stress-induced apoptosis.

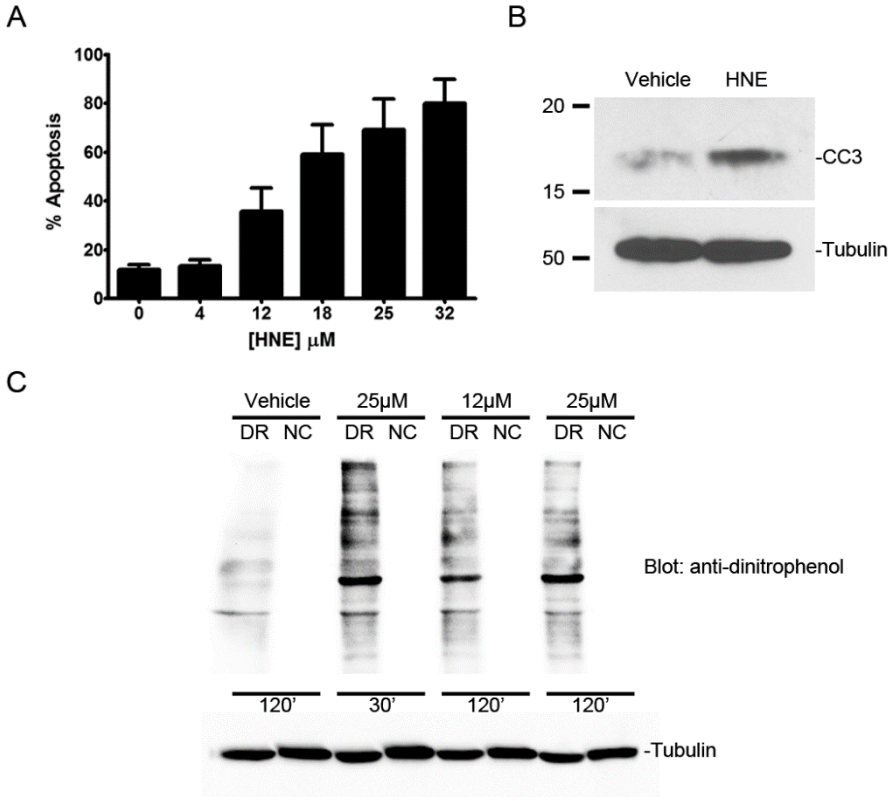

Figure 3: HNE triggered apoptosis of sympathetic neurons. (**A**) Quantification of neuronal death elicited by HNE (n=3). After exposing rat sympathetic neurons to various concentrations of HNE for 20 hours, the cells were fixed, immunostained with the neuron-specific marker TUJ1, and labeled with the nuclear stain DAPI. TUJ1-positive neurons were blindly scored as apoptotic or non-apoptotic based on the appearance of the nuclei. (**B**) Representative western blot of cleaved caspase-3 (CC3) from lysate of rat sympathetic neurons treated for 12 hours with 25 μM HNE (n=3). (**C**) Carbonylation of proteins, detected by Oxyblot™ Protein Oxidation Detection Kit, in lysates of sympathetic neurons exposed for 30 minutes or two hours with 12 μM or 25 μM HNE (DR=derivatization reaction, NC= negative control, n=3).

The $p75^{NTR}$ has been implicated as a mediator of apoptosis in many pathological conditions involving oxidative stress (60,167,197,282-284). We therefore studied sympathetic neurons exposed to HNE to evaluate whether $p75^{NTR}$ contributes to oxidative stress-induced neuronal apoptosis. Sympathetic neurons were cultured from *$p75^{NTR}$* knockout or wildtype mice and assessed for survival following exposure to various concentrations of HNE. Compared to neurons from wildtype mice, sympathetic neurons lacking $p75^{NTR}$ were significantly protected from HNE-induced apoptosis (Fig. 4, *a* and *b*). These findings indicate that $p75^{NTR}$ contributes to neuronal apoptosis induced by HNE.

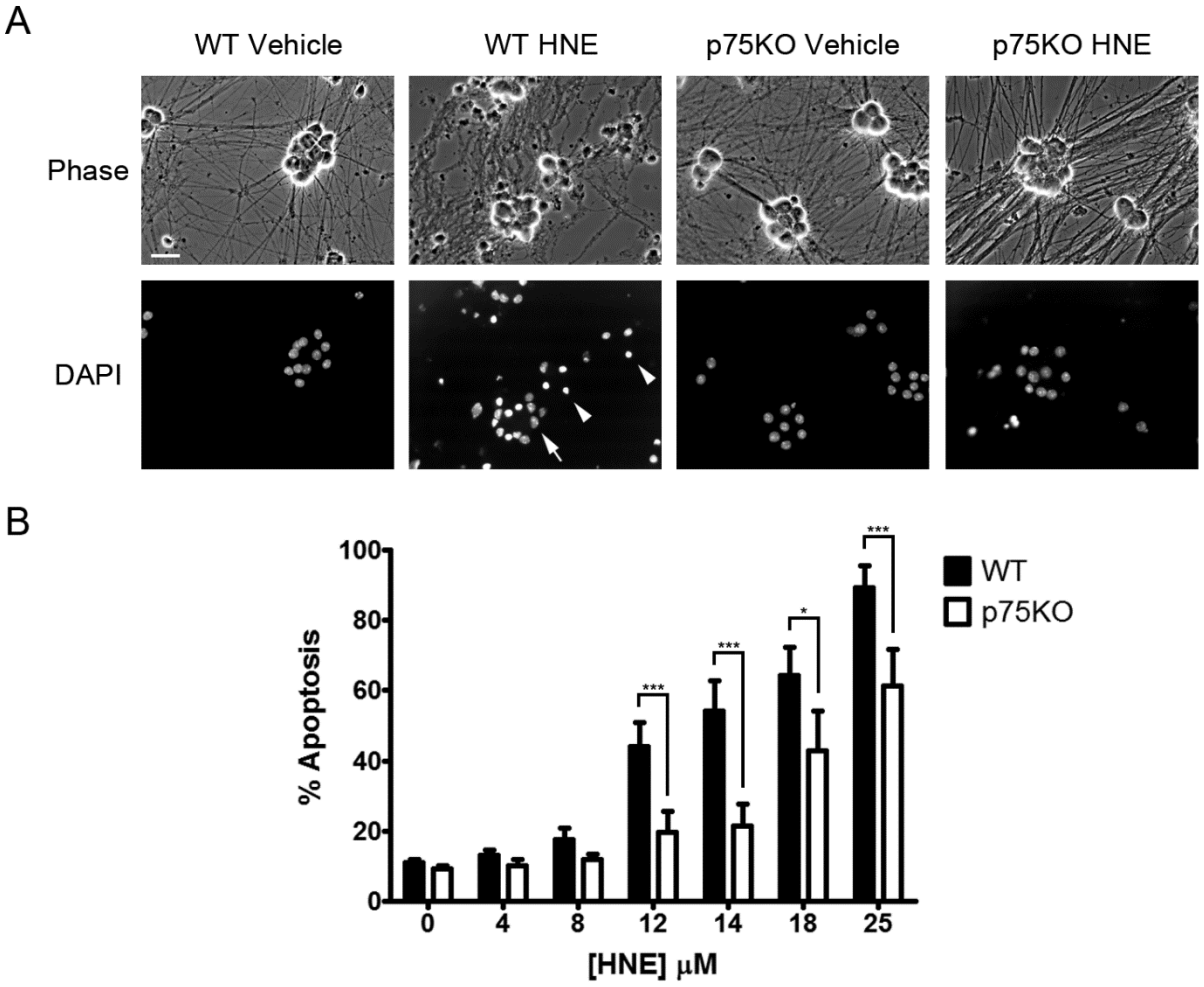

Figure 4: HNE-induced apoptosis is mediated by p75^{NTR}. (**A**) Microscopy images of sympathetic neurons from wildtype or *p75^{NTR}*-/- mice after 20 hours of treatment with vehicle or 12 μM HNE. The nuclei were labeled by DAPI staining and scored as apoptotic or non-apoptotic (Arrow, healthy nucleus. Arrowhead, apoptotic nucleus. Scalebar, 25 μm). (**B**), Quantification of apoptosis of wildtype or *p75^{NTR}*-/- sympathetic neurons treated with different concentrations of HNE. A significant reduction in HNE-induced apoptosis was observed in neurons lacking expression of p75^{NTR} (n=6, mean ± S.E., *** $p < 0.001$, * $p < 0.05$. ANOVA with Bonferroni post-hoc analysis).

HNE Stimulates p75^{NTR}-dependent Neurite Degeneration

During survival analysis of sympathetic neurons exposed to 12 μM HNE, we observed extensive fragmentation of neuronal processes throughout the culture despite less than maximal cell death. While the ability to induce neuronal apoptosis has been the most studied function of p75^{NTR}, recent investigations have also demonstrated a function for the receptor in promoting axonal degeneration (280,281,284). Due to our observations, and because numerous pathological conditions related to oxidative stress have also been associated with axonal degeneration (293,294), we hypothesized that p75^{NTR} mediates the degeneration of axons caused by HNE. Therefore, sympathetic neurons were treated with 12μM HNE and axonal degeneration was quantified from phase-contrast images. Using an automated method of image analysis, we measured the "degeneration index," the ratio of fragmented neurite area over total neurite area (288-290,295). Remarkably, while HNE-treated neurons from wildtype animals had substantial neurite fragmentation, the processes from cells lacking p75^{NTR} were healthy and intact (Fig. 5*a*). Indeed, based on the degeneration index, the *p75^{NTR}-/-* neurons were significantly protected (Fig. 5*b*). These results reveal that p75^{NTR} is necessary for HNE-induced neurite degeneration and suggest that oxidative stress invokes p75^{NTR} signaling to promote axon fragmentation.

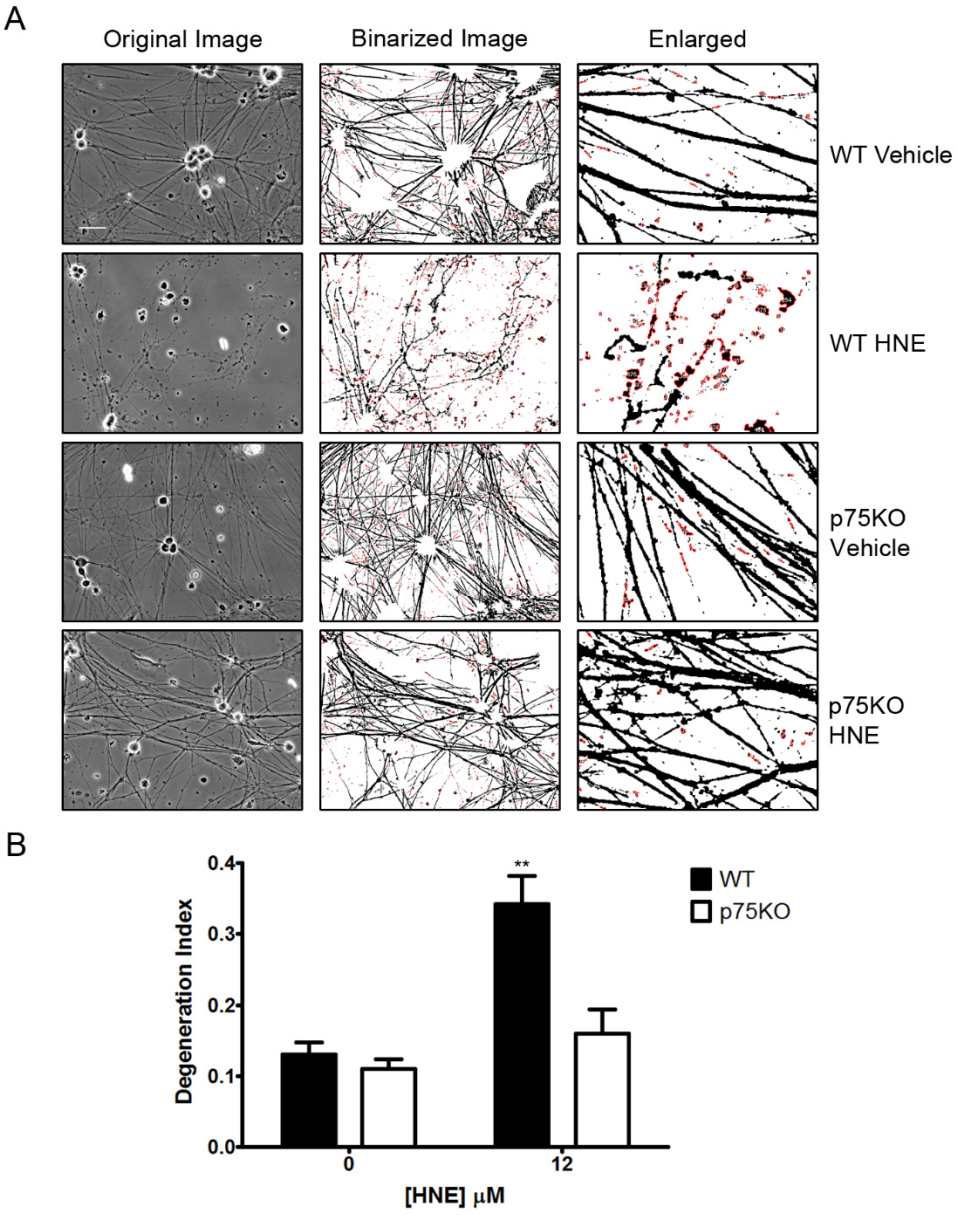

Figure 5: HNE stimulates p75^{NTR}-mediated neurite degeneration. (**A**) Images of neurites from wildtype or *p75^{NTR}*-/- (p75KO) sympathetic neurons exposed for 20 hours to vehicle or 12 μM HNE. (Left, phase-contrast image of the neurons. Middle, binarized image of the neurons with cell bodies removed and neurite fragments outlined in red. Right, 340% enlarged region from binarized images of neurites with outlined neurite fragments. Scalebar, 50 μm). (**B**) Quantification of the results in A. The degeneration index is a measure of neurite fragmentation calculated by dividing the total area covered by neurite fragments by the total neurite area. While the degeneration index of wildtype neurites robustly increased after 20 hours of HNE treatment, p75KO neurites had significantly reduced axonal degeneration (n=4, mean ± S.E., **p < 0.01, ANOVA with Bonferroni post-hoc analysis).

Oxidative Stress-associated Axonal Degeneration Requires p75^{NTR} in vivo

We next sought to evaluate the effects of p75^{NTR} in axonal degeneration induced by oxidative stress *in vivo*. Since our aim was to promote oxidative stress specifically in neurons, we chose to use 6-hydroxydopamine (6-OHDA), which is selectively taken up in cells expressing catecholinergic transporters (296), rather than HNE, which reacts with a wide variety of cell types (260). 6-OHDA is a neurotoxin that has long been used systemically to selectively induce degeneration of sympathetic axons, which express the norepinephrine transporter (297,298). It is thought to promote degeneration of catecholaminergic neurons primarily by increasing intracellular levels of reactive oxygen species, partially due to its tendency to undergo auto-oxidation to generate the hydroxyl radical, quinones, and other reactive species (296,299,300). We administered 6-OHDA to adult wildtype or p75^{NTR} knockout mice to promote degeneration of sympathetic axons *in vivo*. One week after 6-OHDA treatment, marked loss of TH-ir axons in the spleen was observed in wildtype mice (Fig. 6*a*).

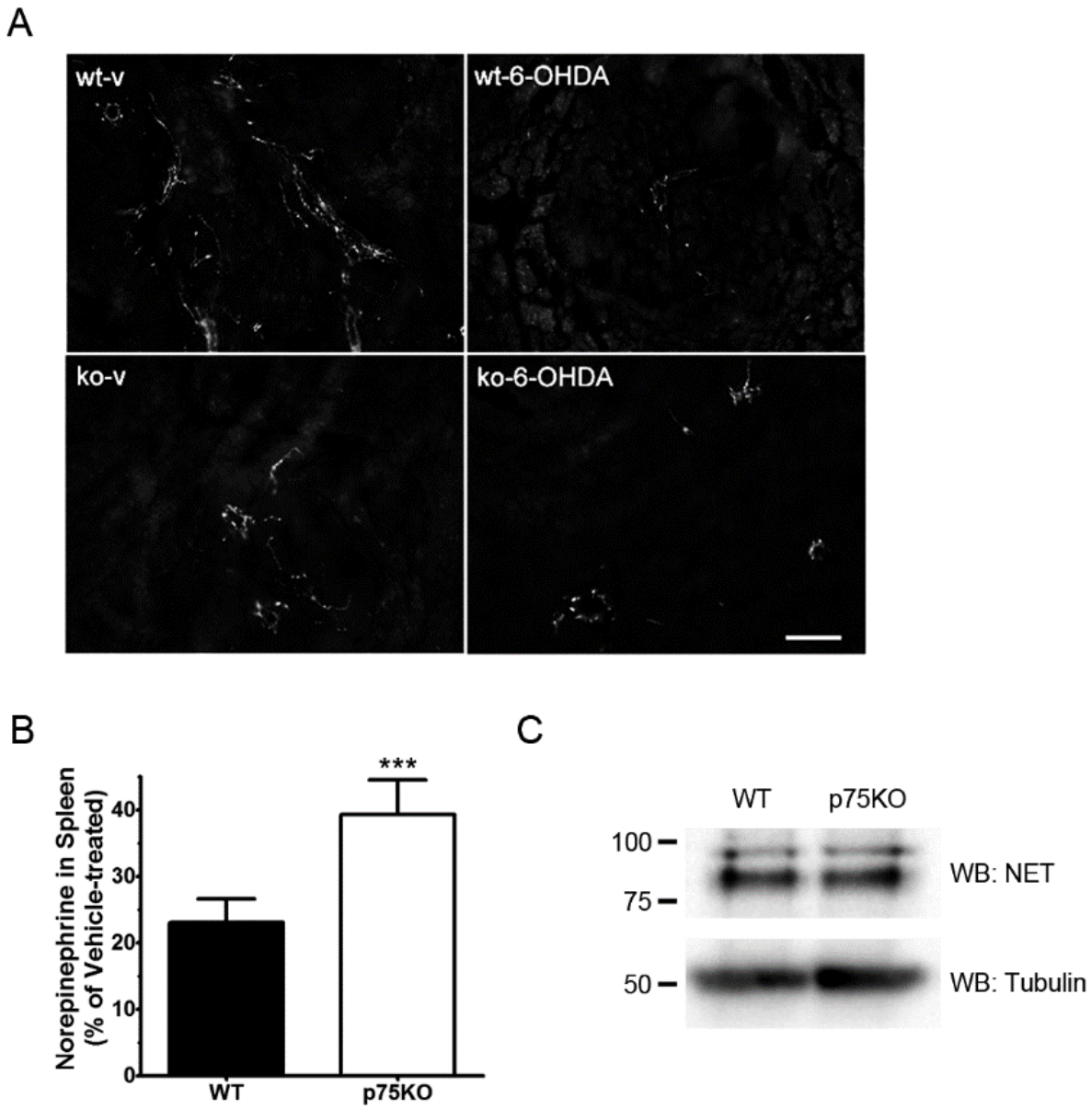

Figure 6: Oxidative Stress-associated Axonal Degeneration Requires p75^{NTR} *in vivo*. (**A**) Representative images of tyrosine hydroxylase immunofluorescence in spleens of wildtype (WT) or *p75^{NTR}*-/- (ko) mice after intraperitoneal administration of vehicle or 100 mg/kg 6-OHDA. Daily administrations were performed for two consecutive days, followed by tissue collection and analysis one week later. (**B**) Quantification of norepinephrine in spleens of wildtype (WT) or *p75^{NTR}*-/- (p75KO) mice following treatment as described in A. Relative to vehicle-treated control animals, administration of 6-OHDA caused a significantly greater loss of splenic norepinephrine in WT mice than in p75KO mice (n=7, mean ± S.E., *** $p < 0.001$, ANOVA). (**C**) Representative western blot of norepinephrine transporter from lysates of superior cervical ganglia collected from adult wildtype (WT) or *p75^{NTR}*-/- (p75KO) mice (n=3).

Interestingly in *p75^{NTR}-/-* mice treated with vehicle, the density of TH-ir axons in the spleen appeared lower than in vehicle-treated wildtype control animals. However, there also appeared to be partial protection from 6-OHDA-induced degeneration in the *p75^{NTR}*-null mice (Fig. 6a). To quantitatively assess the level of the sympathetic innervation, we determined splenic norepinephrine content by HPLC. Administration of 6-OHDA caused a significantly greater loss of splenic norepinephrine in wildtype mice than in p75^{NTR} knockout mice (Fig. 6*b*), thus indicating that p75^{NTR} contributes to 6-OHDA-induced axonal degeneration *in vivo*. Additionally, expression of norepinephrine transporter was similar in sympathetic ganglia of wildtype and *p75^{NTR}-/-* mice (Fig. 6c), and therefore protection of the null animals from 6-OHDA-induced axonal degeneration was unlikely the result of altered 6-OHDA transport. We also sought to determine whether *p75^{NTR}*-null mice would be protected from apoptosis induced by 6-OHDA *in vivo*. Intriguingly, however, our pilot studies revealed that, despite extensive axonal degeneration, no increase in cell death was observed in wildtype mice following administration of 6-OHDA (Fig. 7). Overall, the results of these *in vivo* studies, together with the findings obtained from cultured sympathetic neurons, suggest that oxidative stress promotes activation of p75^{NTR}, thereby leading to axonal degeneration and neuronal apoptosis.

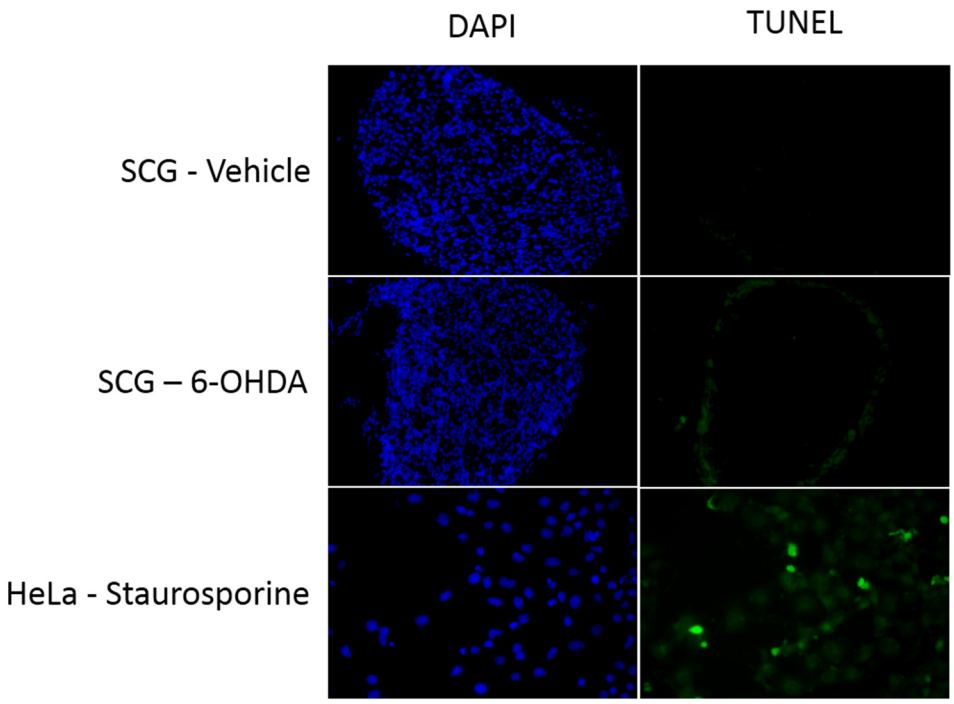

Figure 7: Intraperitoneal administration of 6-OHDA does not induce apoptosis of superior cervical ganglion neurons. Representative images of 4',6-diamidino-2-phenylindole (DAPI, left) staining or Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, right) of superior cervical ganglion from wildtype C57Bl6 mice after intraperitoneal administration of vehicle or 100 mg/kg 6-OHDA. Daily administrations were performed for two consecutive days, followed by tissue collection and analysis one week later. No increase in TUNEL labeling was observed in superior cervical ganglia from mice administered 6-OHDA. As a positive control for apoptosis, HeLa cells were treated for 20 hours with 1 µM staurosporine.

Discussion

The p75^{NTR} is upregulated in response to a variety of conditions involving oxidative stress (60,167,197,199,282,283), suggesting that the receptor may contribute to the associated cell death; however, a direct role for p75^{NTR} as an apoptotic mediator in response to oxidative stress had not been established. Here, we demonstrate that 4-hydroxynonenal (HNE), an endogenous product of oxidative stress, induces p75^{NTR}-mediated axonal degeneration and neuronal apoptosis. Oxidative stress can promote death through a variety of cell signaling mechanisms; thus, blocking an individual pathway may not be sufficient to confer significant protection. That neurons lacking p75^{NTR} were significantly protected from axonal degeneration and apoptosis induced by HNE is therefore quite remarkable, as it suggests that p75^{NTR} is a critical regulator of neuronal responses to oxidative stress. Nevertheless, other signaling pathways also likely contribute to HNE-induced apoptosis, as some apoptosis, although reduced, was still detected in cultures of *p75^{NTR}*-/- neurons exposed to the highest-tested concentrations of HNE.

Our results suggest that sympathetic neurons lacking p75^{NTR} are not only resistant to neurodegeneration induced by HNE in culture; loss of p75^{NTR} also protected sympathetic axons from oxidative stress-associated degeneration induced by 6-OHDA *in vivo*. Sympathetic neurons are susceptible to a variety of neurodegenerative conditions; for example, they develop neurofibrillary tangles in association with tauopathies or myotonic dystrophy (301,302). Additionally, apart from the lower brainstem and olfactory bulb, peripheral autonomic nuclei are among the earliest cell populations affected by Parkinson's disease (PD), and sympathetic neurons of PD patients are susceptible to

Lewy pathology and progressive neurodegeneration (303-305). The oxidant 6-OHDA has long been used to mimic PD in the CNS and was initially characterized for its ability to selectively induce degeneration of sympathetic nerve terminals (296,305). Our findings suggest that the activation of $p75^{NTR}$ by this oxidant plays a key role in promoting the breakdown of these axons. Interestingly, $p75^{NTR}$ has also been detected in neurons of the substantia nigra (200), and the receptor was reported to be upregulated in a mouse model with Parkinsonian-like neuronal loss and motor deficits (199,306). Oxidative stress is widely regarded as a contributing factor to the pathogenesis of Parkinson's disease (277), and thus induction of $p75^{NTR}$ signaling by reactive oxygen species may contribute to neurodegeneration caused by the disorder.

While previous studies have demonstrated that $p75^{NTR}$ mediates axonal degeneration as part of developmental pruning (281), our findings indicate that this function of the receptor is also engaged in response to oxidative stress. Interestingly, while neuronal death and axonal degeneration were correlated in our *in vitro* studies, administration of 6-OHDA *in vivo* caused axonal loss without leading to apoptosis of sympathetic neurons (data not shown). These findings are in agreement with earlier studies of 6-OHDA administration, in which axonal degeneration was detected without sympathetic neuron loss (297,298,307). Thus, these two functions of the receptor appear to have similar upstream components, yet in particular situations produce different functional outcomes. Further studies are needed to understand how $p75^{NTR}$'s degenerative signaling can be confined such that axonal regression occurs without neuronal apoptosis.

CHAPTER III

PROTEOLYSIS OF $p75^{NTR}$ CONTRIBUTES TO OXIDATIVE STRESS-ASSOCIATED NEURODEGENERATION

Introduction

Although $p75^{NTR}$ has been studied for over 20 years, its signaling mechanisms remain poorly understood, primarily due to the complexity of ligands, co-receptors, and cytosolic interactors that regulate $p75^{NTR}$ in a cell-specific manner (308). However, one established mechanism of $p75^{NTR}$ signaling occurs through regulated proteolysis of the receptor (131-133). Within this process, $p75^{NTR}$ is first cleaved in its extracellular domain by the metalloprotease TNF-α converting enzyme (TACE, also known as ADAM17). Subsequently, the remainder of the membrane-bound receptor, termed the c-terminal fragment ($p75^{NTR}$-CTF), is cleaved within its transmembrane region by the γ-secretase complex, thereby releasing the cytosolic intracellular domain ($p75^{NTR}$-ICD). These cleavage events promote a variety of downstream signals with differing cellular functions, including nuclear translocation of neurotrophin receptor-interacting factor (NRIF) (135) and prolonged activation of c-jun N-terminal kinase (JNK) (87) to promote apoptosis, activation of the small GTPase Rho to inhibit neurite outgrowth (137), and enhancement of Trk receptor signaling to promote cell survival (232). While the studies in Chapter II reveal a role for $p75^{NTR}$ in axonal degeneration and apoptosis induced by oxidative stress, the mechanisms through which reactive oxygen species activate $p75^{NTR}$ and whereby the receptor promotes neurodegeneration remained unknown. Here, we demonstrate that

oxidative injury promotes $p75^{NTR}$-mediated neurodegeneration via a neurotrophin-independent mechanism which induces proteolytic cleavage of the receptor.

Experimental Procedures

Sympathetic Neuron Culture

All experiments with animals were approved by the Animal Care and Use Committee at Vanderbilt University. Superior cervical ganglia were dissected from postnatal day 5/6 Sprague Dawley rats, C57BL/6J mice, or C57BL/6J *$p75^{NTR(exonIII)-/-}$* mice and dissociated with 0.08% trypsin (Worthington) and 0.3% collagenase (Sigma). Dissociated cells were then plated at a density of 5000-7000 neurons per 0.7 mm^2 on 8-well chamber slides (Thermo Scientific) or cell culture plates coated with poly-D-lysine (MP Biomedicals) and laminin (Invitrogen). All neurons were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco), 40 ng/ml Nerve growth factor (Harlan), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco**)**, and 100 µg/ml streptomycin (Gibco**)**. To inhibit the proliferation of non-neuronal cells, the neurons were treated with 5-10 µM cytosine arabinofuranoside [(Ara-C), Sigma] 24 hours after plating. Following 3 days of exposure, Ara-C was removed for 24 hours, and the neurons were then treated with the indicated concentrations of HNE or different pharmacological reagents. During pilot studies, we observed increased toxicity of HNE at lower cell densities, as has been reported for 6-hydroxydopamine (6-OHDA) (40), and therefore for all experiments neuron plating densities and Ara-C exposures were equivalent across all experimental conditions.

Cell Treatments

HNE was produced as previously described (41) as well as obtained from Calbiochem, and its concentration was determined by measuring optical density at 224 nm and using an extinction coefficient of 13,750/M. HNE is a highly reactive lipid aldehyde, and some variability in toxicity was observed between different batches of the compound. Every effort was therefore made to limit exposure of the HNE to oxygen, including its storage at -80° C under inert gas. For experiments with 6-OHDA (Sigma), the chemical was dissolved in cold phosphate buffered saline with 0.02% ascorbate immediately prior to each experiment. For assessment of neurotrophin involvement in HNE-induced neurite degeneration and apoptosis, C57BL/6J sympathetic neurons were pretreated for 30 minutes with control serum or with 9650 immune serum containing ligand-blocking antibody specific for the $p75^{NTR}$ extracellular domain (α-p75-ECD, 1:500) (42). The neurons were then exposed to 12 μM HNE for 20 hours before fixation and quantification of neurite degeneration or cell survival. For assessment of whether JNK is required for HNE-induced cleavage of $p75^{NTR}$, Sprague Dawley rat sympathetic neurons were pretreated with DMSO or JNK inhibitor SP600125 (ToCris, #1496) for 1 hour, followed by treatment with 25 μM HNE for 12 hours and analysis for $p75^{NTR}$ cleavage as described below.

Western Blot Analyses

After treating rat sympathetic neurons as indicated with HNE, 6-OHDA, DAPT (Millipore), or TAPI (Millipore), the neurons were lysed in NP-40 lysis buffer [25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1% NP-40, 10% glycerol,] supplemented with Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche) and PhosStop-

Phosphatase Inhibitor Cocktail Tablet (Roche). Cell lysates were subjected to western blot analysis using antibodies to Nerve Growth Factor (1:200, Alomone, AN-240), Brain-Derived Neurotrophic Factor (1:300, Millipore, AB1534), Neurotrophin-3 (1:1000, Alomone, ANT-003), Neurotrophin-4 (1:200, Alomone, ANT-004), and Tubulin (1:1000, Calbiochem, #CP06). To detect $p75^{NTR}$ cleavage fragments, neurons were treated with the proteosome inhibitor ZLLLH (Peptide Institute Inc., 10μM) 45 minutes prior to cell lysis and analyzed by western blot using $p75^{NTR}$-ICD antiserum [1:3000, generated as previously described (6)].

Quantification of Neurite Degeneration

Analyses of neurite degeneration were performed as previously described (43-45), with slight modification. Following the indicated treatments, sympathetic neurons in 8-well chamber slides were fixed with 4% paraformaldehyde and visualized via a 20x optical lens on a Leica inverted fluorescence microscope. Phase-contrast images of five fields of view per well were captured with 16 ms exposure by a Nikon DXM1200C digital camera. To ensure accurate measurement of neurites, images were captured from blindly-selected regions with well-separated axon tracts. Using an automated method of image analysis, the fragmentation of the neurites was then measured. Levels of neurite degeneration are reported as a degeneration index (DI), which is the ratio of fragmented neurite area over total neurite area. To process images for DI calculation, the auto-level function of the software GNU-image Manipulation Program (GIMP) was first used to adjust image gray levels to objectively provide uniform background intensity to all of the images. ImageJ software was then utilized to binarize the image and to remove all cell bodies, rendering an image composed of black neurites on white background. While

healthy neurites appear continuous, degenerating neurites have a disrupted, particulate structure due to blebbing and fragmentation. To measure the area of fragments from degenerating neurites, the Particle Analyzer algorithm of ImageJ was applied to identify regions of fragmentation based on size (20-10,000 pixels) and circularity (0.2 – 1.0). The total area of these detected neurite fragments was then divided by the total black, neurite area to determine the DI. In agreement with other studies (43), a DI of 0.2 or greater accurately indicated neurite degeneration, while a DI of 1.0 would theoretically represent neurites that have completely degenerated into fragmented particles.

Quantification of Neuronal Death

Following the indicated treatments, sympathetic neurons in 8-well chamber slides were fixed with 4% paraformaldehyde and immunostained with neuron-specific anti-TUJ1 primary antibody (Neuronal Class III β-Tubulin, 1:500, Covance, #MMS-435P) and Alexa Fluor® 488 secondary antibody (1:1000, Life Technologies, #A11001). Slides were then mounted using Vectashield with 4,6-diamidino-2-phenylindole (Vector Labs), and the neurons were blindly scored as apoptotic or non-apoptotic based on the appearance of the nucleus, apoptotic nuclei being condensed or fragmented. At least 75 TUJ1-positive neurons were counted per condition in all experiments.

Results

Induction of p75^{NTR}-mediated Neurite Degeneration and Apoptosis by HNE Occurs Through a Ligand-Independent Mechanism

Due to the effects of p75^{NTR} on HNE-induced neurite degeneration and apoptosis, we speculated that oxidative stress promotes neurotrophin or pro-neurotrophin release,

thereby leading to autocrine or paracrine activation of $p75^{NTR}$. We considered BDNF the most likely candidate since BDNF can be produced by sympathetic neurons (70,309) and can promote their apoptosis through activation of $p75^{NTR}$ (57,87,135). Therefore, we collected lysates from neurons treated with 25 µM HNE, the maximally effective dose, and measured BDNF by western blotting. Surprisingly, however, no BDNF was detected, even after treatment with HNE (Fig. 8). We next analyzed other neurotrophins. The precursor form of NGF, proNGF, is a known pro-apoptotic ligand for $p75^{NTR}$ (151-153), while mature NGF is a well-defined pro-survival factor for sympathetic neurons (2,4,5). We detected no proNGF in the neurons and found only low levels of mature NGF, likely due to its internalization from the media, which were unchanged in sympathetic neurons treated with vehicle or HNE (Fig. 8). Similar analyses revealed that sympathetic neurons also do not produce NT-3 or NT-4 in response to HNE (Fig. 8).

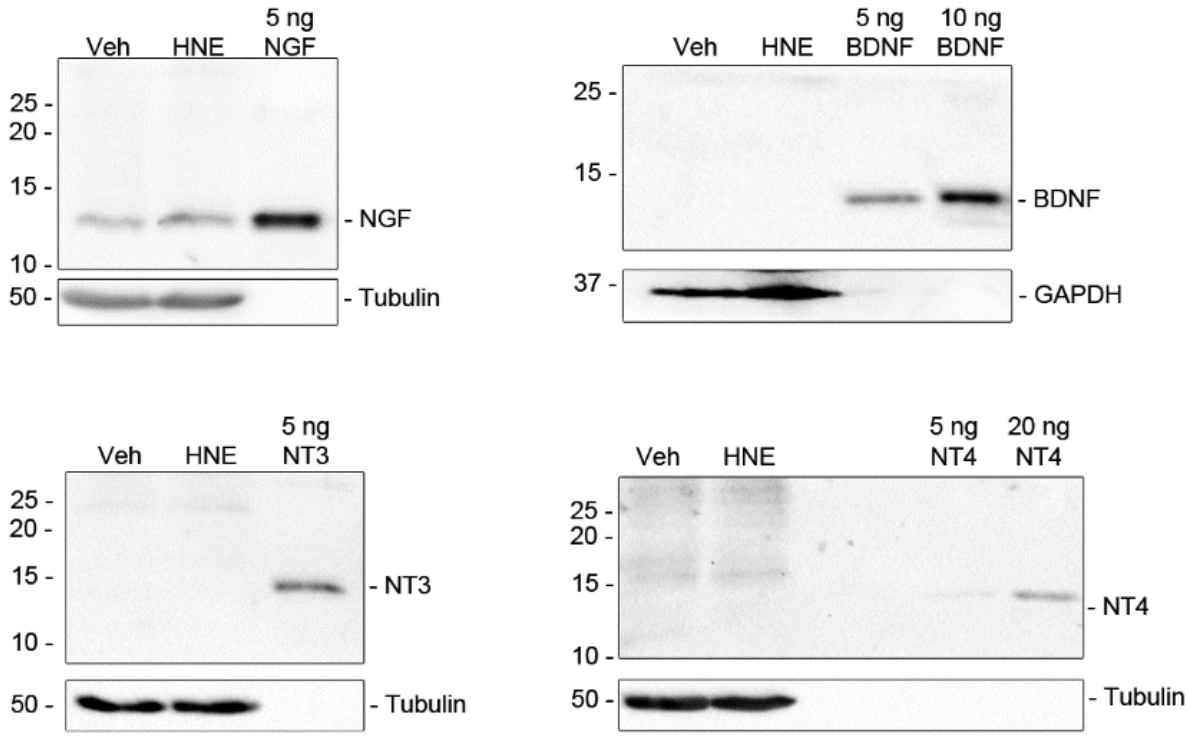

Figure 8: HNE Exposure Does Not Induce Production of Neurotrophins. Representative western blot of BDNF, NGF, NT3, or NT4 from lysate of rat sympathetic neurons treated for 6 hours with vehicle or 25 μM HNE (80 μg protein lysate per lane, n=3). Sensitivity of the antibodies was verified by loading 5 ng, 10 ng, or 20 ng of purified BDNF (Regeneron), NGF (Harlan), NT3 (Regeneron), or NT4 (Alomone). No induction of neurotrophin expression was observed in cultured sympathetic neurons in response to treatment with HNE (n=3).

Although substantial levels of pro-apoptotic neurotrophins would need to be present in order to induce neuronal death in the presence of NGF, which was in the media, it is theoretically possible that neurotrophins remaining in the neurons were below our detection limit. Therefore, we next used an antibody to the extracellular domain of $p75^{NTR}$ that blocks neurotrophin-mediated activation of the receptor to further explore whether HNE-induced axon degeneration and apoptosis requires activation of $p75^{NTR}$ by neurotrophins. As observed in previous studies (97), blockade of the extracellular domain with the $p75^{NTR}$ antibody prevented BDNF-induced death of sympathetic neurons; however, the antibody failed to prevent HNE-induced neurite degeneration and apoptosis (Fig 9, *a* and *b*). Together, these data suggest that oxidative stress promotes $p75^{NTR}$-mediated axonal degeneration and apoptosis through a ligand-independent mechanism.

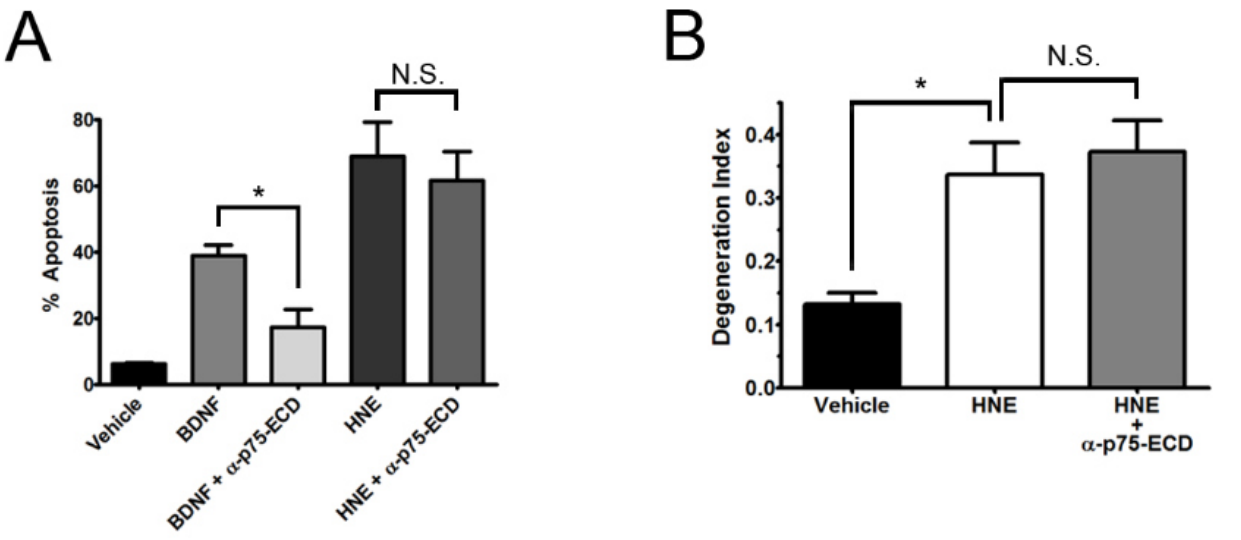

Figure 9: Induction of p75^{NTR}-mediated neurite degeneration and apoptosis occurs through a ligand-independent mechanism. (**A**) Quantification of neuronal apoptosis induced by HNE after pretreatment with ligand-blocking α-p75-ECD antibody. Sympathetic neurons were pretreated with control serum or α-p75-ECD for 30 minutes and then exposed to 12 μM HNE for 20 hours. The neurons were then fixed, labeled with DAPI, and scored for apoptosis. To control for antibody efficacy, sympathetic neurons were similarly pretreated with α-p75-ECD before exposure to BDNF. After maintaining the neurons in 20ng/mL NGF, the neurons were rinsed to remove the NGF and, to promote survival, refed with medium containing 12.5 mM KCl. The neurons were then pretreated with α-p75-ECD for 30 minutes, followed by 200ng/mL BDNF for 24 hours. Though α-p75-ECD significantly blocked BDNF-induced apoptosis, no significant effect of α-p75-ECD on HNE-induced cell death was observed (n=3, mean ± S.E., * $p < 0.05$, NS - not significant, two-tailed t-test). (**B**), Quantification of neurite degeneration after treatment of sympathetic neurons with 12 μM HNE for 20 hours following pretreatment with control serum or immune serum containing antibody specific for the p75^{NTR} extracellular domain (α-p75-ECD) for 30 minutes. Pretreatment with α-p75-ECD caused no significant change in neurite degeneration after exposure to 12 μM HNE (n=3, mean ± S.E., * – $p < 0.05$, N.S. – not significant, ANOVA with Bonferroni post-hoc analysis).

HNE Stimulates Proteolytic Cleavage of p75^{NTR}

Since our results indicated that the effects of HNE did not require ligand binding to p75^{NTR}, we hypothesized that oxidative stress triggers intracellular receptor signaling. We previously demonstrated that p75^{NTR}-mediated apoptosis in sympathetic neurons requires proteolytic cleavage of the receptor, first by the metalloprotease TACE/ADAM17, followed by γ-secretase (87,135). Therefore, we investigated whether HNE stimulates p75^{NTR} proteolysis. Sympathetic neurons were treated with various concentrations of HNE and subjected to western blot analysis using an antibody that recognizes the intracellular domain of p75^{NTR}. Compared to neurons treated with vehicle, HNE-treated neurons had a robust and dose-dependent increase in the 25 kDa and 20 kDa fragments of p75^{NTR} corresponding to the p75^{NTR}-CTF and p75^{NTR}-ICD, respectively (Fig. 10*a*). Cleavage of p75^{NTR} in response to HNE was observed even after just 6 hours of treatment (Fig. 10*b*), which was before apoptosis was visually apparent (data not shown), suggesting that proteolysis of the receptor precedes cell death.

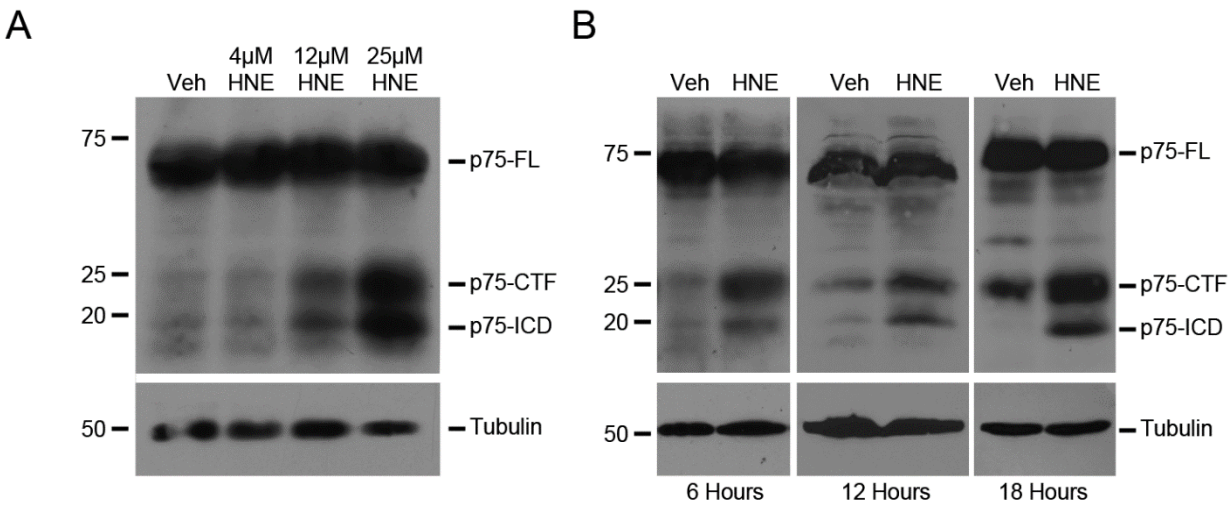

Figure 10: HNE induces proteolytic cleavage of p75^{NTR}. Rat sympathetic neurons were exposed to the indicated concentrations of HNE. To detect p75^{NTR} cleavage fragments, the neurons were treated with the proteosome inhibitor ZLLLH 45 minutes prior to lysis and then subjected to western blot analysis using an antibody specific for the p75^{NTR}-ICD. A, Representative western blot of p75^{NTR}-ICD from lysate of rat sympathetic neurons treated for 18 hours with the indicated concentrations of HNE (n=3). B, Representative western blot of p75^{NTR}-ICD from lysate of rat sympathetic neurons treated for 6 hours, 12 hours, or 18 hours with 25 μM HNE (n= 3-5).

To explore whether proteolysis of $p75^{NTR}$ occurs as a specific response of sympathetic neurons to HNE exposure, or, instead, as a general response to oxidative stress, we next analyzed the effects of other agents known to induce oxidative stress. To our surprise, high concentrations of the mitochondrial complex I inhibitor rotenone failed to cause neurodegeneration of cultured sympathetic neurons, and a similar lack of neurite fragmentation or neuronal death was observed following treatment of cultured sympathetic neurons with super-physiological concentrations of hydrogen peroxide (data not shown). However, exposure to the neurotoxin 6-OHDA induced death of sympathetic neurons (Fig. 11*a*). As we observed following treatment with HNE, exposure of sympathetic neurons to 6-OHDA caused cleavage of $p75^{NTR}$, promoting a robust increase in $p75^{NTR}$-CTF and $p75^{NTR}$-ICD fragments (Fig. 11*b*). These results indicate that $p75^{NTR}$ cleavage is a response to multiple types of oxidative insults.

Previous studies have demonstrated that cleavage of $p75^{NTR}$ in response to neurotrophin stimulation requires activation of c-jun N-terminal kinase (JNK) (87). Because JNK is a stress-induced kinase known for its activation in response to oxidative stress (264,272,273,310,311), we analyzed the requirement of JNK for HNE-induced $p75^{NTR}$ cleavage. However, pretreatment with the JNK inhibitor SP600125 failed to prevent cleavage of $p75^{NTR}$ in sympathetic neurons exposed to HNE, thus suggesting that JNK is not necessary for cleavage of the receptor in response to oxidative stress (Fig 11*c*). Because JNK mediates neurotrophin-induced cleavage of $p75^{NTR}$, these results also further support that proteolysis of the receptor in response to HNE occurs via a ligand-independent mechanism.

We next considered whether cleavage of $p75^{NTR}$ in response to oxidative stress occurs due to upregulation of the receptor, since increased expression of $p75^{NTR}$ is associated with a variety of pathological conditions (60,194,199,201). However, no change in the total expression level of $p75^{NTR}$ was observed in sympathetic neurons exposed to HNE (Fig. 11*d*), indicating that cleavage of $p75^{NTR}$ occurs through regulated activation of proteases rather than due to upregulation of the full-length receptor.

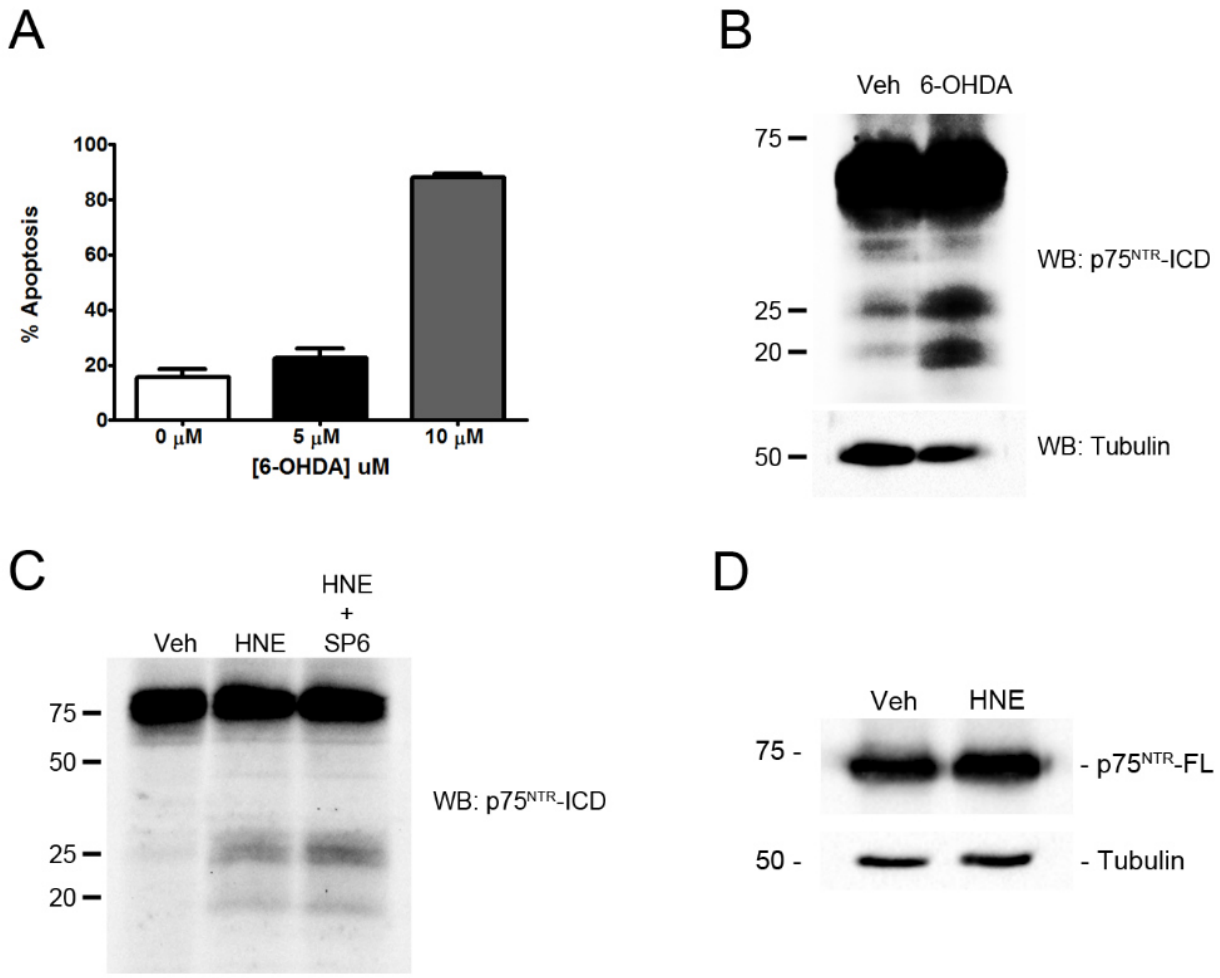

Figure 11: Oxidative stress-induced cleavage of p75^{NTR} does not require activation of JNK. (**A**) Quantification of apoptosis of sympathetic neurons treated with different concentrations of 6-OHDA. Cultured sympathetic neurons were exposed to vehicle, 5 µM 6-OHDA, or 10 µM 6-OHDA for 20 hours and evaluated for apoptosis based on the appearance of the nuclei. (n=3) (**B**) Representative western blot of p75^{NTR} cleavage products from lysates of rat sympathetic neurons treated for 18 hours with vehicle or 15 µM 6-OHDA. To detect cleavage fragments, the neurons were treated with ZLLLH for 45 minutes prior to lysis, followed by western blot analysis using an antibody specific for the p75^{NTR}-ICD (n=3). (**C**) Representative western (n=2) blot of p75^{NTR} cleavage products from lysates of rat sympathetic neurons treated for 12 hours with vehicle or HNE following 1 hour pretreatment with or without the JNK inhibitor SP600125 (SP6). (**D**) Representative western blot of full-length p75^{NTR} from lysates of rat sympathetic neurons treated for 6 hours with vehicle or 25 µM HNE (n=3).

Since TACE and γ-secretase have been shown to mediate cleavage of $p75^{NTR}$ in response to neurotrophins, we hypothesized that similar enzymatic activities may be induced by oxidative stress. Treatment of sympathetic neurons with the TACE inhibitor TAPI-1 or with the γ-secretase inhibitor DAPT blocked HNE-induced cleavage of $p75^{NTR}$ (Fig. 12, *a* and *b*), thus indicating that HNE stimulates proteolytic cleavage of $p75^{NTR}$ by TACE and γ-secretase. While pretreatment of sympathetic neurons with TAPI prevented HNE-induced accumulation of both the $p75^{NTR}$-CTF and $p75^{NTR}$-ICD, inhibition of γ-secretase only reduced accumulation of the $p75^{NTR}$-ICD (Fig. 12, *a* and *b*). These results suggest that HNE-induced cleavage of the receptor by γ-secretase requires prior proteolysis by TACE, as has been observed with neurotrophin-induced $p75^{NTR}$ proteolysis (87).

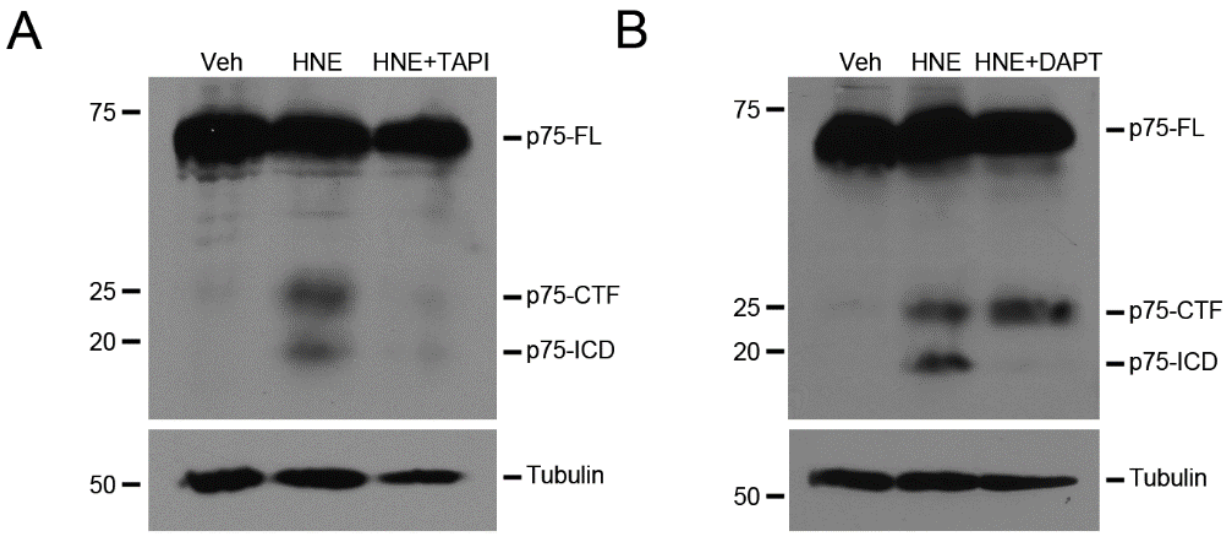

Figure 12: HNE induces sequential proteolytic cleavage of $p75^{NTR}$ by a metalloprotease and γ-secretase. (**A**) Representative western blot of $p75^{NTR}$-ICD from lysate of sympathetic neurons treated for 18 hours with vehicle or 25 μM HNE after one hour pretreatment with the control solvent dimethyl sulfoxide (DMSO) or with 10 μM TAPI-1 (n=3). (**B**) Representative western blot of $p75^{NTR}$-ICD from lysate of sympathetic neurons treated for 12 hours with vehicle or 25 μM HNE after one hour pretreatment with DMSO or DAPT (n= 3).

Cleavage of p75^{NTR} is Required for HNE-induced Neurite Degeneration and Apoptosis

To determine whether proteolysis of p75^{NTR} is required for HNE-induced axon degeneration and apoptosis, we next blocked cleavage of p75^{NTR} by pretreating sympathetic neurons with the TACE inhibitor TAPI-1 and then assessed neurite integrity and neuronal death following exposure to HNE. Compared to neurons pretreated with vehicle, HNE-induced neurite fragmentation was dramatically reduced in sympathetic neurons pretreated with TAPI-1 (Fig. 13, *a* and *b*). Similarly, HNE-induced apoptosis was significantly decreased in neurons pretreated with TAPI-1 (Fig. 13*c*). Hence, receptor proteolysis is required for p75^{NTR}-mediated axon degeneration and apoptosis induced by HNE.

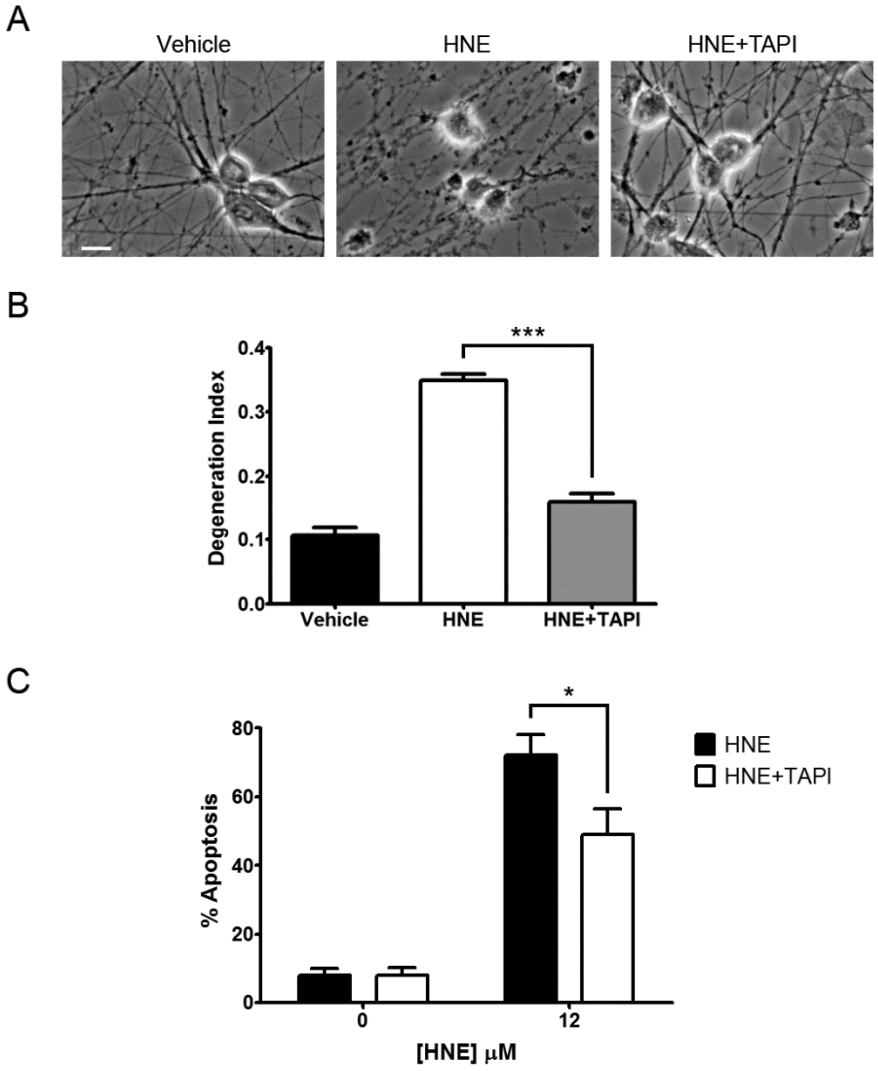

Figure 13: Cleavage of p75^{NTR} is required for HNE-induced neurite degeneration and apoptosis. (**A**) Phase contrast microscopy images of C57Bl6 sympathetic neurons treated with vehicle or 12 μM HNE for 20 hours following 1 hour pretreatment with DMSO or 10 μM TAPI-1 (Scale bar, 12.5μm). (**B**) Measurement of neurite degeneration after 20 hour exposure of sympathetic neurons to vehicle or 12 μM HNE following pretreatment with DMSO or 10 μM TAPI-1 (n= 3, mean ± S.E., *** $p < 0.001$, ANOVA with Bonferroni post-hoc analysis). (**C**) Quantification of apoptosis of sympathetic neurons treated for 20 hours with 12 μM HNE after one hour pretreatment with DMSO or 10 μM TAPI-1 (n= 3, mean ± S.E., *$p < 0.05$, ANOVA with Bonferroni post-hoc analysis).

Discussion

While most investigations of $p75^{NTR}$-mediated cell death have focused on neurotrophin- or proneurotrophin-induced apoptosis, studies over expressing recombinant $p75^{NTR}$ or its cleavage fragments have revealed the potential for ligand-independent apoptotic signaling by the receptor (34,53,88,243,312). Other non-apoptotic functions of $p75^{NTR}$ have also been reported to occur independently of neurotrophin binding, such as inhibition of fibrinolysis through downregulation of the serine protease tissue plasminogen activator (tPA) (313). We did not observe any induction of NGF, BDNF, NT-3, or NT-4 expression in response to HNE, and use of a ligand-blocking antibody failed to prevent HNE-induced neurite degeneration and apoptosis, thus suggesting that initiation of these functions by $p75^{NTR}$ occurs through a ligand-independent mechanism.

Interestingly, we did not observe an upregulation of the $p75^{NTR}$ in response to HNE. This finding was surprising given the numerous reports of increases in $p75^{NTR}$ expression associated with different pathological conditions (60,194,199,201). Because our studies utilized antimitotics to achieve nearly-pure cultures of sympathetic neurons, these findings suggest that the reported increases in $p75^{NTR}$ expression observed under conditions associated with oxidative stress are unlikely due to oxidants acting directly on neurons, but instead are the result of neighboring glial cell activation, leading to the production of cytokines. Friedman's group has shown that proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1ß (IL-1ß), which can be released by microglia and astrocytes (314,315), upregulate the expression of $p75^{NTR}$ (175,316).

Numerous studies have demonstrated that reactive oxygen species (ROS) activate TACE. For example, H_2O_2 was found to activate TACE through a mechanism suggested to involve oxidative disruption of inhibitory interactions between the TACE pro-domain and the Zn^{2+}-containing catalytic site (317). More recently, a study by Walcheck and colleagues revealed that the activity of purified TACE lacking its pro-domain and intracellular region is enhanced by H_2O_2. Their results indicated that oxidation of conserved cysteine-X-X-cysteine motifs within the extracellular domain of TACE promotes its activation (318). These and other studies (319,320) demonstrate that ROS can activate TACE through multiple mechanisms, and we therefore hypothesized that similar mechanisms could link oxidative stress to cleavage of $p75^{NTR}$. Fitting with this hypothesis, treatment of sympathetic neurons with HNE promoted robust cleavage of $p75^{NTR}$, indicating that oxidative stress promotes activation of the receptor's regulatory proteases. Pretreatment with the matrix metalloprotease and TACE inhibitor TAPI-1 or the γ-secretase inhibitor DAPT blocked HNE-induced $p75^{NTR}$ cleavage. Additionally, blocking cleavage of $p75^{NTR}$ with TAPI-1 significantly protected sympathetic neurons from HNE-induced neurite degeneration, as well as apoptosis, indicating that proteolysis of the receptor is required for oxidative stress-induced neurodegeneration. These results provide the first evidence that $p75^{NTR}$-mediated axonal degeneration requires receptor proteolysis, similar to $p75^{NTR}$-mediated inhibition of axon outgrowth and neuronal apoptosis. While cleavage of the $p75^{NTR}$ extracellular domain by metalloproteases other than TACE is also feasible, previous work has demonstrated that TACE is required for $p75^{NTR}$ cleavage in sympathetic neurons (87). Induction of $p75^{NTR}$ cleavage was observed not only after exposure of sympathetic neurons to HNE, but also after treatment with

6-OHDA, indicating that different oxidants are capable of initiating $p75^{NTR}$ signaling. Thus, our results support a model in which oxidative stress promotes ligand-independent cleavage of $p75^{NTR}$ by TACE and γ-secretase, leading to axonal degeneration and programmed cell death (Fig. 14).

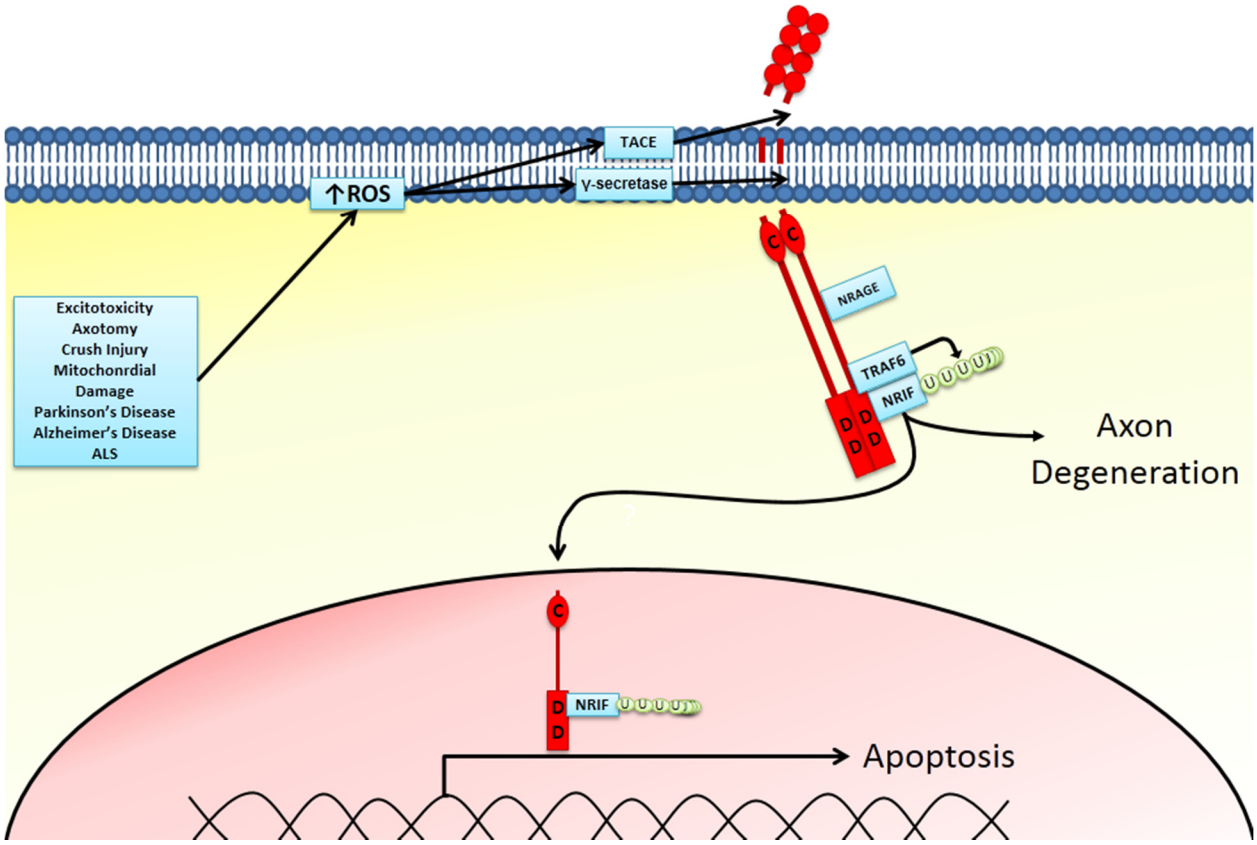

Figure 14: Model of oxidative stress-induced apoptotic signaling by p75^{NTR}. Increased formation of ROS occurs in response to deleterious conditions such as axotomy, crush injury, excitotoxicity, mitochondrial damage, and DNA damage. Through a ligand-independent mechanism, ROS induce proteolysis of p75^{NTR} by TACE and γ-secretase, thus leading to release of the p75^{NTR}-ICD to the cytosol. Subsequently, cleavage of p75^{NTR} promotes TRAF6-mediated ubiquitylation and nuclear translocation of NRIF, as well as prolonged JNK activation, thereby leading to apoptosis.

Although our findings demonstrate that oxidants can trigger activation of $p75^{NTR}$-mediated apoptotic signaling in neurons, a previous report using PC12 cells found the receptor's intracellular domain to have anti-oxidant capability, thereby conferring resistance to ROS (321). Because $p75^{NTR}$ has been shown to have cell-specific effects on survival, cleavage of the receptor in response to oxidative stress may confer death in specific populations of post-mitotic neurons, yet similar signaling mechanisms may lead to cell survival in other non-mitotic cell types. Fitting with this hypothesis is the fact that $p75^{NTR}$ cleavage has been reported to promote cell survival by enhancing Trk receptor signaling in PC12 cells (231,232), yet in sympathetic neurons cleavage of $p75^{NTR}$ induces programmed cell death (87,135). Thus, $p75^{NTR}$ may regulate cell survival in different cell populations through similar proteolytic signaling mechanisms that lead to cell-specific physiological responses. Further studies are needed to understand how signals downstream of $p75^{NTR}$ cleavage are differentially regulated within specific cell types to produce diverse functional outcomes.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Limitations and Remaining Questions

While the results in Chapters II and III provide exciting insight into the mechanisms through which $p75^{NTR}$ contributes to neurodegeneration after cellular injury, many questions remain to be answered. One reported factor linking $p75^{NTR}$ to numerous pathological conditions is increased proneurotrophin expression in disease models or in samples of disease-affected human tissues. However, determining which brain regions may secrete neurotrophins in their uncleaved form – and furthermore, without the proneurotrophins being immediately processed by matrix metalloproteases or other enzymes within the extracellular milieu – has proven difficult. Indeed, the best evidence of proneurotrophin secretion currently comes from reported detection of proNGF in the cerebral spinal fluid of mice following lesion of the internal capsule (153) or after induction of seizures (60). These data indicate that proneurotrophins can certainly persist in the extracellular environment in their uncleaved form, but whether this can occur in all neurotrophin-expressing brain regions after injury or whether this ability is restricted to a few specific neuroanatomical areas remains a mystery. Our results provide a novel mechanism of $p75^{NTR}$ induction after injury: activation of the receptor by oxidative stress. While this mechanism of $p75^{NTR}$ activation was discovered in sympathetic neurons, further investigations are needed to determine whether the receptor is similarly invoked by oxidative stress in neurons of the brain, particularly in regions such as the basal forebrain or ventral midbrain where the receptor may contribute to oxidative stress-

associated disorders such as Alzheimer's disease or Parkinson's disease. Importantly, this mechanism of receptor activation could potentially function in any type of cell expressing $p75^{NTR}$, regardless of the neurotrophin expression of cells within the tissue. Additionally, while proneurotrophins would theoretically have to function as a diffusible factor that can affect all $p75^{NTR}$-expressing cells within a particular microenvironment, ligand-independent activation of $p75^{NTR}$ by intracellular oxidative stress could potentially serve as a more specific signal, promoting the degeneration of individual cells subjected to oxidative stress without causing death of adjacent cells expressing $p75^{NTR}$. Thus, dual mechanisms of $p75^{NTR}$ activation may ensure removal of damaged neurons or neuronal projections, with activation of the receptor by proneurotrophins serving to promote more widespread removal of cells in response to tissue damage, while ligand-independent activation of $p75^{NTR}$ by reactive oxygen species induces a confined degeneration signal that is specific to individually injured neurons or neuronal projections.

While examining $p75^{NTR}$-mediated axonal degeneration and apoptosis in response to oxidative stress-inducing agents, we observed a curious inconsistency: 6-OHDA induced both axonal degeneration and neuronal apoptosis of cultured sympathetic neurons, but administration of 6-OHDA *in vivo* caused only axonal degeneration without neuronal death. These observations indicate that, despite the strong correlation between axon fragmentation and cell death in neuronal cultures, $p75^{NTR}$ can promote axonal degeneration in response to oxidative stress without necessarily inducing apoptosis. Signals downstream of $p75^{NTR}$ cleavage may diverge to produce these two effects, or, alternatively, $p75^{NTR}$ signaling in response to oxidative stress may lead to distinct biological outcomes simply depending upon the cellular location of the signaling

cascades. For example, one explanation could be that $p75^{NTR}$ activation near the soma of the cell leads to apoptosis, while signaling at the distal ends of axons leads to neurite degeneration. Therefore, 6-OHDA exposure to sympathetic neurons in mass culture led to both death and axon degeneration, while selective uptake of 6-OHDA near axon terminals *in vivo* caused only axon degeneration. Further studies are needed to determine the relationship between axonal degeneration and apoptosis mediated by $p75^{NTR}$, with particular emphasis on identifying the downstream factors required for each of these receptor functions.

Our studies suggest that oxidative stress promotes neurotrophin-independent cleavage of $p75^{NTR}$, thereby leading to axonal degeneration and neuronal apoptosis. However, the mechanism through which reactive oxygen species trigger proteolysis of the receptor remains to be determined. Our current hypothesis is that direct oxidation of CXXC motifs within the extracellular domain of TACE enhances its enzymatic activity, thereby leading to increased ectodomain shedding of the $p75^{NTR}$. However, other signaling events would likely also be required to promote formation of the $p75^{NTR}$-ICD, since we have previously found that overexpression of TACE alone is insufficient for further cleavage of the receptor by γ-secretase (unpublished data). Therefore, other events leading to activation of γ-secretase are likely necessary as well. Another possibility is that oxidative stress promotes cleavage of $p75^{NTR}$ by first triggering activation of the stress-activated kinase JNK. We have previously demonstrated that JNK has biphasic roles in $p75^{NTR}$-mediated apoptosis: rapid JNK activity after neurotrophin stimulation is necessary for cleavage of $p75^{NTR}$, while persistent activity of the kinase in response to $p75^{NTR}$ cleavage functions as a downstream apoptotic signal (87). Additionally, JNK

signaling is associated with a number of conditions involving oxidative stress (264,272,273), and importantly, is a known mediator of apoptosis induced by HNE. By directly interacting with the kinase, HNE has been found to induce translocation of JNK to the nucleus (322). Therefore, the kinase may have a significant role in sensing oxidative stress and promoting cleavage of $p75^{NTR}$. Preliminary experiments discussed in Chapter III indicated that cleavage of $p75^{NTR}$ in response to HNE is not prevented by the JNK inhibitor SP600125, thus suggesting that the kinase is not required for $p75^{NTR}$ signaling in response to oxidative stress. However, these results were only preliminary, and further experiments are needed to verify whether SP600125 properly inhibits JNK following HNE exposure, as it is possible that HNE disrupts the inhibitor or that HNE stimulates JNK activity through a mechanism that is not efficiently prevented by SP600125. Additionally, multiple mechanisms may be involved in promoting $p75^{NTR}$ signaling in response to oxidative stress, and therefore inhibiting JNK may be insufficient to prevent such signaling.

The Vps10p family member sortilin has been identified as a $p75^{NTR}$ co-receptor that is necessary for $p75^{NTR}$-induced apoptosis in many different biological contexts. Whether sortilin is required for oxidative stress-induced neuronal death has yet to be investigated. Interestingly, sortilin can also undergo regulated cleavage by a metalloprotease and γ-secretase, though the functional role of such proteolysis is not currently understood (323). In cells co-expressing $p75^{NTR}$ and sortilin, oxidative stress would likely induce cleavage of both receptors, and proteolysis of sortilin may modulate redox-associated $p75^{NTR}$ signaling. Additionally, whether sortilin is required for $p75^{NTR}$-induced axonal degeneration has not been determined, and thus further studies are

needed to evaluate the cellular localization of $p75^{NTR}$-sortilin complexes and to determine whether sortilin contributes to $p75^{NTR}$-mediated degenerative signaling at distal axons.

Another remaining question pertains to the events downstream of $p75^{NTR}$ cleavage which mediate oxidative stress-induced neurodegeneration. We have previously demonstrated that the DNA-binding protein NRIF is a critical signal downstream of $p75^{NTR}$ cleavage that is necessary for apoptosis induced by BDNF (135). NRIF has also been demonstrated to have a similar role in inducing neuronal death in response to seizures (60). Interestingly, seizures and excitotoxicity have been associated with oxidative stress in numerous contexts (324), and, in addition to the known apoptotic role of NRIF within the nucleus, we have recently identified expression of NRIF in distal axons of sympathetic neurons (data not shown). NRIF is therefore a strong candidate for a downstream mediator of $p75^{NTR}$ signaling which facilitates axonal degeneration or neuronal apoptosis in response to oxidative stress. Apart from NRIF, however, several other $p75^{NTR}$ interactors could potentially mediate this neurodegenerative signal. In addition to the aforementioned possibility that JNK stimulates cleavage of $p75^{NTR}$, the kinase is also a known downstream mediator of $p75^{NTR}$-induced apoptosis and therefore could function in such a capacity in response to oxidative stress as well. Other factors, such as the lipid signaling molecule ceramide or the cytosolic protein neurotrophin receptor-interacting MAGE homologue (NRAGE) have also been reported to facilitate $p75^{NTR}$-induced apoptosis. Further studies are needed to determine whether these or other interactors mediate neurodegeneration in response to oxidative stress-induced activation of $p75^{NTR}$.

Summary and Conclusions

This dissertation project sought to understand the role of $p75^{NTR}$ in neurodegeneration caused by oxidative stress. Our work revealed that the receptor has a critical role in promoting axonal degeneration and apoptosis of sympathetic neurons subjected to oxidative injury by exposure to the toxin 6-OHDA or the lipid peroxidation product HNE. Further investigations revealed that activation of $p75^{NTR}$ in response to oxidative stress occurs via a neurotrophin-independent mechanism which promotes sequential cleavage of the receptor by a metalloprotease and γ-secretase. These findings provide a novel mechanism of $p75^{NTR}$ activation which could potentially contribute to neurodegeneration associated with a wide variety of tissue injuries or pathological conditions. Further studies are needed to explore the molecular mechanisms underlying proteolysis of $p75^{NTR}$ during oxidative stress, to identify downstream mediators of $p75^{NTR}$-induced neurodegeneration, and to determine the role of such signals in injuries or disorders of the central nervous system.

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