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The Effects Of Oxidative Stress On P75ntr Signaling In Dopaminergic Neurons

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BY

CASSANDRA ESCOBEDO

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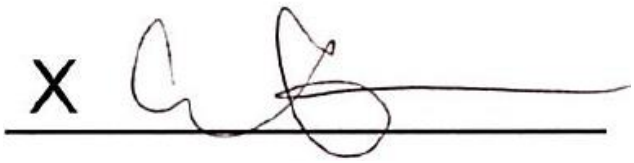

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BY

CASSANDRA ESCOBEDO

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DEDICATION

To my wonderful family, for never allowing me to steer myself away from my dreams; and to my adorable dog, Kobi, whose constant company and unconditional love kept me sane as I prepared this thesis.

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ABSTRACT

The p75 neurotrophin receptor ($p75^{NTR}$) is responsible for implementing cellular death during embryonic development and in response to cellular injury. The receptor has been recognized as a contributor of neurodegeneration in numerous pathological conditions. Cleavage of $p75^{NTR}$ by Tumor Necrosis Factor converting enzyme (TACE) and γ-secretase has been observed to be associated with an increase in neurodegeneration. In a previous study, $p75^{NTR}$ was discovered to become activated in sympathetic neurons in response to oxidative stress induced by 4-hydroxynonenal (HNE) in a ligand-independent mechanism. Furthermore, cleavage of the receptor was demonstrated to contribute to the death of sympathetic neurons following oxidative insult. This study investigates the effects of oxidative stress on $p75^{NTR}$ signaling in dopaminergic neurons. Because dopaminergic neurons only compromise a very small percentage of the ventral midbrain, the Lund Human Mesencephalic cell line (LUHMES) was used in our study. LUHMES cells can be differentiated into mature dopaminergic neurons following a two-step differentiation procedure. The $p75^{NTR}$ was found to be activated and cleaved in response to 6-hydroxydopamine (6-OHDA), a neurotoxin frequently used to mimic oxidative stress in dopaminergic neurons. Furthermore, the pretreatment of dopaminergic neurons with a ligand-blocking antibody specific for the extracellular domain of $p75^{NTR}$ (α- $p75^{NTR}$ ECD) failed to protect neurons from 6-OHDA-induced death. Our results suggest that $p75^{NTR}$ may contribute to the death of dopaminergic neurons exposed to oxidative stress in a ligand-independent manner.

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6-OHDA	6-Hydroxydopamine
AD	Alzheimer's disease
APAF-1	Apoptotic Activating Protease Factor-1
BDNF	Brain-Derived Neurotrophic Factor
COMT	Catechol-O-methyltransferase
CTF	C-terminal Fragment
DAPI	4, 6-diamidino-2-phenylindole
DAT	Dopamine transporter
EAE	Encephalitis
ECD	Extracellular Domain
ERK	Extracellular signal-related Kinase
GIRK	G-protein-coupled inwardly rectifying potassium
HNE	4-hydroxy-2-nonenal
ICD	Intracellular Domain
JNK	c-jun N-terminal Kinase
LUHMES	Lund Human Mesencephalic cells
MAGE	Melanoma-associated antigen
MAO	Monoamine Oxidase
NADE	$p75^{NTR}$ Associated Death Executioner
NF-κB	Nuclear factor-κB
NGF	Nerve Growth Factor
NRAGE	Neurotrophin receptor-interacting MAGE homolog
NRIF	Neurotrophin Receptor-Interacting Factor
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
$p75^{NTR}$	p75 Neurotrophin Receptor
PD	Parkinson's disease

PI3K	Phosphatidylinositol-3-kinase
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TACE	Tumor Necrosis Factor Converting Enzyme
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAF	TNF Receptor Associated Factor
Trk	Tropomyosin Related Kinase
VMAT	Vesicular monoamine transporter

CHAPTER 1

INTRODUCTION

1.1 Neurotrophins

The majority of neurons that compromise both divisions of the nervous system, the central and peripheral nervous systems, are made up of a cell body and its appendages called dendrites and axons. Neurons are responsible for receiving information through their dendrites, which can lead to the stimulation or inhibition of neuron electrical impulses. In an excited neuron, an action potential is generated, which is conducted down the axon. Axons are insulated with myelin, a fatty substance that wraps around the axon and enhances action potential propagations quickly and sufficiently. At the end of the axon, there are branches called axon terminals, which are responsible for communicating to specific target cells and tissues. These neuronal connections are termed synapses, and these are the sites in which information is carried from the first neuron to the target neuron, also known as the pre- and postsynaptic neurons, respectively. The overall health of neurons is reliant on proper growth, differentiation, and function which can be sufficiently provided by specific growth factors.

Neurotrophins are a family of neuronal growth factors that regulate neuronal growth, development, differentiation, and plasticity(E. J. Huang & Reichardt, 2001; Sorkin & von Zastrow, 2002). Their presence has been characterized as a necessity for the survival of developing neurons in which limited quantity of neurotrophins during development can severely affect the number of surviving neurons(Skaper, 2018). Additionally, alterations of neurotrophin levels have been connected to neurodegenerative diseases such as Alzheimer's and Huntington's disease, as well as mental illnesses like substance abuse and depression(M. V. Chao, Rajagopal, & Lee, 2006). The four members of the neurotrophin family include Nerve Growth Factor (NGF), Brain-Derived Neurotrophin Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4). Initially, all four neurotrophins are synthesized as precursors

until they are enzymatically cleaved to yield the mature neurotrophin form (Ramee Lee, Kermani, Teng, & Hempstead, 2001). Within the Golgi apparatus and endoplasmic reticulum, proneurotrophins can be proteolytically cleaved by furin and pro-protein convertases (Seidah et al., 1996). Alternatively, proneurotrophins can be secreted into the extracellular cleavage and be cleaved by plasmin and matrix mellatoproteases thereby releasing mature neurotrophins (R Lee, Kermani, Teng, & Hempstead, 2001). At this point, mature neurotrophins can then exert their survival-promoting effects by binding to one or more of the three members of the Tropomyosin Related Kinases (Trk) receptor family, TrkA, TrkB, or TrkC (M. V. Chao et al., 2006; E. J. Huang & Reichardt, 2001; Sorkin & Zastrow, 2002).

Nerve Growth Factor (NGF) was the first neurotrophin to be discovered and it is the most well characterized member of the neurotrophin family (M. V. Chao et al., 2006). Upon NGF's discovery, the role of cellular interactions was revealed and the "neurotrophic hypothesis" was established (Raff et al., 1993). The neurotrophic hypothesis entails that during nervous system development, a retrograde flow of neurotrophic factors is formed, in which NGF travels from the post-synaptic neuron or target tissue to the cell body of the presynaptic neuron. Neurons that successfully form this retrograde flow survive developmental death while neurons that fail to do so die. This retrograde flow of NGF must consistently occur throughout life in order for the neuron to remain functional. Confirmations of the neurotrophic hypothesis have been investigated, in which removal of post-synaptic neurons results in the death of the developing pre-synaptic neurons that should innervate the target (Raff et al., 1993). Additionally, augmenting NGF to the target area of innervation rescued neurons that would have otherwise resulted in death (Hamburger, Brunso-Bechtold, & Yip, 1981).

NGF serves as a trophic factor that is able to bind and activate TrkA to mediate the proper development, maintenance, and survival of various types of neurons such as sympathetic, peripheral sensory and cholinergic neurons(Dreyfus, 1989). For

example, NGF has been shown to regulate proper differentiation of neurons during development (Skaper, 2018). Furthermore, NGF has been characterized as a promoter of neurite outgrowth in sympathetic and sensory ganglia during development(Viktor & Rita, 2018). As neurons mature, they begin to lose their dependency on NGF for survival. However, NGF has also been shown to promote survival effects in adult neurons. For example, NGF has been suggested to promote the survival and regeneration of injured neurons (Korsching, 1993). Following peripheral nerve injury, the release of cytokines has been demonstrated to promote the synthesis of NGF in Schwann cells, fibroblasts, and mast cells within the injured nerve (Korsching, 1993). Furthermore, NGF has been shown to induce sensitization of nociceptive neurons during inflammation by increasing the expression of proinflammatory molecules and specific ion channels (Vivas, Kruse, & Hille, 2014). NGF has been shown to modulate synaptic transmission of cholinergic neurons by promoting the synthesis of Acetylcholine (Hefti, 1986), a neurotransmitter responsible for stimulating muscle contraction.

Aside from NGF, neurons have also been shown to also express other neurotrophins such as NT-4 and BDNF(Ibáñez, 1995), which bind and activate TrkB with similar affinity(Proenca et al., 2017). However, the functions they each mediate are distinct. For example, BDNF is notoriously recognized as a critical modulator of synaptic plasticity(Ibáñez, 1995; Proenca et al., 2017) and higher order cognitive functions(Zhe-Yu Chen et al., 2006) whereas the role of NT-4 in synaptic transmission is less known. NT-4 has been revealed as a regulator for the proper development and maintenance of preganglionic sympathetic neurons extending from the spinal cord to innervate their sympathetic targets(Roosen et al., 2001; Schober et al., 1998). There is evidence indicating that the reason behind the diverse biological functions of BDNF and NT-4 is due to the difference in endocytic sorting of the TrkB receptor once bound and activated(Proenca et al., 2017). BDNF has also been observed to promote stem cell

differentiation in rat adipose derived stem cells by exerting a synergistic effect with NT-3 (Ji, Zhang, Ji, Wang, & Qiu, 2015).

The neurotrophic activity of NT-3 is broad, ranging from involvement in peripheral nerve repair (Midha, Munro, Dalton, Tator, & Shoichet, 2003; Sterne, Brown, Green, & Terenghi, 1997), to enforcing the formation of neuronal synapses (Maisonpierre et al., 2018). NT-3 has been described to have an important role in the development and differentiation of enteric neurons and glial cells, as well as the promotion of mitosis in neural crest cells (Gao, Wei, Wang, Wu, & Zhu, 2012). NT-3 is commonly used to induce the differentiation of stem cells into neurons (Gao et al., 2012). A partial reason as to why the biological functions of NT-3 are so broad may be due to its broad specificity for more than one Trk receptor. Typically, members of the neurotrophin family bind and activate only one of the three members of the Trk family. NT-3 characteristically binds and activates TrkC. However, NT-3 has been observed to activate other Trk receptors with less efficiency (Reichardt, 2006). For example, in a study conducted by Yancopoulous and colleagues, the specificity of NT-3 was assessed by comparing the ability of NT-3 to promote neurite outgrowth with NGF and BDNF in two different neuronal cell populations (Maisonpierre et al., 2018). BDNF and NGF were shown to only have the ability to promote neurite outgrowth in one of the two neuronal cell populations (Maisonpierre et al., 2018). However, NT-3 was able to promote neurite outgrowth in both cell populations. These results indicate that NT-3 likely has a broader binding specificity compared to other neurotrophins, meaning the neurotrophin can bind to more than one type of Trk receptor. Nonetheless, NT-3 has been dubbed as an essential protein for the survival and growth of developing neurons during the embryonic stages of life(Fox & McAdams, 2010; Sahenk et al., 2010).

1.2 Neurotrophin Receptors

Trk receptors are a class of receptors that belong to the larger family of Receptor Tyrosine Kinases (RTKs) (Sorkin & von Zastrow, 2002). Trk receptors exist as

dimers and have intrinsic tyrosine kinase activity. The structure of Trk receptors includes an extracellular domain containing a cysteine rich cluster followed by three leucine rich repeats, a second cysteine rich cluster, and two immunoglobulin-like domains (M. V. Chao & Hempstead, 1995; Iacaruso et al., 2011). The cytoplasmic region of Trk receptors contains a tyrosine kinase domain which can phosphorylate and dimerize in response to neurotrophin binding (Sorkin & von Zastrow, 2002). The binding of neurotrophins to Trk receptors has been shown to lead to the activation of the proteins Ras, phosphatidylinositol 3-kinase (PI3-K), and phospholipase C-γl, as well as signaling pathways that include these proteins (Skaper, 2018).

The mechanics of Trk signaling have been widely established (Zweifel, Kuruvilla, & Ginty, 2005), which involves the retrograde transport of neurotrophins from postsynaptic neurons to the cell bodies of presynaptic neurons by way of the axon (Vivas et al., 2014). Experiments utilizing compartmentalized neuronal cultures demonstrated that the direct application of NGF to distal axons supported the extension of axons to their final targets (Zweifel et al., 2005). In contrast, the direct application of NGF to cell bodies prevented axons from being able to innervate their targets leading neurons to death (Zweifel et al., 2005). The removal of NGF from distal axons caused axons to retract and degenerate, ultimately causing neuronal death. However, the direct application of NGF to cell bodies allowed neurons to survive. Therefore, it was established that in order for neurons to survive and successfully innervate their specific targets, neurotrophins must be retrogradely transported from distal axons to neuronal cell bodies (Zweifel et al., 2005). The mechanism at which this retrograde signaling occurs has been demonstrated to occur by way of the endocytic pathway. A significant amount of evidence indicates that the binding of neurotrophins to a Trk receptor causes the ligand-bound receptor to become internalized and retrogradely transported to the neuronal cell body through the axon via a ‘signaling endosome’ (Zweifel et al., 2005). However, the exact consensus of the signaling endosome remains vague.

Nevertheless, Trk receptor activation has been shown to stimulate endocytosis leading to the engulfment of activated Trk receptors by clathrin-coated pits (Saxena et al., 2005; Sorkin & von Zastrow, 2009). The internalized Trk receptors are then passed on to sorting endosomes where they meet their fate via a recycling or degradative pathway (Saxena et al., 2005; Sorkin & von Zastrow, 2009). If Trk receptors are recycled, they are returned to the plasma membrane of the cell where they can then undergo ligand induced activation once again. The degradative pathway leads Trk receptors to multivesicular bodies, also known as late endosomes, which are believed to terminate the functions of the receptor by blocking any potential neurotrophin binding (Saxena et al., 2005). The catalytic activity of Trk receptors has been observed to correlate with endocytosis in which this correlation has been described as a "homeostatic regulatory loop"(Sorkin & von Zastrow, 2009). Endocytosis is said to control cell signaling by regulating the number of available Trk receptors within the plasma membrane for activation. Comparatively, activated Trk receptors trigger endocytosis to occur to help prevent excessive cell signaling activation (Sorkin & von Zastrow, 2009). Additionally, endocytosis is believed to alter the concentration of ligand required for Trk receptor activation in order to control the strength or duration of many cell signaling pathways (Sorkin & von Zastrow, 2009).

The signaling activities of Trk receptors, particularly TrkA, can be enhanced under the presence of a second receptor known as the p75 neurotrophin receptor ($p75^{NTR}$) (Iacaruso et al., 2011). The initial discovery of NGF led to the encounter of $p75^{NTR}$ due to the observation that NGF could bind two distinct sites, one of high affinity and the other of low affinity (Sutter, Riopelle, Harris-Warrick, & Shooter, 1979). After numerous investigations, scientists began to question if the two receptors worked together to mediate the survival effects of NGF since both receptors are co-expressed in many cell types. Interestingly enough, co-expression of $p75^{NTR}$ and TrkA was identified to produce a high affinity binding for NGF which led to the enhancement of Trk signaling (M. V. Chao & Hempstead, 1995). Remarkably, the

differential expression of $p75^{NTR}$ was shown to alter Trk receptor signaling. For example, the reduction of $p75^{NTR}$ expression (Benedetti, Levi, & Chao, 1993) or the inhibition of $p75^{NTR}$ with functional-blocking antibodies (Barker & Shooter, 1994; Philippe P Roux & Barker, 2002) led to a reduction in Trk receptor expression as well as in a reduction of Trk receptor responsiveness to NGF. Furthermore, the coexpression of $p75^{NTR}$ and TrkA has been suggested to contribute the massive loss of peripheral neurons and decreased skin innervation observed in adult $p75^{NTR}$ knockout mice (K. Lee et al., 1992).

Although there is an extensive amount of research stating that the interactions between $p75^{NTR}$ and TrkA result in increased responsiveness for NGF, the exact mechanism through which these two receptors interact with each other has not been completely illuminated. However, there are two proposed mechanisms for the two-receptor system. The first, referred to as the 'ligand passing mechanism,' suggests that the expression of $p75^{NTR}$ with TrkA allows the two receptors to interact with each other, leading to an increase in the local concentration of NGF. NGF will initially bind to $p75^{NTR}$, but then bind to TrkA with greater stability (Barker & Shooter, 1994). The second mechanism, termed the 'heterodimer mechanism,' hypothesizes that $p75^{NTR}$ and TrkA form a heterodimer that leads to an induction of NGF. The presence of NGF causes the two receptors to dissociate, further leading to TrkA to undergo a conformational change that is highly favored by NGF. Consequently, this allosteric regulation allows NGF to only bind to TrkA (Esposito et al., 2001).

A variety of characteristics about $p75^{NTR}$ led investigators to further explore the receptor's individual role in neurotrophin signaling. For example, the level of co-expression of TrkA has a major impact on the survival signals $p75^{NTR}$ mediates. In the absence of TrkA, the binding of NGF to $p75^{NTR}$ initiates apoptosis (María Frade, Rodríguez-Tébar, & Barde, 1996). However, co-expression allows $p75^{NTR}$ to mediate cell differentiation and neuronal cell survival (María Frade et al., 1996). Additionally, $p75^{NTR}$ is widely distributed among numerous cell types, such as fibroblasts

(Dobrowsky, Jenkins, & Hannun, 1995), oligodendrocytes (Casaccia-Bonnefil, Carter, Dobrowsky, & Chao, 1996), Schwann cells (Carter et al., 1996), as well as throughout both divisions of the developing nervous system (Ernfors et al., 1991). In contrast, TrkA expression is limited to cholinergic neurons of the basal forebrain (Holtzman et al., 1995). Although, $p75^{NTR}$ is recognized to lack a signaling domain (Dobrowsky et al., 1995), the receptor has been shown to mediate several cellular responses such as myelination(Chan et al., 2004), axonal outgrowth (Marushige, Raju, Marushige, & Koestner, 1987), cell cycle regulation (Verbeke et al., 2010), and Schwann cell migration (Anton, Weskamp, Reichardt, & Matthew, 1994), as well as initiating the activation of several intracellular signaling pathways such as NF-κB (Carter et al., 1996), c-jun terminal kinase (JNK) (Casaccia-Bonnefil et al., 1996), and caspases (Gu, Casaccia-bonnefil, Srinivasan, & Chao, 1999).

1.3 Structure of $p75^{NTR}$

The $p75^{NTR}$ is a Type I transmembrane protein that belongs to the tumor necrosis factor receptor (TNFR) superfamily (Rodriguez-Tébar, Dechant, & Barde, 1990; Squinto et al., 1991) and it has the ability to bind all four members of the neurotrophin family (Rodriguez-Tébar et al., 1990). The extracellular domain (ECD) of $p75^{NTR}$ is made up of four cysteine rich domains, which serves as the ligand-binding domain of $p75^{NTR}$ and is a distinguished feature of all TNFR family members (Barker, 1998). Similar to Trk receptors, $p75^{NTR}$ undergoes ligand-induced dimerization which can lead to the activation of various intracellular signaling pathways (Yano & Chao, 2000). This mechanism through which $p75^{NTR}$ becomes activated in response to neurotrophin binding has been proposed as “the snail-tong model” (Marçal Vilar et al., 2009). Within this model, the activation of p75 is suggested to occur through a ligand-induced rearrangement of disulfide-linked receptor subunits in a scissors-like movement. The proposed mechanism states that when the neurotrophin binds to the ECD of p75, the two ECD subunits move closer together while the two ICD subunits separate. The disulfide-linked subunits of the transmembrane domain are thought to act as a

fulcrum. The separation of the two ICD subunits is suggested to facilitate the activation of various downstream signaling events (Marçal Vilar et al., 2009).

Although $p75^{NTR}$ has been recognized to lack intrinsic catalytic activity, the intracellular domain of the receptor (ICD) has been suggested to contain regions that can interact with various adaptor proteins to mediate several down-stream signaling events leading to cell death (Philippe P Roux & Barker, 2002). For example, the ICD of $p75^{NTR}$ contains an additional notable feature of the TNFR family known as the 'death domain' (Barker, 1998; Philippe P Roux & Barker, 2002) The death domains of the related TNFR family members Fas and TNFR1 have been identified to interact with adaptor proteins that link the receptors to caspase activation, ultimately resulting in programmed cell death (Hofmann & Tschopp, 1995). However, the death domain of $p75^{NTR}$ has been shown to function differently. For example, the death domains of TNF1 and Fas are able to interact with death domain effector proteins such as TRADD and FADD, leading to the activation of the extrinsic apoptotic signaling cascade. Analysis of the $p75^{NTR}$ death domain using NMR spectroscopy revealed that the receptor's death domain is unable to interact with death domain effector proteins (Liepinsh, Ilag, Otting, & Ibáñez, 1997). Furthermore, $p75^{NTR}$ has been shown that it is actually unable to cause activation of the extrinsic apoptotic signaling cascade (Gu et al., 1999). Despite functional differences between the death domain of $p75^{NTR}$ and the death domains of other TNF receptors, the $p75^{NTR}$ death domain has been shown to be necessary for the induction of apoptosis. A significant reduction of neuronal death in the hippocampus, somatosensory, piriform, and entorhinal cortices was observed in mutant mice lacking the $p75^{NTR}$ death domain compared to wild-type controls (Tanaka, Kelly, Goh, Lim, & Ibáñez, 2016).

The death domain of $p75^{NTR}$ is connected to the transmembrane domain of the receptor by a flexible juxtamembrane region termed the 'chopper domain.' This region of $p75^{NTR}$ has also been implicated in promotion of cell death through caspase activation in a variety of neuronal and non-neuronal cell types such as dorsal root

ganglion, Schwann cells, and fibroblasts (E J Coulson et al., 2000). The chopper domain has been suggested to be necessary for the initiation of $p75^{NTR}$-mediated cell death due to the inability of peripheral neurons transfected with mutant constructs lacking the chopper domain to undergo apoptosis (E J Coulson et al., 2000). However, there still remains much controversy regarding the juxtamembrane of the receptor and the structural component of $p75^{NTR}$ responsible for mediating cell death since a recent investigation conducted by Ibanez and colleagues revealed that a $p75^{NTR}$ construct deficient in the chopper domain but abundant in the death domain resulted in the inability of $p75^{NTR}$ to induce cell death (Tanaka et al., 2016).

1.4 $p75^{NTR}$ Mediates Cell Death

There are numerous *in vivo* and *in vitro* studies showing that $p75^{NTR}$ has a role in promoting apoptosis, but the precise mechanism underlying this function remains unclear. Due to the ability of $p75^{NTR}$ to attenuate Trk receptor function, it has been often difficult to interpret whether the receptor can actually facilitate pro-apoptotic effects on its own. However, the pro-apoptotic functions of $p75^{NTR}$ have been better understood by the initiation of neurotrophin withdrawal (Rabizadeh et al., 2017). The expression of $p75^{NTR}$ in immortalized neural cells cultured in medium containing serum had no effect on death. However, when serum was withdrawn, the expression of $p75^{NTR}$ in these cells resulted in death (Rabizadeh et al., 2017). Remarkably, the addition of NGF to the cells following serum withdrawal lead to suppressed cell death (Rabizadeh et al., 2017). Similar results were seen in sympathetic neurons (Rukenstein, Rydel, & Greene, 1991) and differentiated PC12 cells (Rabizadeh et al., 2017). The withdrawal of NGF from culture medium led to the death of PC12 cells and sympathetic neurons. However, the addition of NGF following withdrawal resulted in suppressed cell death (Rabizadeh et al., 2017; Rukenstein et al., 1991).

The ability of $p75^{NTR}$ to induce cell death has been further exemplified in knockout mouse models (Miller & Kaplan, 2001). The four cysteine rich domains localized on ECD of $p75^{NTR}$ serve as the ligand binding domain for the receptor and are

encoded by exonIII. The first $p75^{NTR}$ knockout mouse model was generated by abolishing the exonIII gene (K. Lee et al., 1992). As a result, newborn $p75^{NTR/exonIII}$ pups exhibited a greater number of sympathetic neurons, providing the first line of evidence that $p75^{NTR}$ plays a role in apoptosis during development (K. Lee et al., 1992). Naturally occurring cell death is a significant feature of nervous system development. The process ensures appropriate neuronal connections are made and that the appropriate cell number is generated among neural tissues in order to cultivate a properly functioning nervous system. Further investigations utilizing $p75^{NTR}$ knockout mice established the receptor as a critical regulator of neurodevelopment through the promotion of naturally occurring cell death within the basal forebrain (Naumann et al., 2002), superior cervical ganglion (Bamji et al., 1998), spinal cord (Frade & Barde, 1999), and retina(María Frade et al., 1996) during embryonic development. Deletion of the receptor within the basal forebrain resulted in an increased number of cholinergic neurons (Naumann et al., 2002), thereby suggesting that $p75^{NTR}$ helps mediate normal developmental death within this tissue region.

The pro-apoptotic function of $p75^{NTR}$ has also been identified as a response to cellular injury. During development, the expression of $p75^{NTR}$ is elevated but once adulthood is reached, levels of the receptor decrease. This observation initially led to the idea that the receptor had limited function in the adult nervous system. However, the expression of $p75^{NTR}$ has been shown to increase in response to various forms of cellular injury such as spinal cord injury (Chu, Yu, & Fehlings, 2007), seizures (Özbas-Gerçeker et al., 2004; P P Roux, Colicos, Barker, & Kennedy, 1999) and neuronal axotomy (Johnson, Hökfelt, & Ulfhake, 1999; Zhou, Rush, & McLachlan, 1996). Furthermore, reexpression of the receptor correlated with the induction of apoptosis. For example, following spinal cord injury, a higher percentage of cell death was observed in rat oligodendrocytes expressing p75 compared to oligodendrocytes not expressing the receptor (Chu et al., 2007). Additionally, rats subjected to pilocarpine-induced seizures resulted in increased expression of $p75^{NTR}$ within the hippocampus,

entorhinal cortex, and the piriform cortex which was accompanied with apoptosis (P P Roux et al., 1999).

1.5 Apoptotic Signaling of $p75^{NTR}$

Caspases are inactive proenzymes known as cysteine proteases. In order for caspases to become activated and induce apoptosis, they must first be cleaved at specific aspartic acid residues (Aggarwal, 2000). There are currently 14 known members of the caspase family (Gu et al., 1999), which are divided into two main groups known as upstream initiators (caspase-2, -8, -9, and -10) and downstream executioners (caspase-3, -6, and 7) (Aggarwal, 2000). Upstream initiator caspases undergo auto-cleavage to induce their own activation, but they are also responsible for cleaving and activating the downstream executioner caspases which ultimately trigger the execution phase of apoptosis (Boatright et al., 2003).

There are two major apoptotic signaling pathways, the extrinsic and intrinsic pathways. The two pathways are distinguished by the presence or absence of ligand-induced activation of the apoptotic signaling cascade(Elmore, 2007). For example, TNF-induced apoptosis has been implicated to be associated with the extrinsic apoptotic pathway, which involves the binding of a lethal ligand such as TNF-α or Fas, to a TNFR related receptor (Hongmei & Vogt, 2012). Once a death signal has bound the receptor, various cytoplasmic adaptor proteins, such as TRADD or FADD (Aggarwal, 2000), are recruited to the receptor's death domain resulting in the formation of a death-inducing signaling complex (DISC) (Hongmei & Vogt, 2012). Consequently, caspase-8 activation occurs, followed by apoptotic cell death.

The intrinsic pathway triggers apoptosis through a series of mitochondria related events initiated by a wide range of non-receptor mediated stimuli including radiation, toxins, hypoxia, and free radicals (Elmore, 2007). Each of these stimuli can produce intracellular signals that permeabilize the mitochondrial membrane causing the release of the small hemeprotein, cytochrome-c (Elmore, 2007). After its release, cytochrome-c joins procaspase-9 to bind to the apoptosis protease activation factor-1

(Apaf-1) to produce a wheel-shaped protein complex called the apoptosome (Boatright et al., 2003). The apoptosome serves as a large caspase activating complex which first cleaves and activates caspase-9 followed by caspase-3 cleavage and activation (Li et al., 1997). Cytochrome-c release has also been shown to be regulated by the Bcl-2 protein family (Cory & Adams, 2002). The protein family consists of members that either promote cell survival or promote cell death (Shamas-Din, Brahmbhatt, Leber, & Andrews, 2011). For example, the pro-apoptotic Bcl-2 family members Bax and Bak can reduce mitochondrial integrity and cause cytochrome-c release (Gross, McDonnell, & Korsmeyer, 1999).

The cellular mechanisms responsible for initiating the $p75^{NTR}$-mediated apoptotic death cascade remains poorly understood. However, it is almost certain the cascade involves caspase activation since caspase inhibitors targeted against caspases-1, -3, -6, -7, and -9 prevented $p75^{NTR}$-mediated cell death from occurring (Gu et al., 1999; Troy, Friedman, & Friedman, 2002). The apoptotic pathway induced by $p75^{NTR}$ is unique compared to its TNFR relatives. The death domain of $p75^{NTR}$ is unable to bind TRADD or FADD, and caspase-8 activation is not apparent in the $p75^{NTR}$-mediated apoptotic cascade (Becker, Howell, Kodama, Barker, & Bonni, 2004; Gu et al., 1999). Furthermore, $p75^{NTR}$ activation was shown to cause mitochondrial release of cytochrome-c as well as caspase-9 activation within oligodendrocytes and striatal neurons (Becker et al., 2004; Gu et al., 1999). Dominant negative forms of caspase-9 were shown to attenuate $p75^{NTR}$-induced death, further indicating that $p75^{NTR}$ initiates cell death differently than other TNFR family members (Becker et al., 2004). Intriguingly, the $p75^{NTR}$-apoptotic cascade of oligodendrocytes was shown to consist of the same pattern of caspase activation as radiation-induced apoptosis, which requires p53, a tumor suppressor protein (Liebermann, Hoffman, & Steinman, 1995).

The p53 tumor suppressor protein has been indicated as an essential downstream component of $p75^{NTR}$-mediated cell death in sympathetic neurons (Aloyz et al., 1998). Typically, p53 initiates apoptosis in response to DNA damage or cellular

stress in order to regulate the cell cycle and ultimately prevent tumor formation. However, levels of p53 have been shown to increase in response to $p75^{NTR}$ activation during neuronal apoptosis (Aloyz et al., 1998). Upregulation of p53 has been implicated as a necessity for the occurrence of apoptosis in sympathetic neurons, in which inhibition of p53 expression results in the prevention of apoptosis (Aloyz et al., 1998). Elevated levels of p53 have been shown to correlate with increased levels of the Bcl-2 pro-apoptotic protein, Bax, as well as with the hyperphosphorylation of c-jun kinase (Aloyz et al., 1998). Consequently, $p75^{NTR}$ is suggested to initiate cell death via the JNK-p53-Bax pathway even though phosphorylation of c-jun is not required for the induction of cell death in sympathetic neurons (Palmada et al., 2002).

1.6 Signaling Pathways of $p75^{NTR}$

The ability of $p75^{NTR}$ to activate sphingomyelin hydrolysis and promote the production of ceramide, a second messenger molecule (Dobrowsky et al., 1995), was the first piece of evidence demonstrating the receptor could facilitate cellular signaling. Before this, $p75^{NTR}$ was thought to only serve as a binding partner for Trk receptors that helped lead to Trk receptor activation (M. V. Chao & Hempstead, 1995). However, in response to neurotrophin binding, $p75^{NTR}$ activation can lead to the production of a ceramide, a bioactive sphingolipid metabolite (Dobrowsky et al., 1995). Ceramide has been demonstrated to regulate antimitogenic pathways that result in cell growth inhibition, cell differentiation, and apoptosis (Hannun & Linardic, 1993). Additionally, ceramide production has been found to result in activation of c-jun N-terminal kinase (JNK) (Verheij et al., 1996), a stress-activated protein that plays a role in triggering apoptosis (Xia Z, Dickens M, Raingeaud J, Davis Rj, & Greenberg Me, 1995). In response to neurotrophin binding, JNK activation has been observed in $p75^{NTR}$-mediated apoptosis within a variety of cell types including oligodendrocytes (Casaccia-Bonnefil et al., 1996), PC12 cells (Philippe P Roux, Bhakar, Kennedy, & Barker, 2001), sympathetic neurons (Aloyz et al., 1998), and hippocampal neurons (Friedman, 2000). The use of JNK inhibitors further confirmed the role of JNK in $p75^{NTR}$-mediated

cell death, in which inhibition of upstream JNK activity protected hippocampal neurons from $p75^{NTR}$-mediated cell death (Friedman, 2000).

Intriguingly, the activation of JNK could only be observed in cells that solely expressed $p75^{NTR}$ whereas cells coexpressing TrkA and $p75^{NTR}$ had suppressed JNK activity in response to NGF binding (Yoon, Casaccia-Bonnefil, Carter, & Chao, 1998). A similar effect was observed in the case of sphingomyelin hydrolysis, in which cells only expressing $p75^{NTR}$ were able to produce ceramide (Dobrowsky et al., 1995). Furthermore, cells expressing both TrkA and $p75^{NTR}$ failed to undergo sphingomyelin hydrolysis (Dobrowsky et al., 1995) and there was an increase in mitogen-activated protein kinase (MAPK) activity (Yoon et al., 1998), which is a notable cellular effect induced by Trk receptors. These observations led to the speculation that TrkA may modulate the signaling activities of $p75^{NTR.}$

Consistent with other TNFR family members, $p75^{NTR}$ has the ability to activate the Nuclear Factor kappa B (NF-κB) pathway (Carter et al., 1996). Once activated, the NF-κB transcription factor has the ability to translocate to the nucleus and promote the transcription of various anti-apoptotic genes (Serasanambati & Chilakapati, 2016). In Schwann cells, the binding of NGF to $p75^{NTR}$ caused the activation of the NF-κB (Carter et al., 1996). Fascinatingly, ceramide treatment was also able to activate the NF-κB pathway in $p75^{NTR}$-expressing Schwann cells (Carter et al., 1996), further suggesting the role of NF-κB as an anti-apoptotic signal under stressful cellular conditions. A study conducted by Chao and colleagues (Yoon et al., 1998) proposed TrkA is able to modulate the survival pathways activated by $p75^{NTR}$. For example, the lone expression of $p75^{NTR}$ in oligodendrocytes led to an increase in JNK activity following NGF binding. However, when $p75^{NTR}$ was coexpressed with TrkA, JNK activity was suppressed (Yoon et al., 1998). This result corresponds with the ability of TrkA to produce sphingosine-1-phosphate, a sphingolipid that has been shown to support cell survival as well as inhibit JNK activity (X. Huang, Withers, & Dickson, 2014). In contrast, $p75^{NTR}$ was shown to activate the NF-κB pathway in response to ceramide synthesis in

the presence or absence of TrkA expression in oligodendrocytes (Yoon et al., 1998). These results suggested that $p75^{NTR}$ survival signals can be controlled by TrkA. Ultimately, these results suggested that $p75^{NTR}$ is able to signal through two different pathways, JNK and NF-κB, which is characteristic of other TNFR family members. For example, the tumor necrosis factor-1 (TNF-1) receptor is able to activate both JNK and NF-κB and promote various cellular effects such as cell proliferation, differentiation, and apoptosis (Smith, Farrah, & Goodwin, 1994).

1.7 $p75^{NTR}$ Cytosolic Interactions

The death domain of $p75^{NTR}$ is structurally homologous to the death domains of the TNFR family members, TNFR-1 and Fas (Chapman, 1995). In order to induce cell death, most other TNF receptor family members must first dimerize with themselves via their death domains. This self-association forms a receptor complex that enables the recruitment of death-domain containing adaptor proteins such as TRADD, TRAF, RAIDD, FADD, or RIPP (Dempsey, Doyle, He, & Cheng, 2003). The recruitment of these adaptor proteins leads to caspase activation and ultimately cell death. Despite structural similarities, $p75^{NTR}$ was observed to not induce cell death through the same mechanism. Using a yeast-two hybrid assay, $p75^{NTR}$ failed to undergo homodimerization as well as failed to interact with FADD, TRADD, RIPP, and RAIDD (Nichols, Martinou, Maundrell, & Martinou, 1998). However, $p75^{NTR}$ has been shown to successfully bind all six members of the TNFR associated factor (TRAF) protein family, leading to either NF-κB activation or cell death induction (Ye et al., 1999).

The TRAF protein family was the first group of cytosolic interactors identified for the p75 neurotrophin receptor (Rothe, Wong, & Goeddel, 1994). The TRAF proteins were isolated based on their ability to interact with the cytoplasmic domains of specific TNFR family members (Rothe et al., 1994). TRAF proteins are characterized as signal transducers, but not every member of the TRAF protein family mediates the same signaling effects. For example, TRAF2, TRAF4, and TRAF6 each modulate NF-κB activation. However, the individual coexpression of each of the three TRAF members

with $p75^{NTR}$ leads to discrete NF-κB activity. The coexpression of $p75^{NTR}$ with TRAF2 or TRAF6 in HEK 293T induced a synergistic effect that results in a significant amount of upregulation of NF-κB activation (Ye et al., 1999). Surprisingly, the individual coexpression of TRAF6 with $p75^{NTR}$ resulted in a greater synergistic effect on NF-κB activation compared to TRAF2 coexpression. In contrast, individual coexpression of TRAF4 with $p75^{NTR}$ induced a complete inhibitory effect on $p75^{NTR}$-mediated activation of NF-κB (Ye et al., 1999).

The interactions of TRAF proteins with $p75^{NTR}$ remain elusive. However, TRAF6 has gained much recognition as a requirement for the activation of both the NF-κB and JNK pathways as well as the activation of $p75^{NTR}$-mediated apoptosis. The role of TRAF6 became more evident when Carter and colleagues showed $p75^{NTR}$ signaling deficiencies in in TRAF6 knockout mice (Yeiser, 2004). Wild-type and heterozygous TRAF6 mice treated with NGF led to the activation of NF-κB and phosphorylation of JNK whereas TRAF6 null mice did not experience the same effects. To ensure that the lack of NF-κB and JNK activation were mediated by $p75^{NTR}$, Carter and colleagues treated Schwann cells with TNF, a bioactive cytokine known to activate the NF-κB and JNK pathways independent of $p75^{NTR}$ and TRAF6. Remarkably, TRAF6 deficient Schwann cells experienced the same amount of NF-κB activation and JNK phosphorylation as wild-type Schwann cells (Yeiser, 2004). To further characterize the role of TRAF6 in $p75^{NTR}$-mediated apoptosis, the amount of developmental death that occurred within the sympathetic cervical ganglion of TRAF6 knockout mice and wild-type mice was analyzed. TRAF6 deficient mice experienced a significantly lower amount of naturally occurring death compared to $TRAF6^{+/+}$ counterparts. TRAF6 knockout mice were observed to have 125% more neurons per ganglia compared to wild-type mice (Yeiser, 2004). Astonishingly, these results were similar to those observed in $p75^{NTR}$ knockout mice in which there were 130% more neurons per ganglia observed in $p75^{NTR}$ null mice compared to their wild-type littermates (Yeiser, 2004).

Another adaptor protein found to interact with the intracellular domain of $p75^{NTR}$ is the neurotrophin receptor interacting factor (NRIF). NRIF was first identified as a necessity for programmed cell death due to the observation of mice lacking the NRIF gene experienced a significant reduction in apoptosis within developing retinal cells (Casademunt et al., 1999). This result was noticeably similar to $p75^{NTR}$ null mice (Frade & Barde, 1999), thus implicating a role for NRIF in developmental programmed cell death. NRIF has also been recognized to possibly play a role in initiating death through $p75^{NTR}$ in response to injury (Volosin et al., 2008). Interestingly, NRIF knockout mice displayed an increase in $p75^{NTR}$ expression in response to seizures, but the neurons failed to undergo apoptosis compared to wild-type mice. NRIF was also found to participate in $p75^{NTR}$-mediated cell death in Human embryonic kidney (HEK) 293 cells by binding to the receptor's intracellular domain in response to neurotrophin. The relevance of this physiological interaction was confirmed by deleting the binding region of NRIF to $p75^{NTR}$ which resulted in a reduction of cell death within embryonic retina (Casademunt et al., 1999).

The exact functions of NRIF in $p75^{NTR}$ signaling are not well understood, but it is believed that NRIF functions as a transcription factor that translocates to the nucleus in order to initiate cell death. An investigation conducted by Carter and colleagues displayed a functional interaction between TRAF6 and the NRIF that influenced the translocation of NRIF to nucleus as well $p75^{NTR}$ signaling (Gentry, Rutkoski, Burke, & Carter, 2004).The direct association between TRAF6 and NRIF was shown to dramatically increase the nuclear localization of NRIF as well enhance TRAF6-mediated activation of JNK (Gentry et al., 2004). Additionally, $p75^{NTR}$ was only able to induce the activation of JNK in response to NGF binding under the presence of both TRAF6 and NRIF (Gentry et al., 2004). Thus, the interactions between NRIF and TRAF6 are suggested to play an important role in $p75^{NTR}$-mediated activation of JNK. It has been suggested that in response to $p75^{NTR}$ activation, NRIF must first undergo

polyubiquination in order to associate with TRAF6 and then translocate to the nucleus (Geetha, Kenchappa, Wooten, & Carter, 2005).

1.8 Proteolytic Cleavage of $p75^{NTR}$

One of the most well-established signaling mechanisms of $p75^{NTR}$ is its proteolytic cleavage. The receptor undergoes regulated intramembrane proteolysis (RIP), which is carried out by two enzymes, TNFα-converting enzyme (TACE) and γ-secretase (K. Jung et al., 2003). The extracellular domain of $p75^{NTR}$ is first cleaved by TACE, thereby causing the release of the receptor's ectodomain (ECD) as well as producing a 25 kDa C-terminal fragment (CTF). Next, the transmembrane region of $p75^{NTR}$ is cleaved by γ-secretase, causing the release of a 20 kDa intracellular domain fragment (ICD) (K. Jung et al., 2003). The order that mellatoproteinases cleave $p75^{NTR}$ is consistent, with TACE-mediated cleavage occurring prior to γ-secretase-mediated cleavage (Zampieri, Xu, Neubert, & Chao, 2005). These sequences of events were established with the use of inhibitors, in which cleavage of $p75^{NTR}$ by γ-secretase was prevented when TACE activity was blocked. In contrast, the inhibition of γ-secretase did not affect TACE function (Zampieri et al., 2005).

Cleavage of $p75^{NTR}$ has been demonstrated to contribute to the induction of $p75^{NTR}$-mediated cell death during development as well as in response to cellular injuries. For example, $p75^{NTR}$ cleavage has been shown to contribute to developmental apoptosis that occurs within the superior cervical ganglion. In rats, superior cervical ganglion experience on-going apoptosis at postnatal day 4, but apoptosis subsides at postnatal day 24. Remarkably, the CTF and ICD fragments of $p75^{NTR}$ were observed in superior cervical ganglia of postnatal day 4 rats but not postnatal day 24 (Kenchappa et al., 2006). Proteolytic cleavage of $p75^{NTR}$ has also been observed in neurons subjected to injuries such as pilocarpine-seizures (Volosin et al., 2008) and oxidative stress(Kraemer et al., 2014). Pilocarpine-seizures have been demonstrated to cause the induction of proNGF, a pro-apoptotic ligand and precursor of mature NGF, in hippocampal neurons (Volosin et al., 2008). The treatment of hippocampal neurons

with proNGF or NGF resulted in cleavage of $p75^{NTR}$ followed by cell death. Remarkably, the application of TACE or γ-secretase inhibitors led to an attenuation of apoptosis in hippocampal neurons (Volosin et al., 2008). Similar results were observed in sympathetic neurons exposed to oxidative stress (Kraemer et al., 2014), a common feature of pathological conditions. These results indicate $p75^{NTR}$ proteolytic cleavage must occur in order for $p75^{NTR}$-mediated cell death to be facilitated.

The death-inducing ability of the CTF of $p75^{NTR}$ has not been completely elucidated, but it has been demonstrated that the juxtamembrane region of the CTF, termed the "chopper domain," activates an apoptotic protease activating factor (APAF-1)-dependent death pathway via potassium ionic flux (Elizabeth J Coulson et al., 2008). This potassium ionic flux has been observed to occur through G-protein-coupled inwardly rectifying potassium (GIRK) channels (Elizabeth J Coulson et al., 2008). The GIRK channels are potassium-selective ion channels that are widely expressed within the developing and adult nervous systems and have been shown to cause neuron excitability when activated (S. C. Chen, Ehrhard, Goldowitz, & Smeyne, 1997; Karschin & Karschin, 1997; Signorini, Liao, Duncan, Jan, & Stoffel, 1997; Wickman, Karschin, Karschin, Picciotto, & Clapham, 2000). Upon neurotrophin binding to p75NTR, GIRK channel activation occurs, followed by potassium efflux through the channels. A reduction in cytosolic potassium transpires, ultimately promoting the formation of the apoptosome and caspase activation (Elizabeth J Coulson et al., 2008).

The cleavage fragments produced by regulated intramembrane proteolysis of $p75^{NTR}$ have been suggested to serve multiple functions. Firstly, the ECD of $p75^{NTR}$ has been suggested to have a neuroprotective role against amyloid-beta (Aβ) toxicity (Yao et al., 2015). The restoration of $p75^{NTR}$-ECD in Alzheimer's disease mouse models resulted in the attenuation of neurite degeneration, neuronal death, and Tau phosphorylation (Yao et al., 2015). Secondly, the CTF of $p75^{NTR}$ has been shown to promote cell death when it is over-expressed in sensory neurons (E J Coulson et al., 2000) and PC12 cells (Matusica et al., 2013). For example, PC12 cells overexpressing a

protein mimicking the CTF of $p75^{NTR}$ experienced an increased amount of cell death compared to several other $p75^{NTR}$ variant constructs (Matusica et al., 2013). Likewise, Aβ protein-mediated cell death was observed to correlate with an increased production of the $p75^{NTR}$ C-terminal fragment (Sotthibundhu et al., 2008).

Proteolytic cleavage of $p75^{NTR}$ has been suggested to regulate a variety of cell signaling events following generation of the ICD. The ICD of $p75^{NTR}$ has been suggested to bind various intracellular proteins and then translocate to the nucleus to initiate many downstream signaling events. For example, the ICD has been suggested to initiate JNK activation, NRIF translocation (Volosin et al., 2008), and cell cycle arrest (Ceni et al., 2010). The inhibition of TACE by TAPI or γ-secretase by DAPT was shown to block nuclear translocation of NRIF (Volosin et al., 2008). In addition, the ICD has been suggested to play an important role in facilitating Trk signaling events. For instance, the overexpression of the $p75^{NTR}$ ICD in PC12 cells was observed to cause Akt phosphorylation and PI3K activation (Ceni et al., 2010). When $p75^{NTR}$ expression was depleted, there was a significant reduction in Akt phosphorylation. However, the restoration of $p75^{NTR}$ ICD expression was able to rescue the signaling defect (Ceni et al., 2010). Barker and colleagues have proposed a mechanism at which these events occur (Kommaddi, Thomas, Ceni, Daigneault, & Barker, 2011a). The proposed model suggests that Trk receptor activation causes the phosphorylation of TACE in order to initiate $p75^{NTR}$ cleavage. Consequently, $p75^{NTR}$ ICD accumulation occurs, enabling the small fragment to facilitate Trk signaling and neurotrophin survival (Kommaddi et al., 2011a).

1.9 Proneurotrophins

All neurotrophins are initially synthesized as larger precursors of approximately 30 to 34 kDa (Suter, Heymach, & Shooter, 1991). These larger precursors, known as proneurotrophins, can be cleaved by furin and convertases within the endoplasmic reticulum or the Golgi apparatus (Heymach & Shooter, 1995) to yield mature neurotrophins of approximately 13 kDa (Suter et al., 1991). In addition,

proneurotrophins are able to be secreted into the extracellular matrix and then cleaved by plasmin and matrix mellatoproteases to yield mature neurotrophins (R Lee et al., 2001). Proneurotrophins were initially believed to be biologically inactive. Neurotrophin prodomains were initially recognized to only ensure proper protein folding and neurotrophin secretion, whereas the mature domain was commonly viewed as the secreted ligand responsible for carrying out neurotrophic effects (Suter et al., 1991). However, the perplexing ability of neurotrophins to initiate pro-survival or pro-survival effects led to the hypothesis that $p75^{NTR}$ could be activated by ligands other than neurotrophins (Friedman, 2000). The discovery of proNGF (Ramee Lee et al., 2001) and proBDNF (H. K. Teng et al., 2005) to selectively bind and activate $p75^{NTR}$ to elicit death provided better insight that various neurotrophin isoforms are able to mediate a diverse variety of functions.

Proneurotrophins are unable to bind and activate Trk receptors, but they have been demonstrated to have a high-affinity for $p75^{NTR}$ to induce apoptosis (K. K. Teng, Felice, Kim, & Hempstead, 2010). Thus, the biological functions of neurotrophins are said to be regulated by their proteolytic cleavage, with immature forms preferentially binding to $p75^{NTR}$ to induce death and mature forms binding Trk receptors to induce cell survival. Proneurotrophins are able to induce cell death by binding to the high affinity receptor complex consisting of $p75^{NTR}$ and Sortilin, a member of the Vps10p-domain receptor family (Nykjaer, Lee, Teng, & Jansen, 2004). Sortilin recognizes and binds the prodomains of proneurotrophins whereas the mature domains of proneurotrophins bind to $p75^{NTR}$. Consequently, the high affinity receptor complex is formed and is then able to convey apoptotic signaling through the intrinsic death pathway (Nykjaer et al., 2004).

ProNGF is widely recognized as a pro-apoptotic ligand of $p75^{NTR}$. The ligand is able to mediate its pro-apoptotic signals by binding to the $p75^{NTR}$-Sortillin complex (Nykjaer et al., 2004). In Schwann cells, it has been observed that if one of the receptors is absent, then cells are resistant to proNGF-mediated apoptosis (Nykjaer et

al., 2004). The death inducing abilities of proNGF have linked to cellular injury and disease progression. For example, a study conducted by the Friedman lab demonstrated that proNGF is secreted in response to pilocarpine-induced seizure (Volosin et al., 2008). Similarly, proNGF secretion could be detected after corticospinal injury (Harrington et al., 2004). The induction of proNGF after injury was found to cause neuronal death within the hippocampus (Volosin et al., 2008) and the spinal cord (Harrington et al., 2004) in which blockage of the ligand's prodomain was able to significantly protect neurons from death (Harrington et al., 2004; Volosin et al., 2008). Intriguingly, anti-proNGF treatment was able to prevent $p75^{NTR}$ upregulation from occurring within the hippocampus after seizure (Volosin et al., 2008), suggesting the pro-apoptotic ligand is able to regulate the induction of the receptor in order to elicit apoptosis. Further characterization of the role of proNGF in $p75^{NTR}$-mediated cell death involved $p75^{NTR}$ knockout mice. ProNGF treatment of oligodendrocytes deficient in the receptor resulted in a significant amount of protection from proNGF-mediated cell death (Beattie et al., 2002).These results indicate that in order for proNGF to execute its death signal, it must bind to $p75^{NTR}$.

Similar to proNGF, proBDNF also exhibits high affinity binding to the $p75^{NTR}$-Sortilin complex via its prodomain to elicit cell death (H. K. Teng et al., 2005). However, proBDNF has also been categorized as a modulator of synaptic plasticity. The conversion of proBDNF to mature BDNF has been shown to promote TrkB-dependent long term potentiation (LTP) (Pang et al., 2004). LTP is defined as the strengthening of neuronal synaptic connections, and it has been established as the cellular basis of memory in the mammalian forebrain (Adams & Dudek, 2005). In contrast, proBDNF has been observed to regulate NMDA-dependent long term depression through $p75^{NTR}$ (Woo et al., 2005). LTD is the opposing process of LTP, due to it selective weakening of neuronal synaptic connections. The application of proBDNF to hippocampal slices expressing $p75^{NTR}$ enhanced LTD, therefore suggesting that proBDNF could act as an endogenous ligand to directly regulate LTD (Woo et al., 2005). Due to the expression of

p75^{NTR} being developmentally regulated, it is proposed that different forms of synaptic plasticity may be controlled by various BDNF isoforms. Thus, proBDNF induces LTD in the developing hippocampus and mature BDNF induces LTP in the adult brain (K. K. Teng et al., 2010).

Although the majority of studies have focused on the biological functions of proNGF and proBDNF, there has been speculation that the precursors of NT-3 and NT-4 exhibit biological actions as well. ProNT3 has been shown to exhibit pro-apoptotic effects within sympathetic neurons (Yano, Torkin, Andres-Martin, V Chao, & K Teng, 2009) and developing spiral ganglion neurons (Jacob et al., 2011) by binding to the p75^{NTR}-Sortilin high affinity complex. Additionally, proNT3 may play a role in responding to cellular injuries. Following barotrauma injury, proNT3 expression could be detected alongside p75^{NTR} and Sortilin in spiral ganglion neurons (Jacob et al., 2011). Furthermore, upregulation of proNT3 and p75^{NTR} could be observed in photoreceptors after selective Müeller cell ablation whereas NT3 expression was significantly reduced (Shen, Zhu, Lee, Chung, & Gillies, 2013). Müeller cells are the principal glial cells of the retina and their loss or dysfunction has been linked to multiple retinal diseases such as macular telangiectasia type 2 and diabetic retinopathy (Baumann et al., 2017). The intravitreal injection of mature NT3 and p75^{NTR} rabbit polyclonal antibody was able to significantly protect photoreceptors from degeneration (Baumann et al., 2017). Currently, there are not any reports indicating if proNT4 demonstrates pro-apoptotic activity or any other biological actions. However, the prodomain of NT4 is smaller than other neurotrophin prodomains, and it is unable to bind to Sortilin (Z.-Y. Chen, 2005). Therefore, NT-4 is suggested to only exist as a ligand for TrkB.

1.10 Role of p75^{NTR} in Pathology

As the nervous system matures, the expression of p75^{NTR} is highly down regulated, with the exception of sympathetic and basal forebrain cholinergic neurons (Meeker & Williams, 2014). Due to this change, p75^{NTR} was initially believed to have

limited function in the adult nervous system. However, $p75^{NTR}$ has been shown to modulate hippocampal synapse modifications (Woo et al., 2005) and neurogenesis (Bernabeu & Longo, 2010) within the adult brain. Additionally, numerous studies have displayed that $p75^{NTR}$ expression can rapidly upregulate in response to a variety of pathological conditions in both the central and peripheral nervous systems. For example, increased $p75^{NTR}$ expression has been observed following neuronal axotomy (Harrington et al., 2004), seizure (P P Roux et al., 1999; Volosin et al., 2008), ischemia (Kokaia, Andsberg, Martinez-Serrano, & Lindvall, 1998), and elevated intraocular pressure (Wei et al., 2007).

The induction of $p75^{NTR}$ reexpression during the adult life is thought to be a part of a process that promotes apoptosis of damaged tissues. Following seizure, the expression of $p75^{NTR}$ dramatically increased within cortical neurons subjected to pilocarpine-induced seizure, in which the reexpression of $p75^{NTR}$ correlated with cortical neuron cell death (P P Roux et al., 1999). Similarly, $p75^{NTR}$ expression was induced and associated with apoptosis of cholinergic neurons exposed to the excitotoxin, kainic acid (Oh, Chartisathian, Chase, & Butcher, 2000). Death of cholinergic neurons could be prevented by the function-blocking antibody, Rex, thereby signifying the contribution of $p75^{NTR}$ signaling in excitotoxin-induced death of within this neuronal subpopulation.

In addition to neurons, upregulation of $p75^{NTR}$ has been observed in Schwann cells (Taniuchi, Clark, & Johnson, 1986), the cells of the peripheral nervous system responsible for the regeneration of damaged nerve fibers. Schwann cell migration has been suggested to be regulated by $p75^{NTR}$ due to the cells' migration being prevented by the Rex antibody (Anton et al., 1994). This effect could only be observed in damaged sciatic nerves rather than healthy sciatic nerves, thus $p75^{NTR}$ may help regulate the movement of Schwann cells to injured areas. Intriguingly, $p75^{NTR}$ expression has also been found to increase in immune cells of the monocyctic lineage

(Dowling et al., 1999). However, little is known about the precise effects of $p75^{NTR}$ on cells of the immune system.

Increased $p75^{NTR}$ expression has been suggested to contribute several neurodegenerative diseases. Over the last few decades, there have been several studies suggesting a link between $p75^{NTR}$ and Alzheimer's disease (AD). Principally, $p75^{NTR}$ is vulnerable to the same proteolytic cleavage as amyloid precursor protein (APP) (K. M. Jung et al., 2003; Kanning et al., 2003), the protein from which Aβ derives. Both APP and $p75^{NTR}$ are proteolytic substrates for presenilin-1, γ-secretase, and α-secretase. The highest levels of $p75^{NTR}$ expression in the adult brain are found in cholinergic neurons of the basal forebrain (Rabizadeh et al., 2017), which are cells severely damaged by AD. In AD animal models, Aβ, the main component of plaques found within AD patients, serves as a ligand of $p75^{NTR}$ capable of initiating apoptosis (Yaar et al., 1997). Furthermore, the brains of AD patients have been shown to express increased levels of proNGF (Peng, Wuu, Mufson, & Fahnestock, 2004). Remarkably, proNGF isolated from these brain samples induced $p75^{NTR}$-mediated cell death in sympathetic neurons (Pedraza et al., 2005). Thus, upregulation of proNGF in AD may lead to $p75^{NTR}$ apoptotic signaling which could contribute to the neurodegeneration within the AD brain.

The $p75^{NTR}$ has also been claimed to cause the degeneration of motor neurons during the progression of amyotrophic lateral sclerosis (ALS). Reexpression of the receptor has been detected in spinal motor neurons of an ALS mouse model as well as in spinal cord samples from human ALS patients. Knockdown of the receptor in ALS animal models has been shown to reduce neural damage as well as delay the progression of the disease. For example, in the superoxide dismutase 1 (SOD1) mutant mouse, the antisense knockdown of $p75^{NTR}$ resulted in a delay of ALS onset and progression (Turner et al., 2003). Furthermore, extended survival of SOD1 double transgenic mice genetically deficient in $p75^{NTR}$ was observed. Although these results indicate $p75^{NTR}$ contributes to the progression of ALS, further studies are needed to

determine the role of $p75^{NTR}$ in ALS since the SOD mutation only represents a small population of ALS patients.

1.11 Oxidative Stress

Among all of the pathological conditions in which $p75^{NTR}$-mediated degeneration has been observed, nearly all are associated with elevated reactive oxygen species (ROS) (Jenner Peter, 2003). The production of ROS is a natural incidence during mitochondrial electron transport. When produced in excess, the highly reactive molecules can cause a range of deleterious events. Detoxification of ROS is paramount to the survival of all aerobic life forms. All cells obtain a variety of antioxidant enzymes that are able to eradicate the harmful effects of ROS. However, if an imbalance occurs between excessive ROS production and the inability of antioxidant enzymes to detoxify the reactive species, then oxidative stress occurs. This imbalance could result from limited antioxidant production or limited antioxidant activity.

The term ROS collectively refers to both free radical and non-radical derivatives of molecular oxygen (Halliwell, 2006). The superoxide anion serves as a precursor for other ROS species including hydrogen peroxide, hydroxyl radical, hydroperoxyl radical, and nitric oxide. Superoxide anion is a product of a one-electron reduction of molecular oxygen and can be further reduced by one electron to yield hydrogen peroxide (Halliwell, 2006). Hydrogen peroxide is very unstable and it can easily diffuse through the plasma membrane of cells to cause cellular destruction and death (Fisher, 2009). The mitochondrial electron transport chain is a major source of ROS production due to the numerous redox reactions that occur during the process. Normally, electrons pass through a series of electron carrier proteins to ultimately reduce molecular oxygen to water. However, if an electron leakage transpires, molecular oxygen can instead be reduced to form superoxide anion. Nevertheless, superoxide anion and other ROS are produced as necessary intermediates during enzymatic reactions (Jastroch, Divakaruni, Mookerjee, Treberg, & Brand, 2010)

There are various antioxidant defense mechanisms cells can utilize in order to control and counterattack the toxic effects of ROS accumulation. Superoxide dismutase (SOD) is able to catalyze the breakdown of superoxide anion to oxygen and hydrogen peroxide (Dasuri, Zhang, & Keller, 2013). Peroxide is then targeted and eliminated by peroxidase enzymes. Additionally, hydrogen peroxide can be eradicated by catalase, an enzyme that converts the reactive molecule to water and oxygen by using manganese or iron as a cofactor (Dasuri et al., 2013). In the brain, catalase is localized in peroxisomes (Dasuri et al., 2013). *In vivo* models of Alzheimer's disease have shown that catalase is neuroprotective, in which impairments of the enzyme resulted in increased susceptibility to neuronal injury by Aβ production (Clausen, Bi, & Baudry, 2012; Mao et al., 2012).

In addition, glutathione (GSH) and glutathione peroxidase (GPX) have been implicated to play important roles in modulating levels of oxidative stress within the brain (Peter Klivenyi et al., 2000; M. Lee et al., 2010). Both enzymes are part of the selenium-containing enzyme family, and they each mediate the removal of hydrogen peroxide by ultimately catalyzing the oxidant's reduction to water (Halliwell, 2006). The two enzymes work together to remove hydrogen peroxide by coupling the reduction of hydrogen peroxide with the oxidation of GSH. This reaction forms oxidized glutathione (GSSG), which can be converted back to GSH by glutathione reductase enzymes (Halliwell, 2006). The inhibition of GSH synthesis in human glial cells by buthionine sulfoximine resulted in a neuroinflammatory response (M. Lee et al., 2010). Furthermore, when oxidative stress was induced in wild-type and GPX knockout mice by malonate injection, there was a significant amount of hydroxyl radical generation in GPX knockout mice compared to wild-type control mice (P Klivenyi et al., 2000). Due to these observations, GSH and GPX have been implicated as important hydrogen peroxide eliminators.

The brain is highly susceptible to oxidative stress for several reasons. Firstly, the brain constitutes 2% of human body weight, but the organ utilizes approximately

20% of the body's oxygen consumption (Dasuri et al., 2013). The primary reason for the high consumption of oxygen is due to the massive amounts of ATP needed to maintain neuronal intracellular ion homeostasis for proper action potential propagation and neurotransmitter secretion (Dasuri et al., 2013). Secondly, the brain is rich in iron due to the presence of important iron-containing proteins such as cytochromes, ferritin, and tyrosine hydroxylases (Dasuri et al., 2013). Therefore, ROS can easily be generated by way of the Fenton reaction (Kanti Das, Wati, & Fatima-Shad, 2014). The Fenton reaction is defined as the reaction of ferrous iron (Fe^{2+}) with hydrogen peroxide to produce ferric iron (Fe^{3+}) and the highly reactive hydroxyl radical (Kanti Das et al., 2014).

1.12 Dopaminergic Signaling

Dopamine serves as an additional source of ROS production within the brain due to the natural production of ROS during dopamine degradation (Muñoz, Huenchuguala, Paris, & Segura-Aguilar, 2012). Dopamine is classified as a catecholamine neurotransmitter that is secreted by dopaminergic neurons and it has been shown to regulate a variety of functions including voluntary movement, addiction, memory, learning, and attention (Vogt Weisenhorn, Giesert, & Wurst, 2016).

Within the brain, dopaminergic neurons can be found within the mesencephalon (also known as the midbrain), diencephalon, and the olfactory bulbs (Arias-Carrián, Stamelou, Murillo-Rodríguez, Menéndez-Gonzlez, & Pöppel, 2010). However, the majority of all dopaminergic neurons within the central nervous system are located in the ventral part of the mesencephalon (ventral midbrain) (Arias-Carrián et al., 2010). Dopaminergic neurons within in the ventral midbrain, also known as mesencephalic dopaminergic neurons, can be found in three midbrain structures known as the substantia nigra pars compacta (SNc), the ventral tegmental area (VTA), and the retrorubral field (RRF) (Arias-Carrián et al., 2010). The integrity of these

structures is critical for proper dopaminergic signaling. For example, the degeneration of the substantia nigra is the ultimate cause of Parkinson's disease.

There are four main pathways in which dopamine transmission can undergo. The four pathways include the mesolimbic pathway, the mesocortical pathway, the tuberoinfundibular pathway, and the nigrostriatal pathway (Agid, 1991). The cell bodies of dopaminergic neurons of the mesolimbic pathway originate in the VTA and their axons project and terminate in the ventral striatum (Adinoff, 2004). This pathway modulates reward and pleasure. The mesocortical pathway, which regulates emotion-related behavior, contains dopaminergic neurons that originate in the VTA and project to the prefrontal cortex (Arias-Carrián et al., 2010). Dopaminergic neurons of the tuberoinfundibular pathway originate in the arcuate nucleus of the hypothalamus and project to the median eminence (Weiner & Ganong, 1978). This pathway controls the secretion of prolactin from the anterior pituitary gland. The nigrostriatal pathway consists of dopaminergic neurons whose cell bodies originate in the substantia nigra and terminate in the dorsal striatum (Arias-Carrián et al., 2010). This pathway is responsible for regulating voluntary movement. The degeneration of neuronal projections within the nigrostriatal pathway has been shown to cause the characteristic tremors seen in Parkinson's disease patients (Barbeau, 1962).

1.13 Dopamine Metabolism

Dopamine is synthesized in a sequential reaction within the cytosol of catecholaminergic neurons (Meiser, Weindl, & Hiller, 2013). First, L-tyrosine is hydroxylated by tyrosine hydroxylase (TH) to yield dihydroxyphenylalanine (DOPA). DOPA is then decarboxylated by L-amino acid decarboxylase to yield dopamine (Meiser et al., 2013) (Fig. 1). Upon excitation of presynaptic dopaminergic neurons; dopamine is released from synaptic vesicles and released into the synaptic cleft to bind to postsynaptic dopaminergic receptors. The physiological actions of dopamine are mediated by five G protein-coupled receptors (GPCRs) named D1, D2, D3, D4, and D5 (Beaulieu & Gainetdinov, 2011).

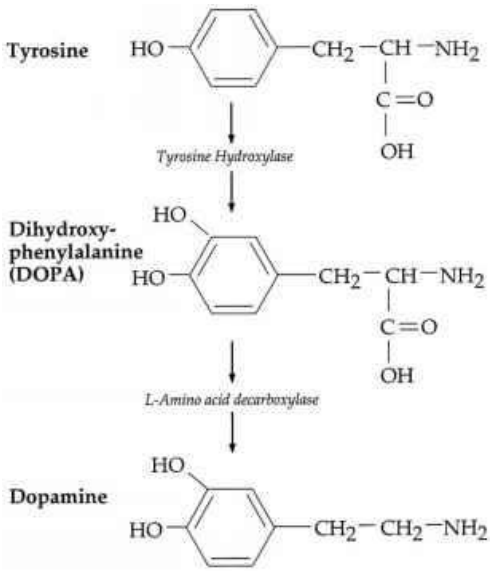


Figure 1: ***The Dopamine Synthesis Pathway*** (Meiser et al., 2013)

Dopaminergic signaling is terminated by two pathways, reuptake or degradation. These pathways help prevent the oxidation of dopamine to aminochrome, the precursor of neuromelanin and a molecule known to be neurotoxic (Graumann et al., 2005). Dopamine reuptake is mediated by the dopamine transporter (DAT), a transmembrane protein located on the presynaptic dopaminergic neuron (Vaughan & Foster, 2013). Molecules of dopamine are translocated from the synaptic cleft into the presynaptic neuron and then repackaged into synaptic vesicles by vesicular monoamine transporters (VMATs) (Beaulieu & Gainetdinov, 2011). VMATs mediate dopamine translocation into vesicles by generating a proton gradient (Muñoz et al., 2012). For every one molecule of dopamine taken up by VMAT, two protons are released. The degradation of dopamine is mediated by two enzymes, monoamine oxidase (MAO) and catechol-o-methyltransferase (COMT). MAO degrades excess dopamine by catalyzing the oxidative deamination of dopamine to 3, 4-dihydroxyphenylacetaldehyde (DOPAL) and hydrogen peroxide (Meiser et al., 2013; Muñoz et al., 2012). DOPAL is then converted to 3, 4-dihydroxyphenylacetic acid (DOPAC), which can then be converted to homovanilic acid by COMT (Muñoz et al., 2012).

Interestingly, both enzymes are expressed in all neurons of the central nervous system (Segura-Aguilar et al., 2014). MAO enzymes are localized within the outer membranes of mitochondria in neurons and glial cells. The two isoforms of COMT, soluble-COMT and membrane-bound-COMT, are found in the cell body, axons, and dendrites of neurons (Segura-Aguilar et al., 2014). Despite the two enzymes' ubiquitous expression within various types of neurons, MAO is less prevalent in substantia nigral neurons and COMT is not detected at all in this neuronal subpopulation (Meiser et al., 2013).

1.14 Dopamine Oxidation

Dopamine is vulnerable to oxidation due to its electron-rich catechol moiety (Meiser et al., 2013). Under aerobic conditions, free cytosolic dopamine is able to spontaneously oxidize to dopamine *o*-quinones in the absence of metal catalysts (Meiser et al., 2013; Muñoz et al., 2012; Segura-Aguilar et al., 2014). The one-electron oxidation of dopamine generates dopamine *o*-semiquionone and one molecule of superoxide. Subsequently, dopamine *o*-semiquionone is oxidized to dopamine *o*-quionone due to the reduction of two oxygen molecules to superoxide radicals (Segura-Aguilar et al., 2014). Dopamine *o*-quinone is unstable in the cytosol at physiological pH, therefore the radical's amino chain cyclizes to form aminochrome (Meiser et al., 2013; Muñoz et al., 2012; Segura-Aguilar et al., 2014).

Aminochrome serves as the precursor to neuromelanin, a pigment mostly found within the substantia nigra (Meiser et al., 2013). Similar to melanin, neuromelanin is accountable for the dark color of the substantia nigra. Studies of post mortem brain tissue of healthy subjects has recognized neuromelanin formation as a normal process that occurs over time and accumulates with age (Muñoz et al., 2012). Despite the pigment's supportive role in dopamine oxidation, neuromelanin has been indicated to have a neuroprotective role as a metal chelator (Gerlach et al., 2003). However, the presence of neuromelanin supports the occurrence of dopamine oxidation due to the initial presence of its precursor, aminochrome.

Aminochrome has been reported to induce mitochondrial dysfunction by forming protein adducts with protein complexes I and III of the electron transport chain (Van Laar, Mishizen, Cascio, & Hastings, 2009). Additionally, aminochrome has been found to be disruptive to cytoskeleton networks in a substantia nigra-derived cell line by forming aggregates with α- and β-tubulin (Paris et al., 2010). Interestingly, its precursor, dopamine *o*-quinone has been reported to form adducts with a variety of proteins such as tyrosine hydroxylase, glutathione peroxidase, dopamine transporter, superoxide dismutase, and mitochondrial complexes I, III, and V. Increased levels of dopamine and glutathione peroxidase adducts have been detected in Parkinson's disease (Spencer et al., 1998). However, due to the rapid cyclization of dopamine *o*-quinone, it is unclear if the damaging effects resulting from dopamine oxidation are caused by dopamine *o*-quinone or aminochrome (Segura-Aguilar et al., 2014).

The oxidation of dopamine can also be catalyzed by enzymes with peroxidase activity such as cyclooxygenases and cytochrome p450 isoforms (Muñoz et al., 2012). Additionally, a variety of transition metals such as copper, iron, and manganese can catalyze the oxidation of dopamine (Muñoz et al., 2012). For example, the autooxidation and MAO-mediated degradation of dopamine generates the production of hydrogen peroxide, which can easily be reduced to the hydroxyl radical by way of the Fenton reaction (Hermida-Ameijeiras, Méndez-Álvarez, Sánchez-Iglesias, Sanmartín-Suárez, & Soto-Otero, 2004). In addition, the reaction of dopamine *o*-quinone with iron can lead to the production of the neurotoxin 6-hydroxydopamine (6-OHDA; Fig. 2) (Napolitano, Pezzella, & Prota, 1999).

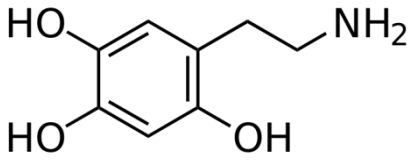


Figure 2: ***6-Hydroxydopamine (6-OHDA) Chemical Structure*** (Napolitano et al., 1999)

6-OHDA is a hydroxylated analogue of dopamine (Blum et al., 2001) and is frequently used to model Parkinson's disease *in vitro* and *in vivo* (Ungerstedt, 1968).

Due to its structural similarities to dopamine, 6-OHDA is able to travel from the cytosol and into the presynaptic terminal of dopaminergic neurons by way of the dopamine transporter (Luthman, Fredriksson, Sundström, Jonsson, & Archer, 1989). Consequently, the toxicity of 6-OHDA is selective for catecholaminergic neurons of both the central and peripheral nervous systems. In addition, the contribution of 6-OHDA to oxidative stress is widely established. Firstly, 6-OHDA is able to auto-oxidize to generate hydrogen peroxide, superoxide anion, and catecholamine quinones (Padiglia et al., 1997). Similarly, the oxidation of 6-OHDA by MAO produces hydrogen peroxide, which stimulates the production of additional reactive species (Cohen, 1984). Furthermore, 6-OHDA has been shown to reduce the enzymatic activity of glutathione peroxidase and superoxide dismutase, thereby promoting neuronal damage induced by ROS (Perumal, Gopal, Tordzro, Cooper, & Cadet, 1992). 6-OHDA is also able to impair neuronal structure and metabolism by interfering with the normal functions of complex I of the electron transport chain (Cleeter, Cooper, & Schapira, 1992). As a result, the formation of superoxide radicals occurs, causing additional neuronal damage.

1.15 Oxidative Stress Mediates Programmed Cell Death

The uncontrollable accumulation of ROS within a variety of cell types has been found to play a vital role in mediating programmed cell death at high concentrations (Sinha, Das, Pal, & Sil, 2013). ROS are able to promote the release of cytochrome c by catalyzing the oxidation of cardiolipin, an anionic phospholipid that functions to attach cytochrome c to the inner mitochondrial membrane (Hirsch et al., 1997). After its release, cytochrome c can then interact with Apaf-1 and procaspase-9 to form the apoptosome, which ultimately leads to caspase activation, followed by apoptosis (Ott, Robertson, Gogvadze, Zhivotovsky, & Orrenius, 2002). The JNK signaling pathway is also regulated by oxidative stress (M. C. Wang, Bohmann, & Jasper, 2003). When activated, JNK signaling is able to promote programmed cell death. JNK is able to promote apoptosis by way of the intrinsic pathway by causing the release of

cytochrome c from the mitochondria (Sinha et al., 2013). The inhibition of JNK activity in fibroblast cells prevented cytochrome c release in response to TNF-α treatment (Kamata et al., 2005), thereby preventing caspase-3 cleavage and ultimately apoptosis from occurring. JNK is also able to indirectly activate apoptosis by activating a variety of transcription factors, such as c-jun, which in turn initiate the upregulation of various pro-apoptotic genes such as Bak, TNFα, and Fas-L (Sinha et al., 2013).

Surprisingly, JNK has also been described as protective response that works to alleviate the toxic effects of ROS (M. C. Wang et al., 2003). For example, the introduction of a mutation in *Drosophila melanogaster* that augmented JNK signaling resulted in less oxidative damage and increase lifespan compared to wild-type flies (M. C. Wang et al., 2003). Additionally, the inhibition of JNK activity in human fibroblasts cells exposed to ultraviolet light resulted in a 30% increase in cell death compared to controls (Courtial et al., 2017). These results suggest that JNK activity is necessary for the repression of ROS accumulation. However, the association between oxidative stress and JNK signaling remains elusive.

1.16 Oxidative Stress and p75^{NTR}

It is widely established that Parkinson's disease (PD) results from the degeneration of ventral midbrain dopaminergic neurons, also known as mesencephalic dopaminergic (mesDA) neurons (Vogt Weisenhorn et al., 2016). Consequently, a diminished amount of dopamine is released, leading to motor deficits. The underlying molecular mechanisms that cause the degeneration of dopaminergic neurons are unknown, but the progression of PD has been highly suggested to be associated with oxidative stress. The presence of oxidative stress in PD is supported by analyses of postmortem brain tissues of PD patients which have shown increased levels of protein carbonylation (Floor & Wetzel, 2002) as well as the DNA and RNA oxidation products 8-hydroxy-deoxyguanosine (Alam et al., 2002) and 8-hydroxy-guanosine (J. Zhang et al., 1999), respectively. Additionally, postmortem brain tissue from PD patients has shown a decreased amount of glutathione (Sian et al., 1994), a compound that functions as an

antioxidant molecule within the mitochondria. The link between oxidative stress and Parkinson's disease is further supported by the observation that toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sedelis, Schwarting, & Huston, 2001), rotenone (Y. Wang et al., 2017), and 6-hydroxydopamine (6-OHDA) (Tieu, 2011) are capable of causing PD-like motor symptoms seen in rodents.

Intriguingly, $p75^{NTR}$ has been implicated to play a role in the degeneration of mesencephalic dopaminergic neurons *in vitro*. A variety of studies conducted by Simon and colleagues have indicated the homeodomain transcription factors, *Engrailed-1* and *Engrailed-2*, are critical for the survival and maintenance of mesDA neurons during PD (Alberi, 2004; Simon, Bhatt, Gherbassi, Sgadó, & Alberí, 2006; Simon, Saueressig, Wurst, Goulding, & O'Leary, 2001). In fact, *Engrailed* double mutant mice (*$En1^{-/-}$; $En2^{-/-}$)* experienced a progressive loss in mesDA neurons as well as PD-like motor deficiencies (Sgadò et al., 2006). Additionally, *Engrailed* double mutant mice exhibited increased expression of $p75^{NTR}$ within the ventral midbrain (Alavian, Sgadò, Alberi, Subramaniam, & Simon, 2009). The knockdown of $p75^{NTR}$ expression or the application of a $p75^{NTR}$-inhibiting antibody significantly protected *Engrailed* double mutant mesDA neurons from apoptosis (Alavian et al., 2009). These results were the first line of evidence suggesting $p75^{NTR}$ contributes to mesDA degeneration during PD. However, it is still unknown if $p75^{NTR}$ is directly responsible for causing PD-associated nigral neurodegeneration since double mutant *Engrailed* neurons are vulnerable to death regardless. Nonetheless, $p75^{NTR}$ appears to function as a stress-activated receptor that promotes neuronal degeneration in response to injury.

A previous report using PC12 cells discovered that $p75^{NTR}$ is protective against oxidative stress, thereby characterizing the receptor as anti-apoptotic under oxidative conditions (Tyurina et al., 2005). The antioxidant capability of $p75^{NTR}$ was demonstrated to be associated with glutathione oxidation due to the increased amount of oxidized glutathione observed following exposure to oxidative stress (Tyurina et al., 2005). These results were each observed in the presence of serum, a

protein-rich liquid that contains several neurotrophins that are able to bind to $p75^{NTR}$. Therefore, each of these results was replicated in the absence of serum to determine if $p75^{NTR}$ mediates its cellular response to oxidative stress in PC12 cells in the presence or absence of a ligand. Remarkably, the survival rates and the amount of oxidized glutathione were unchanged (Tyurina et al., 2005). Thus, it was determined that $p75^{NTR}$ is able to execute its protective activity against oxidative stress in ligand-independent manner.

Investigations of sympathetic neurons have found that $p75^{NTR}$ is activated and cleaved in response to ROS (Kraemer et al., 2014). Using the lipid peroxidation product 4-hydroxynonenal to model oxidative stress, Kraemer et al. discovered the ability of $p75^{NTR}$ to induce neuronal death and axonal fragmentation under these conditions. The neurodegenerative effects were attenuated in $p75^{NTR}$ deficient mice, thereby confirming the receptor's destructive role during oxidative stress (Lotharius, 2005). Additionally, cleavage of $p75^{NTR}$ was observed to be associated with $p75^{NTR}$-mediated neurodegeneration due to the significant protection of sympathetic neurons from apoptosis after TACE or DAPT pre-treatment. Surprisingly, the neurodegenerative effects mediated by $p75^{NTR}$ were not associated with the induction of neurotrophins or neurotrophin binding (Kraemer et al., 2014). Blockade of the extracellular domain of $p75^{NTR}$ had no significant effect on HNE-induced neurite degeneration and apoptosis. Thus, $p75^{NTR}$ exhibited the ability to become activated and cleaved in a ligand-independent manner under conditions of oxidative stress (Kraemer et al., 2014).

1.17 Thesis Goals: Investigating links between Dopaminergic Neuron Degeneration and $p75^{NTR}$

Although there is evidence showing oxidative stress can cause the activation and cleavage of $p75^{NTR}$ in sympathetic neurons (Kraemer et al., 2014), it is currently unknown if similar events occur within brain regions vulnerable to oxidative stress. The $p75^{NTR}$ has been demonstrated to contribute to cell death caused by a variety of cellular injuries and in neurodegenerative disease models associated with oxidative

stress, but the deteriorating effects of the receptor have not been clearly defined within the dopaminergic neuron population. Expression of $p75^{NTR}$ has been suggested to contribute to the degeneration of dopaminergic neurons of the substantia nigra (Alavian et al., 2009), a region of the brain that is highly degenerated by Parkinson's disease. However, the direct contribution of $p75^{NTR}$ to dopaminergic neuron degeneration has not been investigated. A previous report utilizing sympathetic neurons demonstrated that $p75^{NTR}$ signaling is able to become activated by the catecholaminergic specific neurotoxin, 6-OHDA (Kraemer et al., 2014). This neurotoxin is frequently used to model oxidative stress in dopaminergic neurons to investigate the biochemical dysfunctions that occur in Parkinson's disease. Therefore, my thesis project involved the exploration of how oxidative stress affects $p75^{NTR}$ signaling in dopaminergic neurons to better understand the underlying mechanisms that lead to dopaminergic neuron degeneration.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 Cell Culture

2.1.1 LUHMES Cells

Lund Human Mesencephalic (LUHMES) cells were purchased from ATCC (CRL-2927). For all experimental procedures using LUHMES cells, all cell media was placed in the incubator for at least 15 minutes in order for media to reach proper pH. T175 cell culture flasks (VWR) were pre-coated with 7 mL of poly-ornithine (50 μg/mL) in water at room temperature overnight. The poly-ornithine coating solution was removed and the flask was washed two times with water and air-dried. Next, the cell culture flask was coated with 5 mL of fibronectin (1 μg/mL) in PBS (1X) for 3 hours at 37℃ in a humidified 95% air, 5% CO_2 atmosphere. The fibronectin coating solution was removed and the flask was washed three times with water and air-dried before plating. Complete growth medium (19 mL) was used to allow proliferation of the LUHMES cells after plating. Complete growth medium consisted of Dulbecco's modified Eagle's medium/F12, N2 supplement (100x), glutamine (100x), and 40 ng/mL recombinant human fibroblast growth factor (R&D Systems). LUHMES cells were grown at 37℃ in a humidified 95% air, 5% CO_2 atmosphere.

Proliferating LUHMES cells received a half-half exchange of complete growth medium every two days until they were ready for passaging. When proliferating cells reached 80% confluency, they were dissociated with 0.25% trypsin and then subjected to centrifugation (1150 RPM for 4 minutes at 23℃). Cells were then passaged onto poly-ornithine/fibronectin coated cell culture plates and 8-well chamber slides. LUHMES cells were differentiated into mature dopaminergic neurons with differentiating medium, which consisted of Dulbecco's modified Eagle's Medium/F12, N2 supplement (100x), glutamine (100x), dibutyryl-cAMP (50X), human recombinant-glial derived neurotrophic factor (2 ng/mL), and tetracycline (1 μg/mL). Differentiating

LUHMES cells received a half-half exchange of differentiating medium every two days until treatment.

2.1.2 Primary Hippocampal Culture

For primary hippocampal neuronal cultures, 8-well chamber slides were coated with poly-d-lysine/laminin solution (300 µL per well) overnight at room temperature. The next day, wells were washed with sterile water three times and then allowed to air dry before use. Primary hippocampal neurons were isolated from postnatal day 0 wild-type C57BL6 pups and plated into 8-well chamber slides using Neurobasal medium supplemented with B-27 and glutamine. Neurons received a half-half media exchange every two days. On their 5th day in culture, hippocampal neurons were ready for cell treatment.

2.2 Cell Treatments

2.2.1 6-Hydroxydopamine (6-OHDA)

6-OHDA was dissolved in phosphate-buffered saline (1X PBS) containing 0.02% ascorbate. Due to the high reactivity of 6-OHDA with oxygen, aliquots of 6-OHDA were stored at -80℃ under inert gas. Differentiated LUHMES cells were treated on their 5th day in culture.

For dose-response assays, LUHMES cells were plated in 8-well chamber slides with complete growth medium and then differentiated with differentiating medium when they reached 50 to 60% confluency. For treatment, 3.5 mL of differentiating medium was used to treat cells with various concentrations of 6-OHDA (2.5, 5, 7.5, and 10 µM) for 24 hours (Figure 3). First, 600 µL of differentiating media was reserved for

	Untreated 300 µL	2.5 µM 6-OHDA 300 µL	5 µM 6-OHDA 300 µL	10 µM 6-OHDA 300 µL
	Untreated 300 µL	5 µM 6-OHDA 300 µL	7.5 µM 6-OHDA 300 µL	10 µM 6-OHDA 300 µL

Figure 3: Visualization of 6-OHDA treatment in 8-well chamber slide

untreated cells. Next, 2 µL of 10 mM 6-OHDA was added to 2 mL of differentiating medium to make 10 µM 6-OHDA. To make 5 µM 6-OHDA, 500 µL of 10 µM 6-OHDA was added to 500 µL of plain differentiating media. The 7.5 µM 6-OHDA solution was made by taking 300 µL of 10 µM 6-OHDA and combining it with 300 µL of 5 µM 6-OHDA. To make the 2.5 µM 6-OHDA solution, 300 µL of 5µM 6-OHDA was combined with 300 µL of plain differentiating media. Each well contained 300 µL of its indicated concentrated solution. After treatment, cells were fixed with 4% paraformaldehyde (PFA) for 25 minutes at room temperature. Following PFA fixation, cells were rinsed with PBS (1X) two times at room temperature and then stored at 4℃ in 200 µL of PBS (1X).

For western blot analyses of $p75^{NTR}$ cleavage fragments, LUHMES cells were plated in 6-well plates with complete growth medium and then differentiated with differentiating medium when they reached 50 to 60% confluency. Cells were then treated with 6-OHDA on their 5^{th} day of treatment. To prepare contents for 6-OHDA treatment, 6 mL of this media was reserved for cells that would be untreated and the other 6 mL of media was reserved for 6-OHDA treatment. To prepare the 10 µM 6-OHDA solution, 6 µL of 10 mM 6-OHDA was placed in 6 mL of differentiating media. Cells were then treated with 10 µM 6-OHDA for 12 or 18 hours. Following treatment, cells were lysed using a cell scraper and then triturated in 50 µL of Nonidet P-40 (NP-40) lysis buffer supplemented with a Complete Mini EDTA-free protease inhibitor tablet (Roche) and PHOS-STOP (Roche). To extract protein from the cell lysates, sonication was conducted, followed by protein clarification using a centrifuge (12,000 RPM for 10 minutes at 4℃). Lysates were then stored at -80℃.

For assessment of neurotrophin involvement in 6-OHDA induced apoptosis, neurons were pretreated with 9650 immune serum containing ligand-blocking antibody specific for the $p75^{NTR}$ extracellular domain (α-$p75^{NTR}$ ECD, 1:500) for 45 minutes. Neurons were then exposed to 5 or 10 µM 6-OHDA for 24 hours followed by PFA fixation. For western blot analyses of neurotrophin involvement in the induction

of $p75^{NTR}$ cleavage following 6-OHDA treatment, neurons were pretreated with the 9650 antibody for 45 minutes and then treated with 10 µM 6-OHDA for 12 hours.

2.2.2 4-Hydroxynonenal (HNE)

HNE was dissolved in absolute ethanol (200 proof) to create three aliquots of 5mM stock solution and one aliquot of 0.5mM stock solution. Hippocampal neurons were treated with various concentrations of HNE (vehicle, 0.5, 1, 5, 10, 15 µM) for 24 hours (Figure 4). Vehicle solution was prepared by adding 2 µL of absolute ethanol to 0.5 mL of plating media. To prepare the 0.5 µM solution of HNE, 1 µL of 0.5mM HNE was added to 1 mL of plating media. To prepare the 1 µM HNE solution, 1 µL of 0.5 mM HNE was added to 0.5 mL of plating media. To prepare the 5 µM HNE solution, 1 µL of 5 mM HNE was added to 1 mL of plating media. The 10 µM HNE solution was prepared by adding 1 µL of 5 mM HNE to 0.5 mL of plating media. The 20 µM HNE solution was prepared by adding 2 µL of 5 mM HNE to 0.5 mL of plating media. Each well contained 300 µL of treated solution.

	Vehicle	**0.5 µL HNE**	**1 µL HNE**	**10 µM HNE**
	Vehicle	**1 µL HNE**	**5 µL HNE**	**20 µM HNE**

Figure 4: Visualization of HNE treatment in 8-well chamber slide

2.3 Western Blot Analyses

Total protein of LUHMES cell lysates was quantified by the Bradford assay. The Bradford assay was conducted by first placing 200 µL of Bradford solution (1X) in columns 1 through 6 and rows A through C of a 96-well plate. A new pipette tip was used for each column. Next, 1 µL of NP-40 was mixed in each row of column 1. This column was designated as the “blank.” Subsequently, various volumes of Bovine Serum Albumin (BSA; 2mg/mL) were placed into each column of the 96-well plate

(Figure 5). Column 7 was used to mix 1 µL of the 'Untreated' cell lysate. Column 8 was used to mix 1 µL of '10 µM 6-OHDA'. The Bradford assay was then placed in a microplate reader to calculate the absorbance of each sample at 595 nm. The absorbance values of each column were averaged together and then used to generate a standard curve with the dependent variable (BSA mg/mL) on the X-axis and the

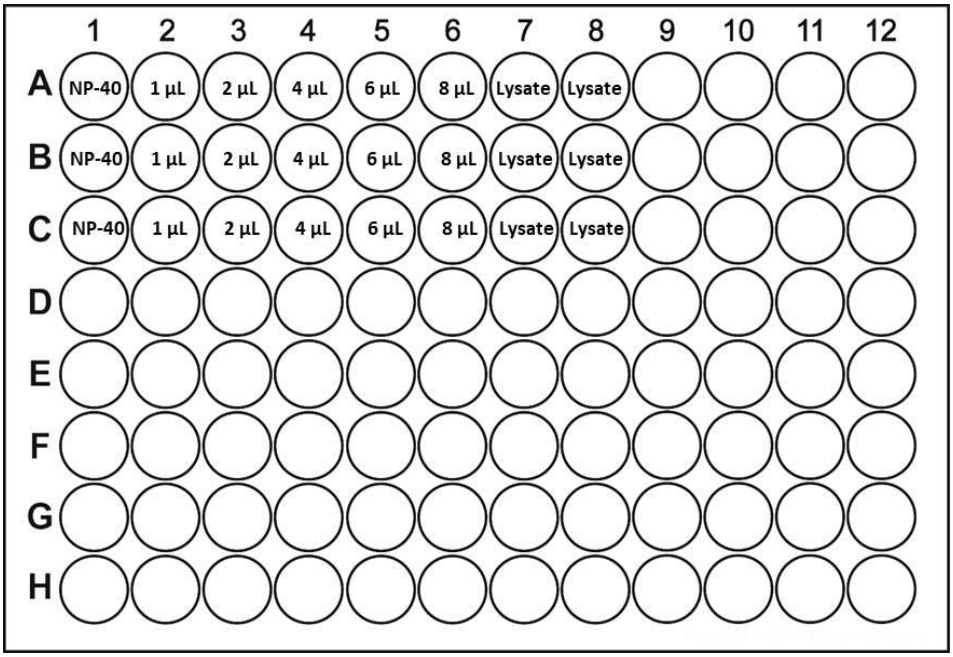


Figure 5: Bradford Assay (volume of BSA per well)

independent variable (absorbance) on the Y-axis. A linear equation was then generated using Excel and the protein concentration for each cell lysate was calculated by solving for X.

LUHMES protein samples (40 µg) were prepared in Laemmli sample buffer (6X) and boiled for 10 minutes at 98℃. Samples were then loaded into a 15% acrylamide gradient gel and separated by SDS PAGE using 5X running buffer at 150 millivolts. After successful protein separation, protein was then transferred to a PVDF membrane using the standard wet transfer method for 18 hours at 111 milliamps. After transfer completion, membranes were blocked in 5% dry milk dissolved in 0.1% Tween-PBS (PBS-T), followed by overnight incubation with primary $p75^{NTR}$ ICD anti-serum (1:3000) at 4℃. Subsequently, membranes were washed of primary antibody multiple times

with PBS-T, and then incubated with secondary anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:10,000) at room temperature for 2 hours. Once secondary incubation was complete, membranes were washed and developed using ECL for visualization of the $p75^{NTR}$ full length receptor FEMTO was used to visualize the $p75^{NTR}$ C-terminal fragment and the $p75^{NTR}$ intracellular domain. Image acquisition was conducted using the Bio-Rad ChemiDoc MP System. To assess equal gel loading among protein samples, Actin was used as a loading control. Blots were stripped for 12 minutes using stripping buffer and then washed 8 times with PBS-T. Blots were then blocked in 5% milk for 1 hour and then incubated with Actin (1:1000) overnight at 4℃. Following primary antibody incubation, blots were then washed several times with PBS-T and then incubated with secondary anti-mouse HRP antibody (1:5000) for 2 hours. ECL was used to for Actin visualization. For assessment of ERK proteins (1:1000), protein samples were loaded into 10% acrylamide gradient gels and then incubated in secondary anti-rabbit HRP antibody (1:10,000) for visualization with ECL. Actin was also used as a loading control for ERK blots.

2.4 Quantification of Neuronal Cell Death

Following 6-OHDA treatment, LUHMES cells in 8-well chamber slides were fixed with 4% paraformaldehyde for 25 minutes at room temperature. Cells were then washed of PFA two times with PBS (1X). In order for immunostaining antibodies to successfully bind to their targets, cells were permeabilized with 0.1% Triton-PCS for 30 minutes and then washed three times with PBs (1X). Next, cells were incubated in blocking solution (10% normal goat serum diluted in 0.1% Triton-PBS) for one hour at room temperature. Subsequently, cells were incubated with the neuron-specific β-III tubulin antibody, TUJ1 (1:1000), overnight at 4℃. After primary incubation, slides were washed three times with 0.1% Triton-PBS and then two times with PBS (1X). Cells were then incubated with the rabbit anti-mouse Alexa Fluor 568 secondary antibody (1:1000) for 2 hours at room temperature. The secondary antibody was washed 7 times with PBS (1X) followed by nuclei labeling with the nucleic acid stain

4',6-Diamidino-2-Phenlindole (DAPI; 5 μg/μL) for 5 minutes. DAPI was then washed off with PBS (1X) three times. The 8-well chamber slides were then prepared for coverslip mounting by removing the chambers of the 8-well using a Chamber removal tool. Once the chambers were removed, slides were then mounted with a coverslip using Permount. Permount was allowed to dry overnight to ensure the coverslip was strongly adhered to the slide. Nuclei were blindly scored as apoptotic or non-apoptotic based on the appearance of nuclei being condensed or fragmented. Confocal images were collected on a ZEISS LSM 800 laser scanning confocal microscope using a 40X objective.

2.5 Statistical Analyses

All data were expressed as the mean ± standard error means (SEM). Statistical comparisons between the $p75^{NTR}$ cleavage fragments of 6-OHDA treated and untreated LUHMES neurons were performed using the Student's *t*-test. Statistical significance was set at $p < 0.05$.

CHAPTER 3

EVALUATING PROTEOLYSIS OF P75^{NTR} WITHIN DOPAMINERGIC NEURONS SUBJECTED TO OXIDATIVE STRESS

3.1 Introduction

Despite the variety of ligands, co-receptors, and adaptor proteins that have been reported to activate p75^{NTR}-mediated signaling pathways, the precise molecular mechanisms to which p75^{NTR} signaling becomes activated remains poorly understood. However, the regulated intramembrane proteolysis of p75^{NTR} has been firmly established among all p75^{NTR} signaling mechanisms. During this process, the extracellular domain of p75^{NTR} is cleaved by the mellatoprotease TNF-α converting enzyme (TACE), leaving the remaining portion of the membrane-bound receptor, the C-terminal fragment (CTF), vulnerable to a second proteolytic cleavage. The γ-secretase complex further cleaves the CTF within its transmembrane region, thereby releasing the intracellular domain (ICD) of p75^{NTR}. These cleavage events have been demonstrated to promote the activation of various signaling pathways and cellular physiological responses such as c-jun N-terminal kinase (JNK) activation (Kenchappa et al., 2010), nuclear translocation of neurotrophin receptor interacting-factor (NRIF) (Volosin et al., 2008), apoptosis (Friedman, 2000; Kenchappa et al., 2006; Kraemer et al., 2014), cell cycle arrest (Ceni et al., 2010), and Trk-mediated activation of survival signaling pathways (Ceni et al., 2010; Kommaddi, Thomas, Ceni, Daigneault, & Barker, 2011b). The proteolytic cleavage of p75^{NTR} has been demonstrated to occur in sympathetic neurons exposed to the lipid peroxidation product, 4-hydroxynonenal (HNE). These events were shown to occur in a ligand-independent manner, indicating the role of p75^{NTR} in sympathetic neuron degeneration is novel. However, it is unknown if similar events occur in dopaminergic neurons.

Primary cultures of midbrain neurons comprise a mixture of different cell populations, in which only 1% of those populations are dopaminergic neurons. This small population percentage is unsuitable for analysis of proteins by western blot

analysis. Therefore, our studies utilized the Lund Human Mesencephalic (LUHMES) cell line, a conditionally-immortalized human midbrain cell line, attributable to their expression of a tetracycline-responsive v-myc gene that can be differentiated into mature dopaminergic neurons (Lotharius, 2005). Within several days of incubation in cell culture medium containing tetracycline, cyclic-AMP (cAMP), and glial derived neurotrophic factor (GDNF), LUHMES cells can be fully differentiated into mature dopaminergic-like neurons. The dopaminergic neuron-related features of differentiated LUHMES cells have been confirmed by their expression of dopaminergic biomarkers including tyrosine hydroxylase and dopamine transporter (X. M. Zhang, Yin, & Zhang, 2014). Cytotoxicity has been observed within the LUHMES cell culture system after treatment with MPP^+, a dopaminergic neuron specific toxin known to be uptaken by the dopamine transporter to induce its toxic effects (X. M. Zhang et al., 2014). Similar to human dopaminergic neurons, LUHMES cells are able to release and uptake dopamine as well as conduct action potentials (Scholz et al., 2011).

The physiological relevance, large culture scale, and homogenous population of LUHMES cells make them an efficient model for studying the effects of degenerating dopaminergic neurons. Furthermore, the cell line is human-derived, thereby making degenerative effects more relevant to human neurodegenerative diseases. Therefore, our investigations have utilized the LUHMES cell line. In this study, we exposed LUHMES cells to 6-OHDA, a neurotoxin used to model oxidative stress, in order to assess if $p75^{NTR}$ proteolysis occurs under these conditions. 6-OHDA is dopaminergic specific due to its ability to enter dopaminergic neurons via the dopamine transporter (Luthman et al., 1989). Once inside, 6-OHDA has been shown to deplete proteasome activity (Elkon, Melamed, & Offen, 2004), activate cell death pathways by causing caspase-3 elevation (Hanrott, Gudmunsen, O'Neill, & Wonnacott, 2006), and interfere with normal mitochondrial function by impairing protein complexes of the electron transport chain (Cleeter et al., 1992). The auto-oxidation of 6-OHDA has been shown to promote the generation of various reactive oxygen species such as hydrogen peroxide,

superoxide anion, hydroxyl radicals, and quinones (Hanrott et al., 2006). Furthermore, hydrogen peroxide has been implicated as a key mediator of 6-OHDA-induced cell death due to the attenuation of catalase activity which led to a significant amount of protection of PC12 cells from 6-OHDA-induced cell death (Hanrott et al., 2006).

In order to understand the pathogenicity of neurodegenerative conditions such as Parkinson's disease, scientific investigations must identify the triggers and mechanisms that are involved in the progression of the disease. By understanding how Parkinson's disease begins, therapeutic treatments can successfully slow or stop neurodegeneration from occurring. There are a wide range of cellular models that have been used to study Parkinson's disease such as toxin-based models and genetic models (Potashkin, Blume, & Runkle, 2011). Ideally, a model of Parkinson's disease should allow the characteristic of Parkinson's disease progression to occur in order to assess the onset of neurodegeneration. This is one reason why genetic models of Parkinson's disease, such as the *Engrailed* deficient model, are useful because they provide the opportunity to intervene with the progression of neurodegeneration (Potashkin et al., 2011). In contrast, toxin-based models, such as 6-OHDA, induce immediate neurodegeneration, thereby preventing assessment of neurodegeneration progression (Potashkin et al., 2011). However, numerous reports have showed that 6-OHDA toxicity is able to induce molecular alterations that are similar to those seen in Parkinson's disease (Potashkin et al., 2011). Therefore the 6-OHDA-induced model of Parkinson's disease is beneficial to explore the mechanisms of neurodegeneration in Parkinson's disease.

Due to a previous report showing that $p75^{NTR}$ is able to become activated and cleaved in response to oxidative stress, as well as mediate the degeneration of sympathetic neurons exposed to oxidative stress, these results provide evidence that there may be a link between $p75^{NTR}$ signaling and oxidative stress. Furthermore, these results suggest that $p75^{NTR}$ signaling could be one mechanism by which neurodegeneration occurs in sympathetic neurons exposed to oxidative stress. These

findings discovered by Kraemer et al. enhance the understanding of how $p75^{NTR}$ signaling can be activated as well as how the receptor may contribute to the etiology of neurodegenerative conditions. Whether $p75^{NTR}$ can become activated by oxidative stress in dopaminergic neurons is unknown. By using 6-OHDA to induce a well-established model of oxidative stress in dopaminergic neurons, a better understanding of $p75^{NTR}$ signaling in the brain will be achieved.

3.2 Experimental Procedures

3.2.1 Cell Culture

Please refer to Chapter 2, section 2.1, for information regarding cell culture procedures.

3.2.2 Cell Treatments

Please refer to Chapter 2, section 2.2, for information regarding cell treatment procedures.

3.2.3 Western Blot Analyses

Please refer to Chapter 2, section 2.3, for information regarding Western blot analyses.

3.2.4 Quantification of Neuronal Death

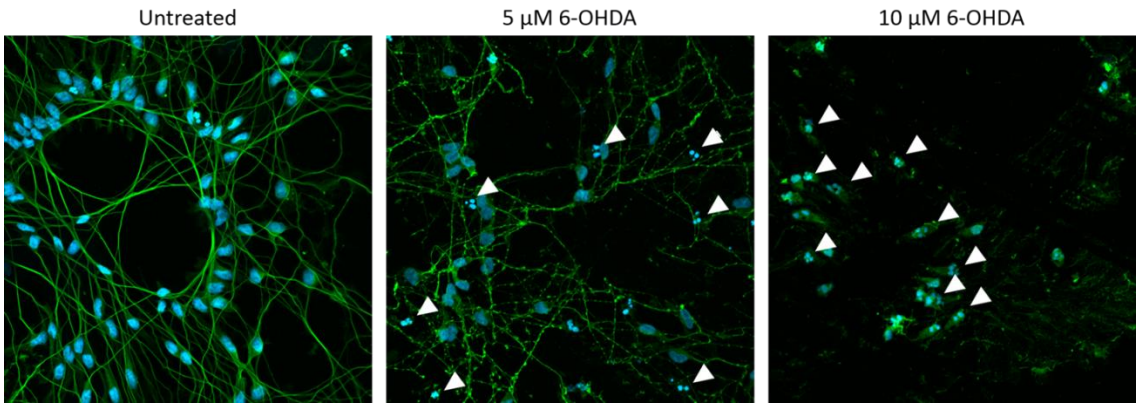
Please refer to Chapter 2, section 2.4, for information regarding quantification of neuronal death.

3.3 Results

3.3.1 Cleavage of $p75^{NTR}$ is stimulated by 6-OHDA

The 6-OHDA neurotoxin is commonly used to induce degeneration of dopaminergic neurons in order to investigate motor and biochemical dysfunctions that occur in Parkinson's disease. To examine the effect of 6-OHDA on the viability of our culture system, LUHMES neurons were treated with a range of 6-OHDA concentrations for 24 hours and then blindly scored for death based on nuclear morphology. 6-OHDA induced death of differentiated LUHMES cells in a dose-dependent manner, with 10 µM 6-OHDA being the maximally effective dose (Figure 6).

A



B

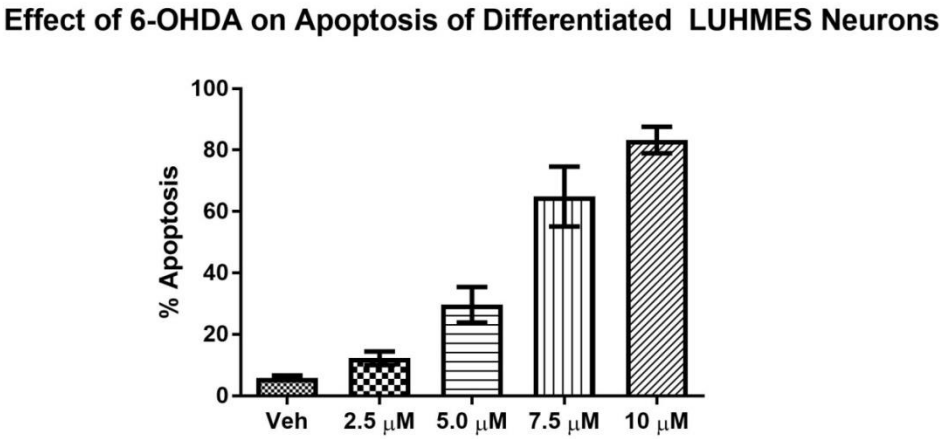


Figure 6: ***6-OHDA Promotes Dose-Dependent Apoptosis of Differentiated LUHMES Neurons.*** **A**; Confocal images of differentiated LUHMES cells treated with the indicated concentrations of 6-OHDA for 24 hours. Neurons were stained for the neuronal marker ß-III tubulin (displayed in green). Nuclei were labeled with the nucleic acid stain 4', 6-Diamidino-2-Phenylindole (DAPI) (displayed in blue) and scored as apoptotic or non-apoptotic. **B**; Quantification of apoptosis induced by various concentrations of 6-OHDA (*n*=6 for each treatment group). An increased percentage of apoptosis was observed as the concentration of 6-OHDA increased. Results are expressed as the mean ± S.E.M.

Using the maximally effective dose of 6-OHDA (10 µM), we investigated whether 6-OHDA is able to stimulate proteolytic cleavage of $p75^{NTR}$. LUHMES cells were treated with 10 µM 6-OHDA for 12 or 18 hours and then subjected to Western blot analysis using an antibody specific for the intracellular domain of $p75^{NTR}$ (1:3000). Cleavage of $p75^{NTR}$ could be observed after 12 hours (Figure 7) in which there was a significant difference between the treated and untreated $p75^{NTR}$ full-length receptor and the $p75^{NTR}$ ICD.

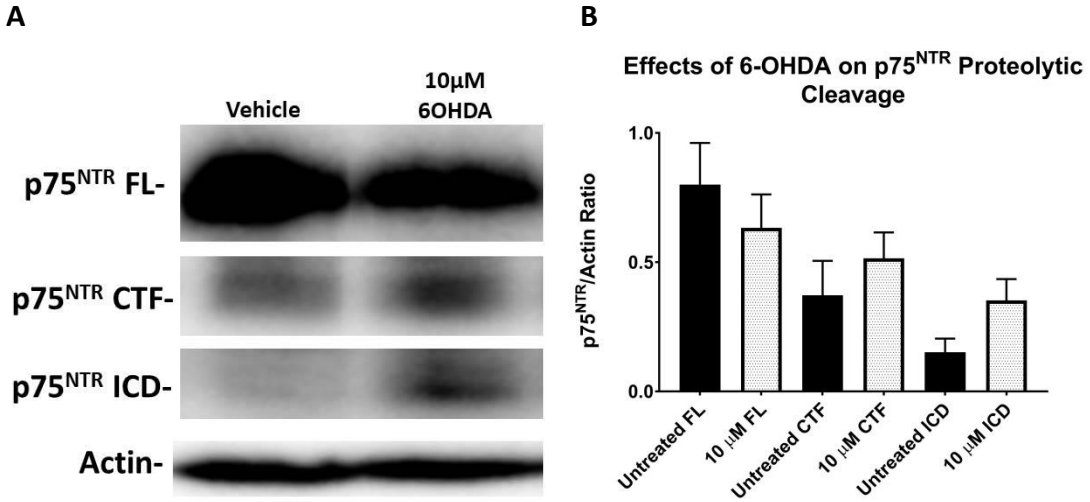


Figure 7: ***6-OHDA Promotes Proteolytic Cleavage of p75^{NTR} after 12 hours:*** **A;** Differentiated LUHMES cells were treated on their 5th day of differentiation with 10 µM of 6-OHDA for 12 hours (*n*=5). Cells were then subjected to lysis and western blot analysis using an antibody specific for the intracellular domain of p75^{NTR}. FL, full length; CTF, C-terminal fragment; ICD, intracellular domain. **B;** Quantification of p75^{NTR} fragments following 10 µM 6-OHDA treatment for 12 hours (*n*=5 independent experiments conducted). Results are expressed as the mean ± S.E.M. Student's *t*-test (FL data; significant; *p*=0.0194) (CTF data; not significant; *p*=0.1355) (ICD data; significant; *p*=0.0640).

However, cleavage fragments were more apparent in LUHMES neurons treated with 6-OHDA for 18 hours (Figure 8). Interestingly, the total expression level of p75^{NTR}, represented by the 75-kDa molecular weight marker, appeared to decrease in response to 6-OHDA treatment. This result suggests that 6-OHDA treatment causes the full-length receptor to become down regulated due to the increase in cleavage activity by the mellatoproteinases, TACE and γ-secretase. Thus, 6-OHDA stimulates the proteolytic cleavage of p75^{NTR}.

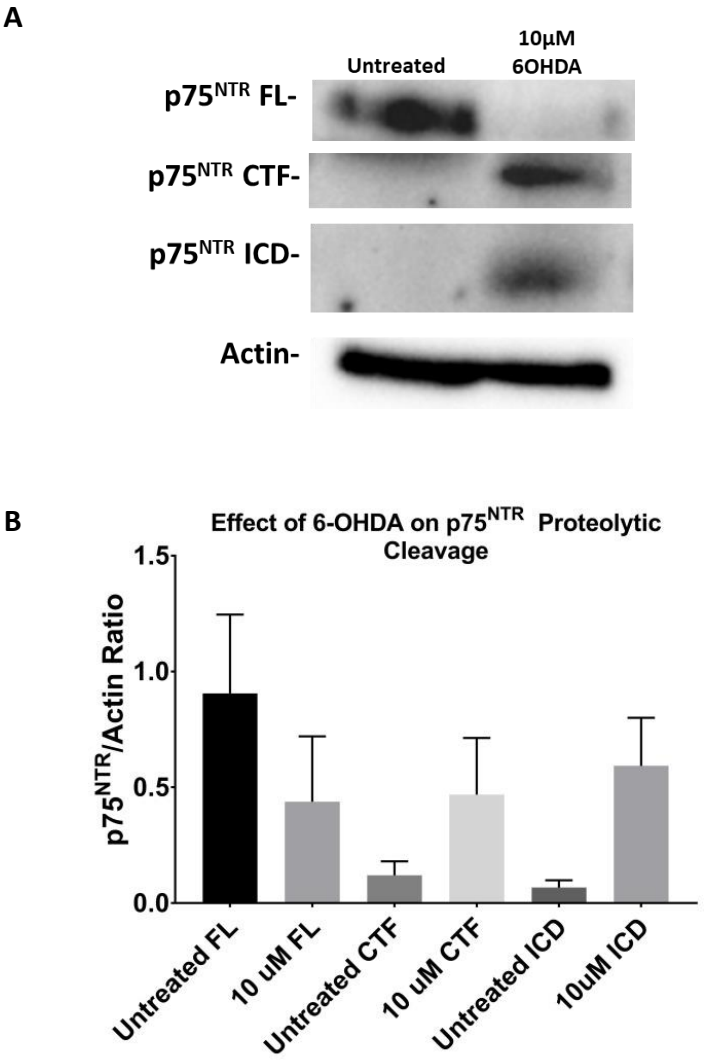


Figure 8: ***6-OHDA Promotes Proteolytic Cleavage of p75^{NTR} after 18 hours:***
A; Differentiated LUHMES cells were treated with 10 µM 6-OHDA for 18 hours (*n*=5 independent experiments conducted). Cells were then subjected to lysis and western blot analysis using an antibody specific for the intracellular domain of p75^{NTR}. FL, full length; CTF, C-terminal fragment; ICD, intracellular domain. **B;** Quantification of p75^{NTR} fragments following 10 µM 6-OHDA treatment for 12 hours (*n*=5). Results are expressed as the mean ± S.E.M. (FL data; not significant; *p*=0.0588) (CTF data; significant; *p*=0.0330) (ICD data; significant; *p*=0.0066).

3.3.2 Ligand-Mediated Activation of P75^{NTR} is not Required for Proteolytic Cleavage or Apoptosis Induction

Due to the ligand-independent mechanism at which p75^{NTR} becomes cleaved in sympathetic neurons subjected to oxidative stress, we investigated whether similar events occurred in dopaminergic neurons exposed to 6-OHDA. Neurons were pre-treated with the 9650 immune serum containing ligand-blocking antibody specific for the extracellular domain of p75^{NTR} (α-p75^{NTR} ECD) for 45 minutes, followed by exposure to 6-OHDA (10 µM) for 12 hours. Cells were then subjected to Western blot analyses using an antibody specific for the p75^{NTR} ICD. Surprisingly, proteolytic cleavage of p75^{NTR} was able to occur regardless of ligand binding (Figure 9).

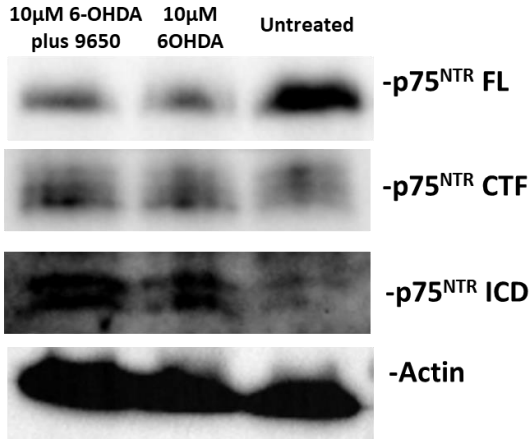


Figure 9: *6-OHDA induces p75^{NTR}proteolytic cleavage in a ligand-independent mechanism:* LUHMES neurons were pre-treated with ligand-blocking α-p75^{NTR} ECD antibody for 45 minutes and then exposed to 6-OHDA (10 µM) for 12 hours (*n*=1 independent experiment conducted). Neurons were then subjected to Western blot analysis using an antibody specific for the p75^{NTR} ICD.

Proteolytic cleavage of p75^{NTR} has been implicated to contribute to cell death in sympathetic(Kenchappa et al., 2006; Kraemer et al., 2014) and hippocampal neurons (Friedman, 2000). Within these cell types, inhibition of p75^{NTR} cleavage using TACE and γ-secretase inhibitors significantly protected neurons from apoptosis. Due to the ability of p75^{NTR} still being able to undergo proteolytic cleavage in the absence of ligand binding, we next wanted to see if blocking the ligand-binding domain of p75^{NTR}

could protect cells from the toxic effects of 6-OHDA. Neurons plated into 8-well chamber slides were pre-treated with α-$p75^{NTR}$ ECD for 45 minutes, followed by 5 µM 6-OHDA treatment for 24 hours. Remarkably, inhibition of the extracellular domain of $p75^{NTR}$ failed to significantly protect neurons from 6-OHDA-induced death (Figure 10). Collectively, these data suggest that oxidative stress promotes $p75^{NTR}$-mediated cleavage and induction of apoptosis in dopaminergic neurons in a ligand-independent manner.

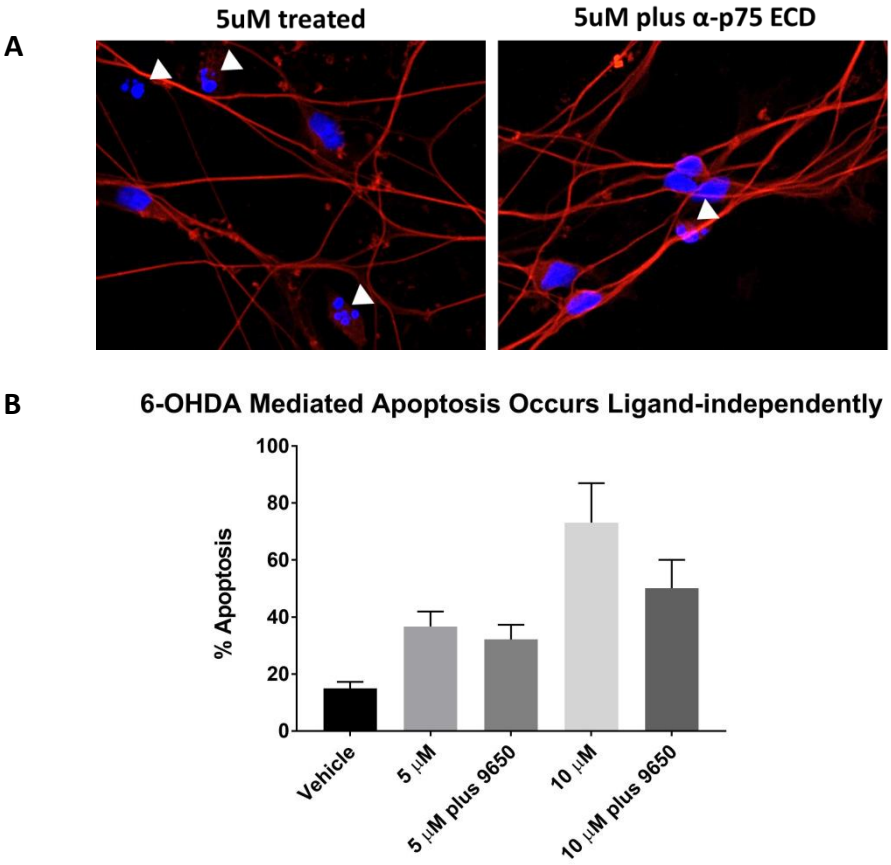


Figure 10: ***Treatment of differentiated LUHMES neurons with 9650 ligand-blocking α-p75 ECD antibody protects LUHMES cells from 6-OHDA-induced death:*** **A;** Confocal images of LUHMES cells exposed to 5µM 6-OHDA for 24 hours following a 45 minute pretreatment with immune serum containing a ligand blocking antibody specific for the extracellular domain of $p75^{NTR}$. Neurons were stained for the neuronal marker, ßIII-tubulin (red). Nuclei were labeled with DAPI stain and scored as apoptotic (indicated by arrowheads) or non-apoptotic. A reduction in apoptosis was observed in cells pre-treated with the ligand-blocking antibody compared to cells lacking pretreatment. **B;** Quantification of apoptosis induced by 6-OHDA after a 45 minute pretreatment with immune serum containing a ligand blocking antibody specific for the extracellular domain of $p75^{NTR}$ (*n*=4 for each treatment group). Pretreatment with the ligand-blocking antibody caused a substantial reduction in apoptosis induced by 6-OHDA.

3.3.3 6-OHDA Suppresses ERK Activity

An investigation conducted by Alvian and Simon et al. showed that ERK phosphorylation could be detected within wild-type mesDA neurons but not in double mutant *Engrailed* neurons, which contain elevated levels of $p75^{NTR}$ expression[175]. These results led our lab to investigate whether oxidative stress is able to suppress the phosphorylation of ERK. LUHMES cells were treated with 6-OHDA (10 µM) for 18 hours and then subjected to cell lysis for western blot analyses. Remarkably, there was a suppression of ERK phosphorylation following 6-OHDA treatment despite equal levels of total ERK protein (Figure 11).

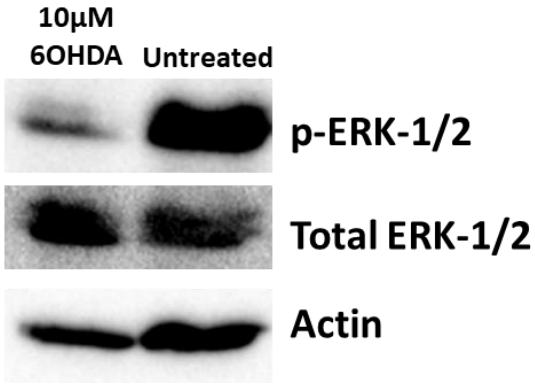


Figure 11: ***Oxidative stress does not induce ERK-1/2 Activation in LUHMES neurons.*** Representative Western blot of ERK-1/2 phosphorylation in untreated and 6-OHDA (10 µM for 18 hours) treated LUHMES neurons.

3.3.4 HNE Causes Dose-Dependent Death of Hippocampal Neurons

In addition to our experiments utilizing dopaminergic neurons, our lab also began exploring the effect of oxidative stress on hippocampal neurons, another population of cells that are located within a region of the brain that is highly degenerated by Alzheimer's disease. To mimic oxidative stress in hippocampal neurons, our lab used the lipid peroxidation product 4-hydroxynonenal (HNE) due to its wide recognition as a key mediator of neuronal apoptosis induced by oxidative stress. We observed that HNE was able to cause death of hippocampal neurons in a dose-dependent manner (Figure 12). Furthermore, we noticed that the 20 μM concentration of HNE was able to cause almost 100 percent cell death of hippocampal neurons.

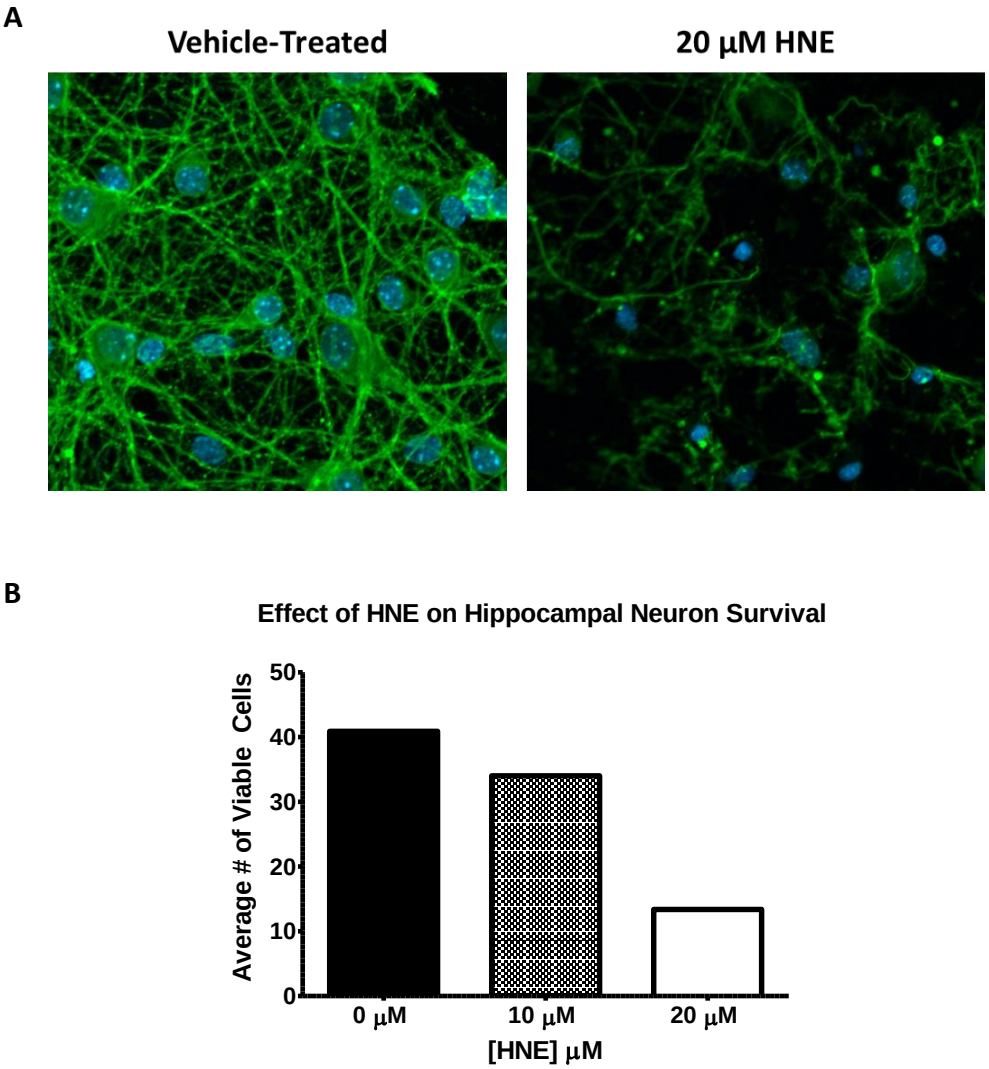


Figure 12: ***HNE Promotes Dose-Dependent Apoptosis of Hippocampal Neurons.*** **A**; Fluorescent microscope images of hippocampal neurons treated with the indicated concentrations of HNE for 24 hours. Neurons were stained for the neuronal marker ß-III tubulin (displayed in green). Nuclei were labeled with the nucleic acid stain 4', 6-Diamidino-2-Phenylindole (DAPI) (displayed in blue) and scored as apoptotic or non-apoptotic. **B**; Quantification of apoptosis induced by various concentrations of HNE (*n*=1 for each treatment group). An increased percentage of apoptosis was observed as the concentration of 6-OHDA increased. Results are expressed as the mean.

3.4 Discussion

The role of $p75^{NTR}$ as an apoptotic mediator in response to oxidative stress has been established in sympathetic neurons (Kraemer et al., 2014). The lipid peroxidation product, HNE, has been demonstrated to induce $p75^{NTR}$-mediated apoptosis and axonal degeneration of sympathetic neurons. Neurons lacking the receptor were significantly protected from HNE-induced axonal degeneration and apoptosis, thereby suggesting $p75^{NTR}$ as a regulator of neuronal responses to oxidative stress. Here, we demonstrate that $p75^{NTR}$ is expressed in fully differentiated LUHMES cells exposed to 6-OHDA neurotoxicity. Therefore, $p75^{NTR}$ may regulate responses to oxidative stress in dopaminergic neurons as well.

The expression of $p75^{NTR}$ is ubiquitous during embryonic development; however, numerous investigations have demonstrated that reexpression of the receptor during adulthood is associated with various cellular injuries and neurodegenerative conditions, leading to cell death. Reexpression of $p75^{NTR}$ has been observed to occur in response to seizures (P P Roux et al., 1999; Volosin et al., 2008), ischemia (Kokaia et al., 1998), neuronal axotomy (Harrington et al., 2004), as well as within models of Alzheimer's disease (Murphy et al., 2015) and Amyotrophic sclerosis (Lowry et al., 2001; Turner et al., 2004), conditions highly associated with oxidative stress. It is currently unknown why reexpression of $p75^{NTR}$ expression occurs in response to various injuries, but a few hypotheses have been made suggesting the receptor may promote positive effects following injuries. For instance, the expression of $p75^{NTR}$ has been previously demonstrated to increase following axonal injury and induce apoptosis(Harrington et al., 2004). However, there have been reports that $p75^{NTR}$ signaling is able to promote remyelination of injured sciatic nerves. Adult mice deficient in $p75^{NTR}$ experienced a reduction in the number of myelinated axons following sciatic nerve lesion compared to wild-type controls (Song, Zhou, Zhong, Wu, & Zhou, 2006). Furthermore, the transplantation of $p75^{NTR}$ deficient Schwann cells in mice resulted in a significant impairment in motor neuron recovery following sciatic

nerve injury (Tomita et al., 2007). The $p75^{NTR}$ deficient Schwann cells failed to promote remyelination, axonal regrowth, and radial growth in axon calibers compared to wild-type controls (Tomita et al., 2007). Therefore, the receptor might initiate apoptosis in injured sciatic nerves in order to provide a more suitable environment for remyelination.

The pro-apoptotic role of $p75^{NTR}$ has also been suggested to control inflammation by encouraging the elimination of damaged cells. For example, the induction of encephalomyelitis (EAE) in has been demonstrated to cause an upregulation of $p75^{NTR}$ in endothelial cells, leading to cell death (Copray et al., 2004). However, when EAE was induced in mice deficient for $p75^{NTR}$, the mice developed increased levels of inflammation within the central nervous system compared to wild-type controls (Copray et al., 2004). It is widely established that without inflammatory control, widespread tissue damage can contribute to the pathogenesis of numerous diseases (Rock & Kono, 2008). Therefore the detrimental effects of $p75^{NTR}$ may be a part of a homeostatic mechanism that goes out of control. Supportive evidence of this theory includes a study conducted by Miller and colleagues (Park, Grosso, Aubert, Kaplan, & Miller, 2010) demonstrating that $p75^{NTR}$ regulates the specificity of neural connectivity in basal forebrain axons by inducing axonal degeneration.

The mechanism of $p75^{NTR}$ proteolytic cleavage is well-established, but its functional significance has not been fully elucidated. Pharmacological investigations have demonstrated that $p75^{NTR}$ proteolytic cleavage relates to the induction of cell death in hippocampal (Kenchappa et al., 2006) and sympathetic neurons (Volosin et al., 2008) after seizure and proBDNF treatment, respectively. Likewise, $p75^{NTR}$ cleavage was detected in sympathetic neurons subjected to oxidative stress, which corresponded with neuronal apoptosis and degeneration. The induction of $p75^{NTR}$ cleavage has been observed in sympathetic neurons following HNE and 6-OHDA treatment, indicating multiple oxidants are able to initiate $p75^{NTR}$ signaling

mechanisms within these cells. Although $p75^{NTR}$ activation could be observed in response to oxidative stress in sympathetic neurons, further investigations are needed to determine if $p75^{NTR}$ can be similarly activated by oxidative stress in neurons of the brain, especially in brain regions vulnerable to oxidative stress such as the ventral midbrain. Furthermore, investigating whether $p75^{NTR}$ contributes to the degeneration of neurons in the ventral midbrain could provide insight that the receptor is linked to oxidative stress-associated disorders such as Parkinson's disease. Due to the abundance of studies reporting 6-OHDA induces oxidative stress and apoptosis in dopaminergic neurons, we wanted to investigate if 6-OHDA is able to stimulate proteolysis of $p75^{NTR}$ within the dopaminergic neuron population.

The ventral midbrain contains a basal ganglia structure known as the substantia nigra which is highly degenerated by Parkinson's disease, a neurodegenerative condition associated with oxidative stress. The utilization of the LUHMES culture system, an immortalized human mesencephalic cell line that can be differentiated to mature dopaminergic neurons, allowed us to investigate the effects of oxidative stress on $p75^{NTR}$ proteolytic cleavage and dopaminergic neuron viability. Our results demonstrated $p75^{NTR}$ undergoes proteolysis in response to 6-OHDA treatment within the LUHMES cell culture system. The cleavage fragments of 6-OHDA treated LUHMES cells were noticeably prominent compared to untreated cells, which was similar to the results of sympathetic neurons treated with HNE. Our assessment of $p75^{NTR}$ proteolysis was conducted by exposing dopaminergic neurons to 6-OHDA treatment for 12 hours, which was consistent with previous treatment times utilizing sympathetic neurons treated with HNE. Proteolytic cleavage of $p75^{NTR}$ in sympathetic neurons was characterized as an event that needed to occur prior to the induction of programmed cell death. Inhibition of $p75^{NTR}$ cleavage by TACE inhibitors significantly protected sympathetic neurons from HNE-induced apoptosis (Kraemer et al., 2014). Due to the similarities in CTF and ICD in dopaminergic neurons and sympathetic neurons following

12 hour exposure to oxidative stress, we hypothesize that $p75^{NTR}$ proteolysis will be the initiating event of 6-OHDA-induced cell death in dopaminergic neurons.

Both $p75^{NTR}$ cleavage fragments have been described as necessary components for $p75^{NTR}$-mediated cell death, but there is much controversy on which fragment is actually the main regulatory component of $p75^{NTR}$-mediated cell death. Coulson and colleagues propose the CTF is primarily responsible for the death inducing abilities of $p75^{NTR}$ due to the presence of a 29 amino acid sequence in the $p75^{NTR}$ CTF juxtamembrane membrane region, known as the "chopper domain"(E J Coulson et al., 2000). On the other hand, the $p75^{NTR}$ ICD has been heavily suspected as a critical regulator of $p75^{NTR}$-mediated apoptosis due to its expression of a "death domain," as well as its ability to interact with adaptor proteins that regulate apoptotic signaling, such as TRAF6, NRIF, NRAGE, NADE, and MAGE homologs. Our dose response assays showed that the maximally effective dose of 6-OHDA (10 µM) caused almost 100 percent cell death. In analyses of $p75^{NTR}$ cleavage, the maximally effective dose of 6-OHDA induced an abundant expression of $p75^{NTR}$ ICD compared to $p75^{NTR}$ CTF in dopaminergic neurons. Furthermore, a reasonable amount of CTF expression could still be detected in untreated cells, thereby suggesting $p75^{NTR}$ undergoes low basal rates of TACE cleavage. We speculate 6-OHDA has a marked effect on γ-secretase cleavage compared to TACE cleavage. Inhibition of γ-secretase cleavage has previously been shown to prevent $p75^{NTR}$-mediated apoptosis (Kenchappa et al., 2006). Since γ-secretase cleavage leads the generation of the $p75^{NTR}$ ICD, we also speculate that the $p75^{NTR}$ ICD may have a critical role in mediating dopaminergic neuron death in response to oxidative insult compared to the $p75^{NTR}$ CTF. However, further investigations are needed to examine the downstream effects of $p75^{NTR}$ cleavage in dopaminergic neurons.

Interestingly, total expression of $p75^{NTR}$ full length (represented by band at the 75 kDa weight marker) decreased in 6-OHDA treated cells compared to untreated cells. Our results contrasted with those of numerous investigations indicating that

expression of $p75^{NTR}$ is upregulated following several injuries. The expression of $p75^{NTR}$ has been shown to upregulate in a time-dependent manner following axotomy (Harrington et al., 2004), spinal cord injury (Beattie et al., 2002), seizure (P P Roux et al., 1999), and kainic acid toxicity (Y.-Q. Wang, Bian, Bai, Cao, & Chen, 2008). However, the expression of $p75^{NTR}$ peaked several days after injury (Beattie et al., 2002; Harrington et al., 2004; P P Roux et al., 1999) or until neurons could no longer undergo cell death (Harrington et al., 2004). In our experiments, downregulation of $p75^{NTR}$ full length receptor could be observed following 12 hours of treatment with the 6-OHDA maximally effective dose. Relative to prior research (Harrington et al., 2004), these results may indicate that after 12 hours of 6-OHDA treatment, LUHMES neurons are unable to undergo cell death any further, thereby initiating downregulation of $p75^{NTR}$. We currently have not assessed expression of the receptor prior to 12 hours of 6-OHDA treatment, therefore it is possible $p75^{NTR}$ expression could be upregulated before then. Increased expression of $p75^{NTR}$ could be detected in dopaminergic neurons after 30 minutes of exposure to kainic acid, so it is likely $p75^{NTR}$ initiates an immediate and rapid response in these cells following injury. However, further investigations are needed to understand the contribution of $p75^{NTR}$ signaling to the degeneration of dopaminergic neurons.

Transgenic mouse models have revealed that the transcription factors *Engrailed-1*/*Engrailed-2* (collectively called *Engrailed* or *En1/2*), are critical for the survival and maintenance of mesDA neurons during late embryonic life (Rekaik, Blaudin de Thé, Prochiantz, Fuchs, & Joshi, 2015). *Engrailed* double mutant (*En1-/-; En2-/-)* mesDA neurons have been observed to die by apoptosis during the stage when the induction of *Engrailed* expression begins (Alberi, 2004; Simon et al., 2001). Remarkably, immunohistochemistry experiments revealed *Engrailed* deficient mesDA neurons exhibit elevated levels of $p75^{NTR}$ along with caspase-3 and caspase-9 activation (Alavian et al., 2009). These results suggest if $p75^{NTR}$ contributes to the death of *Engrailed* deficient neurons then the receptor must do so by way of the

intrinsic apoptotic signaling cascade. Similarly, 6-OHDA treatment of *Engrailed* heterozygous mice induced caspase-9 activation (Alavian et al., 2009). Relative to our investigations, these results suggest that the intrinsic apoptotic signaling cascade is responsible for killing LUHMES neurons exposed to oxidative stress. Furthermore, previous investigations have shown that the intrinsic apoptotic signaling cascade is a downstream signaling pathway of $p75^{NTR}$ due to the significant protection of hippocampal neurons from programmed cell death following Apaf-1 and caspase-9 downregulation (Troy et al., 2002). Therefore, it is possible $p75^{NTR}$ mediates the death of LUHMES neurons through the intrinsic death pathway following 6-OHDA treatment. However, further research is needed to clarify the apoptotic signaling cascade that is mediated by $p75^{NTR}$ in these cells.

Previous investigations have shown that $p75^{NTR}$ is able to augment Trk signaling[107, 192] thereby promoting cellular survival. The coexpression of $p75^{NTR}$ with TrkA in PC12 cells was shown to delay the internalization and degradation of TrkA thereby prolonging TrkA signaling events due to TrkA being able to remain on the cellular surface following neurotrophin binding (Makkerh et al., 2005). Furthermore, Barker and colleagues have demonstrated that Trk receptors are able to promote proteolytic cleavage of $p75^{NTR}$ following neurotrophin binding, further leading to the phosphorylation of the Akt and ERK proteins following generation of the $p75^{NTR}$ ICD by γ-secretase (Ceni et al., 2010). However, high expression of $p75^{NTR}$ has been shown to suppress the phosphorylation of ERK in double mutant *Engrailed* mesDA neurons (Alavian et al., 2009). Double mutant *Engrailed* neurons have been shown to express high levels of $p75^{NTR}$ that correlate with the induction of cell death. Additionally, these neurons exhibited a suppression of ERK activity compared to wild-type mesDA neurons (Alavian et al., 2009). These results suggest that the suppression of ERK activity could promote $p75^{NTR}$-mediated cell death. Similarly, our experiments revealed the treatment of LUHMES neurons with 6-OHDA resulted in a suppression of ERK activity. However, due to the downregulation of the $p75^{NTR}$ full-length receptor following 6-

OHDA treatment, it cannot be determined whether ERK suppression is mediated by $p75^{NTR}$. The knockdown of $p75^{NTR}$ expression in double mutant *Engrailed* neurons allowed ERK activation to occur, thereby suggesting a relationship between the loss of ERK activity and upregulation of $p75^{NTR}$ expression (Alavian et al., 2009). Therefore, further investigations utilizing $p75^{NTR}$ knockout neurons are needed in order to determine if $p75^{NTR}$ suppresses ERK activity in dopaminergic neurons exposed to oxidative stress.

The regulation of $p75^{NTR}$-mediated cell death has been heavily indicated to occur in response to neurotrophin or proneurotrophin binding. However, overexpression of $p75^{NTR}$ cleavage fragments or $p75^{NTR}$ recombinant mutants have displayed the ability to induce apoptotic signaling pathways such as JNK phosphorylation and caspase-3 cleavage in a ligand independent manner (M. Vilar et al., 2009). Relative to these results, sympathetic neurons pre-treated with a ligand-blocking antibody specific for the $p75^{NTR}$ extracellular domain (α-$p75^{NTR}$ ECD) failed to protect neurons from HNE-induced apoptosis, thereby revealing $p75^{NTR}$ mediates these events in a ligand-independent mechanism. In our investigations, we observed similar results when LUHMES neurons were exposed to 6-OHDA toxicity. Blockage of the $p75^{NTR}$ ligand-binding domain using α-$p75^{NTR}$ ECD failed to protect LUHMES neurons from 6-OHDA-induced death. Thus, our results suggest if $p75^{NTR}$ is responsible for promoting apoptosis of dopaminergic neurons in response to oxidative stress, then the mechanism at which this occurs is ligand-independent. Previous investigations have demonstrated $p75^{NTR}$ activation can occur in the absence of ligands due to the expression of a highly conserved cysteine residue 257 (Cys^{257}) in the transmembrane domain of the receptor (M. Vilar et al., 2009). It was initially believed $p75^{NTR}$ could only dimerize in response to neurotrophin binding by way of the proposed 'snail-tong' model (Marçal Vilar et al., 2009). This model suggests that when a neurotrophin binds to $p75^{NTR}$, the receptor dimerizes, causing the intracellular domains to separate in a scissors-like movement. However, the Cys^{257} residue has been shown to enable $p75^{NTR}$

to dimerize at the plasma membrane independent of ligand-binding due to disulfide bonding of the juxtamembrane of the receptor. The precise reason as to why this occurs is unknown, but it is believed that various cysteine residues, such as Cys^{257} may induce different degrees of separation between the $p75^{NTR}$ intracellular domains. Additionally, it is suggested that $p75^{NTR}$ may undergo an array of conformational changes that each have its own particular function (M. Vilar et al., 2009). Therefore, it is possible that oxidative stress stimulates $p75^{NTR}$ to undergo a conformational change that highly favors the induction of cell death.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

This thesis project aimed to investigate the activation of $p75^{NTR}$ in dopaminergic neurons subjected to oxidative stress. Our findings exhibit a novel mechanism of $p75^{NTR}$ activation in dopaminergic neurons exposed to oxidative stress. The induction of $p75^{NTR}$ cleavage occurred following 6-OHDA toxicity along with dopaminergic neuronal cell death. Furthermore, inhibition of the $p75^{NTR}$ extracellular domain failed to protect neurons from 6-OHDA induced death. These results provide evidence that $p75^{NTR}$ could be a potential contributor of the degeneration of dopaminergic neurons in Parkinson's disease. However, further investigations are needed to fully clarify the pro-apoptotic role of $p75^{NTR}$ in this neuronal population under stressful conditions, determine if $p75^{NTR}$ proteolysis contributes to oxidative stress-induced death, and to identify activation of signaling cascades downstream of $p75^{NTR}$.

4.2 Future Studies

Our results provide a novel mechanism of $p75^{NTR}$ activation in dopaminergic neurons exposed to oxidative stress, which is relative to sympathetic neurons exposed to the same conditions. However, the functional role of $p75^{NTR}$ in sympathetic neurons subjected to HNE-induced oxidative stress is more pronounced due to the utilization of cleavage inhibitors and $p75^{NTR}$ knockout mice (Kraemer et al., 2014). These investigations discovered $p75^{NTR}$ proteolytic cleavage contributed to the induction of apoptosis and axonal degeneration in sympathetic neurons by HNE due to the significant amount of protection sympathetic neurons experienced from HNE-induced degeneration following TACE cleavage (Kraemer et al., 2014). Several studies have indicated the functions of $p75^{NTR}$ are cell-type-specific, meaning that the receptor is able to elicit distinct responses in different cell types. For example, hippocampal neurons and cerebral granule neurons exhibit different patterns of $p75^{NTR}$ cleavage,

which is suggested to underlie the different $p75^{NTR}$ signaling activities in the two neuronal cell types (Vicario, Kisiswa, Tann, Kelly, & Ibanez, 2015). Given the discovery that $p75^{NTR}$ cleavage contributes to the induction of HNE-induced death in sympathetic neurons, pharmacological investigations using TACE and γ-secretase inhibitors need to be conducted using dopaminergic neurons in order to understand the functional significance of $p75^{NTR}$ proteolysis in oxidative-stress induced death.

Several investigations have demonstrated that the death signal initiated by $p75^{NTR}$ occurs through the downstream induction of the stress-activated kinase c-Jun N-terminal kinase (JNK) pathway. JNK has been shown to promote pro-apoptotic $p75^{NTR}$ signaling in various cell types such as sympathetic neurons (Palmada et al., 2002), cerebral granule neurons (Vicario et al., 2015), oligodendrocytes (Casaccia-Bonnefil et al., 1996), Schwann cells (Yeiser, 2004), and hippocampal neurons (Friedman, 2000). Therefore, it would beneficial to assess if JNK activation occurs in dopaminergic neurons exposed to oxidative stress. These results could provide evidence that JNK contributes to the degeneration of dopaminergic neurons.

The utilization of $p75^{NTR}$ knockout neurons further confirmed the contribution of $p75^{NTR}$ in the death of sympathetic neurons due to the significant protection of knockout neurons from HNE-induced apoptosis (Kraemer et al., 2014). Therefore, the assessment of death in $p75^{NTR}$ knockout dopaminergic neurons subjected to oxidative stress should be performed. These experiments would further define the contributions of $p75^{NTR}$ signaling in the degeneration of brain regions vulnerable to oxidative stress as well as provide insight on the possible contribution of $p75^{NTR}$ in oxidative stress-associated disorders such as Parkinson's disease. Furthermore, $p75^{NTR}$ knockout neurons would also determine if $p75^{NTR}$ is directly responsible for activating JNK in dopaminergic neurons subjected to oxidative stress.

The ability of $p75^{NTR}$ to induce apoptosis is the most studied function of the receptor. However, $p75^{NTR}$ has also been shown to promote axonal degeneration during the development of sympathetic (Singh et al., 2008) and cholinergic neurons

(Park et al., 2010). Furthermore, $p75^{NTR}$ has been established as a promoter of axonal degeneration in sympathetic neurons exposed to oxidative stress due to the remarkable protection that sympathetic neurons lacking $p75^{NTR}$ experienced following HNE treatment (Kraemer et al., 2014). It has not been determined if $p75^{NTR}$ functions similarly in brain neurons. Axonal degeneration has been characterized as an important step in the pathomechanism of neurodegenerative conditions (Lingor, Koch, Tönges, & Bähr, 2012). The process has been suggested to occur early in the course of neurodegenerative diseases, providing a potential target for therapeutic strategies. Parkinson's disease, a neurological condition related to oxidative stress, has been shown to be associated with axonal degeneration (Lingor et al., 2012). Additionally, axonal degeneration in Parkinson's disease has been suggested to precede neuronal death (Lingor et al., 2012). Therefore it would be essential to investigate if $p75^{NTR}$ contributes to the axonal degeneration of dopaminergic neurons by exposing wild-type and $p75^{NTR}$ knockout dopaminergic neurons to 6-OHDA and then analyzing the amount of axonal degeneration that occurred following treatment.

Trk receptors induce the activation of several cell survival pathways such as PI3K/Akt, Ras/ERK, and phospholipase-C. A previous investigation has exhibited a correlation between high expression of $p75^{NTR}$ and the suppression of ERK activity in mesencephalic dopaminergic neurons deficient in the *Engrailed* genes (Alavian et al., 2009). However, there was not a significant difference in the activation of the Akt pathway in the deficient neurons compared to wild-type neurons. Furthermore, there were not any signs of differential activation in the JNK pathway between *Engrailed* deficient neurons compared to wild-type neurons (Alavian et al., 2009). Our lab has begun investigating the suppression of Trk signaling in dopaminergic neurons exposed to oxidative stress and so far we have observed that 6-OHDA causes a suppression of ERK activity. However, future investigations should include analysis of the Akt and phospholipase-C pathways to further characterize if all Trk signaling pathways are suppressed under conditions of oxidative stress. Furthermore, future investigations

should also utilize dopaminergic neurons deficient in $p75^{NTR}$ to determine if $p75^{NTR}$ plays a role in the suppression of Trk signaling in dopaminergic neurons under conditions of oxidative stress.

Numerous reports have shown Alzheimer's disease is associated with oxidative stress. The hippocampus, a brain region known to play a critical role in learning and memory (Sakaguchi, Kadoshima, Soen, Narii, & Ishida, 2015), is highly degenerated by Alzheimer's disease. Analyses of postmortem brain tissue of Alzheimer's disease patients have shown increased levels of $p75^{NTR}$ within the hippocampus (Ito et al., 2016). Furthermore, the loss of $p75^{NTR}$ expression in a murine model of Alzheimer's disease was shown to reduce Alzheimer's-associated behavioral symptoms (Murphy et al., 2015). Our lab recently began evaluating the effects of HNE on hippocampal neuron viability. HNE is a product of lipid peroxidation and it is commonly used to mimic oxidative stress in neurons due to its reputation as a key mediator of neuronal apoptosis during oxidative stress. Our preliminary results exhibited a dose-dependent effect of HNE on hippocampal neuron survival. Furthermore, we observed the 20 µM concentration of HNE was able to cause almost 100 percent cell death. However, additional experimental trials are needed to confirm 20 µM HNE is the maximally effective dose. This finding will then allow our lab to evaluate the effect of oxidative stress on $p75^{NTR}$ signaling in hippocampal neurons.

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