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Small molecule modulation of the p75 Neurotrophin Receptor attenuates neurodegeneration in an in vitro model of Parkinson's disease

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SMALL MOLECULE MODULATION OF THE P75 NEUROTROPHIN RECEPTOR ATTENUATES NEURODEGENERATION IN AN *IN VITRO* MODEL OF PARKINSON'S DISEASE

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Abstract

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The p75 Neurotrophin Receptor ($p75^{NTR}$) is a multifunctional transmembrane protein expressed in distinct regions of the adult nervous system. $p75^{NTR}$ regulates various functions, including neuronal survival or apoptosis interacting with coreceptors Trk receptors or sortilin. Activation of $p75^{NTR}$ occurs through proteolytic processing, and we previously demonstrated that oxidative stress induces activation of $p75^{NTR}$ in LUHMES cells, a neuronal cell line derived from the human ventral mesencephalon. Dopaminergic neurons of the ventral mesencephalon are vulnerable to oxidative stress and neurodegeneration associated with Parkinson's disease (PD). Thus, we evaluated the mechanisms behind $p75^{NTR}$ activation and its physiological implications in a PD model. Our findings reveal that oxidative stress induces proteolytic processing of $p75^{NTR}$ in LUHMES cells, promoting internalization into endosomes. We also found that pharmacological modulation of $p75^{NTR}$ using LM11A-31 protects LUHMES cells from neurite degeneration and reduces apoptosis associated with oxidative stress, indicating $p75^{NTR}$ as a potential therapeutic target for PD.

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Table of Contents

List of Figures

Chapter 1. Introduction

1.1 Introduction to Neurotrophins

The neurotrophins, a family of closely related protein growth factors exhibiting neurotrophic biological activity, play a pivotal role in regulating the survival, differentiation, and specification of neurons during embryonic and postnatal development. Neurotrophins also modulate synaptic responses in the adult nervous system, consequently influencing higher systemic functions such as behavior, cognition, learning, and memory formation (Dechant & Neumann, 2002). In addition to their conventional impact in promoting neuronal cell survival, neurotrophins have been demonstrated to regulate the growth and restructuring of axons and dendrites, synaptic attributes encompassing number, size, and maturity, as well as neurotransmitter release, long-term potentiation (LTP), long-term depression (LTD), neuronal excitability, and synaptic plasticity (McAllister *et al.*, 1999). A single neurotrophic factor has the potential to impact diverse neurons, and conversely, an individual neuron may be influenced by multiple neurotrophic factors (Korsching, 1993).

In mammals, there are four distinguished neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), all originating from a common ancestral gene, exhibiting comparable sequences and structural homogeneity (Maness *et al.*, 1994). Other neuron-promoting factors have been identified, such as ciliary neurotrophic factor and glial cell line-derived neurotrophic factor (GDNF); however, they do not exhibit homology with neurotrophins (Huang & Reichardt,

2001). In all of these neurotrophins, the active growth factor protein is a homodimer formed by identical peptide chains, and the protein's conformation is stabilized by highly conserved pairs of cysteine residues that form a cysteine knot motif within each monomer (Dechant & Neumann, 2002). Comparison of the entire molecule of mouse NGF, BDNF, and NT-3 proteins revealed a 65%/57% similarity (amino acid sequence similarity/nucleotide sequence identity) between NGF and BDNF, a 70%/61% similarity between NGF and NT-3, and a 68%/58% similarity between BDNF and NT-3. Meanwhile, NT-4 exhibited 60%, 58%, and 51% amino acid identity, respectively (Hallböök *et al.*, 1991) (Figure 1.1). The neurotrophins share numerous characteristics, including comparable molecular weights of around 13.2-15.9 kDa and isoelectric points of 9.0-10.0 (Dawbarn & Allen, 2003; Mowla *et al.*, 2001).

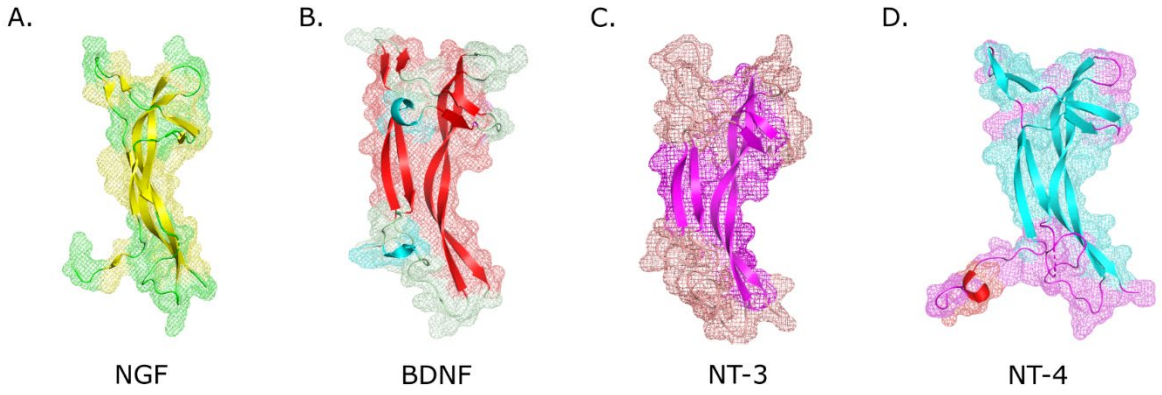


Figure 1.1 Structural representation of Neurotrophins.

A; Structure of nerve growth factor (PDB ID: 1SG1 (Chain A), **B;** Structure of brain-derived neurotrophic factor (PDB ID: 1BND (Chain A)), **C;** Structure of neurotrophin-3 (PDB ID: 1NT3), **D;** Structure of neurotrophin-4 (PDB ID: 1H8M (Chain A)). Figures created using PyMOL.

Protein synthesis of neurotrophins occurs in the rough endoplasmic reticulum, where the pro neurotrophins (approximately 210-270 amino acid residues in length) are assembled into secretory vesicles undergoing processing to form the mature neurotrophins of around 120 residues in length (Bothwell, 2014). Initially synthesized as precursor neurotrophins with an

approximate size of 30 kDa, they undergo cleavage by FURIN or pro-convertases at a highly conserved dibasic amino-acid cleavage site, ultimately yielding carboxyl-terminal mature peptides, each monomer measuring around 12-13 kDa (Chao, 2003; Dawbarn & Allen, 2003). The cleaving of pro-domains can occur at multiple points along both secretion pathways--the regulated pathway, characterized by Ca^{2+}-dependent exocytosis of secretory granules, and the constitutive pathway, which automatically releases the cargo upon reaching the plasma membrane, and in addition, can also occur after the pro-proteins are released, facilitated by extracellular or co-released endopeptidases (e.g., the tissue-plasminogen activator (tPA) plasmin cascade, or matrix-metalloproteinases (MMPs)) (Leßmann & Brigadski, 2009).

Nerve growth factor (NGF), discovered in the 1950s during the exploration of survival factors to understand the detrimental impact of target tissue removal on the subsequent survival of motor and sensory neurons, is the most extensively studied member of the neurotrophin family (Skaper, 2008), and represents the first of the neurotrophins to be described (Maness *et al.*, 1994). NGF has been characterized in neural-crest derivatives, the central nervous system, and in cells of nonneural origin (Levi-Montalcini, 1987). NGF is also recognized as a pleiotropic factor because of its production by several cell types, including structural (epithelial cells, fibroblasts/myofibroblasts, endothelial cells, smooth muscle cells and hepatocytes), accessory (glial cells, astrocytes, and Muller cells) and immune (antigen resenting cells lymphocytes, granulocytes, mast cells and eosinophils) cells (Aloe *et al.*, 2015). BDNF has been identified in most brain areas, including the olfactory bulb, cortex, hippocampus, basal forebrain, mesencephalon, hypothalamus, brainstem, and spinal cord (Bathina & Das, 2015). Higher expression of Neurotrophin-3 has been found in the kidney, liver, lung, spleen, heart, skeletal muscle, and specific brain regions, including the CA1, CA2, and dentate gyrus of the

hippocampus, cerebellum, and placenta (Hohn *et al.*, 1990; Huang & Reichardt, 2001; Jones & Reichardt, 1990). Neurotrophin-4 is highly expressed in the prostate and weakly expressed in the thymus, placenta, skeletal muscle, and testis (Ip *et al.*, 1992).

Above and beyond their central role in cell survival, neuronal growth, and synaptic plasticity, neurotrophins also play a crucial role in initiating apoptotic pathways both during development and following injury, thus exerting a significant influence on apoptotic cell death (Ceni *et al.*, 2014; Shamovsky *et al.*, 2008). Neuronal programmed cell death can be classified into two forms: morphogenetic apoptosis, which occurs during neuronal development, and pathological apoptosis, occurring subsequent to injuries like cerebral ischemia or trauma, as well as in chronic neurodegenerative conditions such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), or Parkinson's disease (PD) (Ichim *et al.*, 2012). Neurotrophin exerts its effects on the responsive neurons by binding to two distinct cell-surface receptors – the Trk receptor tyrosine kinases and the p75 neurotrophin receptor (Chao, 2003; Ebendal, 1992). Both of these receptors have been shown to mediate the biological effects of neurotrophins by either acting together or independently (Simi & Ibáñez, 2010). NGF binding to its receptors has been suggested to form an NGF/receptor complex, which is transported through internalization in a retrograde manner to the cell body, mediating physiological actions (Buck *et al.*, 1988). Studies revealed that proteolytic cleavage of neurotrophins regulates its biological action, such as in the case of pro neurotrophins, previously considered as inactive precursors, which have been shown to induce apoptosis favorably, activating $p75^{NTR}$, whereas the mature forms are thought to promote cell survival by activating Trk Receptors (Mamidipudi & Wooten, 2002).

1.2 Overview of Tropomyosin Kinase Receptors

The Tropomyosin receptor kinases (Trks) are transmembrane proteins, constituting a group of receptor tyrosine kinases crucial for mediating the effects of neurotrophins in the nervous system (Amatu *et al.*, 2016). The Trk family of tyrosine protein kinases functions as signaling receptors for the NGF family of neurotrophins (Barbacid, 1994). The Trk family comprises three receptors: TrkA, encoded by the NTRK1 gene located on chromosome 1q21-q22, TrkB encoded by the NTRK2 gene located on chromosome 9q22.1; and TrkC, encoded by the NTRK3 gene located on chromosome 15q25 (Amatu *et al.*, 2019). TrkA gene is exclusively identified in the sensory cranial and spinal ganglia of neural crest origin; however, it is absent in sensory ganglia of placodial origin (i.e. olfactory, optic, acoustic) throughout development, and more importantly, Trk expression is maintained throughout the life of the organism (Martin-Zanca *et al.*, 1990). On the other hand, the distribution of neurons expressing TrkB and TrkC mRNAs is significantly more extensive throughout the central nervous system (CNS) (Muragaki *et al.*, 1995). TrkB and TrkC exhibit comparable distributions in various brain regions; however, other regions exist, such as the thalamus and habenular nuclei, where their expression is mutually exclusive (Valenzuela *et al.*, 1993).

In mammals, the Trks constitute the second primary class of neurotrophin receptors, distinguished by an extracellular domain featuring a cysteine-rich cluster followed by three leucine-rich repeats, another cysteine-rich cluster, and two Ig-like domains (Reichardt, 2006). This is followed by a single transmembrane region that terminates in a cytoplasmic domain containing tyrosine kinase, surrounded by several tyrosine residues serving as phosphorylation-dependent docking sites for cytoplasmic adaptors and enzymes (D. Skaper, 2011; Reichardt, 2006). The cytoplasmic tail of mammalian Trk receptors contains 10 known conserved tyrosine

residues, some of which undergo phosphorylation upon receptor activation; notably, three of these phosphorylated sites are situated in the auto-regulatory loop of the tyrosine kinase domain, impacting kinase activity upon activation (Uren & Turnley, 2014). The second immunoglobulin-like domain in human Trk receptors has been identified as the site responsible for specific binding to neurotrophins (Urfer *et al.*, 1995).

In general, the structures of Trk receptors exhibit substantial similarity; however, alternate splicing gives rise to two recognized forms of TrkA, three forms of TrkB (including two lacking functional tyrosine kinase domains), and six forms of TrkC (comprising four lacking a functional tyrosine kinase domain and two with small inserts in the tyrosine kinase domain) (Shelton *et al.*, 1995). Data revealed the presence of a 140 kDa TrkA band in all tested cell lines; in addition, a higher molecular weight form of TrkA (~180 kDa), likely indicative of TrkA N-glycosylation, was also observed in SK-BR-3 and BT-474 cell lines (Griffin *et al.*, 2020). TrkB is comprised of three major protein isoforms: the full-length TrkB (TrkB-FL, 145 kDa), along with two alternatively spliced isoforms (95kDa) lacking the tyrosine kinase domain -- TrkB-T1 and TrkB-Shc (Akil, 2016). Similarly, TrkC also exhibits various isoforms; apart from the full-length isoforms containing the tyrosine kinase domain (TrkC-FL) with a molecular weight of 140 kDa, TrkC presents a truncated isoform termed TrkC-T, weighing 90 kDa (Dedoni *et al.*, 2021).

Neurotrophins have been implicated to directly bind to the tyrosine kinases, resulting in dimerization of the receptors and their activation, where TRKA specifically binds to NGF, and TRKB is specific for BDNF and NT-4. In contrast, TRKC is specific for NT-3 (Huang & Reichardt, 2001). However, available reports indicate that neurotrophin NT-3 can also activate TrkA through a molecular mechanism where the NT-3/TrkA complex does not undergo

internalization but rather activates local membrane signaling (Petruska & Mendell, 2017). Neurotrophin receptor multimerization can manifest at different levels, including homodimerization, heteromerization, and engagement with other membrane-associated proteins, and high-resolution structural analyses have confirmed that both Trk and $p75^{NTR}$ receptors interact as homodimers with the mature form of neurotrophins (Simi & Ibáñez, 2010). Following ligand binding, the neurotrophin-receptor complex undergoes internalization, giving rise to "signaling endosomes" (Kruttgen *et al.*, 2003; Pérez *et al.*, 1995) and is retrogradely transported along the axon to the soma (Pérez *et al.*, 1995). The pathways modulated by neurotrophin-mediated activation of Trk receptors encompass a spectrum of cellular processes, including proliferation and survival, axonal and dendritic growth and remodeling, assembly and restructuring of the cytoskeleton, membrane trafficking and diffusion, as well as synapse formation, function, and plasticity (Huang & Reichardt, 2003). TrkA/NGF signaling supports the survival and differentiation of both sympathetic and sensory neurons sensitive to temperature and pain. Conversely, TrkB/BDNF or TrkB/NT-4 and TrkC/NT-3 signaling pathways play roles in supporting sensory neurons responsive to tactile stimuli, motor neurons, and sensory neurons sensitive to limb movement and position, respectively (Nakagawara, 2001).

Neurotrophins, particularly in sympathetic and sensory neurons, play a pivotal role in neuronal survival, and the first neurotrophin-activated signaling protein shown to mediate the survival of these neurons was the small GTP-binding protein Ras, which operates by translating and directing signals initiated by neurotrophins into multiple signaling pathways (Kaplan & Miller, 2000). Comparable to numerous other receptor tyrosine kinases (RTKs), Trk receptors initiate classical signaling pathways, including PLCγ, PI3K, and Ras/MAPK (Patapoutian & Reichardt, 2001). Upon ligand binding, Trk receptors undergo dimerization and

autophosphorylation, activating downstream signaling cascades that encompass the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways (Maruyama, 2014). Activation of these pathways also triggers myriad downstream events, encompassing local signaling to the cytoskeleton, leading to axon branching and inducing transcriptional changes (Barford *et al.*, 2017). Like other tyrosine kinase receptors, the aggregation and tyrosine cross-phosphorylation of Trk receptors are essential for initiating neurotrophin responses (Heumann, 1994). Due to their relevance in neurodegenerative conditions, Trk receptors have been extensively investigated for their responsiveness to neurotrophins in specific groups of central nervous system neurons, such as cholinergic neurons in the basal forebrain and hippocampal neurons (associated with Alzheimer's disease), midbrain dopaminergic neurons (Linked to Parkinson's disease), and spinal and brain stem motor neurons (related to Amyotrophic Lateral Sclerosis) (Snider, 1994).

1.3 Introduction to p75 Neurotrophin Receptor ($p75^{NTR}$)

p75 neurotrophin receptor ($p75^{NTR}$), also known as tumor necrosis factor superfamily receptor 16 (TNFSR16), is a member of the tumor necrosis factor receptor (TNFR) superfamily that includes the Fas (CD95) antigen, CD30, and CD40 (Dechant & Barde, 2002; Simi & Ibáñez, 2010; Zampieri *et al.*, 2005). $P75^{NTR}$ is a pleiotropic receptor playing a crucial role in a myriad range of biological processes such as cell differentiation and growth, scar formation and regeneration, fibrinolysis and tissue fibrosis, obesity and insulin resistance, hypoxia, and various nervous system pathologies, including retinal injuries, Multiple Sclerosis and spinal cord injury, cognitive impairment, Alzheimer's disease, Parkinson's disease, and ALS (Malik *et al.*, 2021). $P75^{NTR}$ is the first discovered member of the superfamily, and it serves as a noncatalytic receptor that has demonstrated the capability to bind each of the neurotrophins with roughly equivalent

nanomolar affinity (D. Skaper, 2011; Simi & Ibáñez, 2010). Both p75NTR and Trk receptors have been shown to bind to neurotrophins individually, prompting independent signaling events. Nevertheless, enhanced trophic signaling has been observed due to the $p75^{NTR}$ interaction mediating at least a 10-fold higher affinity of the Trk receptors for its cognate neurotrophins (Conroy & Coulson, 2022). A mature form of $p75^{NTR}$ (full-length $p75^{NTR}$) is of 75-kDa, whereas an immature under-glycosylated form at 45 kDa, a cytoplasmic fragment ($p75^{NTR}$ CTF) at ~24 kDa, and an intracellular fragment (p75-ICD) at ~19 kDa has been confirmed from immunoblotting results with an antibody directed against the cytoplasmic domain of the receptor (Zampieri *et al.*, 2005).

During development, roughly 50% of the neurons in the central and peripheral nervous systems ultimately degenerate and die due to the occurrence of a cascade of cellular and molecular events (Oppenheim, 1991). $P75^{NTR}$ has been indicated to play a crucial role in regulating neuronal survival, as well as neuronal apoptosis (Bamji *et al.*, 1998). The best-characterized functions of $p75^{NTR}$ include effects on cell survival, apoptosis, and axonal growth, particularly in the neurons of the peripheral and central nervous system; moreover, its well-defined function, including mediation of apoptotic response to high doses of neurotrophins or low doses of pro neurotrophins (Cragnolini & Friedman, 2008). It is widely known that Aβ can bind to the extracellular domain of $p75^{NTR}$, and research indicates $p75^{NTR}$ positive neurites are located in the center of compact senile plaques, therefore advocating the receptor's involvement in the initiation and development of Aβ in the brain (Zhou & Wang, 2011).

$p75^{NTR}$ is widely expressed in the developing nervous system throughout the brain, in the dorsal root ganglia, and Auerbach's plexus of the enteric nervous system, and within the spinal cord, as well as in several non-neural tissues such as glomeruli of the kidney, along nerve fiber

bundles, and muscle anlagen (Ernfors *et al.*, 1991). During development, elevated levels of $p75^{NTR}$ have been detected in numerous central nervous systems, such as spinal motor neurons, brain stem motor nuclei, lateral geniculate nucleus, thalamic nuclei layer, amygdala, cortical subplate neurons, olivary pretectal nucleus, cuneate nucleus, the external granule layer, and deep nuclei of the cerebellum (Barker, 1998). Very High levels of $p75^{NTR}$ expression in motoneurons have been observed during embryonic development, whereas constant reduction of these levels is seen during the early postnatal period (Wiese *et al.*, 1999). $p75^{NTR}$ is also expressed by neurons of the peripheral and central nervous systems, Schwann cells, and various glial cell populations such as oligodendrocytes, microglia, astrocytes, and olfactory ensheathing cells (OECs) (Cragnolini & Friedman, 2008). In the adult nervous system, $p75^{NTR}$ is highly downregulated. In contrast, the expression of the receptor increases drastically in both the peripheral nervous system (PNS) and the central nervous system (CNS) following injury or disease (Meeker & Williams, 2015). In addition, there is a strong upregulation of $p75^{NTR}$ in many neurons and glial cells following injury (Kraemer, Yoon, *et al.*, 2014).

Besides neuronal survival and cell death, widely evident physiological roles of the $p75^{NTR}$ include modulation of neurotrophic responses through the collaboration with Trk receptors. For instance, $p75^{NTR}$ has been recognized to increase TrkA's ability to bind and respond to low NGF levels (Barker, 1998). Assorted studies have indicated that numerous cell populations express Trk receptors without $p75^{NTR}$. Nonetheless, most cells that express $p75^{NTR}$ also express TrkA, TrkB, or TrkC, further suggesting $p75^{NTR}$ functions by modulating Trk activation (Bothwell, 1995). Previous research has established that altered expression of $p75^{NTR}$ may lead to compromised high-affinity NGF binding of the TrkA receptor (Benedetti *et al.*, 1993). p75NTR promotes TrkA signaling through NGF and inhibits TrkA binding with NT-3.

Nevertheless, the downregulation of $p75^{NTR}$ contributes to TrkA signaling through NT-3 (Benedetti *et al.*, 1993). Moreover, TrkB has the ability to bind to BDNF, NT-3, and NT-4/5, but in the presence of $p75^{NTR}$, functional response is mediated only through BDNF binding (Bibel, 1999). Although studies suggest the interaction of $p75^{NTR}$-ECD with NGF and Trk receptors forming a complex, mounting evidence indicates that the Chopper domain of $p75^{NTR}$ is sufficient to promote TrkA and TrkB trophic activity (Conroy & Coulson, 2022). Studies suggested that p75NTR in its monomeric form induces apoptotic induction. However, this effect is barred through dimerization and higher-order receptor multimerization (Wang *et al.*, 2000).

Similar to TNF and Fas receptors, the general assumption remained that p75NTR mediates cell death. However, various experiments have confirmed a different apoptotic signaling pathway by $p75^{NTR}$ (FLee *et al.*, 2001). Sortilin, a member of the Vps10p family, recognized as a sorting receptor, has been implicated in binding pro-NGF molecules with high-affinity (Bronfman & Fainzilber, 2004). The pro-forms of neurotrophins typically induce neuronal apoptosis during development, as well as under pathological conditions or aging, and sortilin has been recognized to bind to the pro-form of NGF and other precursor neurotrophins inducing cell death due to acute or chronic distress of the nervous system (Nykjaer & Willnow, 2012). One-way $p75^{NTR}$ signals apoptosis is through the simultaneous interaction with pro-NGF and sortilin, and proNGF-induced $p75^{NTR}$-mediated pro-apoptotic signal is governed by sortilin acting as a co-receptor of $p75^{NTR}$ (Nykjaer *et al.*, 2004).

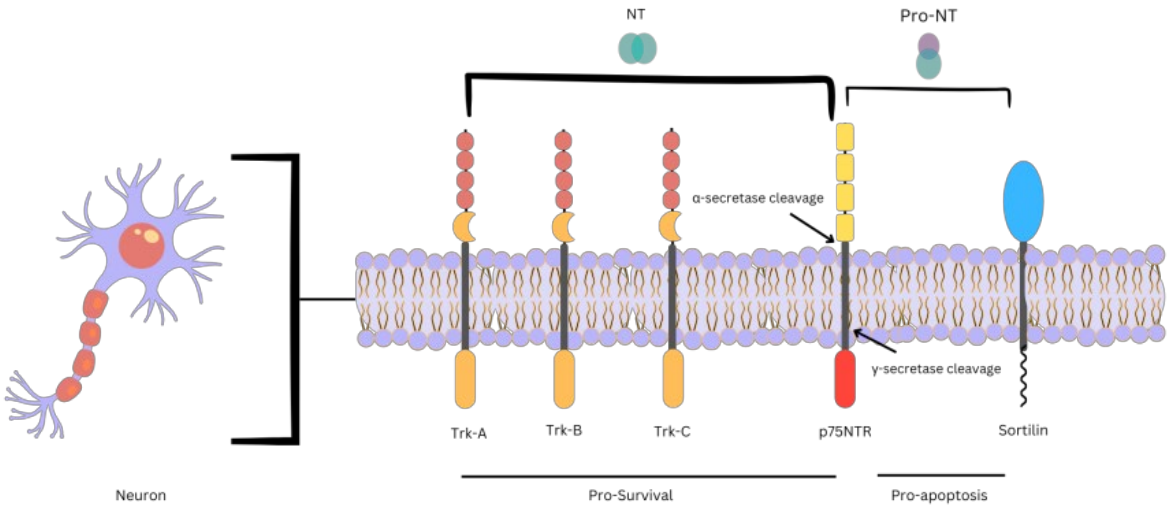


Figure 1.2 Schematic Representation of p75^{NTR} and its Co-Receptors.

p75^{NTR} can interact with all of the Trk receptors and sortilin. NTs' interaction with p75^{NTR} and Trk receptors induces pro-survival signaling, whereas pro-NTs interact with p75^{NTR} and sortilin, inducing pro-apoptotic signaling.

1.4 Structure of p75^{NTR}

The p75^{NTR} was the first identified member of the TNF receptor superfamily, and the distinguishing feature is the presence of tandem arrays of an extracellular motif containing six cysteines and functions as the ligand binding domain (Barker, 1998). Similar to its family of receptors, p75^{NTR} contains a negatively charged four-repeat cysteine-rich extracellular domain, a stalk domain, a single-span helical transmembrane domain, and a noncatalytic cytoplasmic domain consisting of the juxta-membrane domain and a death domain which is highly conserved among species (Dechant & Barde, 2002; Yano & Chao, 2000). The interaction between neurotrophins and p75^{NTR} occurs through the extracellular region's four-repeat cysteine-rich domain (CRD) (Baldwin & Shooter, 1995; Shamovsky *et al.*, 2008a). Besides increasing ligand-receptor selectivity, the p75NTR intracellular domain consists of a death domain similar in sequence to the intracellular domains of the Fas and p55 TNF receptors (Lee *et al.*, 2001). Two

novel genes encode proteins that exhibit considerable sequence homology to $p75^{NTR}$ in both cytoplasmic and transmembrane domains, establishing a gene subfamily referred to as neurotrophin receptor homolog 1 (NTH1) and NRH3 (Hutson & Bothwell, 2001).

The primary structure of the extracellular domain of human $p75^{NTR}$ is highly homologous to that of the mouse. All fragments of the ECD domain have a 100% homology with the amino acid sequences of the mouse $p75^{NTR}$-ECD, except for the fragment containing amino acid sequences 115-122, only differing in two amino acid substitutions (Bobkova *et al.*, 2015). Available evidence implicates areas close to residues Trp 21, Val 22, and residues 25-33 are determinants of the receptor-binding with NGF. Moreover, charged residues Asp 30-Lys 34 that are close to and around the NGF β-hairpin loop 1 may have a role in binding to the acidic $p75^{NTR}$ (McDonald *et al.*, 1991). Experimental results confirmed that residues with a positively charged side chain (25-36) within the NGF β-hairpin loop 1 make contact between NGF and $p75^{NTR}$ and are crucial for the stability, receptor binding, and biological activity of the NGF molecule (Ibáñez *et al.*, 1992). Data obtained through the NMR structure of the p75NTR transmembrane domain indicate that this domain constitutes an alpha helix dimer, where the Cys257-Cys257 disulfide bond drives the dimerization, thus stabilizing weak $p75^{NTR}$ transmembrane dimers (Nadezhdin *et al.*, 2016). The cytoplasmic domain of the $p75^{NTR}$ contains the chopper domain (residues 277-308), a linker region (309-338), and a death domain (DD) (residues 339-417) (Goncharuk *et al.*, 2020) (Figure 3). The extracellular, transmembrane, and intracellular regions of human p75NTR are completely homologous to that of chick and rat; thus, this degree of conservation suggests that these domains possibly play important roles in the $p75^{NTR}$ function (Barker, 1998).

Even though active NGF is characterized by a homodimeric structure containing two potential binding sites, structural evidence suggests its exclusive interaction with only one $p75^{NTR}$ molecule, consequently prohibiting the binding of another. The 2:1 NGF/$p75^{NTR}$ complex is tethered together through two spatially separated binding epitopes where the site I epitope is formed between the first and second CRDs of $p75^{NTR}$ and the top end of the NGF dimer. In contrast, the site II epitope forms the bottom end of the NFG dimer and the junction between the CRD3 and CRD4 regions of the $p75^{NTR}$ extracellular domain (He & Garcia, 2004). The Crystal structure of $P75^{NTR}$ with NGF and NT-3 study suggests the formation of a dimeric complex resulting in a 2:2 ligand-receptor stoichiometry, the native and common mode of ligand binding to glycosylated $p75^{NTR}$, whereas 2:1 complex formation is the result of artificial receptor deglycosylation (Gong *et al.*, 2008). All four cysteine repeats of $p75^{NTR}$ have been implicated to be required for binding with the neurotrophins, the second CRD repeat was found to play the most direct and critical role (Baldwin & Shooter, 1995). Amino acid residues in the β-hairpin loop 30-34 in NGF were found to participate in $p75^{NTR}$ binding, where Lys32 makes the strongest contact. Additionally, results confirmed that NGF residues in regions 25-36 are involved in the stability and receptor binding (Ibáñez *et al.*, 1992).

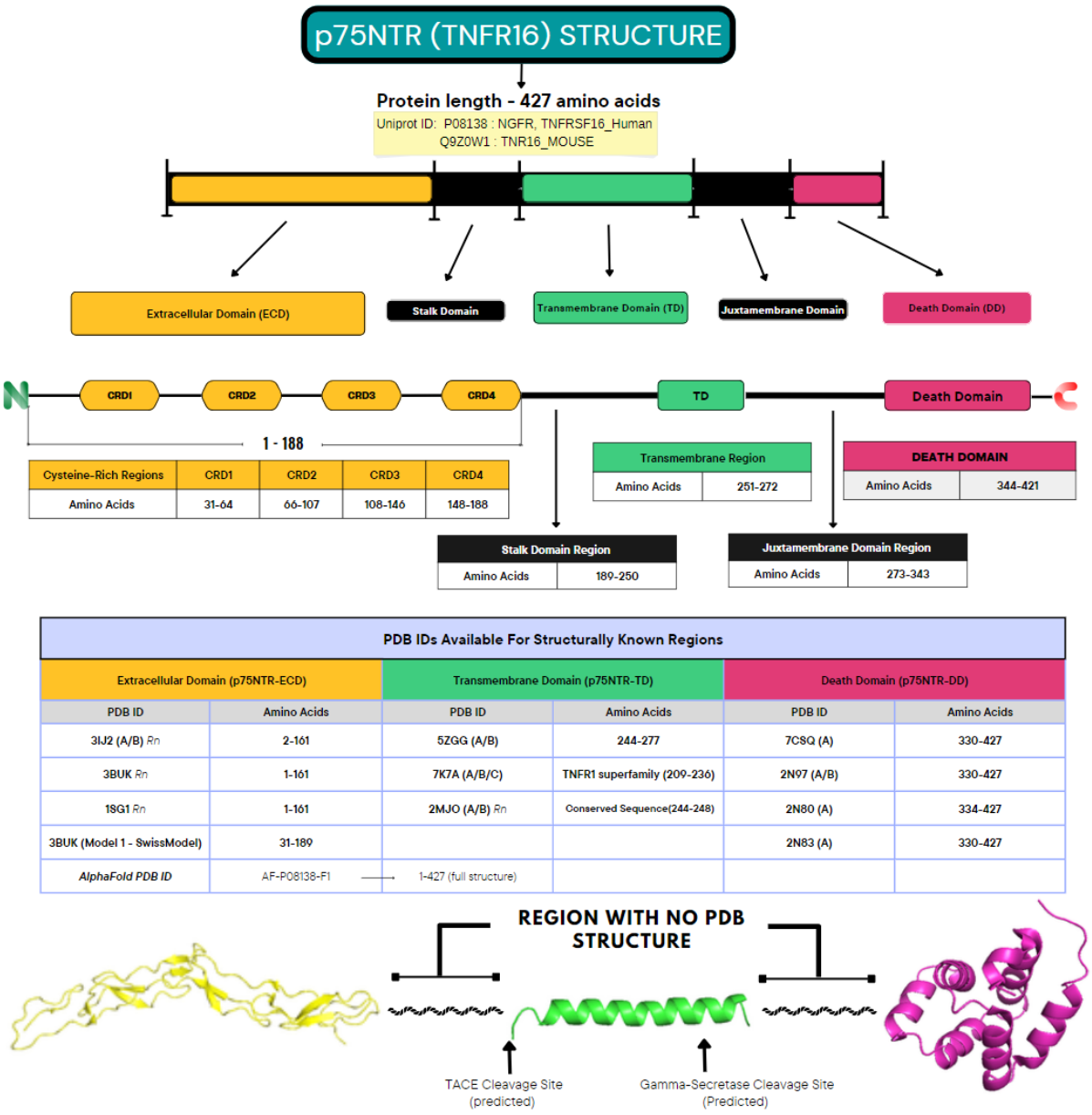


Figure 1.3 Graphical Table Representing p75^{NTR} Structure.

The table presents the receptor's various domains and their available crystal structures in PDB. The stalk domain and the juxtamembrane domain do not possess available structures.

1.5 Regulated Intramembrane Proteolysis (RIP) of p75^{NTR}

Numerous essential membrane glycoproteins undergo proteolytic cleavage of the extracellular domain, releasing them from the cell surface into the extracellular fluid (Zhao *et al.*,

2001). Metalloproteases release several cell surface proteins in response to phorbol ester stimulation, and $P75^{NTR}$ also goes through ectodomain shedding, similar to other receptors such as the p55 TNF receptor and interleukin-6 receptor, and several other proteins, including the amyloid precursor protein, Notch, and Delta (Weskamp *et al.*, 2004). $P75^{NTR}$ undergoes proteolytic processing due to the sequential action of α-secretase and γ-secretase, generating soluble ICD fragments (Kanning *et al.*, 2003). ADAM17, or α-secretase, is a prototypical member of a disintegrin and metalloproteinase family of metzincin proteases, which primarily functions to "shed" cell surface-bound molecules such as EFG receptor ligands, or TNFα (Stawikowska *et al.*, 2013). The cleavage of $p75^{NTR}$ by the metalloproteinase, TACE/ADAM17, produces a soluble extracellular domain, the carboxyl-terminal fragment (CTF), and the released CTF is further processed by gamma-secretase within the transmembrane domain, to release the intracellular domain (ICD) into the cytosol (Kanning *et al.*, 2003). γ-secretase is a large protein complex with an unusual aspartyl protease activity that cleaves various substrates within the transmembrane domain, including APP, Notch, and ErbB4 (Jung *et al.*, 2003). Presenilin-dependent gamma-secretase activity has been shown to be responsible for the intramembrane proteolysis of $p75^{NTR}$, playing a role in the formation or disassembly of $p75^{NTR}$ and Trk receptor complex (Jung *et al.*, 2003).

Study analysis identified that a 15-amino acid region of $p75^{NTR}$ is sufficient for inducing α-secretase cleavage, and this cleavage is required for the subsequent γ-secretase cleavage (Zampieri *et al.*, 2005). The study indicated that TACE-induced RIP of $p75^{NTR}$ along with NgR fragmentation suppresses Rho-A activation and EGFR phosphorylation and the ECD fragments acting as inhibitory signaling antagonists, consequently, p75ECD competitively blocking NgR/$p75^{NTR}$ clustering (Ahmed *et al.*, 2006). $P75^{NTR}$ has been suggested as a co-receptor of

Nogo-66 receptor (NgR), a leucine-rich repeat (LRR) protein containing eight LRRs flanked by cysteine-rich regions, that has been found to bind to three characterized proteins: Nogo, MAG, and OMgp promoting axonal growth cone collapse, and studies have demonstrated $p75^{NTR}$ fraction associated with NgR (McGee & Strittmatter, 2003). In sympathetic neurons, BDNF binding to $p75^{NTR}$ has been proposed to be necessary and sufficient to up-regulate the metalloprotease TACE/ADAM17, resulting in ectodomain shedding of the receptor and, subsequently, by the gamma-secretase releasing $p75^{NTR}$-ICD which then triggers the activation of JNK3, ultimately resulting in cell death (Kenchappa *et al.*, 2010).

1.6 Intracellular $p75^{NTR}$ Interactors

Diverse effects exerted by $p75^{NTR}$ occur due to the activation of several signaling pathways such as Ras homolog gene family, member A (RhoA), Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFkB), which occurs due to direct association of upstream proteins with $p75^{NTR}$ intracellular domain (Verbeke *et al.*, 2013). Numerous adaptor proteins have been reported to bind to $p75^{NTR}$. Three different proteins, NRIF, NADE, and NRAGE, have been indicated to influence apoptosis in immortalized cell lines, whereas other proteins such as RhoA GTPase, Schwann cell factor-1 (SC-1), and NRAGE influence neurite elongation and growth arrest (Lee *et al.*, 2001). The CTF fragment of $p75^{NTR}$ is deemed as a transitional product that is converted to the soluble ICD fragments, able to activate various signaling pathways promoting cell survival, cell death, or even cell invasion (Verbeke *et al.*, 2013). Numerous studies have suggested dimerization of $p75^{NTR}$. However, a recent study found no interaction between the death domains (DDs) of $p75^{NTR}$. This interaction causing dimerization of $p75^{NTR}$ probably occurs due to helper proteins and ligand binding in the complex of two p75NTR molecules, releasing the “helper” and eventually restoring the ability of $p75^{NTR}$

dimer to interact with adapter proteins (Goncharuk *et al.*, 2020). $P75^{NTR}$ interaction with NGF has been suggested to trigger NF-kB, activate JNK, and release ceramide, and in some systems, apoptotic signaling induced by $p75^{NTR}$ is dependent on NGF binding (X. Wang *et al.*, 2001).

$p75^{NTR}$ ICD has been shown to induce signaling to regulate several cell-type-specific physiological functions. In cerebellar granular neurons, the ICD has been shown to inhibit MAG (myelin-associated glycoprotein)-induced cell death; it mediates proapoptotic ligand-induced cell death in sympathetic neurons and also mediates neuronal cell survival interacting with Trk receptors (McGee & Strittmatter, 2003). The intracellular domain of $p75^{NTR}$ has been reported to bind to several proteins such SC-1, NRIF, NADE, ERKs, FAP-1, caveolin-1, NRAGE, and the GTPase RhoA (X. Wang *et al.*, 2001). In general, nine proteins have been identified to be able to interact with the intracellular domain of $P75^{NTR}$. However, NADE is the only protein that has been shown to interact with the death domain of the receptor-initiating cell death (Goncharuk *et al.*, 2020). Furthermore, the Ran-binding protein (RanBPM), has also been recognized to interact with the cytoplasmic domain of p75NTR, triggering apoptosis (Bai *et al.*, 2003).

1.7 Parkinson's Disease

Parkinson's disease (PD), first described by Dr. James Parkinson in 1817 as a "shaking palsy", is a chronic, progressive neurodegenerative disease defined by both motor and nonmotor features (DeMaagd & Philip, 2015). PD is recognized as the second most common neurological disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) in the midbrain (Emamzadeh & Surguchov, 2018) (Figure 1.4). Furthermore, the aggregation of α-Synuclein and the formation of Lewy-body are recognized as the primary pathogenic mechanisms underlying all cases of Parkinson's Disease (Bloem *et al.*, 2021; Spillantini *et al.*, 1997). The majority of PD cases are sporadic, with less than 10% having a

distinct familial origin (De Lau & Breteler, 2006; Thomas & Beal, 2007). To date, genetic studies have associated parkinsonism with proteins involved in lipid and vesicle dynamics (α-Synuclein), the ubiquitin-proteasome system (parkin and UCHL1), MAPKKK signaling (LRRK3), oxidative stress and mitochondrial function (DJ1, PINK1, parkin), and microtubule stability (tau) (Farrer, 2006). Toxic pesticide exposure has also been associated with PD, with specific compounds commonly used as pesticides such as rotenone, paraquat, and the combination of paraquat with maneb or other dithiocarbamates have shown to induce dopaminergic degeneration in the substantia nigra, and motor abnormalities in experimental animals at high doses (Ascherio *et al.*, 2006). Moreover, severe parkinsonism has been associated with 1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), a commercially available compound that is also a by-product in the synthesis of 1-methyl-4-phenyl-4-pro-pionoxy-piperidine (MPPP), a meperidine analog (Langston *et al.*, 1983). Recent findings suggest that the overall prevalence of Parkinson's disease was 572 per 100,000 among persons ages 45 and older, with a notably higher incidence observed among males (Willis *et al.*, 2022). In addition to presenting significant health challenges to an individual, PD contributes substantially to the financial strain, incorporating both direct medical and indirect non-medical expenses. In 2017, the comprehensive economic burden of PD in the U.S. amounted to $51.9 billion, comprising $25.4 billion in direct medical costs and $26.5 billion in indirect and non-medical costs, and is predicted to grow to approximately $79.1 billion by 2037, primarily due to the increasing aging population (W. Yang *et al.*, 2020).

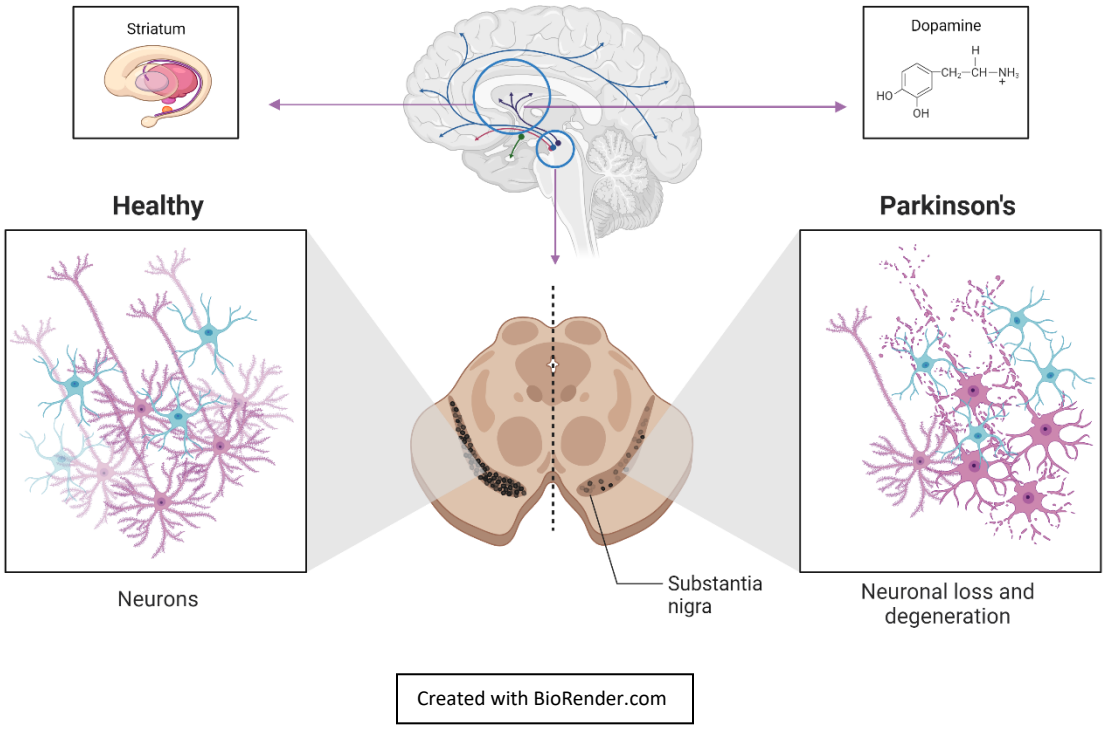


Figure 1.4 Representation of Healthy Brain vs. Parkinson's Patient's Brain.

The figure shows Substantia nigra (SNc) consisting of dopaminergic neurons in a healthy vs Parkinson's brain. The dopaminergic neurons have their cell body in the SNc and project to the striatum, where dopamine is released.

Parkinson's disease is linked to a range of motor symptoms, including tremor, rigidity, akinesia or bradykinesia, and postural instability, along with a spectrum of non-motor symptoms, including but not limited to depression, anxiety, fatigue, hyposmia (lack of smell), rapid eye movement sleep behavior disorder, constipation, urinary urgency, cognitive impairment, and dementia (Connolly & Lang, 2014; Patel & Chang, 2015). Typically, the emergence of PD is subtle, characterized by an asymmetrical and progressively advancing onset, as neuronal dysfunction and cell loss lead to a significant depletion of dopamine in the striatum, a pivotal component of the basal ganglia that is crucial for the initiation and regulation of movement (Farrer, 2006). The onset of PD motor symptoms typically begins in one limb segment due to the

decrease in dopamine concentrations below 60-70% in the contralateral striatum, leading to features like akinesia, bradykinesia, rigidity, and tremor, primarily attributed to dysfunction in the motor circuit associated with dopamine loss in the posterior putamen (Rodriguez-Oroz *et al.*, 2009). The distinctive motor symptoms of PD are also linked to the loss of pigmented cells in the substantia nigra, a nucleus situated in the ventral midbrain, and a decrease of the neurotransmitter dopamine in the striatum. This neuronal loss is believed to be influenced by the action of free radicals and environmental factors (Dawbarn & Allen, 2003).

In Parkinson's disease, neuronal degeneration initiates in dopaminergic terminals and advances towards the soma; early stages are characterized by a rapid decline in the terminals within the striatum, whereas the neuromelanin-laden dopamine neuron in SN exhibits variable density initially, transitioning to gradual and less variable loss in advanced stages (Furukawa *et al.*, 2022). The dendrites of the nigrostriatal dopaminergic neurons possess the ability to synthesize, store, and release DA, and several neuronal pathways projecting to the SN influence directly or indirectly, through local microcircuits, the release of DA from the dendrite (Cheramy *et al.*, 1981). Consequently, the degeneration of neuromelanin-laden dopamine neurons in the SN might be associated with motor deterioration in advanced stages of PD (Furukawa *et al.*, 2022). Immunohistochemical analysis has revealed the presence of neurotrophins and their receptors in a substantial number of substantia nigra neurons. Nonetheless, diminished levels of NGF and BDNF, as well as GDNF and CNTF, have been observed in SNCs affected by PD (Kruttgen *et al.*, 2003; Siegel & Chauhan, 2000).

The pathology in PD is widespread, affecting both the central nervous system (CNS) and the peripheral nervous system (PNS), involving well-documented neuronal loss in the SNc, locus coeruleus (LC), and the dorsal motor nucleus of the vagus (DMV), while Lewy pathology (LP) is

observed in various peripheral neurons; notable losses in the norepinephrine (NE) neurons innervating the heart and skin, and dopamine (DA) neurons of the enteric nervous system (ENS) which may contribute to common symptoms such as orthostatic hypotension, sweating (hyperhidrosis), and constipation (Sulzer & Surmeier, 2013). Various mechanisms, including oxidative stress, mitochondrial dysfunction, protein aggregation and misfolding, inflammation, excitotoxicity, apoptosis and other cell death pathways, and loss of trophic support, have been implicated as essential in PD pathogenesis. Nevertheless, no single mechanism appears to be universally primary in all cases of PD (Yacoubian & Standaert, 2009). Moreover, inflammation also appears to play a crucial role in the pathophysiological pathway of PD, with microglial activation contributing to neuroinflammation, subsequently leading to the expression of cytokines IL-1α, IL2, IL-1β, TNF-α, IL-6, TGF-β, and IFNγ which have been implicated in the degeneration of DA neurons in the Substantia Nigra Pars Compacta (Marogianni *et al.*, 2020). A profound understanding of the mechanisms contributing to the emergence and advancement of PD pathology is crucial for developing neuroprotective therapies (Yacoubian & Standaert, 2009). However, no established therapies currently modify the disease or provide neuroprotection for PD (Connolly & Lang, 2014).

Swedish pharmacologist Arvid Carlsson made a groundbreaking revelation—demonstrating that dopamine serves as a neurotransmitter capable of regulating movement—during his investigation of a novel drug known as reserpine, introduced in the mid-1950s for treating schizophrenia but associated with notable side effects such as parkinsonian symptoms, (Abbott, 2010). Following the 1960 revelation by Ehringer and Hornykiewicz, who pinpointed a substantial deficit of dopamine in the caudate nucleus and putamen while analyzing post-mortem brains of PD patients, thus Levodopa (L-DOPA) was employed for the initial treatment of

parkinsonism (Riederer & Horowski, 2023; Tolosa *et al.*, 1998). While L-DOPA attained the gold standard of anti-Parkinsonian treatment due to its ability to replenish deficient dopamine levels in the brain, it suffered from various disadvantages including a notably brief half-life, variable bioavailability through various metabolic pathways, early adverse effects like nausea, vomiting, and orthostatic hypotension, early morning akinesia, the emergence of motor fluctuations manifesting as wearing off and on-off phenomenon, as well as a heightened risk of dyskinesias leading to the appearance of motor complications (peak-dose dyskinesias) in later stage (Riederer & Horowski, 2023).

Levodopa undergoes metabolism via four major pathways: decarboxylation, O-methylation, transamination, and oxidation, while the predominant route for levodopa metabolism involves the decarboxylation of L-DOPA by aromatic amino acid decarboxylase (AAAD) (or levodopa decarboxylase) (Nutl & Fellman, 1984; Patel & Chang, 2015). Due to extensive first-pass metabolism and rapid plasma clearance by decarboxylation to dopamine, a mere 1% of an orally administered dose of levodopa enters the brain (Abbott, 2010; Hauser, 2009). Additionally, as dopamine cannot traverse the blood-brain barrier, the formulation of levodopa, in conjunction with one of two decarboxylase inhibitors, carbidopa or benserazide, is employed to reduce side effects and enhance the delivery of dopamine to the brain (Patel & Chang, 2015). Levodopa and its various formulations, synthetic dopamine agonists, monoamine oxidase-type B inhibitors, anticholinergics, and amantadine can serve as primary medications, either individually or in combination, for the initial treatment of PD (Fang & Tolleson, 2017).

Stereotactic procedures for alleviating different types of tremors seem to involve ventralis intermedius (VIM) thalamotomy predominantly (Benabid *et al.*, 1987). Thus, another treatment modality to alleviate motor symptoms in PD involves surgical intervention, such as deep brain

stimulation (DBS), which entails the implantation of programmable multi-contact electrodes into precise anatomical targets "deep" within the brain (Fang & Tolleson, 2017). While subthalamic nucleus deep brain stimulation (STN DBS) or conventional deep brain stimulation (cDBS) effectively ameliorates motor symptoms in PD patients, it has limitations, including stimulation-induced side effects like dysarthria, imbalance, and dyskinesia. Consequently, these challenges have spurred the development and sparked growing scientific interest in closed-loop, responsive, or adaptive deep brain stimulation (aDBS) (Habets *et al.*, 2018). In the context of DBS, evidence suggests an intervention-related decrease in impulse control, potentially influenced by surgical procedures and/or chronic stimulation (Janssen *et al.*, 2014). The study also affirms that STN DBS does not appear to alter or prevent cognitive decline over the course of the disease (Janssen *et al.*, 2014; Zangaglia *et al.*, 2012). Disease-modifying treatments for PD aim to impede or arrest the neurodegenerative process by safeguarding and maintaining the remaining DA-producing neurons in the brain (Nakmode *et al.*, 2023). Nonetheless, there is no definitive cure for PD; the existing treatment for PD only offers symptomatic relief, and none have been proven to rescue or regenerate damaged neurons (Sidorova *et al.*, 2019), nor to reduce or prevent the disease's progression (Nakmode *et al.*, 2023). Some of the major therapeutic challenges for PD include the development of disease-modifying treatments to impede or prevent neurodegenerative progression and the development of efficacious interventions for non-motor symptoms (Schapira *et al.*, 2017).

1.8 Oxidative Stress

Oxidative Stress (OS) characterizes an imbalance between the production of reactive oxygen species (ROS) and the biological system's detoxification capacity, resulting in a precarious state that contributes to cellular damage; in the brain, ROS primarily originates from

dopamine metabolism, mitochondrial dysfunction, and neuroinflammation (Dias *et al.*, 2013). While oxygen is crucial for life, participating in signal transduction, gene transcription, and various cellular activities, it concurrently poses a deleterious effect on biomolecules in the form of free radicals and ROS (Singh *et al.*, 2019). Various enzymes are acknowledged as potentially capable of generating ROS, with NADPH oxidase being considered one of the most significant among them (Genestra, 2007). Elevated concentrations of ROS prompt apoptotic cell death in diverse cell types, indicating that ROS play a role in cell death whenever they are produced within the apoptotic process (Genestra, 2007). While the adult human brain represents around 2% of the total body weight, it utilizes roughly 20% of the body's oxygen and glucose to generate energy in the form of ATP (Purdon & Rapoport, 2007). Hence, this organ is especially vulnerable to the repercussions of malfunction in mitochondrial energy metabolism and the resulting harmful transition. Furthermore, the catecholamine metabolism (CA) in catecholaminergic neurons serves as an additional source for ROS production (Meiser *et al.*, 2013). Free radicals, characterized by at least one unpaired electron in their outermost shell, exhibit high reactivity. They include hydroxyl (OH), superoxide ($O_2^{\cdot -}$), nitric oxide ($NO\cdot$), nitrogen dioxide ($NO_2\cdot$), peroxyl ($ROO\cdot$), and lipid peroxyl ($LOO\cdot$) (Chen *et al.*, 2012).

OS arises from an elevated presence of reactive free radicals, stemming either from an overproduction of these free radicals or a breakdown in mechanisms that regulate their accumulation (AlDakheel *et al.*, 2014; Yacoubian & Standaert, 2009). These mechanisms resulting in oxidative stress include depletion of antioxidants, defects in mitochondrial electron transport, neurotoxin exposure, and excessive oxidation of dopamine in patients given L-Dopa (Alam *et al.*, 1997). Recent studies continue to highlight the involvement of oxidative stress in the advancement of several neurodegenerative diseases, such as Alzheimer's and Parkinson's

diseases, glaucoma, and mitochondrial optic neuropathies (Xin *et al.*, 2022). Findings from clinical investigations, postmortem examinations, and animal model studies propose that the initiation and progression of PD involve mitochondrial dysfunction, oxidative stress, compromised proteasomal system, dysfunctional autophagy/mitophagy, and dysregulation of neuroinflammation (Xiao *et al.*, 2022). In both idiopathic and genetic cases of PD, oxidative stress serves as the shared fundamental molecular mechanism, triggering a cascade of molecular reactions that culminate in the selective death of neurons in the substantia nigra and their terminals in the striatum (Makletsova *et al.*, 2019). In post-mortem substantia nigra samples from individuals with PD, evidence of oxidative stress manifest through elevated malondialdehyde and lipid hydroperoxide formation, decreased levels of both reduced and total glutathione, heightened superoxide dismutase activity, increased iron levels with concurrent reduction in ferritin levels (Dexter *et al.*, 1991), as well as a decline in the activity of complex I of the mitochondrial respiratory chain (Dexter *et al.*, 1994).

Oxidant stress has been implicated in Parkinson's disease due to the convergence of four biochemical features--monoamine oxidase activity, autoxidation, accumulation of iron, and the presence of neuromelanin--around the primary site of cell death, notably affecting the dopaminergic neurons, particularly the pigmented ones in the substantia nigra (Fahn & Cohen, 1992). Monoamine oxidases (MAOs) function as oxidoreductases, deaminating catecholamines; in dopaminergic axons, MAO plays a role in retrieving released dopamine (DA) from the extracellular space, either recycling it into vesicles or degrading it (Graves *et al.*, 2020). Two key concepts outline the role of toxic oxygen species and oxidative stress in the degenerative mechanism leading to dopamine cell death in Parkinson's disease: (1) the metabolism of dopamine by MAO generates hydrogen peroxide, causing damage to neurons directly or through

subsequent conversion to hydroxyl radicals, and (2) excessive radical formation results from toxin action, exemplified by the mechanism of action of MPTP (Jenner & Jenner, 1991). Neurotoxicity induced by MPTP is primarily linked with a reduction in dopamine (DA) levels and a decrease in the activity of tyrosine hydroxylase (TH); concurrently, in the brain, MPTP undergoes conversion to the pyridine metabolite, 1-methyl-4-phenylpyridinium (MPP+) through the enzymatic action of monoamine oxidase B (Ali *et al.*, 1994). In aerobic conditions, the mitochondrial respiratory chain stands as one of the cell's most potent sources of free radicals, with enhanced production occurring through inhibition of complex I by rotenone or MPP+ and inhibition of complex III by antimycin A (Cleeter *et al.*, 1992). The production of oxygen-free radicals in MPTP toxicity is linked to altering the midpoint potential of a component within Complex I by MPP+, making it negative enough to interact with oxygen and generate the superoxide radical (Sriram *et al.*, 1997).

To gain deeper insights into the mechanisms associated with oxidative stress in neurodegenerative disorders such as Parkinson's disease, laboratory experiments involve the use of various chemicals and drugs such as 6-Hydroxydopamine, Rotenone, MPTP, Paraquat (Klintworth *et al.*, 2007), 4-hydroxy-2-nonenal (HNE) (Kraemer, Snow, *et al.*, 2014, p. 75), Hydrogen Peroxide (H_2O_2) and FeSO4 (Iron(II) sulfate) (Gambaro *et al.*, 2019). First documented in 1959, the neurotoxin 6-hydroxydopamine (6-OHDA), a structural analog of catecholamines, dopamine, and noradrenaline, has been pivotal in preclinical Parkinson's disease research due to its ability to induce toxic effects on catecholaminergic neurons (Simola *et al.*, 2007). HNE, identified as a lipid peroxidation adduct with physiological relevance to brain metabolism, stands out as one of the most cytotoxic products of lipid peroxidation (Castellani *et al.*, 2002). Studies have indicated the presence of lipid peroxidation adducts, specifically HNE

and Nε-(carboxymethyl) lysine, within Lewy bodies in post-mortem PD brain tissue (Shichiri, 2014).

1.9 Small Molecule Modulator of $p75^{NTR}$, LM11A-31

LM11A-31, [IUPAC: (2S,3s)-2-Amino-3-methyl-*N*-[2-(morpholinyl)ethyl] pentatonic acid amide], an amino acid isoleucine derivative containing a morpholino group (Knowles *et al.*, 2013), identified as a drug-like compound, was discovered by virtual screening of a compound library mimicking NGF β-hairpin loop 1, a domain that interacts with $p75^{NTR}$ (Massa *et al.*, 2006) (Figure 1.5). LM11A-31 is a water-soluble, non-peptide small-molecule modulator of $p75^{NTR}$ (Simmons *et al.*, 2014), which is orally bioavailable, has high blood-brain barrier penetration, and is associated with no known side effects (Xie *et al.*, 2021). Furthermore, LM11A-31 has been found to compete with NGF and proNGF interaction with $p75^{NTR}$ alone and not with TrkA, thus indicating this compound is a $p75^{NTR}$-specific modulator (Massa *et al.*, 2006).

LM11A-31 administration has been suggested to up-regulate survival signaling, whereas down-regulate $p75^{NTR}$-related degenerative signaling, consequently preventing neurodegeneration in in-vitro studies and animal models of multiple neurological diseases, including post-traumatic brain injury and Alzheimer's disease (Simmons *et al.*, 2016). LM11A-31 was found to inhibit pro-NGF binding and recruit survival adaptor to $p75^{NTR}$, interleukin-1 receptor-associated kinase (IRAK), consequently activating downstream AKT and NF-kB signaling (F. M. Longo & Massa, 2013). Studies indicated that $p75^{NTR}$ signaling is modulated by LM11A-31 in a manner different from that of NGF, signifying a novel approach to targeting $p75^{NTR}$ (Knowles *et al.*, 2013).

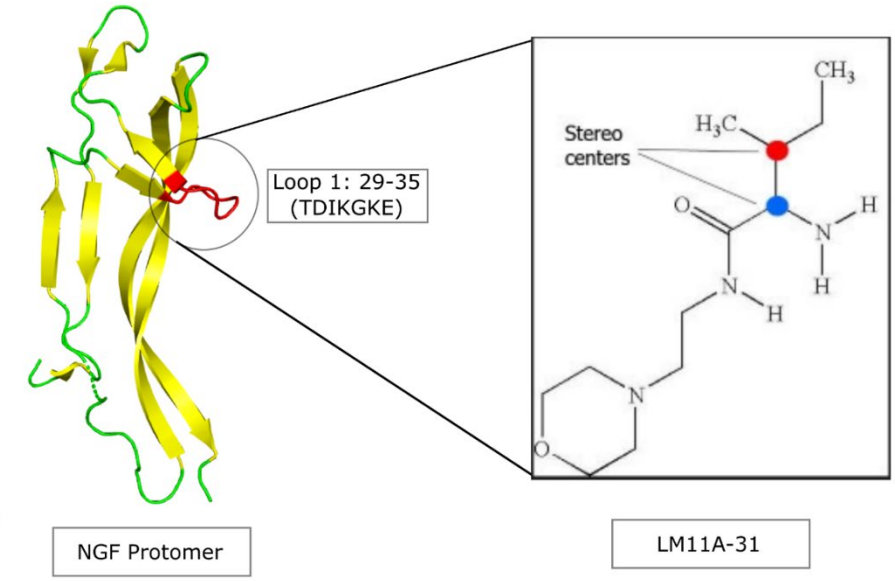


Figure 1.5 Structural Representation of NGF Loop 1 and LM11A-31.

LM11A-31 mimics loop 1 of NGF, which consists of amino acid residues 29-35 (amino acids: TDIKGKE), and this region interacts with $p75^{NTR}$.

In the context of Alzheimer's disease (AD), the compound substantially inhibited neurites and spine degeneration, reduced AD-associated forms of tau induced by Aβ, reduced Aβ-induced spino- and synaptotoxic Fyn kinase activities, and mitigated Rho-family GTPase and cofilin responses to Aβ (T. Yang, 2020). Similarly, in the case of the Huntington's Disease (HD) model, LM11A-31 mitigated intranuclear Htt aggregates and striatal DARPP-32 deficits, improved striatal cholinergic interneuron dendrite structure, reduced microglial activation, and prevented dendritic spine loss in striatum and hippocampus (Simmons *et al.*, 2016). Furthermore, LM11A-31 inhibited proNGF binding to $p75^{NTR}$, promoted motor function, increased myelin sparing in a dose-dependent manner, and inhibited $p75^{NTR}$-mediated JNK3 activation after spinal cord injury (SCI) (Tep *et al.*, 2013).

1.10 Homology Modeling

Homology modeling, or comparative modeling, is a method of computational structure prediction used to determine 3D protein structure from its amino acid sequence based on its template with known 3D structure. It is considered the most accurate structure prediction method (Muhammed & Aki-Yalcin, 2019). Structural biology follows the sequence-structure-function paradigm, which states that protein structure is determined by its sequence, and structure determines its function (Koehler Leman *et al.*, 2023), and thus homology modeling is based on the observation that proteins sharing amino acid sequence similarity/identity also share similar/identical structures (Gromiha *et al.*, 2019). Homology modeling generates a structural model of a protein using evolutionary-related structures (templates) (Biasini *et al.*, 2014). It consists of four major steps, including fold assignment (identification of similarity between the target and its known template), sequence alignment between the target and the template sequences, building a model based on the above facts, and finally, predicting model errors (Webb & Sali, 2014). Homology modeling uses the following steps to obtain the 3D structure of a protein: (i) using the BLAST search to identify the proper template for the provided target sequence, (ii) aligning sequences, (iii) correcting alignment to ensure the arrangement of the conserved or functionally important residues, (iv) generating backbone, (v) modeling loop, (vi) modeling side chain using rotamer libraries, (vii) using energy minimization to optimize the model, and (viii) using stereochemical evaluation such as Ramachandran plot as well as favorable energies to validate the model (Gromiha *et al.*, 2019).

SWISS-MODEL (http://swissmodel.epasy.org), the first fully automated protein homology modeling server, is one of the most widely used structure modeling servers in the world, currently generating over 3000 models a day (Waterhouse *et al.*, 2018). The modeling

workflow in SWISS-MODEL consists of five major steps: 1) data input, where the target protein amino acid sequence is provided, either in FASTA or UniProtKB accession code; 2) template search, where evolutionary-related protein structures are searched against the SWISS-MODEL template library SMTL using the data provided in the previous step; 3) template selection, where various templates are provided and ranked according to the expected quality of the resulting models, as estimated by Global Model Quality Estimate (GMQE) and Quaternary Structure Quality Estimate (QSQE); 4) model building, a 3D protein model is generated for each selected template; and 5) model quality estimation, where QMEAN scoring function is used to quantify modeling errors, thereby estimating expected model accuracy (Waterhouse *et al.*, 2018). SWISS-MODEL server highlights a user-friendly web interface, providing reliably accurate models and the expected accuracy of all generated models in the form of a QMEAN score. Furthermore, CAMEO (Continuous Automated Model Evaluation) continuously monitors the overall accuracy of SWISS-MODEL (Biasini *et al.*, 2014).

1.11 Intrinsically Disordered Regions

Proteins possess specific functions due to their ability to uniquely fold into correct three-dimensional structures, which was a long-standing belief, but multiple proteins are highly flexible or structurally disordered and aren't structured throughout their entire lengths (Uversky, 2013). Proteins vary tremendously in their structure, from securely folded single domains to highly flexible or disordered regions containing multiple domains, to disordered but compact, and to extremely extended, heterogeneous, unstructured states (Dyson & Wright, 2005). Intrinsically disordered protein (IDP) sequence is characterized by the combination of high net charge (causing strong electrostatic repulsion) and low mean hydrophobicity (causing low driving force for protein compaction). Comparison of ordered proteins and domains, IDPs/IDRs

(intrinsically disordered regions) revealed significantly lower numbers of order-promoting amino acids, such as Ile, Leu, Val, Trp, Tyr, Phe, Cys, and Asn, and substantially higher numbers of disorder-promoting amino acids, Ala, Arg, Gly, Gln, Ser, Glu, Lys, and Pro (Uversky, 2013). Similar to ordered/globular proteins, the same physical forces govern the structural organizations of IDPs/IDRs and from a genomic standpoint, multiple gene sequences have been shown to code for functional long amino acid sequences that could either attain a non-globular conformation or exist as IDPs/IDRs as unfolded entities in solution (Trivedi & Nagarajaram, 2022).

Studies have suggested that unstructured proteins/regions do not possess enzymatic activity. Their functions are linked to their structural disorder, which is divided into five broad classes, including entropic chains, molecular recognition, scavenging (storing and/or neutralizing small ligands), assembling (stabilizing and regulating large multiprotein complexes), and mediating regulatory posttranslational modification (such as phosphorylation or limited proteolysis) (Tompa, 2002). Functional elements of IDRs can be classified into three categories: 1) Short Linear Motifs (SLiMs), or MiniMotifs or Linear Motifs (LMs), which are 3-10—-residue long peptide segments containing few highly conserved residues, 2) Molecular Recognition Features (MoRFs) that are 10-70-residue long peptide motifs and are capable of undergoing disorder-to-order transition upon binding with their partners and attain well-defined structures like α-helices (α-MoRFs), β-strands (β-MoRFs), γ-coils (γ-MoRFs), or a combination of all of these conformations (complex-MoRFs), and 3) Intrinsically Disordered Domains (IDDs) that contain long disordered regions (>20 residue) with conserved sequence and function are mostly involved in the DNA, RNA, and protein binding (Trivedi & Nagarajaram, 2022).

The intrinsic structural disorder can be detected using various experimental methods such as X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR), small-angle X-ray

scattering, circular dichroism, and Forster resonance energy transfer, each technique providing researchers a unique insight into the functional mechanisms of IDPs, such as their flexibility, folded-structure upon binding, and conformational heterogeneity. To assess IDP/IDR predictors, critical assessment of protein intrinsic disorder (CAID), a biennial experiment was organized, which represents a community-based effort to develop and implement evaluation strategies to assess (1) clear intrinsic structural disorder (ID) definition and (2) the performance of methods used in ID prediction (Necci *et al.*, 2021). Additionally, DisProt provides annotations of ~2400 IDPs/IDRs consisting of at least 10 residues with biological relevance and is considered the most comprehensive database of disordered proteins (Redl *et al.*, 2023). Various servers are available capable of predicting IDPs/IDRs computationally, each server possessing varied criteria for predicting order and disorder of structure in provided amino acid sequence, which includes PONDR (Predictor of Naturally Disordered Region), ADOPT (Attention-based DisOrder PredicTor), fLDpnn (putative function and linker-based Disorder Prediction using a deep neural network), and Albatross-colab/Metapredict.

PONDRs VLXT, VSL2, and VL3 are meta-predictors developed using combinations of individual predictors that are capable of predicting disordered proteins (Xue *et al.*, 2010). PONDR web server is available through "www.pondr.com," and it consists of various predictors, the most accurate of which is VL-XT, with a 78% success rate on predicting order and a 60% success rate on predicting disorder with a False Negative of 40% (predictor indicating "order" for known disordered regions), and False Positive of 22% (predictor indicating "disorder" for known ordered regions). ADOPT uses AI technology to accurately predict protein disorders from sequences alone. It is comprised of two blocks: a self-supervised encoder and a supervised disorder predictor. The process includes an encoder that uses information from a large sequences

database and generates feature information for every residue of the provided sequence. In contrast, a decoder predicts a disorder score utilizing the information the encoder provides (Redl *et al.*, 2023). FlDPnn is a computational tool that has been implicated in accurately predicting disorder (AUC=0.814) and the fully disordered proteins in CAID. Furthermore, it also provides putative functions for the disordered protein regions of the provided protein sequence involving the four most commonly annotated functions such as DNA-binding, RNA-binding, protein-binding, and the linkers (Hu *et al.*, 2021). Lastly, Metapredict is a stand-alone web server capable of accurately reproducing consensus disorder scores for provided protein sequences. Additionally, it also offers a structural confidence score based on folding propensity derived through AlphaFlold2 predictions, providing per-residue predicted Local Distance Difference Test (pLDDT) scores (Emenecker *et al.*, 2021, 2022).

1.12 Ab-Initio Protein Structure Prediction

A protein spontaneously folds into its native structure due to the interplay of covalent bonds and numerous non-covalent inter-residue interactions such as hydrophobic effects, hydrogen bonds, van der Waals forces, and ionic bonds. Thus, a deep understanding of the protein folding process and the relationship between protein sequences and native structures are immensely important for accurately predicting protein structures (B. Huang *et al.*, 2023). Ab initio prediction is based on the 'thermodynamic hypothesis,' which states that the free energy at the global minimum contributes to the native structure of a protein that is thermodynamically stable in its normal physiological milieu. In a given environment, the native conformation of the protein is determined by all the inter-atomic interactions of the amino acid sequence (Anfinsen, 1973; Xia *et al.*, 2000). Ab initio modeling uses the guidance of the designed energy function to

conduct a conformational search. It generates multiple possible conformations (aka structure decoys), finally selecting the final models (J. Lee *et al.*, 2017).

Numerous methods for ab initio structure prediction are readily available, including I-TASSER, RosettaFold, AlphaFold, and phyre prediction. I-TASSER, an ab initio protein modeling server, provides significant accuracy and reliability in full-length structure prediction for protein targets compared with various useful online structure prediction tools. The methods applied by I-TAASSER can be divided into four general steps: 1) **threading**, a procedure for template protein identification of the query protein sequence using a similar structure or motif from structure databases, and the quality of the template alignments is based on the statistical significance of the best threading alignment, 2) **structural assembly**, where template structures are excised from the built models to produce continuous fragments in threading alignments, and structural conformations of the aligned with the unaligned regions are assembled, 3) **model selection and refinement**, a second iteration process to remove steric clashes and to refine the global topology of the cluster centroids to generate the final structural models through optimization of hydrogen bonding networks, and 4) **structure-based functional annotation**, where the predicted 3D models are structurally matched against the proteins of known structure and function in the PDB to infer the function of the query protein (Roy *et al.*, 2010). I-TASSER constructs the final structural model from the low-energy conformations (J. Yang *et al.*, 2015) and provides a final confidence score (C-score) for the models based on the Monte Carlo simulations structure convergence and the statistical significance of the PPA threading alignments (Zhang, 2008). On the other hand, AlphaFold utilizes neural network architectures and the evolutionary, physical, and geometric constraints of protein structures to predict the 3D model structure of the provided sequence accurately. AlphaFold also provides accurate end-to-

end structure prediction by using embedding multiple sequence alignments (MSAs) and pairwise features, and the final models are generated based on the confidence measure, the predicted local-distance difference test (pLDDT) (Jumper *et al.*, 2021).

1.13 Study Significance and Overarching Hypothesis

$p75^{NTR}$ can bind with all of the neurotrophins and is involved in both pro-survival and pro-apoptotic signaling. Furthermore, the internalization of $p75^{NTR}$ into early endosomes has been observed in PC12 cells due to NGF interaction (Saxena *et al.*, 2005). Another study indicated that JNK activation and Rab5 and dynein promote the retrograde transport of the $p75^{NTR}$-endosome consisting of the full-length receptor ($p75^{NTR}$-FL) (Escudero *et al.*, 2019). Our previous study demonstrated ligand-independent activation of $p75^{NTR}$, where oxidative stress activates JNK signaling, which promotes $p75^{NTR}$ processing (Kraemer *et al.*, 2021). However, the mechanism of proteolytic processing of the receptor induced by oxidative stress occurring in an endosome is poorly understood. Here, we report that receptor internalization is required for the proteolytic processing of $p75^{NTR}$ in LUHMES cells following oxidative stress (Figure 1.6). Furthermore, our study regarding small molecule LM11A-31, modulation of $p75^{NTR}$ demonstrates a shift of $p75^{NTR}$-related signaling towards pro-survival and attenuates neurite degeneration in oxidative-stress-induced neuronal cells. We hypothesize that Oxidative stress induces internalization of plasma membrane-localized $p75^{NTR}$, thereby leading to endosomal processing of the receptor and downstream regulation of neuronal survival.

Our Hypothesis

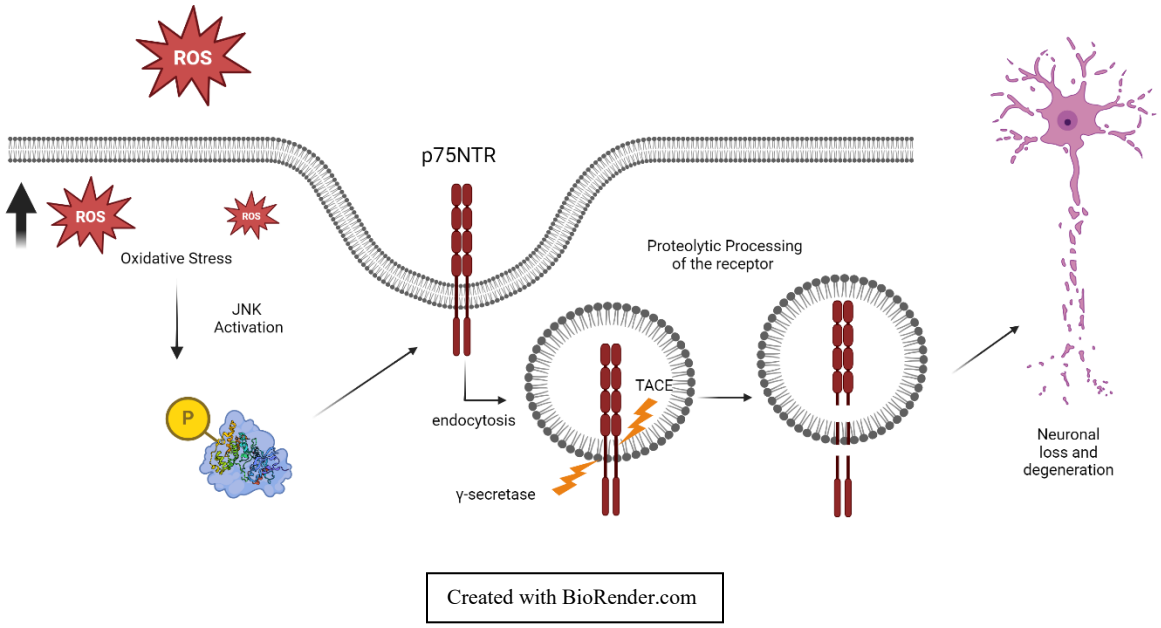


Figure 1.6 Schematic Representation of Our Hypothesis.

Elevated levels of ROS induce oxidative stress, which activates JNK signaling. JNK activation promotes the internalization of $p75^{NTR}$, further facilitating proteolytic processing of the receptor, consequently leading to neuronal cell death and degeneration.

Chapter 2. Experimental Procedures

2.1 Cell Culture

Lund human mesencephalic (LUHMES) cells (ATCC, Manassas, VA, USA, RRID: CVCL_B056) were cultured in 60 mm standard cell culture dishes (USA Scientific, Ocala, FL, USA), and in 8-well chambered slides (Lab-Tek™ II, Nunc™ Roskilde, Denmark) at 37 °C and 5% CO_2. Dishes were coated with 100 µg/mL poly-L-ornithine (Sigma-Aldrich, St. Louis, MO, USA Cat no: P3655) overnight and incubated with 2 µg/mL fibronectin (Sigma-Aldrich, Cat no: F0895) for 3 hours at 37 °C, while the chambered slides were coated with 100 µg/mL poly-L-ornithine (Sigma-Aldrich) overnight and incubated for 3 hours at 37 °C with 2 µg/mL fibronectin (Sigma-Aldrich), 100 ng/mL poly-D-lysine (Sigma-Aldrich, Cat no: P7280), and 10 µg/mL laminin (Corning, NY, USA Cat no: 354232) overnight. For cell proliferation, cells were incubated in a blend of growth medium containing Dulbecco's Modified Eagle Medium with Nutrient Mixture F-12 (DMEM/F12) (Gibco, Waltham, MA, USA Cat no: 11330057), 2 mM glutamine (VWR, Radnor, PA, Cat no: VWRL0131-0100), 1% (v/v) N-2 supplement (Gibco, Cat no: 17-502-048), and 40 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA Cat no: 233-FB). Cells received half-volume growth media change every other day. Once 60% cell confluency was reached, the cells were differentiated to post-mitotic neurons by replacing the growth medium with differentiation media containing DMEM/F12 (Gibco), 2mM glutamine (VWR), 1% (V/v) N-2 supplement, 1 mM N6, 2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate (db-cAMP) (Enzo Life Sciences, Farmingdale,

NY, USA Cat no: BML-CN125-0100), 2 ng/mL glial cell line-derived neurotrophic factor (GDNF) (R&D Systems, Cat no: 212-GD-010), and 1 μg/mL tetracycline (Sigma-Aldrich, Cat no: 87128-25G). A half-volume differentiation media change was performed every other day prior to the treatment. Cell stocks with passage numbers 5 or 6 were used for all the experiments performed to minimize and avoid genetic drift.

2.2 Cell Treatment

After 5 days of differentiation, LUHMES cells were exposed to the indicated concentrations of 6-hydroxydopamine (6-OHDA) (Sigma-Aldrich, Cat no: 162957), (2S,3S)-2-Amino-3-methyl-*N*-[2-(4-morpholinyl) ethyl] pentanamide dihydrochloride (LM11A-31 dihydrochloride) (TOCRIS, Cat no: 5046), Dynasore (Dynamin Inhibitor) (TOCRIS, Cat. No. 2897), or vehicle solution. Preparation of 6-OHDA solution was performed in cold phosphate-buffered saline (PBS) (corning, Cat no: 45000-448) containing 0.02% ascorbic acid (Sigma-Aldrich, Cat no: A5960), LM11A-31 solution was prepared in di-H_20, and Dynasore was dissolved in DMSO (VWR, CAS: 67-68-5). 6-OHDA aliquots were prepared in 0.5 mL brown epi-tubes (Fisher Scientific) under inert gas to protect from light and were stored at -80 °C. LM11A-31 aliquots were prepared in 0.5 mL clear epi-tubes and stored in -20 °C. Dynasore aliquots were prepared in 0.5 ml brown epi-tubes to protect from light and were stored at -20 °C. To assess whether the modulation of $p75^{NTR}$ attenuates cell survival in oxidative-stress-induced dopaminergic neurons, differentiated LUHMES cells were cultured in 8-well chambered slides (Nunc™) and co-treated with 10 nM or 1 nM LM11A-31 (TOCRIS) and 7.5 μM, or 5 μM 6-OHDA (Sigma-Aldrich), or vehicle solution for 24 hours. To determine whether LM11A-31 modulation of $p75^{NTR}$ protects neurons from neurite degeneration, differentiated LUHMES cells were cultured in 8-well chambered slides (Lab-Tek™) and co-treated with 10 pM, or 1 pM

LM11A-31 (TOCRIS), and 5 μM 6-OHDA (Sigma-Aldrich), or vehicle solution for 24 hours. To evaluate whether endocytosis (endosomal activity) is required for oxidative stress-induced processing of $p75^{NTR}$, differentiated LUHMES cells were cultured in 60 mm cell culture dishes (USA Scientific). They were treated with 80 nM Dynasore and 10 μM 6-OHDA (Sigma-Aldrich) or vehicle solution for 18 hours.

2.3 Cell Viability Analysis

Eight-well-chambered slides (LabTek) were used to culture LUHMES cells and were differentiated for 5 days. The cells were then treated with 7.5 μM, or 5 μM 6-OHDA (Sigma-Aldrich), or vehicle solution, or co-treated with 10 nM, or 1 nM LM11A-31 (TOCRIS) and 7.5 μM, or 5 μM 6-OHDA (Sigma-Aldrich) for 24 hours. The slides were then fixed using a 4% PFA solution. Fixed slides were immunostained using TUJ1, an antibody specific for βIII-tubulin, and further stained with DAPI (antibody specific for the Nucleus). The Zeiss LSM 800 confocal microscope system was used to capture images; five images per condition were captured. The nucleus was scored for cell viability to evaluate healthy versus dying cells regarding the appearance of nuclei. Fragmented cells, or cells with low nuclear area and chromatin condensation, were used as the scoring criteria for dying cells. To avoid biases, the obtained images were subjected to blinding (name/number change of the images to blind the treatment condition), and a blinded experimenter counted cells.

2.4 Immunostaining and Confocal Microscopy

Cultured LUHMES cells were differentiated for 5 days on 8-well-chambered slides (Nunc™). Treatments were performed as indicated, and 4% paraformaldehyde (PFA) was used to fix the cells after 24 hours. The fixed cells were permeabilized using PBS containing 0.1%

Triton™ X-100 (Sigma-Aldrich). The cells were then blocked using PBS consisting of 0.1% Triton™ X-100 and 10% goat serum for one hour. Once blocking was completed, primary antibody (α-TUJ1) specific for βIII-tubulin (Covance, Princeton, NJ: 1:500, RRID: AB-2313773) was applied and incubated overnight at 4 °C. Cells were then washed twice with PBS containing 0.1% Triton™ X-100, and two more times with PBS. Following washes, a secondary antibody coupled to Alexa Fluor 488 (Thermo Fisher Scientific; 1:1000. RRID: AB_142495) fluorophore was applied for 1.5 hours. The cells were washed four additional times with PBS. 5 µg/mL 4', 6'-diamidino-2-phenylindole (DAPI) in PBS was applied and incubated for 5 minutes, followed by three further washes with PBS. After the washes, Fluoromount-G® mounting media (Southern Biotech, Birmingham, AL, Cat no: 0100-01) was applied, and a coverslip (Fisher Scientific) was mounted over the slide. Zeiss LSM 800 confocal microscope system (Zeiss, Oberkochen, Germany) and Zen 2 software (Zeiss) were used to capture 1024 x 1024 px images of TUJ1 and DAPI immunostained cells. Image J software was utilized to quantify neurite degeneration (Refer to Figure 7).

2.5 Immunoblotting Analyses

LUHMES cells were cultured in 60 mm cell culture dishes. Treatment was performed as indicated after five days of differentiation. Cells were lysed using Np40 Buffer supplemented with a PhosStop phosphatase inhibitor mixture tablet (Roche, Cat no: 4906837001) and a Complete Mini EDTA-free protease inhibitor mixture tablet (Roche, Basel, Switzerland, Cat no: 11836170001). The lysates were subjected to sonication and clarification. Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA, Cat no: 5000006) was performed to obtain the total protein concentrations of the samples. The lysates were then denatured using SDS sample buffer (58 mM tris-HCL, 60.10 mM sodium dodecyl sulfate, 100.5 mM dithiothreitol, and 29.85 πM

bromophenol blue) by incubation at 95 °C for 5 minutes. SDS/PAGE was used to separate proteins in the cell lysates, and the BioRad Mini Trans-Blot system was utilized to transfer proteins to the PVDF membrane (Sigma-Aldrich, Cat no: ISEQ00010). The PVDF membrane was blocked in 0.5% milk in PBS supplemented with 1% Tween. Western Blot was performed using a primary antibody specific for the intracellular domain of $p75^{NTR}$ (1:3000 ratio, as previously mentioned (Kraemer *et al.*, 2021)), or TrkA (1:1000 ratio, cell signaling, Product No: 2505), or TrkB (1:1000 ratio, cell signaling, mAb#4603 2505), or TrkC (1:1000 ratio, cell signaling, mAb#3376), or Sortilin ((1:1000 ratio, cell signaling, Product No: 20681), and a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, RRID: AB-2307391). BioRad Chemidoc MP imaging system was used for visualization and image attainment of the blots subjected to enhanced chemiluminescent substrate (ECL substrate) (Thermo Fisher Scientific) or Femto Substrate (Thermo Fisher Scientific). Blots were stripped to remove primary and secondary antibodies through 10-minute incubation in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Cat no: PI21059). To verify equal protein loading, Immunoblotting was performed using an anti-β-actin antibody (Sigma-Aldrich, RRID: AB-2223041) and a peroxidase-conjugated secondary antibody (Jackson Immunoresearch Labs, RRID: AB-2307392). The blots were then visualized as previously described. Image Lab Software Version 6.0.0 (Bio-Rad laboratories Inc.) was utilized to measure the band intensities, and values were normalized to actin levels.

2.6 Statistical Analyses

All quantitative data presented were box and whisker plots. The middle line in the Box and whisker plot represents the median, the boxes occupy the 25th-75th percentiles, and the whiskers indicate the 5^{th}-95^{th} percentiles. GraphPad Prism 8.3.0 was utilized to compare statistics

between groups. The Shapiro-Wilk test was performed to verify data normality. RM one-way ANOVA was used with Tukey's multiple comparisons test for normal datasets. Multiple comparison Friedman was used with Dunn's multiple comparisons test for datasets with non-normal distribution. $p < 0.5$ values for Statistical significance between groups were accepted, and no outliers were excluded.

2.7 Study Design and Ethical Statements

Ethical approval of the performed experiments was not required since this study did not involve using animals or samples from human patients. LUHMES cells fully differentiate into dopaminergic neurons after five days of differentiation and thus were used for all the experiments to model Parkinson's Disease. This cell line has not been listed as a commonly misidentified cell line by the International Cell Line Authentication Committee. The cells were purchased from a reputable vendor (ATCC, Manassas, VA), and the authenticity of the cells was further confirmed by evaluating the expression of the neuronal marker βIII-tubulin after maturation into post-mitotic neurons.

2.8 Homology Modeling

The $p75^{NTR}$ (tumor necrosis factor receptor superfamily member 16 precursor [Homo sapiens] sequence was obtained from the National Library of Medicine (NIH) (NCBI Reference Sequence: NP_002498.1) (https://www.ncbi.nlm.nih.gov/protein/NP_002498.1). To model the extracellular domain of Human p75NTR, amino acids 1-189 were used as the target sequence. The sequence was provided in the SWISS-MODEL web server accessible through (https://swissmodel.expasy.org/interactive). Then, the "search for templates" tool was utilized to generate available templates. Out of the 50 templates generated, four templates consisting of

over 90% sequence identity were chosen to build models. The templates used were as follows: 1. Tumor necrosis factor receptor superfamily member 16 isoform X2, AlphaFold DB model of organism *Trichechus manatus latirostris (Florida manatee)* (gene: LOC101358935) (PDB ID: A0A2Y9DNL7.1.A), 2. Nerve growth factor receptor (TNFR superfamily, member 16) (PDB ID: 3IJ2.1.B), Tumor necrosis factor receptor superfamily member 16 (PDB ID: 1SG1.1.C), and Tumor necrosis factor receptor superfamily member 16 (PDB ID: 3BUK.1.C). PYMOL was used to align and superimpose the models generated by SWISS-MODEL.

2.9 IDR Prediction

The $p75^{NTR}$ (tumor necrosis factor receptor superfamily member 16 precursor [Homo sapiens] sequence was obtained from the National Library of Medicine (NIH) (NCBI Reference Sequence: NP_002498.1). The Stalk domain sequence of the $p75^{NTR}$ receptor (aa: 189-240) was provided to various servers to predict disordered regions. Four various web servers were utilized to generate the data: 1. Predictor of Natural Disordered Regions (PONDR) web server accessible through (www.pondr.com), 2. Attention-based DisOrder PredicTor (ADOPT) web server accessible through (https://adopt.peptone.io), putative function- and linker-based Disorder Prediction using deep neural network (flDPnn), and 4. Metapredict, a deep-learning-based consensus predictor of intrinsic disorder and predicted structure, is accessible on a web server (https://metapredict.net/#). Similarly, to understand whether the juxta-membrane domain of $p75^{NTR}$, the region between the transmembrane domain and the death domain in the cytosol, consists of IDR, amino acid sequence from 260-360 was provided to the webservers mentioned above. Furthermore, sequences of the extracellular domain, transmembrane domain, and death domain, for which 3D structures are available, were used to back-test the capability of the servers to predict order in the protein regions.

2.10 Ab Initio Protein Modeling

The amino acid sequence of p75NTR was obtained in Fasta format through the NCBI protein database (NCBI Reference Sequence: NP_002498.1). The Uniprot database (Protein ID: P08138 – TNR16_Human) was utilized to identify the various domains of $p75^{NTR}$ (Tumor necrosis factor receptor superfamily member 16). The stalk domain sequence (amino acid 193-247) was provided to various protein fold prediction web servers to model the stalk domain and the juxta-membrane domain of the p75NTR receptor. The web portals include: 1. I-TASSER (Protein Structure & Function Predictions), accessible through (https://zhanggroup.org/I-TASSER/), 2. Alphafold2, accessible via web address (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) RoseTTAFold, accessible through (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/RoseTTAFold.ipynb), and Phyre2, which can be accessed through (http://www.sbg.bio.ic.ac.uk/phyre2/). Each web server utilizes different scoring techniques to predict the structure of the provided amino acid sequence, and models were generated and ranked with the highest prediction score being the first model and so on. I-TASSER, AlphaFold, and RosettaFold provided 5 structural models The models generated using the above-mentioned web portals were downloaded in PDB Format, and PYMOL and/or MOE were used to evaluate the models.

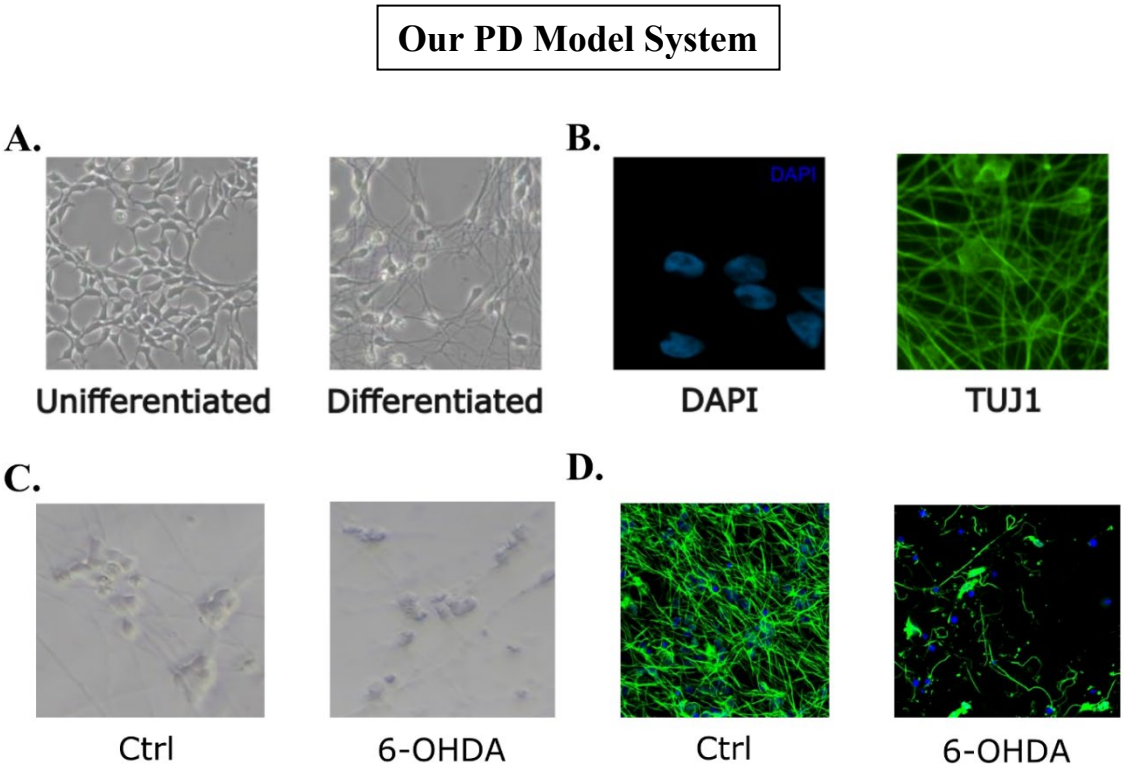


Figure 2.1 Model System Demonstrating LUHMES Cells in Various Conditions.

A; Images representing undifferentiated LUHMES cells (left) or LUHMES cells cultured in differentiation medium for 5 days (right), **B;** Micrographs of differentiated LUHMES cells subjected to immunostaining for β-III tubulin (right) and nuclear labeling with DAPI (left), **C;** Phase contrast image representing differentiated LUHMES cells treated with vehicle (left), or differentiated LUHMES cells treated with 7.5 µM 6-OHDA, **D;** Micrographs of differentiated LUHMES cells treated with vehicle (left) or 7.5 µM 6-OHDA (right) subjected to immunostaining for β-III tubulin and nuclear labeling with DAPI.

Chapter 3. Results

3.1 Receptor Internalization Is Required for Oxidative Stress-Induced p75^{NTR} Processing

Our previous study showed that JNK signaling is required for the cleavage of p75^{NTR} into its constituent fragments in response to oxidative stress in neuronal cells from the ventral mesencephalon (Kraemer *et al.*, 2021). Furthermore, studies of other cell types have suggested that proteolytic processing of p75^{NTR} occurs in the endosome (C. Escudero *et al.*, 2014; Zanin *et al.*, 2019). The expression of p75^{NTR} in cultured LUHMES cells has been confirmed in our previous studies, with the protein located in regions closer to the axon hillock, soma, and distal neurites. Therefore, in the present study, we investigated whether endosomal activity is required for oxidative stress-induced cleavage of p75^{NTR} in neuronal cells derived from the ventral mesencephalon. Lund Human Mesencephalic (LUHMES) cells derived from healthy, eight-week-old human mesencephalic tissue were used to conduct these investigations. These cells are a population of cells that were conditionally immortalized by introducing a tetracycline-responsive v-myc gene (TET-off). LUHMES cells differentiate into mature, post-mitotic neurons after incubation in a differentiation medium containing tetracycline, cyclic AMP (cAMP), and glial-derived neurotrophic factor (GDNF) (Zhang *et al.*, 2014). We cultured LUHMES cells in 60 mm dishes and transitioned them into differentiation media after reaching ~60% confluency. Phase contrast microscopy was used to analyze differentiated cell cultures for neurite growth to validate the maturation state of LUHMES cells (Figure 7). Five days of differentiation produced

uniform neurite growth among cell cultures Differentiated LUHMES cells were exposed to 6-OHDA (6-hydroxydopamine), a neurotoxin that has been shown to promote oxidative damage in catecholaminergic neurons and is frequently used to model Parkinson's disease. Exposure to 6-OHDA has been shown to induce proteolytic processing of $p75^{NTR}$ in cultured LUHMES cells (Kraemer *et al.*, 2021). Thus, cultures were treated with either 6-OHDA or vehicle for control, or cells exposed to 6-OHDA were co-treated for 18 hours with Dynasore (Dynamin inhibitor) to block the internalization of the $p75^{NTR}$ receptor into an endosome. The cells were then lysed and clarified, and immunoblotting was performed using an antibody specific to the intracellular domain of $p75^{NTR}$. Compared to lysates of cells treated with 6-OHDA alone, a significant decrease ($p = 0.0226$) of the p75NTR-C-terminal fragment (p75NTR-CTF) and p75NTR-intracellular domain (p75NTR-ICD) ($p = 0.0089$), as well as recovery of full-length p75NTR ($p = 0.0496$), was detected in lysates of cells co-treated with Dynasore and 6-OHDA in comparison with 6-OHDA (n=7) (Figure 3.1). These data indicate that receptor internalization is required for oxidative stress-induced $p75^{NTR}$ processing.

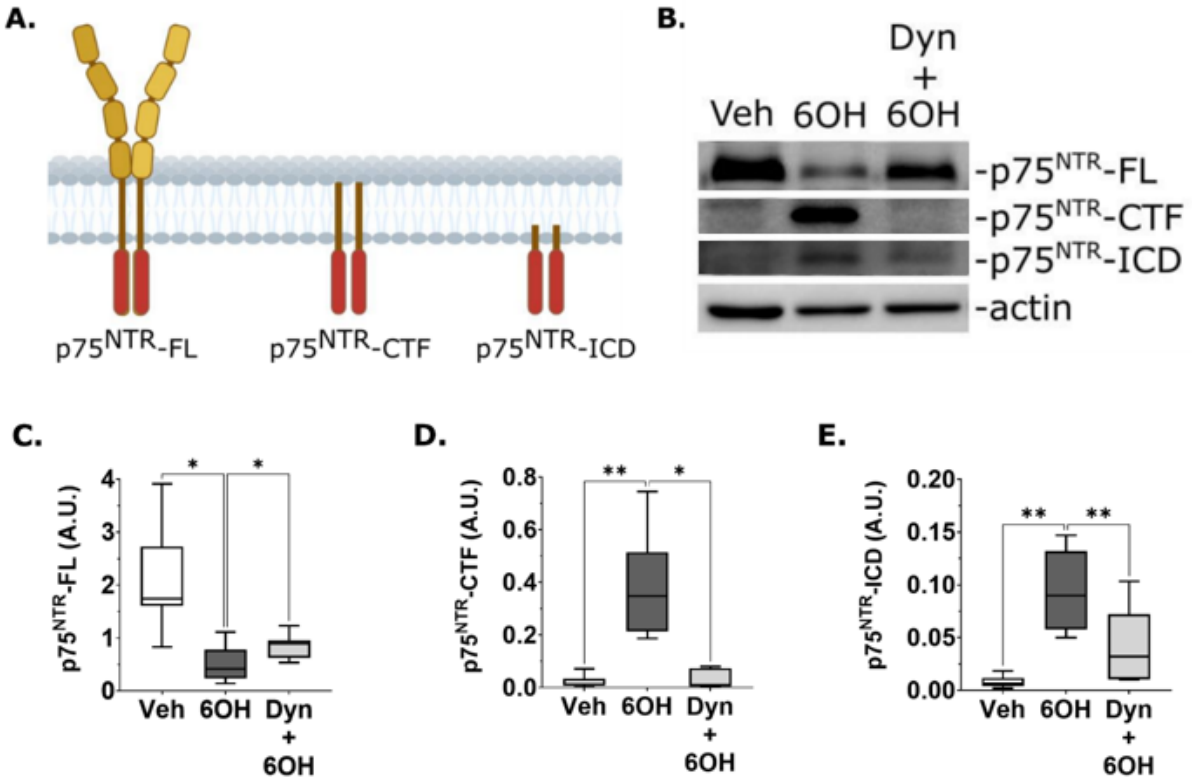


Figure 3.1 Receptor Internalization is Required for Oxidative Stress-Induced $p75^{NTR}$ Processing.

A; Schematic diagram representing receptor fragments produced by regulated intramembrane proteolysis of $p75^{NTR}$, **B;** Differentiated LUHMES cells were treated with vehicle, 10 µM 6-OHDA (6OH), and 10 µM 6-OHDA + Dynasore (Dyn+6OH), for 18 hours. Lysates were then subjected to western blot analysis using an antibody specific for the $p75^{NTR}$-ICD. Immunoblotting for actin was performed as a loading control, **C, D & E;** Quantification results for $p75^{NTR}$ full length (left), $p75^{NTR}$ cytoplasmic fragment (middle), and $p75^{NTR}$ intracellular fragment (right).

3.2 Pharmacological Modulation of $p75^{NTR}$ Reduced Neurite Degeneration and Death Associated with Oxidative Stress

Oxidative stress is known to be one of the major factors responsible for the pathogenesis of almost all neurological disorders. It has been shown to promote alterations in biochemical and biomolecular components, eventually leading to various neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (Behl *et al.*, 2021). Our previous study confirmed that 6-OHDA induces oxidative stress in neuronal cells, leading to neurite degeneration (Clements *et al.*, 2022) and cell death (Kraemer, Snow, *et al.*, 2014). LM11A-31, originally derived as a small, nonpeptide mimic of nerve growth factor (NGF), was found to have pro-survival effects by selectively inhibiting the binding of proNGF and NGF to p75NTR-at higher concentrations and induce $p75^{NTR}$-dependent NFκB and AKT activations. Furthermore, LM11A-31 stimulated proliferation, survival, and differentiation in Hippocampal NPC cultures (Shi *et al.*, 2013). Recent studies have suggested a therapeutic role for the p75 neurotrophin receptor as proNGF/p75NTR modulation with a small molecule LM11A-31 was shown to improve stroke recovery, chronic brain metabolism, post-traumatic brain injury (Shi *et al.*, 2013), and acute ischemic injury in mice (Nasoohi *et al.*, 2023). Additionally, LM11A-31 exhibited favorable brain bioavailability and possessed neuroprotective effects in the APP mice model ((Knowles *et al.*, 2013). Thus, to investigate whether modulating $p75^{NTR}$ attenuates neurite degeneration and cell death associated with oxidative stress in LUHMES cells, we administered LM11A-31 in 6-OHDA exposed cultures. LUHMES cells cultured in 8-well chambered slides were treated with vehicle solution for control or 6-OHDA or co-treated with 6-OHDA and varying doses of LM11A-31 after full maturation to evaluate the effects of $p75^{NTR}$ on cell survival and neurite degeneration. The cells were fixed 24 hours after

treatment and stained with DAPI (4',6-diamidino-2-phenylindole), a DNA-specific fluorescent probe capable of staining the nucleus (Kapuscinski, 1995), and TUJ1 (class III β-tubulin), a biological marker for neural differentiation (S. Lee *et al.*, 2005). Then, confocal microscopy was utilized to capture images of nuclei and neurites. The nuclei were then scored as healthy or dying to analyze cell survival using ImageJ software. Furthermore, neurite degeneration assay was performed using ImageJ software and an automated macroanalysis previously developed by our laboratory (Clements *et al.*, 2022). While 6-OHDA exposure led to cell death and neurite degeneration of over 50% of cultured neuronal cells, co-treatment with LM11A-31 resulted in a significant increase in cell survival (n = 7) and a reduction in neurite degeneration (n=3) (Figure 3.2). Our analyses revealed that $p75^{NTR}$ is vital in regulating viability and neurite degeneration in neuronal cells subjected to oxidative damage.

As previously mentioned, oxidative stress induces the cleavage of the $p75^{NTR}$ receptor in mesencephalic cells. To investigate whether LM11A-31 modulates $p75^{NTR}$ in a manner that blocks the proteolytic processing of the receptor, cells were treated with vehicle, 6-OHDA, or 6-OHDA and LM11A-31, and lysates were subjected to immunoblotting to measure $p75^{NTR}$ fragments. Despite some variability, our preliminary result indicated that LM11A-31 reduces oxidative stress-induced $p75^{NTR}$ processing since we have observed a general trend in which there is recovery of $p75^{NTR}$ full-length. In contrast, a decrease in $p75^{NTR}$ fragments was observed (Figure 9). Furthermore, our analyses also revealed a dose-dependent effect of LM11A-31 where 20 nM concentration showed a greater effect than 2 nM than 200pM than 20pM in attenuating cell survival and neurite degeneration in LUHMES cells. Interestingly, our experiments using a 100nM concentration of LM11A-31 did not show higher protection than a 20nM concentration. Thus, these results revealed that $p75^{NTR}$ regulates the survival of neuronal cells derived from

mesencephalon, and our immunoblotting results suggest the role of proteolytic processing of the receptor in cell survival.

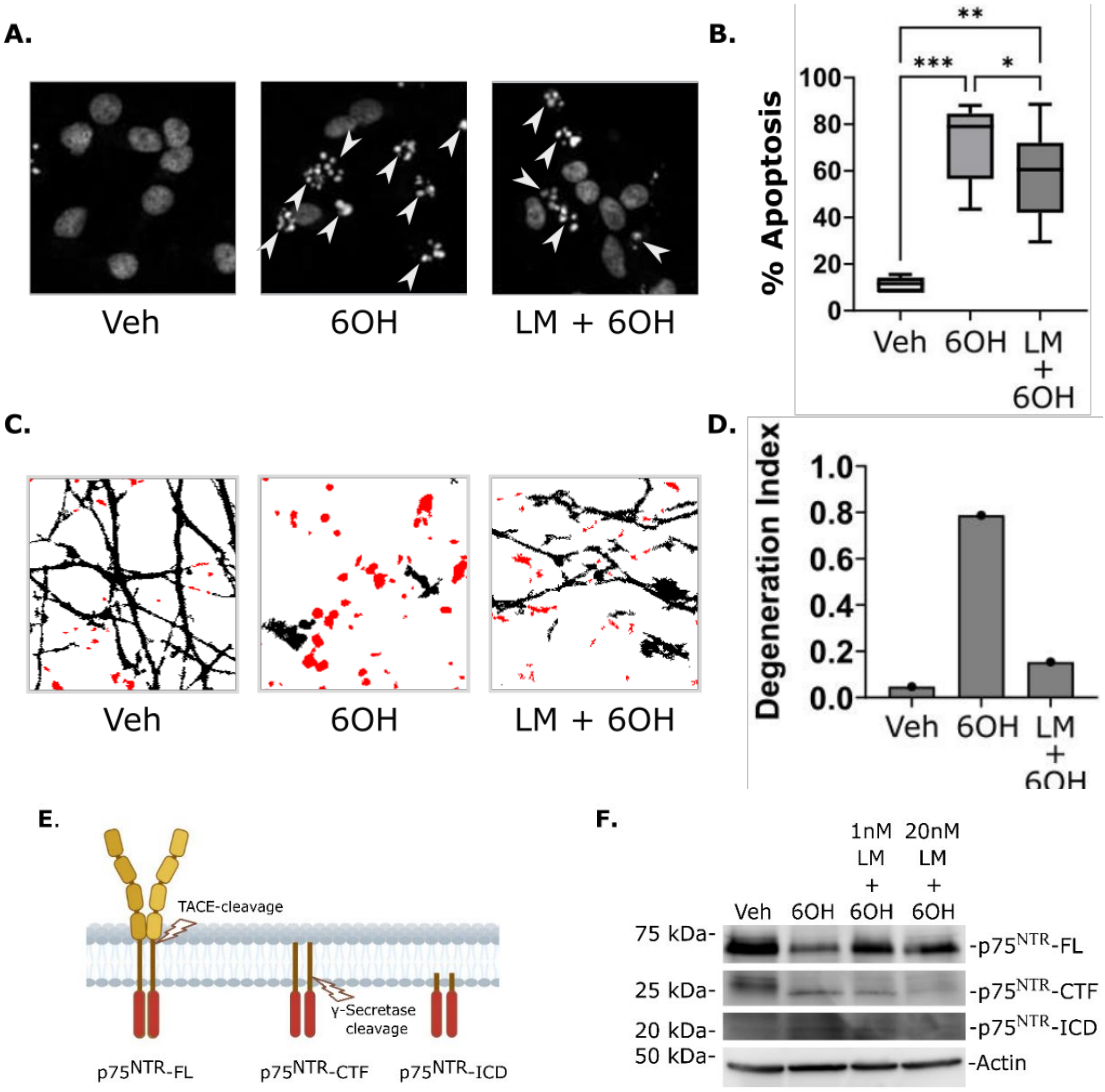


Figure 3.2 Pharmacological Modulation of p75^{NTR} Reduced Neurite Degeneration and Death Associated with Oxidative Stress.

A; Differentiated LUHMES cells were treated with vehicle solution, 5 µM 6-OHDA, or 5 µM 6-OHDA and 10 nM LM11A-31 for 24 hours. Fixed cells were then stained with DAPI and TUJ1, then image captured, and scored for pyknotic nuclei, B; Quantification of cells treated and assessed as described in 'A.' **C-D:** Differentiated LUHMES cells were treated with vehicle solution, 5 µM 6-OHDA, or 5 µM 6-OHDA and 0.1 nM LM11A-31. Micrographs of the cultures were then assessed for neurite degeneration using the ANDI macro for ImageJ, as previously described (clements *et al.*, 2022). **C;** Representative images featuring soma removal, binarization, and highlighting of neurite fragments in red, **D;** Degeneration Index Quantification. **E;** Schematic representation of **p**75^{NTR} fragments. **F;** Immunoblotting result of p75^{NTR} fragments in 1 nM and 20 nM LM11A-31 treated cells.

3.3 Differentiated LUHMES Cells Express Sortilin and TrkA but Lack TrkB and TrkC

$p75^{NTR}$ interacts with all of the neurotrophins and has been shown to influence cellular signaling by interacting with multiple coreceptors, including tropomyosin kinases (TrkA, TrkB, TrkC) and sortilin. Studies suggested that $p75^{NTR}$ functions by modulating Trk activation and expression of TrkA, TrkB, or TrkC has been found in most cells that express $p75^{NTR}$ (Bothwell, 1995). Furthermore, modulating neurotrophin responses in collaboration with Trk receptors has been suggested as one of the chief physiological roles of the $p75^{NTR}$ (Barker, 1998). It is also well established that $p75^{NTR}$ influences Trk, helps form a high-affinity binding site for their cognate neurotrophins, and regulates cell survival and differentiation signaling (Conroy & Coulson, 2022, p. 75). Similarly, sortilin, a Type I transmembrane protein containing a Vps10p domain, interacts with $p75^{NTR}$, forming a high-affinity co-receptor complex that regulates pro-neurotrophin-induced cell death in various neuronal (Ceni *et al.*, 2014), and non-neuronal cell types (Skeldal *et al.*, 2012). However, the role of $p75^{NTR}$ coreceptors in oxidative stress-induced $p75^{NTR}$ cleavage remains poorly understood. Thus, to investigate the expression of Trk receptors and sortilin in fully differentiated mesencephalic cells, LUHMES cells were cultured in 60mm tissue-culture dishes. Differentiated LUHMES cells were treated with a vehicle for control or various concentrations of 6-OHDA (5 μM and 7.5 μM) for 18 hours. Cells were then lysed, and immunoblotting was performed on the cell lysates using antibodies specific to TrkA, TrkB, TrkC, or sortilin to assess their expression. Our results demonstrated that cells derived from mesencephalon express TrkA and sortilin but lack the expression of TrkB and TrkC (Figure 3.3). Furthermore, a significant decrease in TrkA expression in cultures exposed to oxidative stress was observed. However, the expression of sortilin was not affected by the 6-OHDA treatment.

These results suggest that TrkA and sortilin could play a role in influencing $p75^{NTR}$ signaling in mesencephalic cells, while TrkB and TrkC do not.

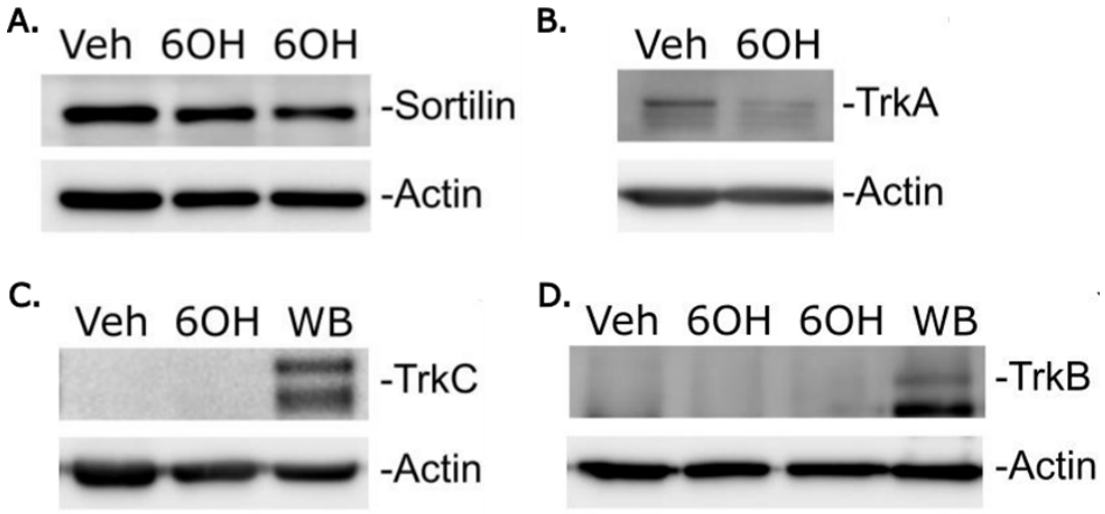


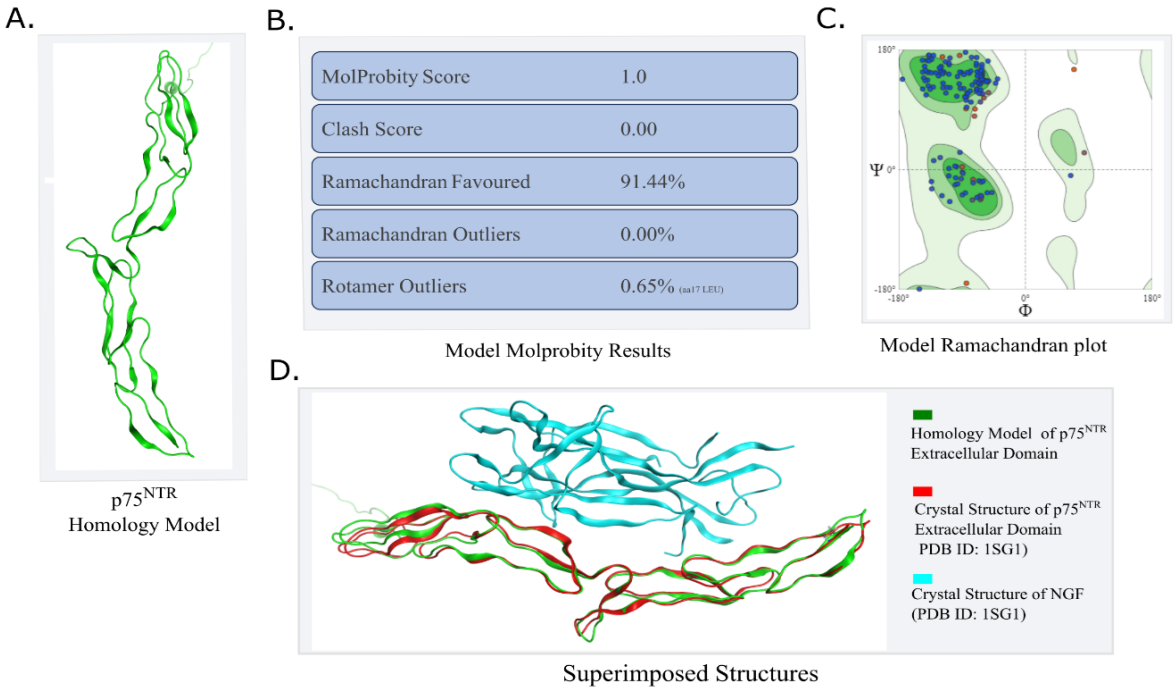
Figure 3.3 Differentiated LUHMES Cells Express Sortilin and TrkA but Lack TrkB and TrkC**.**

A-D; Differentiated LUHMES cells were treated with vehicle or 10 µM 6-OHDA for 18 hours. Lysates were then subjected to western blot analyses for $p75^{NTR}$ co-receptors. to analyze the expression of p75NTR co-receptors. Immunoblotting for actin was performed as a loading control. Whole brain lysate (WB) was used as a positive control for protein detection. Sortilin (**A**) and TrkA (**B**) were detected in differentiated LUHMES cells, while TrkC (**C**) and TrkB (**D**) were not detected.

3.4 Homology Modeling of Human p75^{NTR} Extracellular Domain

p75^{NTR} consists of various domains, and crystal structures have revealed the structure of its extracellular domain, transmembrane domain, and death domain. Various crystal structures of the extracellular domain of p75^{NTR} are available in the protein data bank. Even though the structure of p75^{NTR}-ECD has been deciphered, they do not belong to the extracellular domain of Human p75^{NTR}. Additionally, the extracellular, transmembrane, and intracellular domains of human p75NTR are homologous with that of chick and rat (Barker, 1998). Utilizing the concept that homologous sequences lead to similarity in structure, we sought to model human p75^{NTR}-ECD utilizing the available crystal structures. Thus, to model Human p75^{NTR}-ECD, homology modeling was performed using SWISS-MODEL. The amino acid sequence of p75NTR was retrieved from the NCBI database, and the NCBI blast search was performed utilizing the human version as the template against that of the mouse, chick, and rat. Amino acids (1-189) were provided in the SWISS-MODEL server as a target sequence, and templates were searched. Out of fifty templates generated by the server, four templates with over 90% sequence identity were chosen to build models of human p75^{NTR}. We chose the top two models with the highest QMEAN or GQME scores. The two models, the first being the one derived using AlphaFold DB (PDB ID: A0A2Y9DNL7.1.A) as a template, showed a GQME score of 91.00, with MolProbity score of 1.0 and a Ramachandran favored percentage of 91.44%. The second was generated using PDB ID:1SG1 as a template. The model has a QMEANDisCo Global score of ~0.82, a MolProbity score of 2.02, and a Ramachandran favored percentage of 91.77%, with Ramachandran outliers being 1.90%. Our homology model revealed that the extracellular domain of human p75^{NTR} is indeed homologous in structure with that of the available crystal structure of the mouse (PDB ID: 1SG1) (Figure 3.4). After evaluating the two models, we used

the first model for our studies since it included the N-terminal domain sequences and consisted of amino acid residues 1-189, while the second model only consisted of amino acid sequences from 29-161.



E.

Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
754	754	99%	0.0	92.24%	425	Query_350075

Blast Result: Human vs. Rat p75NTR

Figure 3.4 Homology Modeling of the Structure of Human p75NTR-ECD.

A; human model of p75NTR-ECD generated using homology modeling. **B;** table showing scores for predicted Model. C; Ramachandran plot of the model. D; superposition of human p75NTR-ECD on the crystal structure of rat. E. Blast sequence alignment of Human p75NTR receptor vs. that of the Rat showing a percentage identity of 92.24%.

3.5 P75^{NTR} Stalk Domain Consists of Intrinsically Disordered Regions

p75^{NTR} goes through proteolytic processing, where the extracellular domain gets cleaved, releasing the ectodomain by α-secretase and is further cleaved by γ-secretase in the transmembrane region (Zampieri *et al.*, 2005). To investigate whether a small molecule could be determined to block the ectodomain shedding of p75^{NTR} by the action of α-secretase, initially, we searched for the crystal structure of the stalk domain, a region between the extracellular domain and the transmembrane domain of p75^{NTR} but there are no available structures for the region. Therefore, we sought to use ab initio modeling of the stalk domain. The models generated showed vast regions of the sequences being unstructured. Therefore, we utilized various tools to understand whether the stalk domain region consists of intrinsically disordered regions and provided 54 amino acid sequences in between the extracellular domain and the transmembrane domain to numerous web servers able to predict disorder in protein structures. PONDR VL-XT showed an overall percent disorder of 74% with an average prediction score of 0.7294 (Figure 3.5). Similarly, Adopt prediction and Metapredict also showed most of the regions of the stalk domain being unstructured. Furthermore, flDPnn prediction also predicted the region having no structure in the provided sequence. Therefore, our result suggests that most of the region of the stalk domain is intrinsically disordered. Furthermore, we used various servers such as I-TASSER, Alphafold2, Phyre2, and RosettaFold to model the stalk domain, and our ab initio model showed a small region closer to the transmembrane region being α-helix (Figure 3.6).

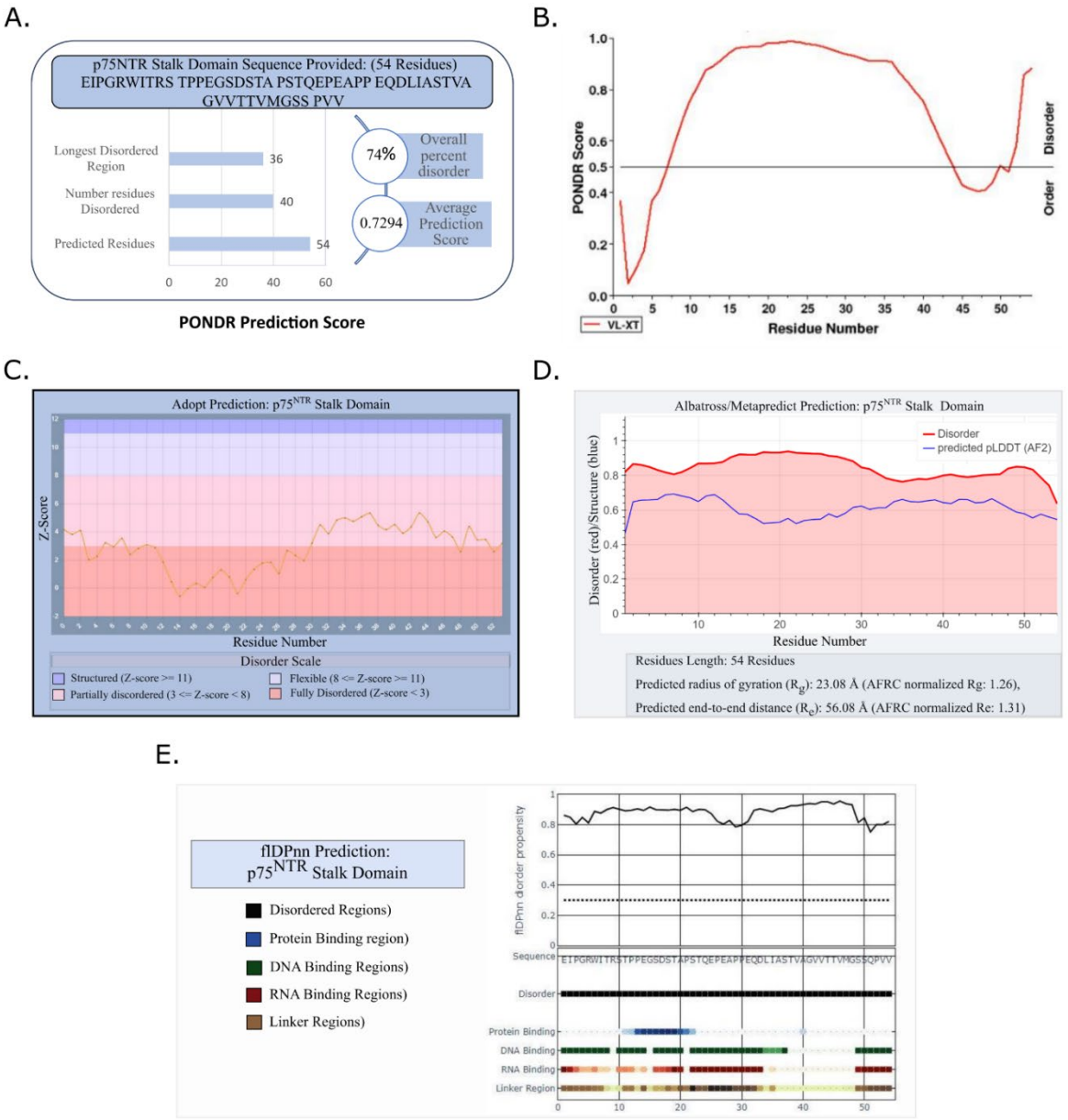


Figure 3.5 The p75NTR Stalk Domain Consists of IDRs**.**

Various IDR-predicting web servers were provided with the p75NTR stalk domain (54 amino acid residues). **A;** PONDR prediction result; **B;** PONDR score per residue. **C;** Adopt IDR prediction plot for each residue. **D;** Metapridict IDR prediction plot per amino acid sequence provided. **E.** flDPnn IDR prediction results for the stalk domain of $p75^{NTR}$.

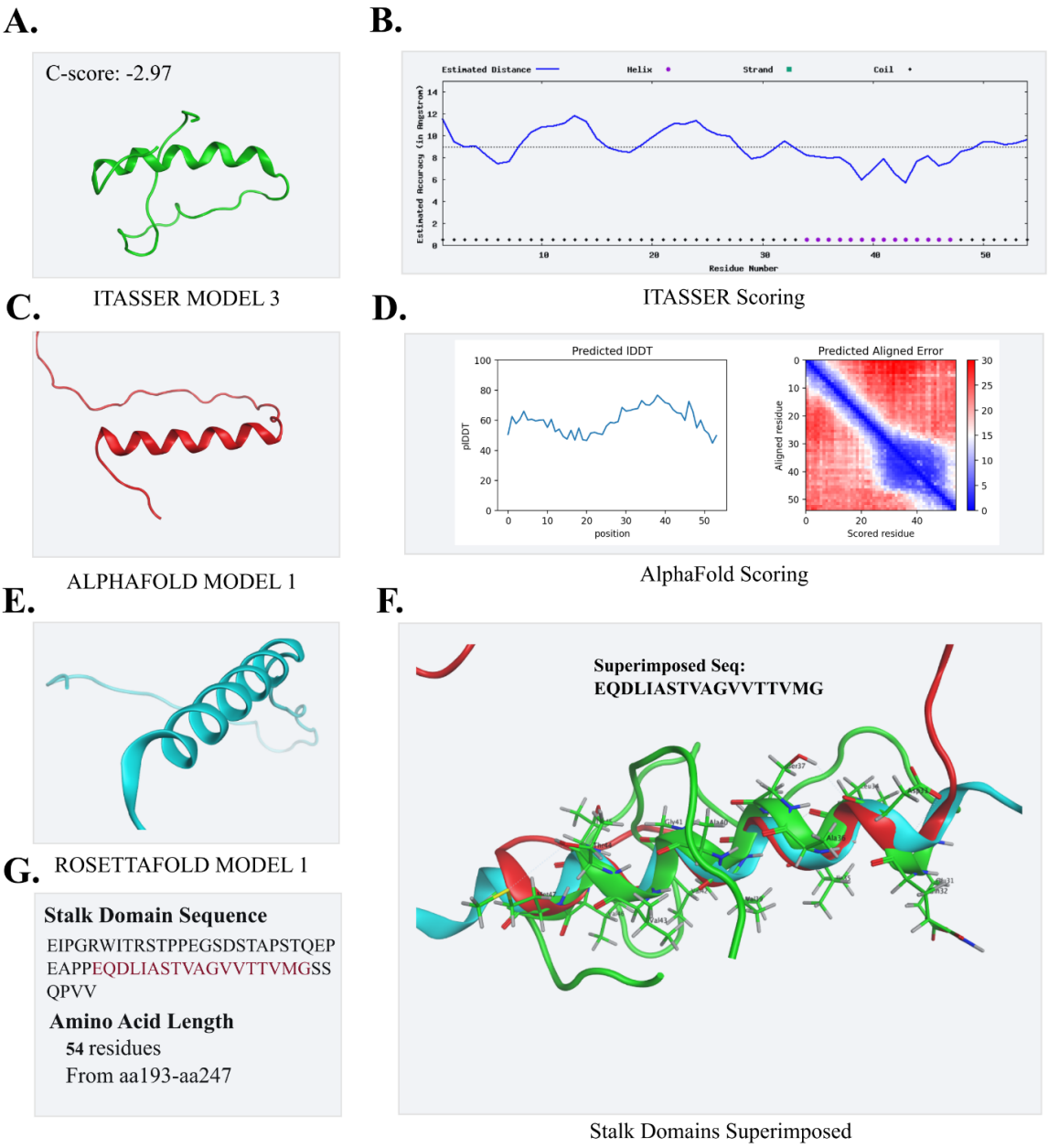


Figure 3.6 Ab Initio modeling of the p75^{NTR} Stalk Domain.

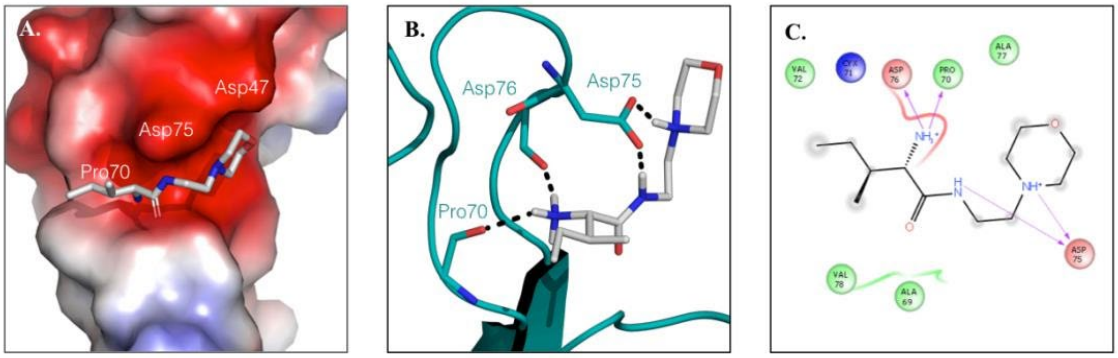
A; I-TASSER structure prediction of p75^{NTR} stalk domain**, B;** I-TASSER score plot**. C**; Alphafold structure prediction model of p75^{NTR} stalk domain**, D;** Alphafold scoring results**, E.** RosettaFold model**. F;** superimposition of the three models **(A,C, and E). G;** p75^{NTR} stalk domain sequence**.**

3.6 LM11A-31 Binds in Non-Canonical Regions in the P75^{NTR} Extracellular Domain Away from the NGF Binding Site I

It is well established that p75^{NTR} activation can influence cellular signaling towards pro-survival or pro-apoptosis. P75^{NTR} is activated through various pathways, and continuous effort has gone into determining the receptor activation and the induction of p75^{NTR}-dependent apoptosis. Data from in vitro studies also show that p75^{NTR} enhances cell survival induced by the interaction of NGF in sympathetic neurons, DRG, primary trigeminal, and Schwann cells (Ceni *et al.*, 2014). However, p75^{NTR} mediates pro-apoptotic effects by forming a complex with sortilin due to the interaction of pro neurotrophins proNGF and proBDNF (Dedoni, 2020). Therefore, the interaction of neurotrophins and/or pro-neurotrophins plays a vital role in p75^{NTR} signaling. LM11A-31, a non-peptide ligand of the p75 neurotrophin receptor, mimics NGF β-hairpin loop 1 consisting of amino acids 29-35, a domain that interacts with p75^{NTR} (F. Longo *et al.*, 2008). Thus, we investigated these sites in human p75^{NTR}-ECD generated using SWISS-MODEL. Our study demonstrated that the residue Asp103 is involved with binding NGF residues Lys 32 and Lys35 (binding site I). Meanwhile, binding site II consists of p75 residues Cys 164 and Glu 147, which create a stabilized hydrogen bond with NGF-Arg114 (Figure 3.7). LM11A-31/p75^{NTR} interaction has not been characterized in the Human model of p75^{NTR}. LM11A-31 structure was downloaded from PubChem, and the docking procedure was performed in MOE (Molecular Operating Environment). The area of interaction between the small molecule LM11A-31 and mouse p75^{NTR}-ECD of the mouse has been demonstrated by Carder in Ph.D. Dissertation (Figure 3.8). Thus, we sought to understand the mechanism of interaction using a human receptor model. The human p75NTR-ECD model was provided as the receptor, and molecular docking was performed in various ways, with LM11A-31 being the ligand. 30 conformations and interactions

were generated, out of which 5 conformations were selected for our study. Our agnostic docking results suggest various non-canonical binding pockets in the receptor. Numerous docking results also suggested that residue Arg126 of p75^{NTR}-ECD interacted with LM11A-31 (Hydrogen bond). Another residue, Asp140, also interacted with the small molecule (Figure 3.9). Previous studies suggested that the ligand could bind at several sites on the same receptor (Massa *et al.*, 2005). Therefore, we hypothesize that LM11A-31 and p75^{NTR} interaction involves multiple binding sites and residues and may also involve conformational changes of the receptor's binding site, disallowing NGF or pro-NGF interaction.

A.



(Synthetic efforts toward P97 AAA+ Atpase and p75 Neurotrophin Receptor Inhibitors, Figure 25 (Putative Binding Model of LM11A-31 at the NGF^{32}KGKE^{35} Recognition Site of p75^{NTR}, PHD Dissertation, Carder, 2011).

B. **C.**

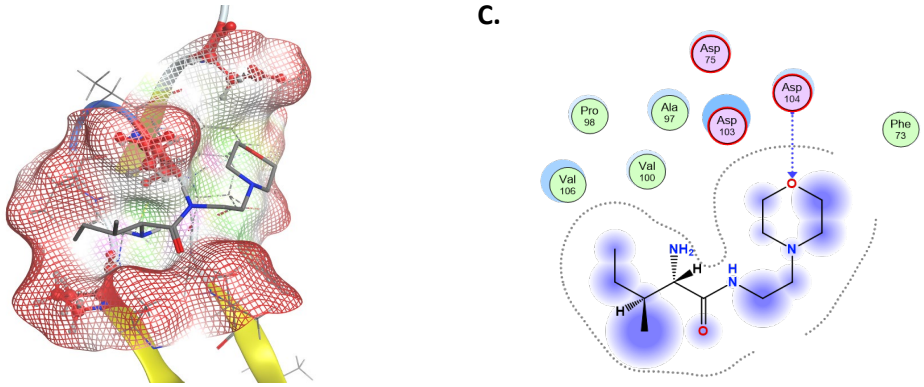


Figure 3.7 Molecular Interaction Between LM11A-31/p75^{NTR}—ECD.

A; Previous study showing LM11A-p75NTR interaction (Carder, 2011). **B-C**: Preliminary docking result targeting previously described binding site, surface map **(B),** and ligand interaction map **(C).**

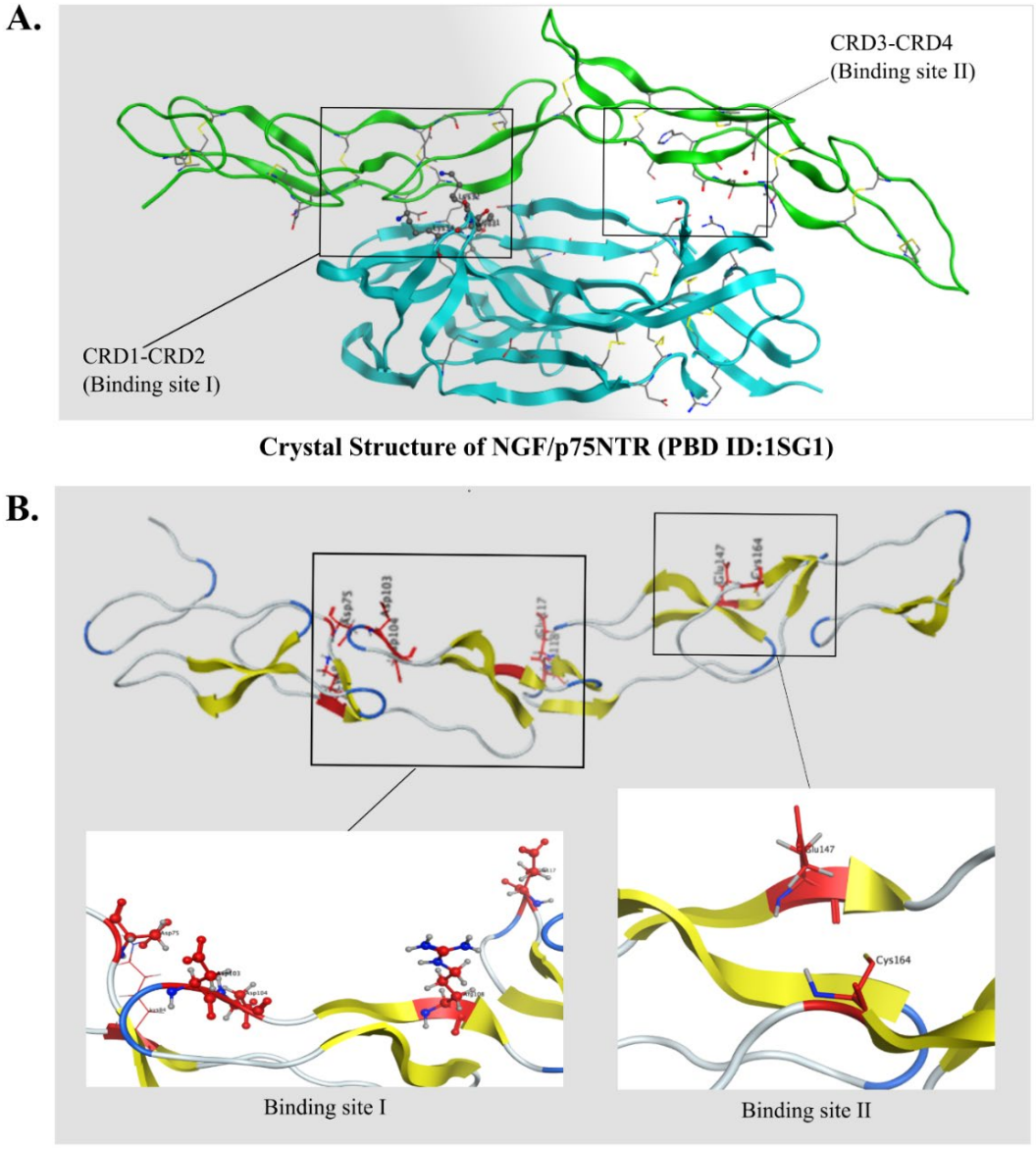


Figure 3.8 Structural Characterization of the NGF Binding Site of the $p75^{NTR}$-ECD.

A; structural representation of the two binding sites of the $p75^{NTR}$ model using the crystal structure of the NGF/p75NTR complex (PDB ID:1SG1). **B;** Elaboration of the binding sites showing the residues involved in the binding of the NGF molecule to the p75NTR-ECD using the human $p75^{NTR}$-ECD model.

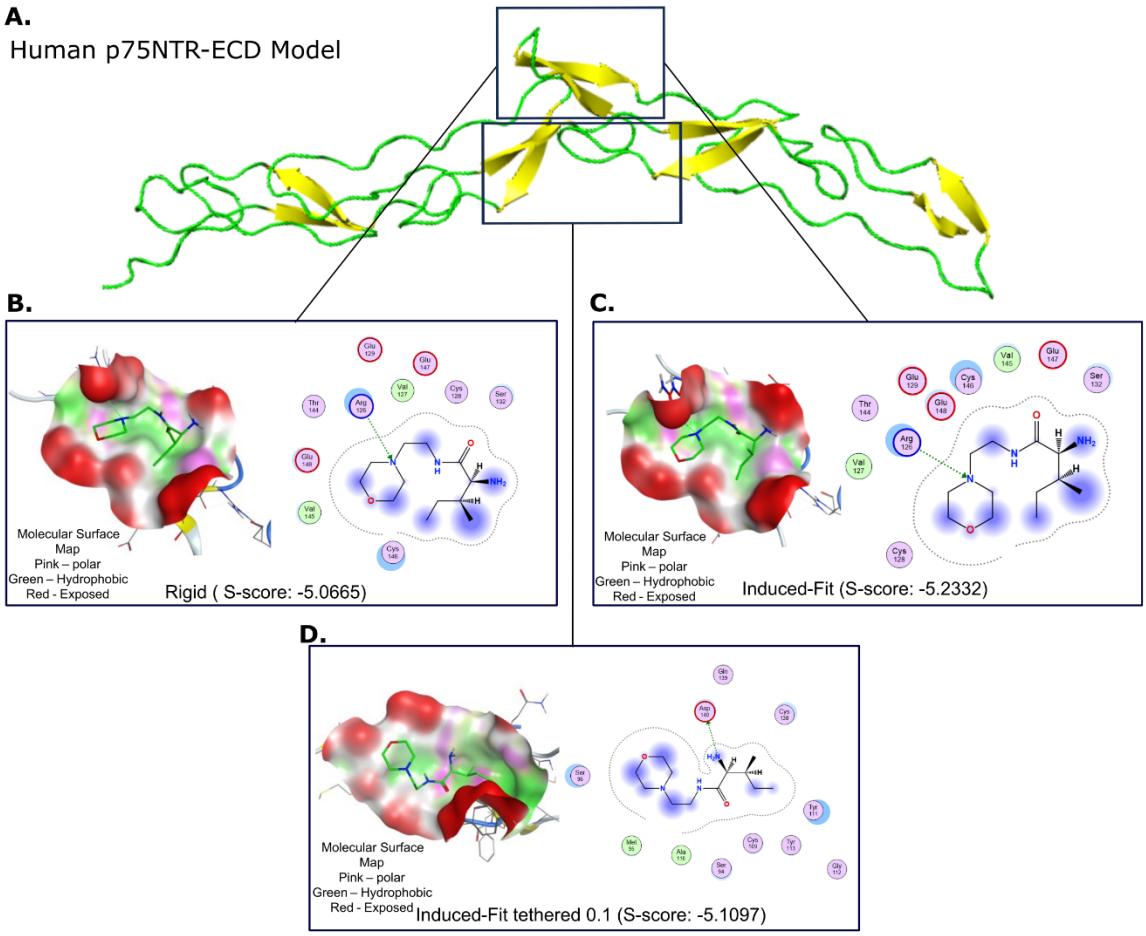


Figure 3.9 Molecular Docking of LM11A-31 in the $p75^{NTR}$ – ECD

The small molecule LM11A-31 was docked in the human p75NTR-ECD model using various criteria. A: The human p75NTR-ECD structure marking the regions of interest. **B-D**: The molecular surface map (left) and the ligand-receptor interaction depiction (right), using rigid docking **(B)**, Induced-Fit docking **(C)**, and Induced-Fit tethered 0.1 **(D)**.

Chapter 4. Discussion

4.1 Role of Receptor Internalization in p75^{NTR} Processing

In many biological contexts, neurotrophin signaling is regulated by endocytosis and intracellular trafficking of the neurotrophin receptors. For example, Trk receptors have been demonstrated to interact with dynein, suggesting a possible trafficking mechanism for Trk-signaling endosomes in sympathetic and sensory neurons (Yano *et al.*, 2001). Similarly, various studies have suggested endosomal activity for the p75^{NTR} receptor in various cell types. For example, in PC12 cells, p75^{NTR} interacts with signaling adaptors in endosomes in a ligand-dependent manner. Additionally, the internalization of the p75^{NTR} induced in this way adheres to the clathrin-mediated internalization pathway to the recycling endosome, distinctively from Trk-signaling endosomes (Bronfman *et al.*, 2003). Furthermore, in compartmentalized sympathetic neuronal cultures, internalization and retrograde trafficking of p75^{NTR} were detected both in response to ligand binding and in a ligand-independent manner without neurotrophin treatment (Escudero *et al.*, 2019). The Rab family of small GTPases are considered prime regulators of membrane trafficking, where Rab5 marks the early/sorting endosome, which frequently contains either adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1(APPL1) or early endosome antigen 1 (EEA1). APPL1 has been implicated in TrkA signaling, and NGF-TrkA function is determined by the multiple pathways involving TrkA trafficking (Barford *et al.*, 2017). P75^{NTR} has been shown to activate Rab5 Family GTPases, Rab5 and Rab31, and the ICD domain of the receptor was found to interact with these binding partners (Baeza-Raja *et al.*, 2012). Given this evidence that endosomal interactions mediate neurotrophin receptor functions, further research evaluating the signaling mechanisms that govern neurotrophin receptor

internalization and trafficking is merited. Such events may underlie the ability of $p75^{NTR}$ to stimulate various signaling cascades and serve distinct physiological roles in different biological contexts.

In PC12 cells, NGF interaction promoted the internalization of $p75^{NTR}$ into early endosomes, but no decrease in the surface levels of the receptor was observed (Saxena *et al.*, 2005). It was also found that the retrograde transport of the $p75^{NTR}$-endosome consisted of the full-length receptor ($p75^{NTR}$-FL) and indicated the requirement of JNK activation and Rab5 and dynein involvement in the death-signaling endosome (Escudero *et al.*, 2019). Furthermore, a study has also suggested that JNK activation may promote receptor internalization (Kenchappa *et al.*, 2010). This data indicates that the proteolytic processing of the p75 neurotrophin receptor leads to a pro-apoptotic pathway occurring in the endosome. However, the association between $p75^{NTR}$ internalization and $p75^{NTR}$ cleavage influenced by oxidative stress has not been evaluated. Furthermore, the mechanism underlying proteolytic processing of the receptor has not been studied in dopaminergic cells/a Parkinson's disease model. Thus, in this study, we revealed that receptor internalization is required for oxidative stress-induced $p75^{NTR}$ processing. Our study uncovers a possible mechanism of oxidative stress-induced $p75^{NTR}$ activation where JNK is activated by $p75^{NTR}$, directing to internalization and proteolytic processing of the receptor, consequently leading to neuronal cell death. Receptor internalization of $p75^{NTR}$ stimulated by the stress-activated kinase JNK has been confirmed in several cell types, such as mouse cortical neurons, PC12 and U373 cells (Bhakar *et al.*, 2003), and sympathetic Neurons, and JNK activation has been demonstrated to induce p75NTR-mediated cell death (Kenchappa *et al.*, 2010). Our previous study demonstrated a novel mechanism of JNK-dependent $p75^{NTR}$ processing stimulated by oxidative stress in mesencephalic cells in a ligand-independent manner

(Kraemer *et al.*, 2021). Based on this evidence, we sought to understand whether internalization of the receptor plays a role in p75NTR cleavage in LUHMES cells exposed to oxidative stress. However, further studies are required to elucidate how much of a role the internalization of the receptor plays in the apoptotic pathway. It is possible that oxidative stress leads to excessive $p75^{NTR}$ processing due to JNK activation and further increases the internalization of $p75^{NTR}$, consequently leading to further proteolytic processing of the receptor. Additionally, an understanding of the events leading to the internalization of the receptor is yet to be further elucidated.

4.2 Small Molecule, LM11A-31 Modulation of $p75^{NTR}$ in Neurological Disorders

We found that a small molecule modulator of $p75^{NTR}$, LM11A-31, significantly reduces cell death and attenuates neurite degeneration in mesencephalic cells exposed to 6-OHDA. LM11A-31 is a non-peptide selected through screening of compounds mimicking an NGF single loop 1 structure (Massa *et al.*, 2006). Widely accepted, $P75^{NTR}$ is a receptor with unique and diverse signaling mechanisms capable of interacting with many proteins, producing pro-survival and pro-apoptosis in a cell-type-specific manner. Moreover, $P75^{NTR}$ has been indicated to be associated with various neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS), schizophrenia, major depressive disorder (MD), posttraumatic stress disorder (PTSD) (Shu *et al.*, 2015, p. 75) as well as cerebrovascular disease, acute or chronic brain injury, spinal cord injury (Xiong *et al.*, 2022). Various compounds, such as LM11A-31 or LM11A-24 and THX-B, have been confirmed as modulators of $p75^{NTR}$, mainly functioning by promoting survival signaling and interfering with proNT degenerative signaling (Xiong *et al.*, 2022). Higher levels of ProNTs have been associated with apoptosis in multiple neuropathologic conditions, including Alzheimer's disease,

CNS trauma, and seizure. Likewise, various forms of Motor neuron death in ALS patients directly correlate to increased proNGF and/or NGF levels and $p75^{NTR}$ re-expression (F. Longo *et al.*, 2008). LM11A-31 has been found to modulate proNGF/p75NTR complex, reduce astrocytic proNGF levels, suppress neural JNK/PARP signaling *in vitro*, and amend acute stroke injury (Nasoohi *et al.*, 2023). Furthermore, LM11A-31 was suggested to inhibit calpain activity and activation of cdk5, JNK, and cofilin, diminish the formation of tau aggregates, and reduce degeneration of synaptic spines and synapses in PS19 mice model (T. Yang *et al.*, 2020). Therefore, the results observed through our study of LM11A-31 support the notion that $p75^{NTR}$ regulates cell survival and neurite degeneration in cells of the mesencephalon exposed to oxidative stress. Yet, further studies are required to understand how LM11A-31 modulates $p75^{NTR}$ towards pro-survival in oxidative stress-induced neuronal cells. Higher Levels of $p75^{NTR}$-ECD are observed in brain cells with injury and neurodegeneration and have also been seen in the urine samples of ALS patients, as well as in HD patients and R6/2 mice models (Simmons *et al.*, 2021). Since $p75^{NTR}$ has been found in various neurological disorders, our data supports the role of $p75^{NTR}$ in neurological signaling pathways. Thus, our findings may not only extend to PD but also assist with other neurodegeneration-related diseases. Higher $p75^{NTR}$-ECD levels also indicate the role of $p75^{NTR}$ proteolytic processing in neuronal cell death. However, it is not well understood whether extracellular domain shedding occurs due to ligand interaction or ligand-independent $p75^{NTR}$-mediated apoptotic death signaling, which unseals other areas of exploration. Current treatments for Parkinson's disease are palliative. We found that LM11A-31 significantly improved cell viability in various doses (1 nM (n=7), 10 nM (n=5), and 20 nM (n=2) in a dose-dependent manner and reduced neurite degeneration in oxidative stress-induced LUHMES cells. These findings indicate that $p75^{NTR}$ regulates mesencephalic cells' cell survival

and neurite degeneration. Therefore, LM11A-31 modulation of $p75^{NTR}$ could be a potential therapeutic target for Parkinson's disease. The possibility exists that the small molecule regulates the signaling of $p75^{NTR}$ by interfering with its co-receptor interaction and/or through other mechanisms not fully understood. It is also possible that the small molecule influences pro-survival through a completely different mechanism, such as interaction with proteins/receptors not involving $p75^{NTR}$. In accordance with the role of $p75^{NTR}$ in cell survival and neurite degeneration in cells derived from the mesencephalon, and LM11A-31 being bioavailable and a potent modulator of $p75^{NTR}$, small molecule LM11A-31 could provide a potential for further exploration in drug discovery studies related to Parkinson's Disease.

4.3 $P75^{NTR}$ Signaling Influencers in the Cells Derived from Mesencephalon

Moreover, $p75^{NTR}$ can interact with various classes of receptors mediating numerous outcomes: interactions of $p75^{NTR}$ and Trk receptors enhance growth signaling and cell survival, interactions of the receptor with sortilin and pro-neurotrophins lead to apoptosis, interactions with the Nogo receptor and Lingo-1 control neuronal growth (Malik *et al.*, 2021; Meeker & Williams, 2015), and interactions with protein kinase A (PKA) regulate cAMP (Malik *et al.*, 2021). Moreover, Trk receptor-mediated signaling activates major pathways such as Ras-ERK, PI3K-AKT, PLC-γ, and their downstream effectors (Huang & Reichardt, 2003). The p75 neurotrophin receptor has been suggested to modulate the function of Trk receptors, either by promoting ligand binding and neurotrophin accessibility or by endocytosis and retrograde transport to specific membrane compartments (Skaper, 2008). However, the roles of coreceptors in $p75^{NTR}$ activation induced by oxidative stress in mesencephalic cells are not known, and p75NTR coreceptor expression has not been characterized in LUHMES cells. $p75^{NTR}$ can interact with various receptors and influence various signaling pathways in a cell-type-specific

manner (Kraemer, Yoon, *et al.*, 2014) and is dependent on the expression of the coreceptors. Our study confirms the expression of TrkA and sortilin, whereas there is a lack of TrkB and TrkC in LUHMES cells. These findings suggest that TrkA and sortilin may regulate $p75^{NTR}$ activation and signaling in cells derived from mesencephalon. Nevertheless, further research is required to understand how $p75^{NTR}$ coreceptors influence its signaling and will be the focus of our future studies.

4.4 Understanding the Structure of $p75^{NTR}$, and the Interaction Between LM11A and $p75^{NTR}$-ECD

Anfinsen dictated that a protein's amino acid sequence determines its 3D structure, and structure determines its function (Guo *et al.*, 2022). Similar sequences fold into identical structures since proteins' structures are more conserved (Muhammed & Aki-Yalcin, 2019). $p75^{NTR}$ structure is characterized by the extracellular domain ($p75^{NTR}$-ECD) consisting of four cysteine-rich repeat regions (termed the extracellular domain) and a stalk domain, a transmembrane domain (TMD) containing highly conserved Cys257, and the intracellular domain ($p75^{NTR}$-ICD) comprised of the juxtamembrane domain (JTM) and the death domain (DD) (Vilar, 2017). The structure of the $p75^{NTR}$-ECD has been solved, and X-ray crystallography structures are available in the protein data bank (RCSB) of *Rattus norvegicus* (PBD ID: 1SG1, 3BUK) and *Mus musculus* (PBD ID: 3IJ2). Similarly, the NMR structure of *Homo sapiens* $p75^{NTR}$-TMD (PBD ID: 5ZGG), *Rattus norvegicus* (PBD ID: 2MJO, 2MIC), and solution NMR structure of the death domain of *Homo sapiens* (PBD ID: 2N83, 7CSQ) are also readily available. Even then, available structures in the RCSB of $p75^{NTR}$-ECD do not belong to that of humans. Furthermore, the structure of the stalk domain (the region between the ECD and the

TMD) and the juxtamembrane domain (the region between the TMD and the DD) has not been solved.

LM11A-31 has been suggested to mimic NGF loop 1 and indicated to bind in the site I region of $p75^{NTR}$-ECD. However, the mechanism of action of the small molecule is not fully understood. Homology modeling is a computational structural method for protein structure prediction that uses the amino acid sequence of a protein to determine its 3D structure based on its template (Muhammed & Aki-Yalcin, 2019). Therefore, we used SWISS-MODEL regarding the importance of P75NTR-ECD in the binding of neurotrophins and LM11A-31. This widely used server utilizes comparative modeling (homology modeling) to predict the 3D structure of human $p75^{NTR}$-ECD, thereby understanding the potential binding site/sites and the mode of action of the small molecule. NCBI blast results of the extracellular domain of the $p75^{NTR}$ showed over 90% sequence identity amino acid sequence between humans and that of the mouse and rat. Consequently, our structural results also indicated that the 3D structure of this region is extremely similar, consisting of almost identical folds. Thus, our study demonstrates that the structure of p75NTR-ECD cysteine-rich repeat is extremely conserved among species. This region consists of the ligand-binding site and provides a potential target in drug discovery studies for neurodegeneration that is now being started in the Kraemer and Baudry laboratories.

Computational Studies to reveal the binding site have been performed on NGF and $p75^{NTR}$-ECD, and the structure of the complex has also been solved through crystallography. He and Garcia described two separate sites: site I (in the CRD1-CRD2 domains) and site II (CRD3-CRD4 junction) in the $p75^{NTR}$-ECD to be involved in the binding of NGF molecules and form an asymmetric receptor and ligand complex (He & Garcia, 2004) (Figure 13). Site-directed mutagenesis results suggested two adjacent hairpin loops of NGF, loop I (residue 23-35) and

loop IV (residues 93-98), where residues Lys 32, Lys34, and Lys95 were found to participate in $p75^{NTR}$ binding. Whereas amino acid residues Asp47, Lys56, Asp75, Asp76, Asp88, and Glu89 of $p75^{NTR}$ were principally involved in the binding (Shamovsky *et al.*, 2008). In terms of small molecule binding, Carter. Evan J., in his ph.D dissertation, showed LM11A-31 bound in the binding site I of $p75^{NTR}$ where the NGF beta-hairpin turn loop 1 (Lys34, Lys32) amino acids interact with Asp75 of $p75^{NTR}$ through a network of hydrogen bonding and electrostatic interactions. (Carter., 2019, Ph.D. dissertation). However, the precise interaction of the small molecule has not been characterized in human $p75^{NTR}$-ECD, thus requiring further clarification. Crystallographic studies of an NGF-p75NTR complex only confirmed K32 and H75 but not the other lysine residues as contact sites of NGF with $p75^{NTR}$ (F. Longo *et al.*, 2008). To understand the molecular interaction between LM11A-31 and $p75^{NTR}$-ECD, we used MOE (Molecular Operating Environment). We docked the small molecule into our human model of $p75^{NTR}$-ECD in three different ways: rigid, induced fit, and induced fit tethered 0.1. Our preliminary data demonstrates various binding regions away from the putative binding site, providing novel interaction regions between LM11A-31 and the human $p75^{NTR}$-ECD. Furthermore, studies thus far have only utilized rat or mouse $p75^{NTR}$-ECD crystal structures to elucidate the interaction site between NGF/$p75^{NTR}$-ECD (He & Garcia, 2004) and LM11A-31/$p75^{NTR}$-ECD (Carter, 2010, Ph.D. Dissertation) (Figure 16).

Primarily, our focus was to screen for small molecules capable of blocking the interaction of ADAM17 (TACE) with the p75NTR receptor. TACE has been found to proteolytically cleave the ECD of the low-affinity neurotrophin receptor $p75^{NTR}$ and initiate regulated intramembranous proteolysis (RIP) of the CTF by influencing γ-secretase activity(Ahmed *et al.*, 2006; Gil *et al.*, 2007). Blocking the ectodomain shedding could provide us with a better

understanding of the mechanism of activation of the receptor and its roles. Specifically, TACE-induced proteolysis occurs in the region close to the transmembrane domain of the receptors (Gooz, 2010). These considerations led us to investigate the substrate recognition site in $p75^{NTR}$ to block sheddase activity. However, no crystal structures are available for the receptor's stalk domain. Therefore, we used ab initio modeling methods to generate model structures for the stalk domain region. Validated through various web servers to predict intrinsically disordered regions in proteins, our results demonstrated vast regions of Intrinsically disordered regions being disordered. Uniprot data on human $p75^{NTR}$ (Uniprot ID: P08138) shows amino acid regions 194-219, as well as 218-338 as disordered. Our results confirm the unstructured region. Interestingly, our ab initio modeling results suggest α-helix structure for a small region in the stalk domain. This region could have greater significance and should prove valuable for therapeutic targets to block $p75^{NTR}$ extracellular domain shedding.

Chapter 5. Conclusion

In conclusion, our recent findings indicate that receptor internalization is required for oxidative stress-induced $p75^{NTR}$ processing. Considered with previous data demonstrating a role for JNK in activating $p75^{NTR}$, these findings support a model in which oxidative stress induces activation of JNK, which subsequently stimulates $p75^{NTR}$ internalization, thereby facilitating proteolysis of the receptor by endosomal proteases. The role of $p75^{NTR}$ coreceptors in oxidative stress-induced $p75^{NTR}$ activation remains poorly understood. However, $p75^{NTR}$ receptor stimulation can occur independently of TrkB and TrkC since differentiated LUHMES cells lack detectable production of these co-receptors. We are currently evaluating the role of TrkA and sortilin in the proteolytic processing of $p75^{NTR}$ induced by oxidative stress. These signaling events may be of central importance in determining the survival of dopaminergic neurons since pretreatment of LUHMES cells with LM11A-31, a small molecule modulator of $p75^{NTR}$, significantly reduced neuronal death associated with oxidative stress. Additionally, our preliminary data indicate that LM11A-31 protects mesencephalic neurons from oxidative stress-induced neurite degeneration, a key, early-stage event associated with Parkinson's disease (PD). Furthermore, the complete structure of the receptor has not been solved yet. The Protein Data bank has structures for the extracellular, transmembrane, and death domains. However, the structure for the stalk domain (domain between $p75^{NTR}$-ECD and $p75^{NTR}$-TMD) is not available. Our study indicates that the stalk domain is mostly intrinsically disordered and may involve conformational changes once activated to play a crucial role in p75NTR signaling, such as forming a recognition site, protein-protein interaction site, or cleavage site. ADAM17 indicated that this region (`eight amino acid region proximal to the TMD) is where the cleavage site for TACE lies (Gooz, 2010). Furthermore, our preliminary study of LM11A-31/$p75^{NTR}$ interaction

revealed multiple potential binding sites, suggesting a mechanism of action involving various binding pockets and the possible mechanism through which the ligand influences $p75^{NTR}$ signaling. Further elucidation of the ligand/receptor interaction will be a focus of our future studies. Altogether, these findings provide novel insight into p75NTR signaling mechanisms and their potential impact on neurodegeneration associated with PD, and this work underscores the need for further studies investigating the therapeutic potential of targeting $p75^{NTR}$ to treat PD. Our ongoing and future studies are designed to identify the impact of $p75^{NTR}$ signaling on dopaminergic neurodegeneration in *in-vivo* models of PD.

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