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Assay Development for Investigating the Contributions of p75 Neurotrophin Receptor (p75NTR) Signaling to Neurodegeneration Associated with Parkinson's Disease

Samantha A. Radomski
Eastern Kentucky University, samantha_radomski@mymail.eku.edu

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Assay Development for Investigating the Contributions of p75 Neurotrophin Receptor ($p75^{NTR}$) Signaling to Neurodegeneration Associated with Parkinson's Disease

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Samantha Radomski

Faculty Mentor

Dr. Bradley Kraemer

Department of Biological Sciences

Assay Development for Investigating the Contributions of p75 Neurotrophin Receptor ($p75^{NTR}$) Signaling to Neurodegeneration Associated with Parkinson's Disease

Samantha Radomski

Dr. Bradley Kraemer, Department of Biological Sciences

Abstract:

The p75 neurotrophin receptor ($p75^{NTR}$) possesses the ability to promote cell survival or apoptosis upon activation. The specific signaling cascades regulated by $p75^{NTR}$ in neurodegenerative pathologies are poorly understood due to the multifaceted nature of the receptor. Prior studies associate oxidative stress, a cellular condition induced by an overwhelming presence of reactive oxygen species, with promoting proteolysis and subsequent activation of $p75^{NTR}$ in dopaminergic neurons. Dopaminergic neurons express $p75^{NTR}$ and are of interest in neurodegenerative research. The degeneration of these neurons occur during the progression of Parkinson's Disease. Here, we optimized an *in vivo* and an *in vitro* protocol for modeling Parkinson's Disease. Another aspect of this research was to optimize an automated method to measure the degeneration index (DI) of neurons. Developing an optimized *in vivo* model of Parkinson's Disease may provide insight into the role of $p75^{NTR}$ regarding the survival and health of dopaminergic neurons within substantia nigra and striatum regions of the brain. Developing an optimized *in vitro* model of Parkinson's Disease may allow for investigation to the effects of $p75^{NTR}$ mediated signaling events associated with

oxidative stress on cellular function. The DI is a calculation of neurite degeneration, which allows for the quantification of neurodegenerative diseases. An optimized method of analysis has provided a streamlined approach to calculating the DI and neurite fragmentation in culture. Preliminary data suggests that our optimized protocols serve as a successful model of Parkinson's Disease and thus allow further analysis into how $p75^{NTR}$ signaling cascades affect cellular outcome in Parkinson's Disease.

Keywords and phrases:

P75 neurotrophin receptor, Parkinson's Disease, neurodegeneration, oxidative stress, dopaminergic neurons, neurotrophins, co-receptors, optimized model, degeneration index, ANDI

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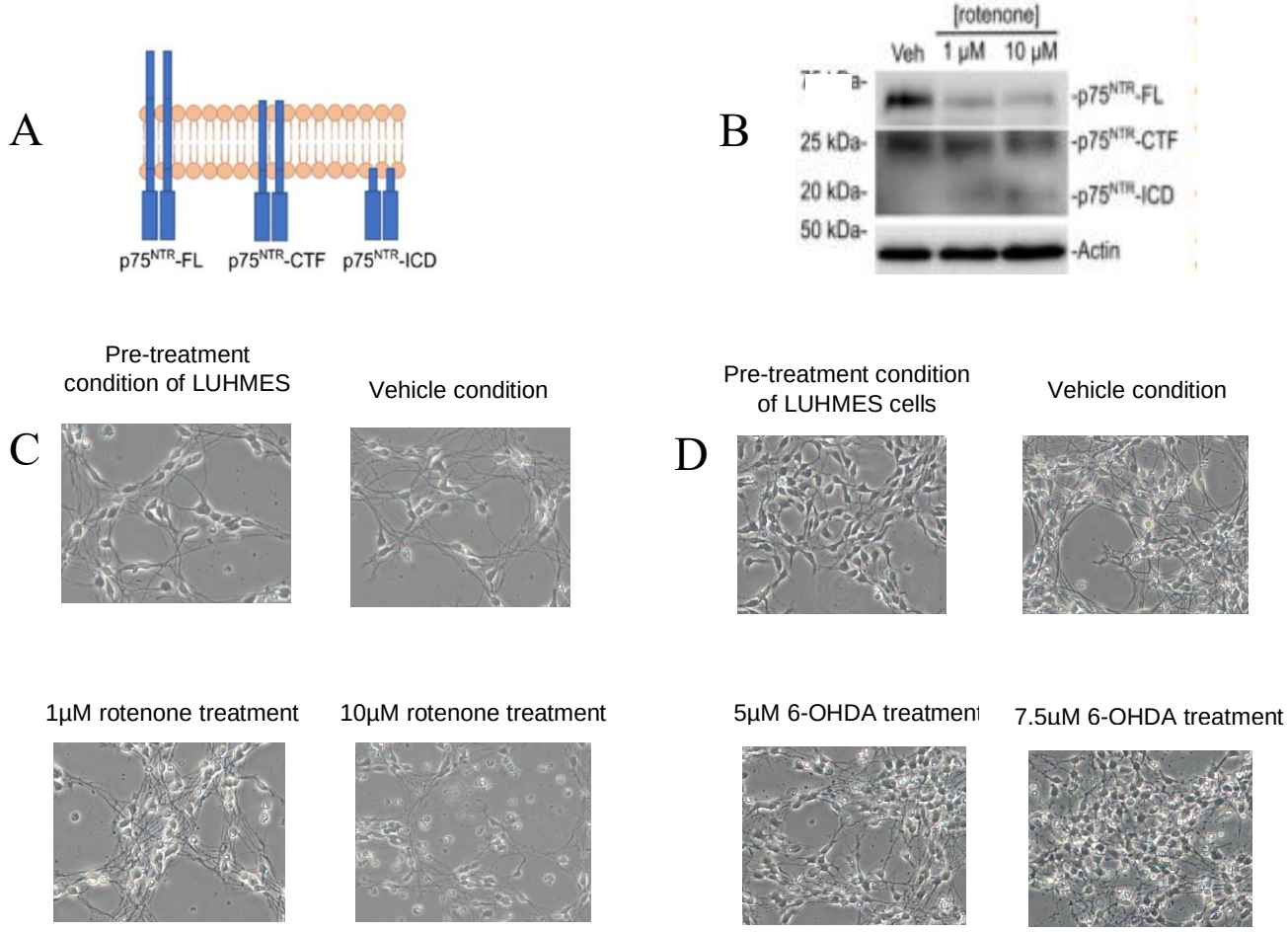

Figure 1: The proteolysis of p75^{NTR} in response to oxidative stress associated with the application of 6-hydroxydopamine or rotenone. A) A representative diagram of the proteolytic event experienced by p75^{NTR} in distinct stages. The full-length region of the protein is cleaved by an enzyme to release the extracellular domain while a secondary enzymatic event serves as a final cleavage event to liberate the intracellular domain. **B)** A representative western blot of rotenone lysates probed for p75^{NTR}. The resulting protein bands suggest that rotenone induces the proteolysis of p75^{NTR} at concentrations of both 1µM and 10µM. The proteolysis event of p75^{NTR} may be observed by matching the protein bands to the specific molecular weight, as each fragment of p75^{NTR} is assigned specific molecular weight. **C)** An example of a rotenone treatment on LUHMES cells. The image each represent a cultured dish and the subsequent degeneration observed 18 hours after the treatment was performed. Each image represents a treatment condition as described in the methods section. **D)** An example of a 6-hydroxydopamine (6-OHDA) treatment on LUHMES cells. Each image represents the effects seen after an 18-hour treatment condition of varied concentrations of 6-OHDA as described in the methods section.

Figure 2: Investigation of p75^{NTR} co-receptors present in cultured cell and whole brain lysates

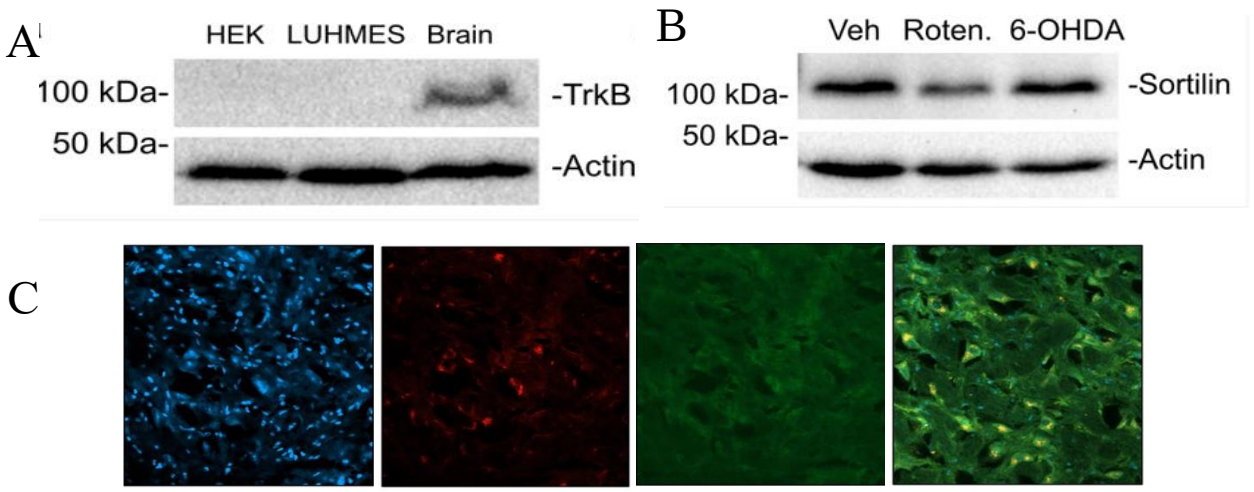

Figure 2: Investigation of p75^{NTR} co-receptors present in cultured LUHMES cell lysates and whole brain lysates. A) A representative western blot utilized to investigate three types of lysates for the presence of the p75^{NTR} co-receptor TrkB. This analysis was conducted to analyze the presence of p75^{NTR} co-receptors within a population of dopaminergic neurons. The HEK (human embryonic kidney cells) were used as a negative control and the whole brain lysate was used as the positive control. When probed for the presence of TrkB, the cultured LUHMES cells did not express a protein band. This suggests that TrkB is not present within the cultured dopaminergic regions, while it may be present in various regions of the brain. **B)** A representative western blot utilized to probe three lysate samples for the presence of p75^{NTR} co-receptor sortilin. All three populations investigated were found to express sortilin. This is an indication that sortilin is present within dopaminergic neurons and may interact with p75^{NTR}. **C)** An immunostaining assay was performed on a population of cultured LUHMES cells to investigate the presence of tyrosine hydroxylase and sortilin. The blue (first) image represents the fluorescent DAPI labeling for the nuclei of the cells. The red (second) image represents the fluorescent labeling for tyrosine hydroxylase commonly found within the dopaminergic neurons in cells. The green (third) image represents the fluorescent labeling for sortilin to further investigate the areas of the cell that possess sortilin. The combination (fourth) image represents the overlay of each stain to provide context of the interactions between each of the components (nuclei, dopaminergic neurons, sortilin).

Figure 3: The effects of p75^{NTR} on dopaminergic neurons and striatal neurodegeneration in a mouse model of Parkinson's Disease

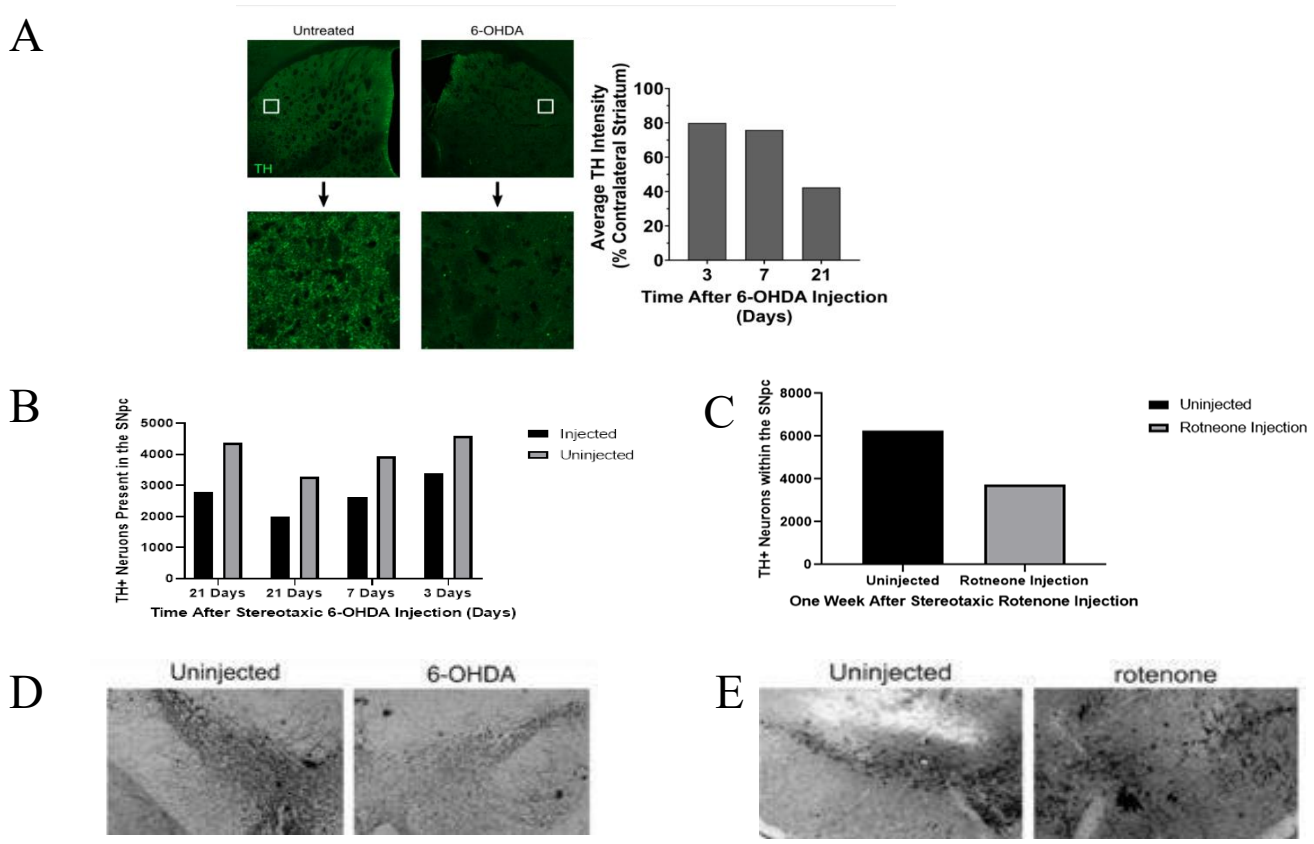

Figure 3: Investigation of dopaminergic neurons in an *in vivo* model of Parkinson's Disease. A) Representative image of fluorescent tyrosine hydroxylase (TH) staining and quantification of dopaminergic neurons within the striatum. To investigate the efficacy of the stereotaxic surgeries, the regions of interest must be isolated and analyzed to evaluate neurodegeneration. There was a visible reduction of staining intensity indicating a loss of dopaminergic neurons within the striatum after being exposed to a dosage of 6-hydroxydopamine (6-OHDA). The quantification was performed to evaluate the average brightness after a series of days after injection. This graphical representation indicates that as more time passes, the less bright the staining. This can be interpreted as the longer the 6-OHDA interacts with the dopaminergic neurons, the more degeneration occurs resulting in less healthy/intact neurons able to express TH. **B)** A representative graph depicting the number of dopaminergic neurons within the region of the substantia nigra that were observed after the surgical procedure introducing 6-OHDA into the brain. **C)** A representative graph depicting the amount of dopaminergic neurons present within the region of substantia nigra after a surgery utilizing rotenone. This analysis was performed by staining the tissue with a TH primary antibody and

subsequent reagents from a VectorElite staining kit. DAB substrate was applied as a secondary condition to develop the intensity of the stained neurons. Following the staining procedure, a stereology program was used to sample regions of the substantia nigra tissue to analyze the number of stained neurons. This analysis allowed for a comparison between the regions of the brain ipsilateral and contralateral to the site of the 6-OHDA treatment. **C)** A representative graph depicting the number of dopaminergic neurons present after one week of being exposed to an injected dose of rotenone. This analysis was performed utilizing methods described above in 3B. **D)** The tissue was stained with a TH primary antibody and subsequent reagents from a VectorElite staining kit. DAB substrate was applied as a secondary condition to develop the intensity of the stained neurons. Representative image of the substantia nigra between the uninjected region and the region injected with 6-OHDA. The image was taken via confocal microscopy and there is a visible reduction in the number of stained dopaminergic neurons, suggesting that the model was successful in inducing Parkinson's Disease. **E)** An immunostaining assay was performed in the same manner as described above in 3D. Representative image of the substantia nigra between the uninjected region and the region injected with rotenone. The image was taken via confocal microscopy and suggests a successful model of Parkinson's Disease.

Figure 4: Optimization of an automated neurite degeneration index (ANDI) from a traditional model for calculating DI

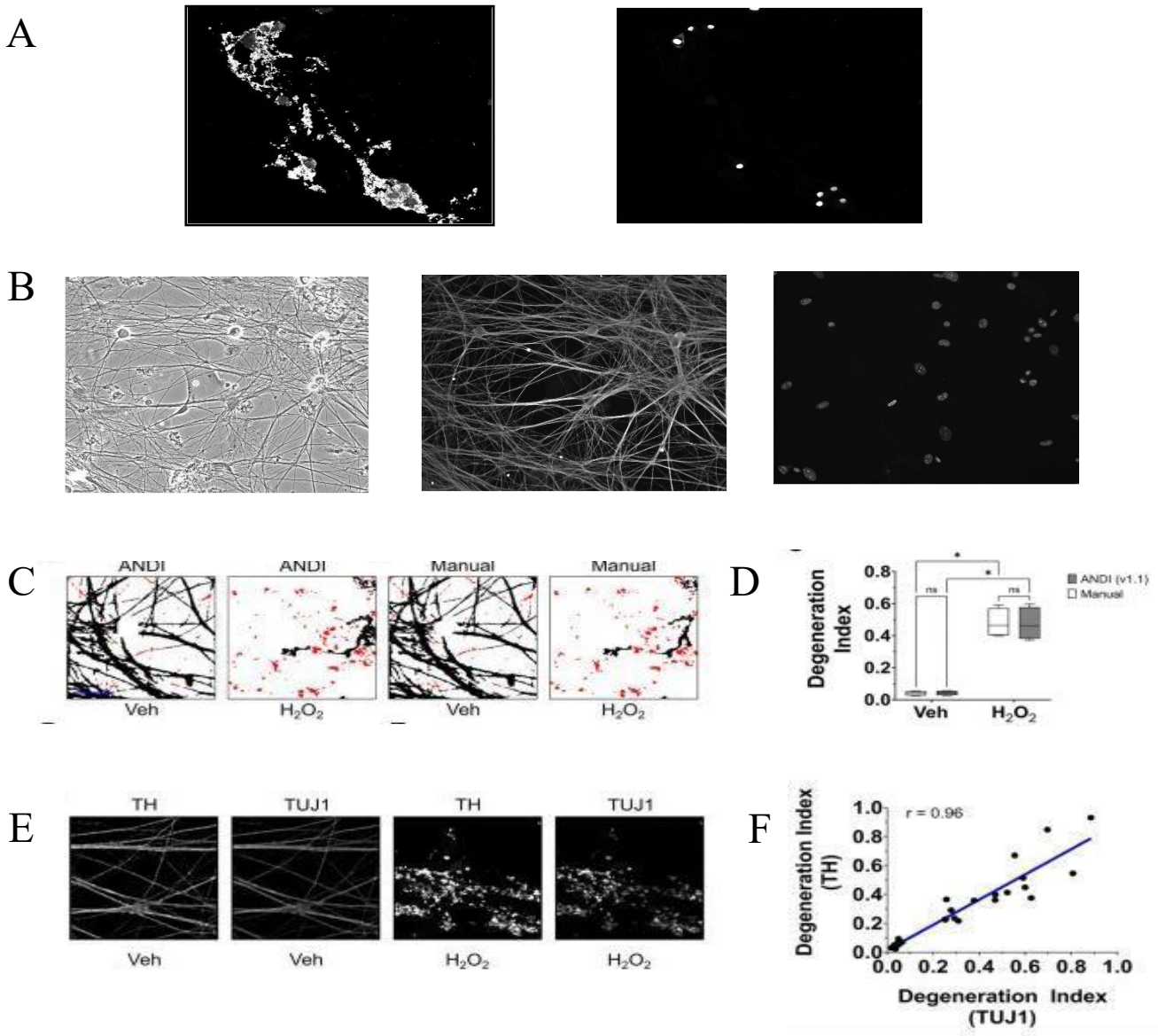

Figure 4: Optimization of an automated neurite degeneration index (ANDI) from a traditional model for calculating DI. A) Representative images were acquired from varied treatment conditions of SCG cultures. The treatment set in this image is an example of a hydrogen peroxide treatment set. Because hydrogen peroxide is known to induce neurite degeneration, it was utilized at various concentrations to observe the effect on neuronal degeneration. This image represents a treatment of 500µM hydrogen peroxide applied to a culture of SCG sympathetic neuronal ganglia. This application was performed as a condition for measuring the degeneration index (DI). The images were collected through Nikon and confocal microscopy after subjection to immunostaining of TH, DAPI, TUJ1, and fluorescent staining. The image sets were run through ImageJ (an open-sourced free software) and were incubated in a primary antibody that targeted a specific component. For example, if TUJ1 was being investigated, a primary antibody was applied to probe for it and if TH was being investigated, a different primary antibody was utilized that specifically targeted TH. A secondary antibody of Alexa Fluorophore 488 was used to perform a fluorescent analysis assay. The image on the left represents the degeneration observed from the application of hydrogen peroxide while the image on the right represents the DAPI labeling of the nuclei **B)** Representative images of a vehicle condition of cultured SCG's that were immunostained and analyzed through ImageJ. To perform a comparative analysis, the vehicle set of cultured SCG ganglia were subjected to a sham treatment of water. After the 18-hour treatment time, the culture was immunostained similar to the process mentioned above in 4A. The image on the left represents a phase contrast image of the in-tact neurites, the middle image represents a TUJ1 staining on the SCG culture, the final image represents the DAPI labeled nuclei of the cells. **C)** Previous experiments using LUHMES cells have indicated that there is not a statistical difference in using the automated method (ANDI) or a manual method of measuring the DI. A benefit of using ANDI is a more streamlined method of investigation as the levels of subjectivity are decreased in using an automated method. These images serve as an investigation if ANDI would accurately measure DI of several diverse types of cells. **D)** A graphical quantification of the staining in 4C. These images allowed for the observation that ANDI accurately measures neurite degeneration as there is no significant statistical difference between ANDI and a manual method. **E)** Representative images to analyze if ANDI would be able to measure across various staining types. The immunostaining assay was conducted for TUJ1 and TH to observe the DI of each across a vehicle and treatment condition. This analysis indicates that ANDI may be used across various cell types and treatments to calculate the DI. **F)** To investigate the efficacy of ANDI in measuring DI analysis through two methods of immunostaining, a graph was constructed to plot the comparative resulting values.

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Introduction:

The p75 neurotrophin receptor:

The p75 neurotrophin receptor ($p75^{NTR}$) is a transmembrane protein that is known to regulate cellular responses to a variety of biological conditions. $P75^{NTR}$ has been investigated as a diagnostic biomarker for neurodegenerative disease. Research has revealed that the receptor possesses the ability to regulate cellular function including programmed cell death, growth of axons, axonal degeneration, myelination, and synaptic plasticity (Kraemer et al 2014). Although $p75^{NTR}$ has been associated with regulating various cellular processes, there remains a poor understanding in the underlying signaling mechanisms of the receptor. It has been reported that the receptor is present within the layers of the neocortex as well as various populations of adult neurogenesis stem cells (DeFreitas et al 2001). These neurogenesis cells are often observed in neurodegenerative pathologies such as Alzheimer's and Parkinson's Disease. Research suggests that $p75^{NTR}$ promotes both cell survival and apoptotic pathways. This curious effect may be further explained as research groups have observed that within the

promotion of nociceptive sensory neurons the activation of $p75^{NTR}$ promotes survival, but the activation of the same receptor promotes apoptosis in sympathetic and motor neurons (DeFreitas et al 2001). In discerning the signaling mechanisms and functionality of $p75^{NTR}$, there may be a breakthrough in understanding the role of the receptor in various pathologies.

Role of $p75^{NTR}$ in development:

There are various stages of physical, cognitive, and social development that are documented and serve as the basic definition of maturation within organisms. Development is important as it allows for intricate biological systems and defining characteristics to begin to emerge. The development of the brain and nervous system is a complex event that occurs over time with observable impact and influence of various genetic and environmental factors. During the process of development, $p75^{NTR}$ has been observed at low levels of expression in the nervous systems aside from hippocampal synapse modification and regulation of neurogenesis (Meeker and Williams 2015). This receptor has been found within both the central nervous system (CNS) and the peripheral nervous system (PNS) with increased expression in the spinal cord, brainstem, cerebellum, hippocampus, and basal forebrain (Meeker and Williams 2015). A population of neurons within the basal forebrain, referred to as cholinergic neurons, have been demonstrated to express $p75^{NTR}$ during all stages of development. Cholinergic neurons are responsible for providing a primary source of acetylcholine to the region of the cerebral cortex. This source of acetylcholine aids in regulation of excitability in the cortex and hippocampus, thus allowing the

body to execute complicated cognitive tasks (Franco et al 2021). The $p75^{NTR}$ has been documented to play a role in pleiotropic effects throughout the developmental and mature CNS (Tang et al 2020). This receptor may contribute to the foundational process of development as it is found within target regions of the brain and nervous system at varied levels of expression through the developmental stages. These levels of expression indicate that $p75^{NTR}$ play hold a role in preserving or promoting age related conditions, such as Parkinson's Disease.

$P75^{NTR}$ and signaling cascades:

The $p75^{NTR}$ has been investigated in regard to its affinity to promote survival or mediate apoptotic pathways upon stimulated induced activation (Pathak and Carter 2017). There have been numerous studies investigating the cellular outcomes when the receptor is targeted with various conditions to promote a proteolytic event. $P75^{NTR}$ possesses a cysteine-rich extracellular domain that allows for ligand binding, a single-transmembrane domain, and an intracellular death domain (Zhong et al 2021). Researchers have identified $p75^{NTR}$ to play a role in mediating the growth, survival, apoptosis, and migration of tumor cells in populations of cancer cells. $P75^{NTR}$ has been investigated to promote cell survival in the nerve sheath tumors known as schwannoma and within breast cancer cells, but receptor has also been observed to inhibit the growth of prostate, gastric, and bladder tumor cells (Zhong et al 2021). Another study implicated the receptor in having the ability to facilitate signaling cascades to induce death in a population of superior cervical ganglia found in primary

trigeminal and hippocampus (Bhakar et al 2003). Additional areas found to be affected by the autonomous apoptotic pathway include the Schwann, oligodendrocyte, and neuroblastoma cells as well as glia and neurons of the spinal cord and brain after a traumatic injury (Bhakar et al 2003). In a comparable way to how there have been a multitude of instances where the apoptotic pathway has resulted in neurodegeneration, there have also been studies that have examined the role of $p75^{NTR}$ in promoting survival. These aforementioned studies have resulted in a collective understanding that cell survival may be promoted by an absence of tropomyosin-related kinase receptors (Trk) interacting with the receptor (Massa et al 2006). A study investigating the interaction between $p75^{NTR}$ and signaling partners (referred to as ligands) found that the receptor may encourage survival by antagonizing apoptotic pathways (Majdan et al 2001). Research has demonstrated that $p75^{NTR}$ possesses a wide range of reactions in response to injury and signaling partners. The receptor may provide a pivotal role in the cellular regulation depending upon the cell type, signaling cascades, ligand presence, and interaction of various co-receptors.

Structure of $p75^{NTR}$:

Understanding the structure of $p75^{NTR}$ is important to gain insight into the overall function and interactions of the receptor. This receptor is classified as a type one integral membrane protein with a helical structure (Mineev et al 2015). $P75^{NTR}$ is a member of the tumor necrosis factor receptor superfamily and possesses four cysteine rich domains (Baldwin and Shooter 1994). When the receptor interacts with neurotrophins, the neurotrophins bind to the cysteine rich

domains 2, 3, or 4 (which are coded in exon III). There are several major sections of the receptor, the cysteine-rich domain of the extracellular domain, the transmembrane domain, and the intracellular domain (Almeida and Duarte 2014). Upon understanding the various regions of $p75^{NTR}$, a greater understanding of the interactions between the receptor and other biological compounds (ligands, co-receptors, etc).

The proteolytic event of $p75^{NTR}$*:*

The proteolysis of $p75^{NTR}$ occurs in two major cleavage events and results in a downstream signaling event. To begin the cleavage of the $p75^{NTR}$, the receptor must be “activated” by an outside stimulus. Once activated, the extracellular domain (ECD) is cut and shed from the full-length receptor by the metalloprotease enzyme ADAM17. The alpha converting enzyme (TACE) releases tumor necrosis factor (TNF) to cleave the cysteine rich region of the ECD from the rest of the receptor. A second cleavage event is induced through the enzyme gamma secretase to release the intracellular domain (ICD) (Ahmed et al 2006). Each cleaved region of the $p75^{NTR}$ receptor has an associated molecular weight, which allows for each region to be tracked and observed through an assay of western blotting. It is known that a full length $p75^{NTR}$ is 75 kDa, the ECD is 55 kDa, the CTF is 25 kDa, and the ICD is 19 kDa. When observing these fragments, a molecular ladder is utilized; the protein bands can be isolated to a specific molecular weight when compared to this ladder. The ICD region within the $p75^{NTR}$ is similar in structure and function to that of the ICD region seen in other members of the TNFR. The ICD of the TNFR is known to

mediate apoptosis within a cell, allowing this region of $p75^{NTR}$ to mediate neurodegeneration. (Kraemer et al 2014). A difference between the ICD of $p75^{NTR}$ and TNFR is the interactions of this region. The ICD of $p75^{NTR}$ does not interact with other death domain proteins, which is a behavior unlike the ICD region of other common members of the TNFR (Coulson et al 2000). In understanding the general method of the proteolytic event of $p75^{NTR}$, one can investigate how the additional binding of neurotrophin ligands and co-receptors impact the cleavage event and expression of the receptor.

Relationship between neurotrophins and $p75^{NTR}$:

Neurotrophins are a family of proteins that are known to induce the survival and development of cells and neurons. Because this family encourages survival and growth, they are classified as growth factors. There are two main classifications of neurotrophins, pro- and mature-. Proneurotrophins are uncleaved biological peptides while mature neurotrophins are considered to be the active form of the protein (Meeker and Williams 2014). The pro-neurotrophins include pro-brain derived neurotrophin (pro BDNF), pro-nerve growth factor (pro NGF), pro-neurotrophin-3 (pro NT3), and pro-neurotrophin-4 (pro NT4) (Volosin et al 2008). The recognized mature neurotrophins are brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). Each of the pro-neurotrophins binds with high affinity to the co-receptor known as sortilin, (a major co-receptor of $p75^{NTR}$). Each of the mature neurotrophins bind with the same affinity to $p75^{NTR}$ but bind to Trk receptors (a major co-receptor of $p75^{NTR}$) with an ordered and selective

specificity. The neurotrophin NGF selectively binds to the $p75^{NTR}$ co-receptor TrkA, the neurotrophins BDNF and NT4 selectively bind to the co-receptor TrkB, and the neurotrophin NT3 selectively binds to the co-receptor TrkC (Shamovsky et al 1999). Additionally, research has demonstrated that $p75^{NTR}$ is fairly localized within specific regions of the brain. The basal forebrain and striatal neurons are two examples of these localized regions where there is a colocalization that occurs between the receptors $p75^{NTR}$ and Trk (Friedman and Greene 1999). The signaling cascade that results from the interaction between $p75^{NTR}$, neurotrophins, and co-receptors is still being investigated to better understand the mechanisms of the receptor.

Relationship between co-receptors and $p75^{NTR}$:

Co-receptors are a broad term for a cellular component that serves as a bridge between a signaling molecule and a primary receptor. Co-receptors of $p75^{NTR}$ are known to bind to the neurotrophins and induce a signaling cascade to occur from the interaction. The main co-receptors of $p75^{NTR}$ are tropomyosin-receptor kinase (TrkA, TrkB, and TrkC), sortilin, and nogo receptors (Meeker and Williams 2014). When mature neurotrophins bind to Trk receptors, there is an activated pathway called the mitogen-activated protein kinase cascade that results from the dimerization and activation of intrinsic tyrosine kinase activity within the Trk receptors. The binding of TrkA and NGF results in the stimulation of neurite growth and innervation (Marlin and Li 2015). Studies have shown that $p75^{NTR}$ plays a significant role in the affinity of neurotrophins to bind with TrkA which occurs as $p75^{NTR}$ is present with TrkA at the plasma membrane and

modulates the endocytosis of the kinase (Perrone et al 2005). TrkB forms an interaction bind with the neurotrophins BDNF and NT4. The result of this interaction has been documented to promote neuronal protection and general survival. A multi-step cleavage event occurring through the nuclear factor kappa light chain reaction (NFkB) when $p75^{NTR}$ and TrkB interact with each other. NFkB is a molecule known as an inducible transcription factor and this molecule has been observed in regulating genes involved in promoting inflammatory responses (Liu et al 2017). Additionally, the JNK pathway was activated by this interaction and thus resulted in a proteolytic cleavage event of $p75^{NTR}$ (Sandhya et al 2013). The interaction between the neurotrophin NT3 and co-receptor TrkC results in neuronal survival and differentiation within peripheral tissue (Sinnappah-Kang et al 2005). Sortilin is part of the vacuolar protein sorting 10 protein family and is located within brain tissue. Sortilin has been observed to contribute towards the inflammation and calcification of arterial walls, dysregulation of metabolism, and vascular disorders. When sortilin and proneurotrophins interact and bind, there is an activation of a death signaling pathway (Goettsch et al 2018). Nogo receptors are known to function as myelin associated proteins that bind to the $p75^{NTR}$ to suppress axonal growth (Meeker and Williams 2014). The relationship between $p75^{NTR}$ and the various co-receptors are observed to result in survival or death via apoptotic pathway.

Relationship between neurodegeneration and $p75^{NTR}$:

Neurodegeneration is a disease state that is a typical application to many conditions that involve the deterioration of neurons, axons, and dendrites. In an

etymological sense, the term “neuro” refers to nerve cells while the term “degeneration” refers to a process of losing structure and function. Examples of neurodegeneration disorders include Alzheimer Disease, Parkinson’s Disease, Huntington’s disease, and Amyotrophic Lateral Sclerosis (Przedborski et al 2003). In the context of Parkinson’s Disease, there is a chronic and progressive disorder resulting in cognitive deficits and mental impairment from a loss of neurons (Ding et al 2015). Neurodegeneration can be observed within nerve cells and can induce a reduction of function and death in a previously healthy system. Modern medicine has provided a method of alleviating the symptoms of Parkinson’s Disease, but not a method of reversing neurodegeneration (which is the basis of Parkinson’s) (Ding et al 2015).

Parkinson’s Disease:

Parkinson’s Disease is classified as a neurodegenerative disease and is often referred to as an age-related disease. Parkinson’s Disease is characterized by physical symptoms along with mental symptoms. Several of these symptoms include tremors, rigidity, memory loss, disordered speech, cognitive impairment, and motor dysfunction (Chen et al 2022). In cases of diagnosed Parkinson’s Disease, there is a progression of symptoms associated with stages of degeneration. As the neurodegeneration progresses, as do the observable symptoms.

Oxidative Stress:

Oxidative stress is a phenomenon where toxic molecules accumulate within a cell thus weakening it by interfering with normal cellular function (Meeker and Williams 2014). In oxidative stress, reactive oxygen species (ROS) are produced as by-products from cellular aerobic respiration. The cell is overwhelmed by the presence of the ROS as the antioxidant compounds are not able to remove the free radical ROS's (Noh and Ha 2011). Research has found that oxidative stress contributes to mitochondrial dysfunction and disruption of normal processes of the cell. Due to deviation from normal function, the cell is more vulnerable to degeneration and death at this stage. Studies have indicated that when experiencing oxidative stress, there is damage done to the DNA, RNA, proteins, and lipids (Sundar et al 2022). Oxidative stress is an event that causes the homeostasis of the cell to become unbalanced and thus results in stressful conditions. The population of dopaminergic neurons are sensitive to oxidative stress and experience damage and increased susceptibility to death when oxidative stress in applied.

Dopaminergic neurons:

Dopaminergic neurons are a specific population of neurons located within the nervous system that are responsible for transmitting dopamine across the synaptic cleft. Dopamine is a neurotransmitter that acts as a chemical messenger to send signals between nerve cells. Midbrain dopaminergic neurons are localized to specific regions of the brain; these two of these regions include the substantia nigra pars compacta and the ventral tegmental area (VTA). The

presence of these dopaminergic neurons are important to note as these neurons are often associated with the regulation of motor and cognitive function (Gale and Li 2008). A hallmark of Parkinson's Disease is a reduction of dopaminergic neurons throughout the regions of the brain (Luo and Huang 2016). Although dopaminergic neurons are localized within the substantia nigra pars compacta, the neurons project into the anterior region of the brain known as the striatum. The myelinated axons of the neurons are projected into a region known as the dorsal striatum located within the subcortex basal ganglia (Heltberg et al 2022). The dopaminergic neurons in the VTA are associated with the reward-motivation centers of the brain. Patients who have been diagnosed with Parkinson's Disease have shown a correlation with increased depression and decreased activity in the motivation-rewards center due to a loss of dopaminergic neurons in the VTA.

Lewy bodies and neurodegeneration:

Lewy bodies are protein aggregates of alpha synuclein located in a buildup of plaque within the neurons. When alpha synuclein is concentrated in a specific area, neurodegeneration is commonly observed. Alpha synuclein is a protein that is expressed in the brain and can be found specifically in the neocortex, hippocampus, substantia nigra, and cerebellum (Emamzadeh 2016). has many functions within the cell including vesicle transport, transcription regulation, and protein folding. Accumulation of alpha synuclein is known to promote the misfolding of the alpha form of proteins. When proteins are subjected to misfolding, there are common issues often documented including

aggregation, loss of function, and neurodegeneration (Timmer et al 2018). The accumulation of the misfolded alpha synuclein forms beta pleated sheets (referred to as amyloid beta plaque sheets). Lewy bodies are the name given to the abnormal deposits of alpha synuclein within the brain. Lewy bodies are associated with neuronal toxicity and result in a depletion of dopaminergic neurons in neurodegenerative disorders such as Parkinson's Disease (Kasen et al 2022). In a diagnosis of Parkinson's Disease, there is often a correlation to the observably high presence of these Lewy bodies.

The substantia nigra pars compacta:

A reduction of dopaminergic neurons within the substantia nigra has been associated with cognitive and motor impairment in Parkinson's Disease (Timmer et al 2018). The substantia nigra pars compacta is a region of ventral midbrain that contains a large source of dopaminergic neurons, thus allowing this region to play a critical role in regulating motor and cognitive function. These dopaminergic neurons project into the striatum to form an interaction known as a nigrostriatal pathway (Tritsch et al 2012). The disruption of this pathway is observed in various neurological disorders as an interruption of this pathway results in the depletion of dopamine thus resulting in a decrease of neuronal executive function within the brain.

The striatum:

The striatum is a region of the subcortical basal ganglia that receives input from dopaminergic neurons. A common side effect of Parkinson's Disease is

motor and cognitive impairment as motor dysfunction is often attributed to a loss of dopaminergic neurons within the dorsal striatum. This region of the brain is the main recipient of the dopaminergic innervation and thus requires a level of healthy dopaminergic neurons to ensure the behavior of motor and cognitive functions occurs normally (Zhai et al 2019).

Importance of the project:

Parkinson's Disease is the second most diagnosed neurodegenerative disease after Alzheimer's. This disease impacts seven to ten million people globally. This disorder stems from a loss of neurons and thus a decrease in dopamine production (Kraemer et al 2021). Parkinson's Disease is associated with neurodegeneration and thus is not reversible as this is an event that is not able to be therapeutically targeted. Symptoms of Parkinson's Disease are often associated with cognitive and motor deficiencies such as trembling, memory loss, and confusion (Meeker and Williams 2015). If the receptor ($p75^{NTR}$) is able to serve as a diagnostic biomarker, it may aid in therapeutic treatment of target neurodegenerative diseases such as Parkinson's Disease.

In vitro experimental design for optimizing protocols:

To investigate the role of $p75^{NTR}$ on neurodegeneration associated with Parkinson's Disease, *in vivo* and *in vitro* experimental models can be utilized. *In vitro* methods of experimental models include utilizing immortalized embryonic cell lines to observe the signaling cascades and mechanism of the $p75^{NTR}$ in response to various stimuli and interactions. The Lund human mesencephalic

cell line (LUHMES) is an immortalized line of embryonic cells. LUMES cells have status of proliferating cells due to the expression of the tetracycline-regulatable (Tet-off) myc transgenes. This type of cell allows for culturing and observing a differentiation process as the cells grow into postmitotic neuronal cells. The cells can be introduced to tetracycline, glial cell-derived neurotrophic factor, and dibutryl cAMP to induce the differentiation stage (Edwards and Bloom 2019). Performing assays such as cell treatments and western blots allow for the cultured neurons to be further investigated and exposed to various conditions in establishing an experimental model of Parkinson's Disease (Zhang et al 2014). When cells are allowed to differentiate and have a treatment of a chemical such as 6-hydroxydopamine (6-OHDA) or rotenone applied to them, a comparative model between cultured neurons and the neurons within a patient with a neurodegenerative disorder is observable. By applying neurotoxins and analyzing the cleavage events of $p75^{NTR}$, one can replicate the conditions of Parkinson's Disease that is often observed in human patients. Another line of cells utilized for Parkinson's research is the neuroblastoma (SH-SY5Y line) This line was established from immortalized bone marrow. These cells are utilized to mimic the effects of Parkinson's Disease in a drug-based approach. When a neurotoxin (such as 6-hydroxydopamine) was introduced into the cell culture, it was observed that the neurotoxin was transported via catecholaminergic neurons via dopamine transporters. An accumulation of neurotoxins within the cell triggers an event of oxidative stress. From this event, there was an observed production of ROS and catecholamine quinones and thus the process in tandem resulted in

neurodegeneration. Another example of a neurotoxin used to model Parkinson's Disease is the pesticide rotenone. This neurotoxin promotes formation of Lewy Bodies and inhibits mitochondrial complex I via induced ROS production. The mechanism of this neurotoxin is to enter the cell independent of transporters and disrupt cell proliferation by inhibiting the mitochondrial respiratory chain complex I (Xicoy et al 2017). Researchers utilize immortalized cell lines to perform assays to provide increased confidence and validity to the study of Parkinson's Disease. Investigators are able to draw conclusions from elucidating signaling cascades and observing biological functions that occur when a neurotoxin is introduced to a culture. It is important to test the results of *in vitro* methods that are obtained against the results of *in vivo* experimentation.

In vivo experimental design for optimizing protocols:

Another method of experimentation includes *in vivo* analysis. This method is commonly carried out through use of a mouse model as there is great genomic similarity between mice and humans. Along with this point, mice are easy to care for and have an expedited breeding cycle making the choice for these animals as experimental subjects a cost and time effective choice. There are many similarities between a mouse model and a human model of Parkinson's Disease including the observable symptoms and morphological characteristics. To investigate a model of Parkinson's Disease, a comparative analysis may be drawn between specific genotypes of mice when a neurotoxin in introduced to a specific region of the brain. Mice studies may result in a greater understanding and transference into human studies. Mice in these studies were given a dosage

of a neurotoxic drug and are left for a specific time course before further tissue analysis was conducted. During this time, the mice were monitored exhibiting any symptoms of Parkinson's Disease including turning, shivering, or tremoring. After allowing the neurotoxic drug to interact naturally within the brain and nervous system of the mouse, the mouse was anesthetized and euthanized prior brain collection. The brain was cryopreserved to prevent tissue damage, and from this point various assays may be performed to investigate the effects of Parkinson's Disease (Rocha et al 2022). Analytical assays commonly associated with this form of experimental design include tissue immunostaining, tissue extraction, quantification of neurons, and statistical analysis to observe the effect of a neurotoxic drug within the *in vivo* model. When performing assays, it is important to analyze a variety of treatment conditions and update protocols to ensure that the proper methodology is carried out. Our lab sought to optimize a protocol of mimicking Parkinson's Disease in mice to further investigate the contributions of $p75^{NTR}$ on neurodegeneration and the promotion of Parkinson's Disease.

Degeneration index calculations:

Parkinson's Disease is known to promote neurodegeneration and thus establishing a uniform method of calculating the degeneration present. This method of calculation is a quantitative method of analysis to observe the neuronal death in a strategic and objective manner. Because this assay measures levels of degermation and is used to assigning numerical values to conditions, it is important to remain objective and perform these calculations in a manner that is not drastically different between experimental groups (Kasap et al

2017). Neurite degeneration results from the introduction of dysfunction and morphological changes resulting in an overall reduction of neurite integrity (Kraemer et al 2022). To analyze and quantify neurite degeneration within a particular cell population, the method of calculating a degeneration index (DI) may be employed. The traditional method for quantifying neurodegeneration by measuring the DI was performed by binarizing an image (obtained via microscopy) and utilizing a software (usually ImageJ) to measure the pixel area of the binarized image. The next step is to calculate the average area occupied by neurite fragments. To perform a DI calculation, the area of neurite fragments is divided by the overall neurite area. The resulting calculation is a measure of neurite fragmentation. The DI value allows a researcher to analyze the impact of the treatment on cell survival based upon the quantifiable death and fragmentation of accompanying neurites (Sasaki et al 2009). Although proving to be a useful tool of analysis, this traditional method of DI calculations is time consuming and often resulted in low contrast images due to the software limitations. By enhancing image contrast and incorporating optimized parameters for the analysis, seen in setting a minimum particle size and pixel parameters for detecting neurite fragmentations and an automated cell removal operation, the time for performing the calculation is reduced while maintaining confidence in the calculated degeneration index. Another method of traditional calculations of neuronal degeneration utilizes a subjective quantification by analyzing specific qualitative criteria in the sample, following this subjective scoring a program was then utilized in sampling the axon and dendrite fields throughout the sample to

compare values (Johnstone et al 2018). Traditional methods of DI calculation involve a method of subjective scoring. Using subjectivity, a researcher was blinded to the conditions of an image set and proceeded to score the images based on a set value range of 0.0-1.0 with the scored values representing a sample of fragmented neurite area (Kraemer et al 2022). This method of analysis allows for a measure of neurite degeneration but is an assay that requires a considerable time commitment and provides images with low levels of contrast.

Aims of developing optimized protocols:

The method of developing assays to investigate the contributions of $p75^{NTR}$ signaling and to neurodegeneration that is often associated with Parkinson's Disease may be accomplished through a variety of experimental and analytical designs. To investigate $p75^{NTR}$ signaling, three main aims are investigated. Aim one is the optimization of an *in vitro* protocol of modeling Parkinson's Disease. This aim centers around utilizing the LUHMES cell culture model exposed to different concentrations of the neurotoxic drugs 6-OHDA and rotenone. After this exposure and subsequent time course, the levels of neurodegeneration are evaluated by performing western blot analysis and immunostaining to investigate the interaction between and induction of $p75^{NTR}$ proteolysis by its associated neurotrophins and co-receptors. This analysis allowed for an investigation of what biological functions are observed with the receptor. This form of investigation allowed for a greater understanding as to how the $p75^{NTR}$ interacts in a model of Parkinson's Disease. Aim two is the optimization of *in vivo* protocols of modeling Parkinson's Disease. To investigate

aim two, a mouse model was utilized. To model Parkinson's Disease, a comparative study was established by using two genotypes of mice and investigating the physiological and morphological differences between the genotypes. The c57bl/6 (wildtype) mice were compared to the $p75^{-/-}$ (p75 Knock Out) genotypes. These mice were utilized at ages between seven months and two years to allow for appropriate natural development to occur. These studies were also conducted in a manner of ensuring the subject of each experiment was matched with an age-match pair, meaning the experiments took place to analyze the effect of neurotoxic drugs on the brain of mice that were born within two weeks of each other. To thoroughly investigate the contributions of $p75^{NTR}$ in the mouse model of Parkinson's Disease, assays and analysis were conducted to collect qualitative and quantitative data. These data allow for multiple methods of observing the effects of $p75^{NTR}$ on various regions of the brain in a simulation of Parkinson's Disease. Specific protocols for immunostaining were optimized and developed, striatal section mean brightness of the TH stain was calculated, and stereology was performed on the sections of substantia nigra to observe present dopaminergic cells. The brightness of the TH stain is important to note as it is a correlation to the amount of dopaminergic neurons present throughout the target region where areas of less staining possess a reduced number of dopaminergic neurons. Together, these methods of analysis provide a basis for if the experimental methods were successful in promoting Parkinson's Disease. Aim three is centers around the development of a program to analyze the degeneration index in a method that is less time consuming and provide high

quality contrasting images of the experimental conditions. This aim was investigated by comparing the original method of calculating a DI to an automated method (ANDI) in order to compare the efficacy, time differences, and subjectivity difference between the two methods. Using a free open-source software FIJI (Fiji is just image J), a program was developed to automatically assess a set of images. This automated method of calculating the DI allowed for a more streamlined method of analyzing levels of neurite fragmentation. Previous studies utilized a traditional method of calculating the DI which was a time-consuming process that involved a multi-step approach to complete. Additionally, issues in regard to optimizing the contrast of sample images were common in using the traditional method of calculating DI (Fuller 2020). When substituting the traditional method for the optimized method established by our lab, it was experimentally observed to provide reliable data equivalent to the data collected in the traditional method. However, when using the automated method, a more streamlined approach was utilized and thus there was a reduction in time and enhanced imagery contrast in investigating levels of DI (Kraemer et al 2022). The automated method (ANDI) allowed for a more streamline The automated method enhanced the overall efficiency of calculating the DI of a sample.

Materials and Methods:

LUHMES cell culture:

Lund Human Mesencephalic cells (LUHMES) are an immortalized cell line of embryonic neuronal precursor cells. These cells are capable of proliferation and growth, allowing the culture to undergo a set of treatment conditions.

LUHMES cells were plated on 60mm cell culture dishes coated with 200 µg/mL poly-L-ornithine in sterile H2O (Sigma-Aldrich, St. Louis, MO) and 2µg/mL fibronectin in sterile PBS (Sigma-Aldrich). Coating allows for an adhesion event to occur between the surface of the plastic dishes and the cell bodies upon plating. The medium of the cells was composed of Dulbecco's Modified Eagle Medium supplemented with Ham's Nutrient Mixture F-12 (DMEM F12) (Gibco, Waltham, MA), 1% (v/v) N2 supplement (Gibco), 2mM Glutamine (VWR, Radnor, PA), and 40 ng/mL Basic Fibroblast Growth Factor (BFGF) (R&D Systems). The media was incubated at 37°C for 20 minutes to allow for proper establishment of 5% CO_2 levels. After plating, the cells were left in the incubator for five days, every two days (or when deemed necessary due to general health and confluence) a ½ media change was performed. After growing to approximately 60% confluency (or reaching day five), the cells were transferred into a coated dish with a differentiation medium. The differentiation medium allowed for cells to develop into the neuronal precursor cells. Differentiation medium was comprised of DMEM F12 (Gibco) was supplemented with 1mM db cAMP (Enzo Life Sciences), 2mM Glutamine (VWR), 1% (v/v) N2 (Gibco), 2ng/mL GDNF (R&D Systems), and 1 µg/mL Tetracycline (Sigma-Aldrich). Unlike the growth media, BFGF was not included in the media, this was done to prevent any additional cell growth. The media was then warmed in the incubator at 37°C for 20 minutes to reach appropriate levels of CO_2.

Cell Treatments

On the fifth day of differentiation, the cultures were subjected to treatment. These treatments were performed to observe the effect of various concentrations of chemicals on the cells. The information from these treatments allowed for analysis of whether the chemicals induced $p75^{NTR}$ proteolysis. Treatments were conducted with 6-hydroxydopamine (6-OHDA) (Sigma-Aldrich) and rotenone (Sigma-Aldrich) to investigate the effect of these chemicals on intracellular interactions within an *in vitro* model of Parkinson's Disease. Because of their reactivity and sensitivity, both 6-OHDA and rotenone were stored under inert (nitrogen) gas in amber epi tubes (to protect from light) at -80°C until required for usage. The 6-OHDA was prepared in a solvent of dimethyl sulfoxide (DMSO) at a stock concentration of 10mM. The rotenone was prepared in a solvent of DMSO at a stock concentration of 100mM. Cell treatments were conducted at various concentrations of each chemical including doses of 1 µM, 2.5 µM, 5 µM, 7.5 µM, and 10 µM concentrations. Each treatment set consisted of one vehicle (addition of the solvent DMSO) to allow for comparisons between treatment groups and a normal cell group. The treatment was applied and allowed to interact with the cell culture for 18 hours before lysis was performed.

Lysate Preparation:

After the 18-hour treatment course, the cells were lysed and analyzed via western blot analysis. Lysates were prepared by allowing the cells to rest on ice and removing the media. Approximately 2mL of PBS was added to the dish and the cells were scraped in a clockwise motion and collected into a 15mL conical

tube. The dish was rinsed with 3mL of PBS and scraped in a counterclockwise motion and once again, the solution was added to the 15mL conical tube. This process was performed with each dish of the treatment. The conical tubes were then centrifuged at 2000G for five minutes at 4°C. After centrifugation, a lysis buffer of Nonidet P-40 (NP40) (Sigma Aldrich) (comprised of a protease inhibitor and a PhosStop phosphatase inhibitor tablet) was added and the solution was triturated to break open the pellet of cells. The NP40 buffer served as a non-ionic and non-denaturing detergent. The lysates were then stored in -80°C until required for further analysis.

Western blot analysis:

Western blot analysis was performed to investigate the proteolysis of $p75^{NTR}$ when the receptor was exposed to a chemical known to promote oxidative stress within cellular populations. This analysis was performed to investigate the roles of co-receptors (TrkB and sortilin) and to analyze the extent of $p75^{NTR}$ fragmentation caused by 6-OHDA and rotenone. The lysates were sonicated and clarified. A Bradford assay was performed to obtain the necessary amount of each lysate to add to ensure proper protein concentrations were achieved. This assay was performed by creating a Bradford curve and calculated R^2 value. A concentration was created from using 5X Bradford assay solution (Bio Rad) and Bovine Serum Albumin (BSA) protein concentration standards (GibCo). After generating a Bradford curve with an R^2 greater than 0.97, a microliter of lysate solution was added to the gradient and the assay was once again run. After running the curve, a calculation (slope equation) was constructed to figure the

amount of protein to add to achieve the desired concentration levels. Additionally, the amount of 6X and 1X buffer to load was also calculated to ensure the aforementioned concentration levels were achieved. The lysates were prepared by adding a Laemmle buffer (Bio Rad) to the lysates and then boiling them for eight minutes and then being added to a gel composed of 1.5M Tris, 30% Acrylamide, SDS, APS, and TMED. The samples were separated by electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The gel membrane containing the lysate samples was blocked in a solution of 5% milk in 0.1% Tween in PBS. After blocking, the primary antibody was introduced to the blot. The primary antibody used was a specific to detecting the intracellular domain of $p75^{NTR}$ and was utilized at a concentration of 1:3000 (in milk) overnight at a temperature of -20°C. The next step utilized an anti-rabbit secondary antibody at a concentration of 1:12000 for an hour and a half at room temperature. After the incubation period, the $p75^{NTR}$ fragmented bands were visualized using enhanced chemiluminescence (ECL) and a ChemiDoc imaging station. After visualization, the blots were stripped and visualized with MAB1501 at a 1:1000 concentration overnight at -20°C. The next step utilized a horseradish peroxidase conjugated anti-mouse secondary antibody at a 1:5000 concentration for an hour and a half a room temperature. The actin band was then imaged using ECL or West Fempto substrate and an analysis program on the ChemiDoc imaging station.

In vivo surgery mouse model:

To investigate the effect of $p75^{NTR}$ expression on dopaminergic neurons within a mouse model of Parkinson's Disease, an *in vivo* surgical procedure was designed. This expression was investigated by exposing the dopaminergic neurons to chemicals intended to induce oxidative stress. Upon the promotion of oxidative stress, the physiological and morphological traits were investigated. Comparisons were drawn between a line of C75bl/6 (WT) and $p75^{NTR\ -/-}$ (P75 ko) mice. To obtain comparable data, studies were conducted on mice that were age matched and were between the ages of seven months and a year and a half. To perform a surgery using 6-OHDA, the mouse was given an intraperitoneal (IP) injection with 2 mg/mL desipramine in PBS (at 10mg/kg concentration) and allowed to sit for 30 minutes. Desipramine is a chemical known to inhibit the transmittance of norepinephrine and thus serves to protect the noradrenergic terminals by blocking the uptake of 6-OHDA into these terminals (Kraemer 2022). During the period of rest, the 6-OHDA was prepared at 2mg/mL using 0.02% PBS ascorbate. The 6-OHDA was diffused at a rate of 0.1 µL/minute.
Although 6-OHDA and rotenone are two neurotoxic chemicals assumed to induce Parkinson's Disease, the methods of doing so differ. Due to the nature of rotenone, a desipramine injection was not needed. Instead, the rotenone was prepared at 2.51 µL/96.5 µL in a solvent of DMSO and the surgical procedure proceeded in a manner identical to that of the 6-OHDA surgery (after the desipramine injection). Both the 6-OHDA and rotenone surgeries were conducted in the same manner after the preparation of the chemical. The next step in the

surgery protocol was to secure the mouse to a stereotaxic rig via a nose cone and ear bars. A subcutaneous injection of Meloxicam was administered at 1 mg/mL by body weight for a final concentration of 5mg/kg. Meloxicam serves to release analgesics in mice and thus serves as a method of pain and infection prevention/management after a surgery has been conducted. A Hamilton syringe was primed with isopropyl alcohol and then rinsed with the solvent before being loaded with the neurotoxic chemical. To ensure a sterile environment, the fur of the mouse was trimmed, and the skin of the mouse was disinfected with 70% ethanol and povidone iodine. A scalpel was utilized to create a vertical midline incision and hydrogen peroxide was utilized to clean the exposed bone plate. The stereotaxic rig was utilized to navigate the tip of the syringe to Bregma. From Bregma, specific coordinates were applied where the anterior-posterior value was set to +0.5mm, the medial-lateral was set to -1.9mm, and the dorsal-ventral was set to +3.5mm. At this location, a small hole was drilled into the skull using a dental drill and the syringe was lowered into the hole. The neurotoxin was administered and after being expelled, the syringe was allowed to sit in the brain for an additional five minutes to prevent capillary action from drawing up the chemical. The mouse was sutured and 200 µL of PBS was administered to replace fluid levels. The mouse was monitored for symptoms and health of the wound site for a time course of 4, 8, and 12 hours after the surgery. Additionally, Meloxicam was administered 24 and 48 hours after the surgery to prevent infection and pain.

Immunostaining striatum:

The tissue was permeabilized with 0.3% Triton-X-100 in sterile 1X PBS. The tissue was blocked with 10% normal goat serum (NGS) in PBS for an hour. The blocking solution was removed and a fresh solution of 10% NGS in PBS was made, and the primary antibody Anti-Tyrosine hydroxylase (AB152) was added in a 1:400 concentration. The solution was applied to the tissue and incubated overnight at 4°C in a humidity chamber. The next day, washes were performed on the tissue and a secondary Alexa fluorophore secondary antibody (Alexa fluor 488) was applied at a concentration of 1:500 in PBS for an hour and a half at room temperature (and protected from light to abnormal excitation of the fluorophore). To stain the nuclei, 4',6-diamidino-2-phenylindole (DAPI) was added at a concentration of 1:200 and allowed to incubate for five minutes at room temperature. The slides were washed, mounted with fluoromount-G (Southern Biotech), and allowed to dry.

Immunostaining substantia nigra:

The tissue was permeabilized with 0.1% Triton-X-100 in sterile 1X PBS. The tissue was incubated in the Vector-elite staining kit blocking serum diluted with sterile PBS for 30 minutes at room temperature. The tissue was then incubated in fresh blocking serum and PBS containing primary antibody (AB152) at a concentration of 1:400 at 4°C overnight. After performing washes, the tissue was incubated for 30 minutes in biotinylated secondary antibiotic serum. The ABC reagent provided by the kit was incubated for 30 minutes. A 3,3'-diaminobenzidine (DAB substrate) was incubated for eight minutes to develop in

intensity. The slide was mounted with fluoromount-G (Southern Biotech) and was allowed to dry.

Image capture:

To investigate the levels of tyrosine hydroxylase staining (TH) and dopaminergic innervation of the striatum, striatum image capture was conducted using a Zeiss LSM 800 confocal microscope. The striatum was imaged using ZEN software and the images were quantified for mean brightness through FIJI (an open-sourced deviation of ImageJ). The images were analyzed in regard to the mean brightness and quantified to obtain analysis of the dopaminergic neurons within the sample of the striatum. Because the samples were collected, immunostained, and imaged as an age match pair, the results of the quantification are comparable. The average intensity of immunostaining was analyzed to investigate the average TH brightness within samples resulting in a comparative investigation into striatal innervation in the presence of $p75^{NTR}$.

Stereology:

To investigate levels of tyrosine hydroxylase staining (TH) and presence of dopaminergic cells within the substantia nigra, the tissue of this region was immunostained and analyzed. The tissue was immunostained to target the TH present in the population of dopaminergic neurons in the region. This immunostaining was then quantified by using a stereology program and Zeiss program through confocal microscopy. Parameters including sample size, sample thickness, and frame size were input into the program. As the program focused through the z-axis, the program allowed the selection of dopaminergic

cells; these cells served as a representative sample of dopaminergic neurons from each tissue sample.

Optimized macro analysis (ANDI) and degeneration index calculation:

To optimize a macro analysis and perform optimized degeneration index calculations, sympathetic neurons were cultured, immunostained, and imaged with both standard and confocal microscopy. Eight-well chamber slides for this experiment were coated with 100 µg/mL poly-L-ornithine (Sigma-Aldrich), 1 µg /mL Fibronectin (Sigma-Aldrich),100 ng/mL poly-D-Lysine (Sigma-Aldrich), and 10 µg/mL Laminin (Corning, Corning, NY). The sympathetic ganglia from mice pups (>7 days old) were collected and plated in growth media. The growth media was composed of DMEM (Gibco), 10% fetal bovine serum (Invitrogen), 2mM Glutamine (Corning), and 40 ng/mL of NGF (Fisher Scientific). A concentration of 5 µM Ara-C (Sigma-Aldrich) was utilized to aid in the removal of non-neuronal cells such as Schwann cells from the culture. The slides of cells were immunostained to investigate levels of βIII-tubulin and TH. In addition to these immunofluorescent labels, a DAPI counterstain was applied to label the nuclei of the cells. After fixing, the cells were permeabilized with 0.3% Triton-X-100. The cells were blocked with 10% NGS in sterile 1X PBS for one hour. The primary antibody (AB152) was added to fresh blocking solution so that the concentration was at a 1:400 concentration at 4°C overnight. The slides were washed and incubated with Alexa fluorophore 568 as the secondary antibody at a concentration of 1:500. DAPI in PBS was added for 5 minutes. The slides were

washed with sterile 1X PBS and were mounted using fluoromount-G (Sourthern biotech).

The treatment, immunostaining, and image capture took place in eight-well chambered slides with each chamber subjected to a specific treatment condition. Analysis was first performed via a Nikon Eclipse II Ti-U Inverted Microscope System employed with a NIS Element Acquisition Software. To optimize the microscope optics, Kohler illumination was performed prior to the image capture section. Cells were imaged under phase contrast microscopy and fluorescent microscopy images were collected on a TUJ1 and TH channel. For the phase contrast images, the microscope was set to 20x magnification with a 1.00x gain and 21 second exposure time. If needed, the gain and exposure time was adjusted so that the brightness of the picture was consistent. Each of the adjustment made in regard to gain and exposure time was noted and kept constant through the image set. A background image was taken to correct for any camera imperfections. Background correction was employed with each phase contrast imaging set. The well was imaged in five different representative spots where the neurites were defined and where cells were evenly distributed. Phase contrast, TUJ1, TH, and DAPI images were collected from each chamber. To further the analysis, the wells were also imaged on the confocal at 0x magnification. Using Nis Element Acquisition software in tandem with Nikon inverted microscope (standard microscopy), an image was collected from each channel. Each of the images was categorized as phase contrast, fluorescent, and DAPI folders and ensured that each set matched up. To calculate the

degenerative index (DI) measurements cited in traditional methods, each image was input into FIJI (open-source ImageJ software). The images were binarized so that the background was white contrasted with the black cells of the foreground. The freehand tool was utilized to trace over cell bodies to convert the mass into the white background. The measure tool was utilized to receive a measurement of the total area occupied by the black background. Neurite fragmentation was measured using parameters in the FIJI analysis. In an analysis of the traditional method of calculating DI, this fragmentation was analyzed by measuring size of fragments between 20-10,000 pixels and with circulatory settings at 0.20-1.00. In an analysis of the optimized automated method of analyzing neurite fragmentation, the analyze particle tool within the FIJI software was utilized with parameters of 0-9 pixels and circulatory parameters of 0.0-1.0. The subsequent calculation to determine the DI was obtained by summing the neurite fragments divided by the total area occupied by the background to provide the DI of the image.

Results:

***In vitro* results:**

LUHMES cells were cultured, differentiated, and treated with 6-OHDA or rotenone to investigate various interactions of $p75^{NTR}$ including the proteolysis, interaction of co-receptors, neurotrophin interaction, and response to oxidative stress. The treatment conditions of rotenone were investigated to observe the effect of the chemical on the proteolysis of $p75^{NTR}$ as a model of Parkinson's

Disease. The event of $p75^{NTR}$ proteolysis is a two-step process (Figure 1A). The experiments (Figure 1C) revealed a dose dependent increase of degeneration and death in the population of LUHMES cells. The lysates procured from the treatments were analyzed through western blot analysis to further investigate the proteolysis and accumulation of $p75^{NTR}$ fragments induced following rotenone application (Figure 1B). We assumed the application of rotenone to a population of cells would mimic oxidative stress and promote the proteolysis of $p75^{NTR}$. This was then investigated when a population of LUHEMS cells (thought to possess dopaminergic characteristics by expressing the $p75^{NTR}$ receptor) was exposed to rotenone and analyzed for the proteolysis of the receptor. We observed the $p75^{NTR}$ ICD fragment to be present in 1µm and 10µm rotenone conditions. The co-receptor TrkB was not present within the lysates of LUHMES or HEK cell culture but was expressed in the lysates of the whole brain (Figure 2A). The co-receptor sortilin was found to be present in vehicle, rotenone, and 6-OHDA treatment conditions (Figure 2B). Sortilin was investigated in a population of LUHMES cells and was found to be present in an immunostaining assay (Figure 2C). TrkB was not present in LUHMES cells while sortilin was found to be present in LUHMES cells after treatment and analysis via western blot analysis.

In vivo results:

To optimize a mouse model of Parkinson's Disease, stereotaxic surgeries were performed with 6-OHDA and rotenone. Using age matched mice of two distinct genotypes (one lacking the $p75^{NTR}$ gene and one exhibiting the $p75^{NTR}$ gene), comparative studies were conducted to determine the physiological and

morphological divergences and effects in regard to $p75^{NTR}$ on neuronal protection and degeneration. It was experimentally observed that an injection of 2µg/mL of 10mM 6-OHDA in 0.02% PBS ascorbate at a rate of 0.1µl/minute resulted in substantial loss of dopaminergic neurons from regions contralateral and ipsilateral of the injection within both the striatum (Figure 3A) and the substantia nigra (Figure 3B). Similarly, rotenone (in a DMSO solvent) was administered at a rate of 0.1µl/minute resulting in loss of dopaminergic cells from the striatum and substantia nigra. In these studies, there was an observable loss of dopaminergic neurons between the ipsilateral and contralateral regions of the injection site for both 6-OHDA and rotenone within the region of the striatum (Figure 3C) (Figure 3D).

Optimized degeneration index results:

Traditional methods of degeneration index (DI) calculations presented with issues in efficiency and image contrast. ANDI was established by optimizing a protocol of DI calculation by adjusting the parameters of the study to quantify neurite degeneration. Image sets of phase contrast, TUJ1, TH, and DAPI were obtained through standard and confocal microscopy. These image sets were done with vehicle wells and with various treatment conditions of hydrogen peroxide (Figure 4A) (Figure 4B). The automated method proved to be an effective method of quantifying neurite degeneration as there was no statistical significance detected between traditional and optimized methods. Although there was not a statistical difference in results, there was a greater efficiency associated with the automated ANDI model in regard to time as well as the ability

to provide a higher contrasting image through the program. This ANDI program utilized particle-remover plugins to automatically remove non-neurite and debris fragments from the images. Measurement and calculation of the DI was obtained by dividing neurite fragment area by total neurite area. A treatment group utilized hydrogen peroxide to establish the automated macro successfully measures the neurite degeneration in sources besides 6-OHDA (Figure 4C) (Figure 4D). The use of SCG cultures to collect primary neurons allows for the exploration of ANDI and measurement of a population other than LUHMES. Upon comparing the calculated DI from studies conducted through the traditional manual method and the optimized ANDI method, there was no statistical difference between the calculation values. The ANDI method was established as a more efficient method of DI calculations as the results were statistically insignificant in comparison to the manual method while the time required to perform the optimized method was reduced in comparison to performing the manual method (Figure 4E) (Figure 4F).

Discussion:

Investigation of the transmembrane protein $p75^{NTR}$ may result in understanding the relationship between its activation and neurodegeneration in regard to the development of Parkinson's Disease. It has been observed that $p75^{NTR}$ is present in many models of neuronal degeneration and thus may be capable of mediating neurodegenerative diseases (Gitler et al 2017). To further investigate the signaling mechanisms and relationships of $p75^{NTR}$, we optimized multiple protocols for investigating the effects of $p75^{NTR}$ expression on dopaminergic neuron degeneration, analyzing the mechanisms of $p75^{NTR}$ upon

exposure to oxidative stress, and establishing an efficient method of calculating the DI. While the specific signaling mechanisms of $p75^{NTR}$ remain unknown, optimizing *in vivo* and *in vitro* methods of modeling Parkinson's Disease will allow for us to understand the contributions of the receptor to the disease. 6-OHDA is known to induce oxidative stress allowing for a method of modeling Parkinson's Disease. Rotenone is a chemical known to promote the formation of Lewy Bodies and oxidative stress is induced allowing for the chemical to be used to model Parkinson's Disease. In establishing an optimized method of calculating the DI, a more efficient method of analyzing the fragmentation and degeneration in the sample. The optimized calculations which have been shown to produce similar values seen in a manual method of calculating the DI. It was not statistically significant between the values from ANDI or manual; thus, the method of ANDI is a more time efficient method of analysis without skewing objectivity of analysis.

The experimental design included *in vitro* and *in vivo* methods. Performing a variety of assays allows for an in-depth analysis of $p75^{NTR}$ under various conditions including interactions between co-receptors, neurotrophins, and oxidative stress. Through past western blot analyses, it was discovered that $P75^{NTR}$ undergoes a proteolytic event when exposed to an outside stimulus such as oxidative stress. To further analyze the signaling cascade of $p75^{NTR}$, 6-OHDA and rotenone were utilized as cell treatments and in an *in vivo* surgical procedure to induce Parkinson's Disease in a mouse model. From these treatments, western blot analysis enables measurement of fragmentation upon proteolysis of the receptor. It was expected that rotenone would cause neurodegeneration;

however, the method through which $p75^{NTR}$ experiences proteolysis and subsequent signaling cascade has not been well documented or understood.

The use of *in vivo* studies are necessary to investigate the role of $p75^{NTR}$ in mice models to further investigate the effect of $p75^{NTR}$ within a biological system. These studies were performed with two chemicals known to induce oxidative stress. Preliminary data suggests that $p75^{NTR}$ may play a role in neuronal protection in Parkinson's Disease. Both 6-OHDA and rotenone were demonstrated to model Parkinson's Disease by promoting neurodegeneration and a general reduction of dopaminergic neurons.

In vitro studies have demonstrated that the LUHMES cells are a model for dopaminergic neurons (Tushaus et al 2021). By utilizing a population of cells that exhibit similar characteristics commonly observed in dopaminergic neurons. These LUHMES cells are able to proliferate and differentiate into postmitotic neuronal cells upon introduction to growth media or differentiation media (Harischandra et al 2020). In utilizing cultures of LUHMES cells, we were able to investigate the relationship between $p75^{NTR}$ and other biological ligands and co-receptors. We found that the co-receptor TrkB is not expressed in dopaminergic cells from the ventral midbrain, but the co-receptor sortilin is. This is interesting as sortilin is a type-1 receptor that is thought to promote apoptotic pathways (Nykajaer and Willnow 2012). The presence of sortilin may form an interaction with $p75^{NTR}$ resulting in apoptosis induction and subsequent neurodegeneration.

An automated neurite degeneration index (ANDI) was created to aid in optimizing an efficient protocol to calculate the degeneration index.

Traditional/manual methods of calculating the degeneration index are time consuming and provide low contrast images. In optimizing the protocol using FIJI and a macro program, we were able to provide efficiency and a greater quality than what was previously able to be achieved. ANDI was shown to provide accurate DI calculations for treatment groups of hydrogen peroxide at various concentration thus allowing a confirmation that ANDI is an optimized method of DI calculations for various cell and treatment types. In regard to statistical accuracy, there was no statistical significance found between the DI calculated through a manual method and through ANDI. This resulted in an indication that ANDI is an optimized protocol for measuring DI calculation as it aids in the overall efficiency and speed of the calculations while also provides a better set of contrast images by adjusting the parameters in FIJI. By adjusting the particle remover tool, pixel function, and circulatory functions, the images were produced with sharper contrast.

Currently, there needs to be further investigation in regard to how $p75^{NTR}$ impacts neurodegeneration and how these models are translational to Parkinson's Disease in the human population. Further studies from our lab may provide an analysis for neuronal and axonal degeneration in response to oxidative stress. Completion of these additional studies will lead to novel insight into the impact of specific $p75^{NTR}$ signaling mechanism on neurodegeneration associated with Parkinson's Disease.

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