TP5: A POTENTIAL THERAPEUTIC DRUG FOR PARKINSON'S DISEASE

INVESTIGATING THE THERAPEUTIC EFFECTS OF TRUNCATED PEPTIDE 5, A CDK5/P25 INHIBITOR, IN AN *IN VITRO* AND *IN VIVO* MODEL OF PARKINSON'S DISEASE

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TITLE: Investigating the Therapeutic Effects of Truncated Peptide 5, a Cdk5/p25 inhibitor, in an *in vitro* and *in vivo* model of Parkinson's Disease

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ABSTRACT

Parkinson's Disease is a chronic progressive neurodegenerative disease that affects approximately 2000 out of 100 000 people over the age of 80. This disease is typically characterized by impaired motor function, as well as cognitive and autonomic dysfunction as the pathology worsens. The two main hallmarks typically associated with PD are the dopaminergic loss in the nigrostriatal pathway and the presence of Lewy bodies. However, a cellular dysfunction has been commonly found in neurodegenerative diseases, known as the hyperactivation of the Cdk5/p25 complex. This complex is an essential target to focus on the protection of neurons and prevent the pathology of PD to worsen as treatments are currently only temporary in symptom relief. Truncated Peptide 5 (TP5) is a Cdk5/p25 inhibitor that has demonstrated potential therapeutic effects in neurodegenerative disease models such as Alzheimer's Disease. Paraquat is an herbicide that has implicated Parkinson's Disease symptoms in those who have been exposed to this toxin. The purpose of this study was to explore TP5's therapeutic effects to determine if TP5 has the potential drug to treat towards PD. TP5 was tested *in vitro* and *in vivo* models that are exposed to paraquat to induce Parkinson's Disease like phenotypes and biochemical features. A truncated fragment of TP5, known as Peptide A, was also further explored to determine potential therapeutic effects like TP5. TP5 has shown its ability to protect differentiated *SH-SY5Y* cells and the dopaminergic morphology and behaviour of *C. elegans* when exposed to paraquat. These results further support that TP5 has neuroprotective effects against models of Parkinson's Disease. TP5 was also able to regulate the physiological mechanism of Cdk5, such as neurite outgrowth, to further

understand its relationship with Cdk5 activity. Lastly, TP5 restored dopaminergic morphology against worms exposed to paraquat. These results suggest TP5 influences the pathology of PD, in both neuroprotective and neurorestorative manner, to confirm that TP5's potential as a therapeutic drug for Parkinson's Disease.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ADE	Anterior deirid neurons
ANOVA	Analysis of Variance
ASN	Alpha-Synuclein
BBB	Blood-brain-barrier
CEP	Cephalic neuron
CDK5	Cyclin-dependent Kinase 5
CIP	125 amino acid residue of p35
CNS	Central nervous system
GSH	Glutathione
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MEF2	Myocyte Enhancer Factor 2
MPTP	1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine
MTT	3-(3,4-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
ONOO-	Peroxynirite anion
PD	Parkinson's Disease
PDE	Posterior deirid neuron
PQ	Paraquat (1,1'-dimethyl- 4,4'-bipyridium)
RA	All-trans-retinoic acid
ROS	Reactive oxygen species
SNpc	Substantia Nigra pars compacta
SynI	Synapsin I
SynIII	Synapsin III
TH	Tyrosine hydroxylase
TP5	Truncated Peptide

Declaration of Academic Achievement

The majority of work described herein was conceived, analyzed, and written by the author of this thesis, in consultation with Dr. R. Mishra. They also performed most of the experiments *in vitro*, such as the cell viability assays and the neurite outgrowth assays. Shane Taylor and Anika Gupta assisted in the *in vivo* experiments, specifically with the imaging of the dopaminergic degeneration of the *C. elegans*.

CHAPTER ONE

INTRODUCTION

1.1 Parkinson's Disease

1.1.1 Prevalence and Symptomology

Parkinson's disease (PD) is a chronic progressive neurodegenerative disease that affects approximately 41 people out of 100 000 over 40 years of age, and 1 900 out of 100 000 over 80, making PD the second most common neurodegenerative disease after Alzheimer's disease (AD) (Cacabelos, 2017). Based on the increased prevalence of PD in older aged groups, age is the predominant factor of developing PD. There are two forms of PD: familial, those who are predisposed to genetic mutations due to family inheritance, and sporadic idiopathic, those who develop the disease due to environmental and/or genetic factors. 15% of cases are in the early onset of familial PD, which is seen around the age of 35 (Camargos et al., 2009) The majority of cases are sporadic idiopathic which usually leads to a later onset of PD. However, an individual can develop this disease from a multifactorial etiology. This means a genetic predisposition and environmental triggers could increase their chances of developing severe pathological and clinical symptoms.

The typical diagnosis of PD consists of impaired motor symptoms such as tremors, bradykinesia, stupor, and rigidity (Kalia & Lang, 2015). Bradykinesia, rigidity, and tremors are initiated in the early stages of PD, whereas dysphagia, postural instability, and freezing of gaits are found in the advanced or later stages. In addition to advanced PD, individuals can also experience psychosis, cognitive impairment, and autonomic dysfunction. Nevertheless, there are clinical symptoms that can act as early indicators before the full onset of typical PD symptoms are present. This is due to the early neurodegeneration of dopaminergic neurons that occurs before the onset of motor

symptoms; when these typical motor symptoms are present, 30% of dopaminergic neurons are already damaged (Cheng, Ulane, & Burke, 2010). The prodromal phase can precede typical symptoms by 20 years or more; symptoms usually consist of constipation and diagnosis of rapid eye movement sleep disorder (Kalia & Lang, 2015). However, the course and prognosis of the disease differs based on the severity of the pathology and the risk of development. Not all individuals will undergo the same symptoms, thus making it difficult to determine their exact etiology and provide proper treatment.

1.1.2 Etiology

The exact etiology of Parkinson's Disease cannot be confirmed due to a variety of genetic and environmental causes that can be potentially involved in the development of this disease. As mentioned before, about 15% of cases are determined to be familial that develop this disease due to genetic mutations (Corti, Lesage, & Brice, 2011). Synuclein Alpha (SNCA) is a major causative gene involved in this form of familial PD (Lücking & Brice, 2000). Other genes that are known to be involved in the development of PD are LRRK2, Parkin, PINK-1 and many more (Kalia & Lang, 2015). Although there have been recent advances towards determining the genetic predisposition for PD, 85% of PD cases are sporadic, which are due to a combination of genetic and/or environmental causes (Rajput, Offord, Beard, & Kurland, 1984). Investigating these environmental risk factors will help identify the possible mechanisms of action and how they lead to the pathogenesis of PD. There is also a strong association of developing PD in rural areas, usage of beta blockers, having an agricultural occupation, and exposure to pesticides (Delamarre & Meissner, 2017).

1.1.3 Pathophysiology

There are two main pathological hallmarks of PD. The first hallmark is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Kalia & Lang, 2015). This leads to the degeneration of the nigrostriatal pathway, specifically between the SNpc and the striatum. These two regions are essential in the regulation of motor control. Typically, the striatum receives the dopaminergic input from the neurons in the SNpc. However, when the dopaminergic neurons are damaged in the SNpc, they are unable to release dopamine into the striatum from the nerve terminals. As a result, dopamine synthesis will be reduced, and the maintenance of motor movement will be dysregulated.

Another well known characteristic is the formation of Lewy bodies and Lewy neurites, which is made up of insoluble aggregates of alpha-synuclein (ASN) (Lücking & Brice, 2000). They can be detected in post mortem tissues of a PD patient's brain (Kellie et al., 2015). In physiological conditions, ASN is believed to be structured as a native unfolded monomer or helically folded tetramer (Ghiglieri, Calabrese, & Calabresi, 2018). ASN proteins are localized in the presynaptic terminal; therefore, it is involved in the regulation of synaptic plasticity (Lücking & Brice, 2000). Although the function of this protein has not been fully established, it is believed that this protein regulates the release and transport of dopamine, induces fibrillization of tau, and inhibits p53 and proapoptotic genes to protect non-dopaminergic neurons (Siddiqui, Pervaiz, & Abbasi, 2016). Due to ASN's complex functions, there are quality control systems to regulate the assembly of the correct form of this protein by inhibiting and controlling the oligomerization of ASN.

However, due to abnormal cellular death signals, the soluble monomer initially misfolds into oligomers, then progressively combine to form small protofibrils and eventually forms large insoluble fibrils within the cell body and processes of neurons (Lücking & Brice, 2000). When ASN becomes dysfunctional, regulation of dopamine is impaired and protection towards neurons is lost due to the increased expression of p53 and proapoptotic genes, leading to neuronal loss.

However, the aggregation of ASN does not only occur from genetic mutations. This aggregation can also occur through cellular dysfunction that takes place in the brain. Oxidative stress, mitochondrial dysfunction, and neuroinflammation are often determinants that can result in the aggregation of ASN as well as the loss of dopaminergic neurons (Ghiglieri et al., 2018). It was also demonstrated that through the progression of age, the efficiency of proteolytic mechanisms decreases, leading to the increased accumulation of ASN in the nigral dopaminergic neurons (Chu & Kordower, 2007). The presence of these hallmarks and cellular dysfunction will also affect non-dopaminergic neurons, such as serotonergic, noradrenergic, and cholinergic neurons, which contributes to the existence of non-motor and autonomic dysfunction that are exhibited in the advanced stages.

1.1.4 Current Treatments

Therapeutic drugs that aid Parkinson's Disease individuals focus on alleviating symptoms by increasing levels of striatal dopamine or stimulating dopamine receptors. Some current treatments consist of dopamine agonists, levodopa, and monoamine oxidase

type B inhibitors. (Ellis & Fell, 2017). Levodopa is the primary treatment for PD and currently alleviates most of the symptoms (Cacabelos, 2017). Levodopa can cross the blood brain barrier (BBB) and is converted into dopamine through aromatic L-amino acid decarboxylase. Due to levodopa and dopamine agonists' mechanism of action to increase dopamine, these treatments are only temporary and may lead to more severe symptoms than the typical symptoms experienced in the advanced stages (Kalia & Lang, 2015). Some treatments can lead to severe side effects; with dopamine agonists, patients can experience nausea and hallucinations. Long term usage of levodopa can lead to motor and non-motor fluctuations and dyskinesia, which is demonstrated in over 80% of patients using levodopa for over 10 years (Ellis & Fell, 2017). Although primary treatments focus on the increase of dopamine, the degeneration of dopaminergic neurons is still present; therefore, storage and release of dopamine will be impaired, resulting in the ineffectiveness of levodopa and dopamine agonists. At present, there are no available treatments that aim to slow the progression or prevent the pathology of this neurodegenerative disease (Kalia & Lang, 2015). Therapeutic drugs should aim to focus on selecting a dysfunctional pathway that is common in most individuals to prevent their symptoms from worsening.

1.2 Toxin-induced models of Parkinson's Disease

Models of PD are required to determine the underlying mechanism of this disease. A common method to induce Parkinson like symptoms and its pathophysiology is through the usage of toxins. A variety of neurotoxins have demonstrated different characteristics

of the disease for potential therapeutic drugs to treat motor symptoms and its pathophysiology (Bové & Perier, 2012). However, an ideal model of PD should consist of not only all the pathological and clinical features, but there should be an age-dependent onset and progressive nature of this disease as well. None of the current toxin-induced models have all these PD features; however, examining different models can further aid towards better understanding how the disease progresses and potential treatments for neuroprotective strategies.

1.2.1 6-hydroxydopamine

6-hydroxydopamine (6-OHDA) is a neurotoxic synthetic organic compound that selectively targets dopaminergic and noradrenergic neurons. Due to its similar chemical structure to the catecholamines, 6-OHDA has a high affinity for the catecholamine transporters, leading to the damage of dopaminergic and noradrenergic neurons (Luthman, Fredriksson, Sundström, Jonsson, & Archer, 1989). 6-OHDA must be directly injected into the brain to activate neuronal death due to its lack of accessibility through the BBB. Direct administration of the neurotoxin into the SNpc will cause dopaminergic degeneration within 24 h (Faull & Laverty, 1969). 6-OHDA's toxicity towards dopaminergic neurons is caused by the increased oxidative stress after entering the neuron through the dopamine transporter (Cohen, 1984). The degeneration of dopaminergic neurons is progressive in PD; however, 6-OHDA has yet to show any signs of the progressive loss of dopaminergic neurons; direct administration of 6-OHDA has only led to immediate neuronal death (Bové & Perier, 2012). In addition, although one of the hallmarks of PD is demonstrated by 6-OHDA, such as the presence of dopaminergic

degeneration, the formation of Lewy Bodies has not been detected (Bové & Perier, 2012) There are many behavioural abnormalities that are influenced by 6-OHDA that fits the Parkinson like model (Jiang & Dickson, 2017). Overall, this model of PD provides enough capacity to test for possible treatments for dopaminergic neuronal death and how it relates to impaired motor symptoms.

1.2.2 1-methyl-4-phenyl-4-propionpiperidine (MPTP)

Another common neurotoxin that has been used to examine PD like pathophysiology and symptoms is 1-methyl-4-phenyl-4-propionpiperidine (MPTP). It was originally intended to be used for recreational drug purposes such as heroin, until it was later discovered the use of MPTP led to the development of bradykinesia that can only be improved with the treatment of Levodopa (Fahn, 1996). Monkeys that were administered with MPTP were also induced with permanent parkinsonian syndrome. Not only did the monkeys have all the cardinal features of PD, such as tremors, rigidity and bradykinesia, there was also the loss of dopaminergic neurons in the midbrain (Langston & Irwin, 1986). The mechanism of the MPTP works through its active metabolite 1-methyl-4-phenylpyridinium (MPP+). This active toxin can specifically target dopaminergic neurons through the dopamine transporter due to its high affinity. MPP+ can then go on to inhibit complex I of the mitochondrial electron transport chain, leading to the impairment of the mitochondria and an increase in oxidative stress. This will result in the neurodegeneration of dopaminergic neurons, leading to those cardinal features of PD. However, as shown in the 6-OHDA induced model of PD, MPTP also does not have a progressive nature over a course of years. Once administered, the neurodegeneration

quickly worsens within a few days and then this effect eventually subsides (Langston et al., 1999). In addition, there have been inconsistent results with the classical hallmark of Lewy bodies in different animal models when induced with MPTP. Although they were observed in older squirrel monkeys, they were not detected in the mouse MPTP model (Pérez-Otan˜o et al., 1991). Overall, this is also a useful model of PD to examine both hallmarks in certain animal models but not all due to some inconsistent results observed.

1.2.3 Rotenone

Rotenone is an insecticide that has also been investigated to develop a better understanding of PD due to its exposure leading to a higher risk of PD (Dhillon et al., 2008). Rotenone has shown to impair oxidative phosphorylation in the mitochondria through the inhibition of NADH, leading to mitochondria dysfunction (Schuler & Casida, 2001). In addition, rotenone can also disrupt the formation of microtubules, leading to the impaired structure of the cytoskeleton (Marshall & Himes, 1978). Rats induced with this insecticide has shown motor impairments such as rigidity, flexed posture, and reduced mobility as expected (Sherer, Kim, Betarbet, & Greenamyre, 2003). However, rotenone is not as specific as the other toxins used to induce Parkinsonian models. Different classes of neurons were degenerated with the influence of rotenone, indicating a larger widespread of neurotoxicity (Höglinger et al., 2003). This makes it difficult to determine if the presence of the impaired motor symptoms occur solely due to the degeneration of the dopaminergic neurons. In addition, although this model is similar to the MPTP model due to its effect in the mitochondria system, this model has not been widely used due to its lack of consistent reproducibility with research groups. However, there have been

reports of the combined loss of neurons, ASN aggregation, and a decrease in gastrointestinal motility, further investigation is still required to ensure the potential of this toxin as a model of PD (Drolet, Cannon, Montero, & Greenamyre, 2009).

1.3 Paraquat

1.3.1 Function

Exposure to an herbicide, 1,1'-dimethyl- 4,4'-bipyridium, also known as Paraquat (PQ), has lead to the symptoms of idiopathic PD (Nandipati & Litvan, 2016). PQ was previously used for maintaining crops; however, it is now banned due to its toxic side effects such as cerebral damage with edema and hemorrhaging (Dinis-Oliveira et al., 2006). PQ can be introduced into the body by inhalation, ingestion, and injection. High doses of PQ will lead to pulmonary toxicity whereas prolonged exposure to low levels will lead to harmful effects in the central nervous system (CNS) (Dinis-Oliveira et al., 2006). When PQ enters the neuronal mitochondrion, a variety of cellular diaphorases catalyze the reduction of PQ by transferring electrons from NAD(P)H in complex I. When O_2 is present, the PQ monocation free radical ($PQ^{\cdot+}$) is reoxidized which causes O_2 to convert into a superoxide radical ($O_2^{\cdot-}$) (**Figure 1**). This leads to a cascade of reactions to generate other reactive oxygen species (ROS) such as H_2O_2 and peroxynitrite anion ($ONOO^-$). The increased $O_2^{\cdot-}$ production will disrupt complex I activity and $ONOO^-$ can inhibit enzymes in the mitochondrial respiratory chain to decrease ATP synthesis. Thus,

resulting in mitochondrial dysfunction and increased oxidative stress (Dinis-Oliveira et al., 2006).

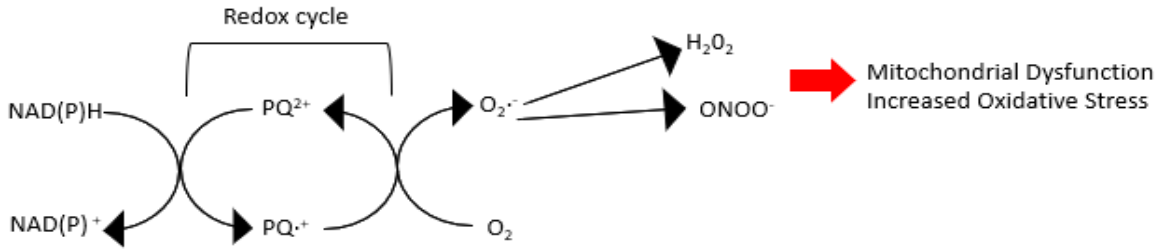

Figure 1. Paraquat's Toxicity Mechanism. Paraquat's redox cycle within the neuronal mitochondrion which leads to the toxicity of neurons through mitochondrial dysfunction and increased oxidative stress.

1.3.2 Model of Parkinson's Disease

Many epidemiology studies have discovered a strong association with the exposure to PQ and the risk of PD. For instance, PD patients in Taiwan who previously used this herbicide for the rice crops in the past have demonstrated two or more cardinal signs of PD, such as resting tremor, bradykinesia, and responsiveness to levodopa. A case control study in British Columbia has also found a correlation with the exposure to PQ and the risk of developing PD (Hertzman, Wiens, Bowering, Snow, & Calne, 1990). These studies further support the idea that PQ can serve as a potential model for PD.

PQ specifically targets dopaminergic neurons due to their susceptibility to oxidative stress. During normal physiological conditions, where synthesis and degradation of dopamine occur, hydrogen peroxide is created as a standard by-product. However, with the addition of PQ, levels of the ROS will rise, increasing the vulnerability

of these neurons. The SNpc also has the lowest concentration of glutathione (GSH), an antioxidant, compared to any other brain regions, making the SNpc relatively more susceptible to oxidative stress. In addition, GSH becomes more depleted as age increases, further increasing the vulnerability of dopaminergic neurons. Lastly, tyrosine hydroxylase (TH), the enzyme that synthesizes dopamine, has been a target for nitration in the presence of ONOO- (Ara et al., 1998). The nitration of TH leads to the loss of its enzymatic activity, causing dopamine synthesis impairment. There are currently no reports that indicate PQ affects different classes of neurons, other than one study showing PQ's lack of effect on GABAergic neurons in mice (Mccormack et al., 2002). This confirms that PQ is an effective representative model towards PD due to dopamine neurons' susceptibility towards PQ.

Many studies investigated PQ's effect in different animal models and cell lines to observe the two hallmarks of PD, as well as clinical symptoms if applicable. Studies have demonstrated that mice exposed to PQ have reduced motor activity due to a loss of dopaminergic neurons (Brooks, Chadwick, Gelbard, Cory-Slechta, & Federoff, 1999; Fernagut et al., 2007). PQ has also demonstrated its influence in ASN aggregation, where the presence of this toxin accelerated the rate of ASN fibril formation *in vitro* and *in vivo* (Manning-Bog et al., 2002) The clinical and pathological characteristics of PD exhibited in these models further validate PQ as a model for studying PD.

1.4 Cyclin-dependent kinase 5

1.4.1 Physiological Function

Cyclin-dependent kinase 5 (Cdk5) is an unusual member of the Cdk family. Although Cdk5 does not regulate the cell cycle like the other members do, the activation of this member is essential for proper regulation of the CNS (**Figure 2C**). Cdk5 is mostly prominent in the CNS and its activity is generally found in post mitotic cells. Cdk5 is a proline directed serine/threonine kinase that is only active when it binds to its neuron specific regulatory partners such as p35 or p39. Cdk5 binding to p35 or p39 is essential for proper regulation of synaptic function, neurite outgrowth, and cell adhesion (Lopes & Agostinho, 2011).

1.4.2 Neurite Outgrowth

One of Cdk5's physiological functions as mentioned above is neurite outgrowth. Neurite outgrowth is essential for controlling the wiring of the CNS during development as well as regeneration after pathological conditions (Miller & Suter, 2018). This critical function determines neuronal connectivity, but impairment of neurite outgrowth can lead to cognitive deficits (Page, Pacico, Ourtioualous, Deprez, & Koshibu, 2015). One component of the neurite outgrowth is the growth cone which is a highly motile structure that is found at the tip of growing axons and dendrites, also known as neurites. There are also two main cytoskeletal proteins that are fundamental in this critical function which are actin filaments and microtubules. The actin cytoskeleton controls numerous proteins that regulate the mechanism of neurite outgrowth such as cofilin, profilin, and gelsolin which

are actin binding proteins (Dos Remedios et al., 2003). Since this is a multifaceted system, there are complexes that can affect the regulation of neurite outgrowth. When Cdk5 binds to p35, there are many substrates that lead to the activation of increased neurite outgrowth, one of which is the phosphorylation of synapsin and Nuerabin-1 (Shah & Rossie, 2018). This is crucial towards the development for neurite formation and branching. In a pathological setting, when the neurite becomes impaired or significantly decreased, then regeneration would take place if there are certain effectors present to ensure that the Cdk5/p35 complex is activated to initiate signaling pathways for the essential proteins to function and remodel the growth cone for neurite outgrowth. However, in extreme pathological conditions due to the influence of toxins, neurite outgrowth has been impaired, which can lead to detrimental neurite effects, such as the impairment of neuritogenesis and synaptogenesis (Alural, Ozerdem, Allmer, Genc, & Genc, 2015). Therefore, it is essential to ensure the Cdk5/p35 complex is regulating appropriately for maintaining these fundamental mechanisms for the CNS.

1.4.3 Pathological Function

In the cellular system, p35 is not the only activator expressed with Cdk5 at the same time. It is balanced with another complex, known as Cdk5/p25, that does not favour neuronal survival. When Cdk5/p35 has more expression than Cdk5/p25, normal physiological conditions can occur (**Figure 2A**). However, when Cdk5/p25 complex is at extremely high levels of activity compared to Cdk5/p35, pathological consequences are present (**Figure 2B**). Many neurodegenerative diseases have been associated with the hyperactivation of Cdk5, which leads to neuronal death (Wilkaniec, Czapski, &

Adamczyk, 2016). If multiple neurotoxic signals are present, such as increased oxidative stress and mitochondrial dysfunction, a Ca^{2+} influx sensor, calpain, will cleave p35 into p25 and p10 (**Figure 2D**). Cdk5 will easily bind to p25 as it lacks a membrane anchoring signal. In addition, p25 has a longer half life than p35, making this complex more constitutively active compared to Cdk5/p35 (Gentry et al., 1999). Post mortem brain tissues of PD patients displayed elevated levels of calpain-related proteolytic formation of p25 as well as an increase in the p25/p35 immunoreactivity ratio (Alvira et al., 2008). The hyperactivation of this complex results in an inhibition of myocyte enhancer factor 2 (MEF2), a transcription factor required for the regulation of neuronal survival, leading to synaptic dysfunction and neuroinflammation (Gong et al., 2003; Lopes & Agostinho, 2011). Cdk5/p25 also phosphorylates parkin, an ubiquitin ligase that has been involved in the aggregation of ASN, implicating Cdk5/p25's role in Lewy body formation (Avraham, Rott, Liani, Szargel, & Engelender, 2007; von Coelln, Dawson, & Dawson, 2004). This emphasizes the focus to target this pathway as the complex can diverge onto many further consequences, such as formation of Lewy bodies and neurodegeneration of dopaminergic neurons.

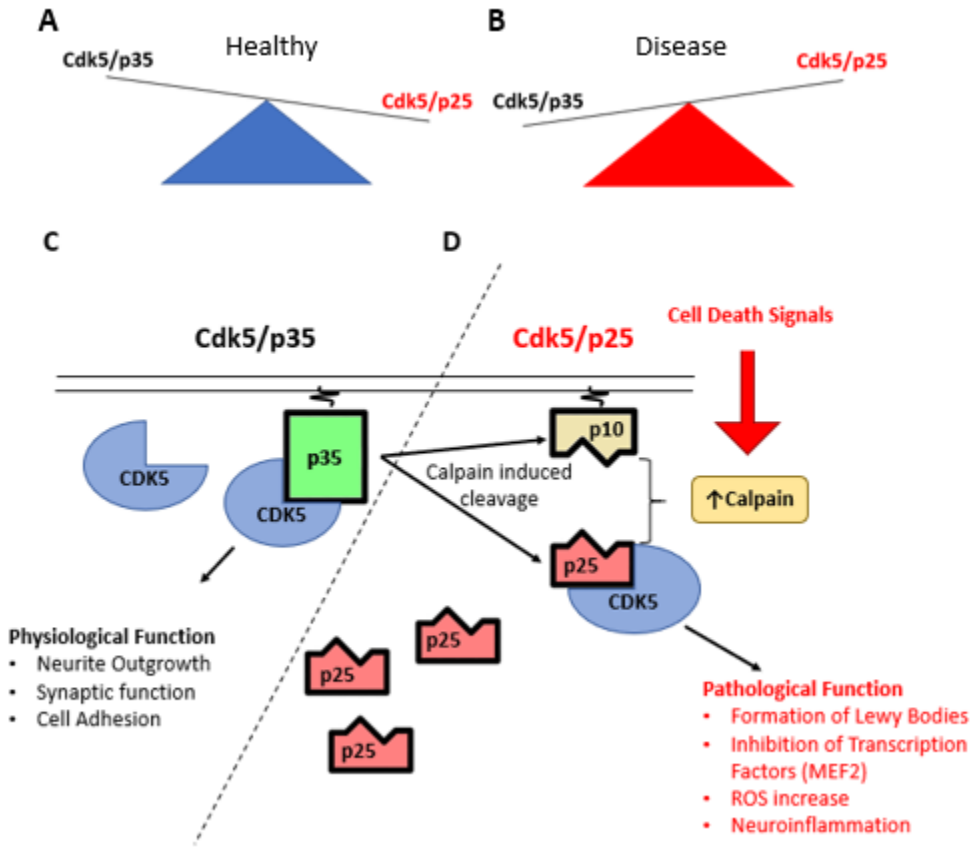

Figure 2. Cdk5 activity between p35 and p25. Healthy state (A) demonstrates the normal balance of Cdk5/p35 compared to Cdk5/p25 activity. Diseased state (B) occurs when there is hyperactivation of Cdk5/p25. The physiological functions of Cdk5/p35 activity (C) and pathological functions of Cdk5/p25 activity (D).

1.5 Truncated Peptide 5: A synthetic Cdk5/p25 inhibitor

1.5.1 Structure and Function

One possible way to treat the progression of PD is to prevent the hyperactivation of the Cdk5/p25 complex; therefore, Cdk5 inhibitors were investigated. Although Cdk5 inhibitors are able to act against Cdk5/p25, these inhibitors are non-specific and would

prevent Cdk5/p35 activity, thereby leading to lethal consequences such as severe defects in neuronal migration (Ohshima et al., 1999). Therefore, to ensure specificity towards the Cdk5/p25 complex, a 125 amino acid residue of p35 (CIP) was synthetically created to demonstrate inhibition of the aberrant Cdk5/p25 activity in cortical neurons **(Figure 3**). It was shown by Zheng et al., 2005 that these neurons were protected against the AD pathology without any consequences towards the Cdk5/p35 activity. These promising results ensure CIP is a potential inhibitor for neurodegenerative diseases with their deregulated Cdk5 activity. However, for therapy application purposes, a peptide consisting of 125 amino acids is too large to pass through the blood-brain-barrier (BBB). Therefore, a variety of smaller sequences derived from CIP were created to determine if they produced the same efficacy as CIP itself. Truncated Peptide 5 (TP5) is a 24 amino acid peptide, with an addition of transactivator of transcription (TAT) protein transduction domain at the C-terminal to cross the BBB and enter cells easily. TP5's main mechanism is the inhibition of Cdk5/p25 activity. Currently, TP5 has demonstrated no effect towards endogenous Cdk5/p35 activity due to the absence of the necessary N- and C- domains from p35 required to activate Cdk5 activity (Amin, Albers, & Pant, 2002). Based on CIP's high affinity towards Cdk5 compared to p25, TP5 has a higher affinity due to shorter amino acid length (Amin et al., 2002).

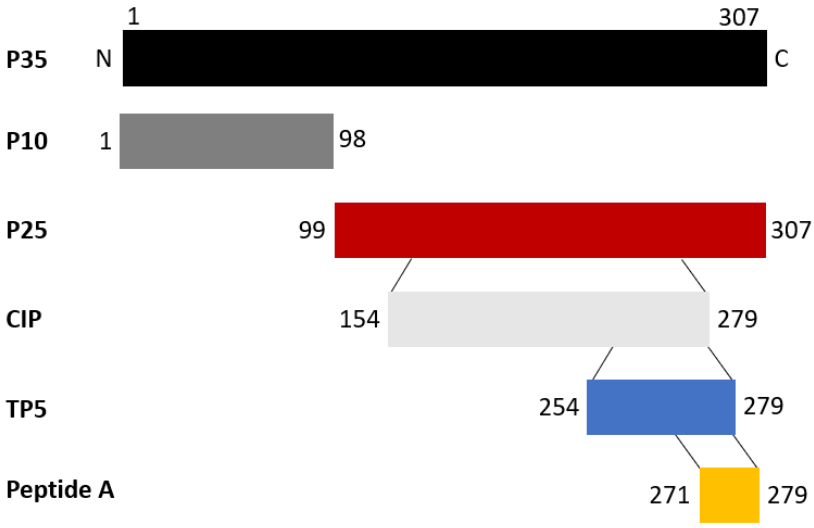

Figure 3. Derivatives of p35.

TP5 was tested initially for AD research, using cortical neurons treated with β-amyloid, a marker of AD that converts p35 into p25, which increased tau hyperphosphorylation and cell apoptosis. Cells with this AD pathology were pretreated with TP5 which was found to specifically reduce this hyperphosphorylation and apoptosis (Zheng et al., 2010a). Further research investigated mice with AD pathology that exhibit neuroinflammation, increased oxidative stress and abnormal Cdk5 activity (Shukla et al., 2013). When mice were pretreated with TP5, these cellular dysfunctions were inhibited, including the hyperactive Cdk5/p25 activity. In addition, behavioural tests demonstrated an improvement in working memory (Shukla et al., 2013). Although TP5's involvement in PD research is fairly recent, TP5 has demonstrated neuroprotective effects in mesencephalic primary cultures and in mice models, where both models were induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin similar to PQ, to generate

PD's pathology (Binukumar et al., 2015). Due to many neurodegenerative diseases that exhibit this aberrant Cdk5 activity, TP5 is a potential therapeutic drug that can prevent this hyperactivation and protect against the pathogenesis of Parkinson's Disease.

1.5.2 Truncated Fragment of TP5

Delivering TP5 into the brain has proven to be challenging due to the inability to pass the BBB without the TAT domain. Therefore, Peptide A, which consists of eight amino acids residues of TP5, was produced to determine if this fragment would have a similar efficacy as TP5 (**Figure 3**). This truncated fragment can potentially inhibit Cdk5/p25 activity as well as protect the neurons against PD pathology. The benefits of having an effective truncated fragment of TP5 will allow the fragment to easily cross through the BBB for individuals with PD for successful therapy treatments.

1.6 *SH-SY5Y* cells

A common *in vitro* model used in PD research is the *SH-SY5Y* human catecholaminergic neuroblastoma cell line that can model dopaminergic neurons. This cell line was originally derived from *SK-H-SH* cells, taken from a bone marrow biopsy of a metastatic neuroblastoma patient and then subcloned three times to become the *SH-SY5Y* cell line (Biedler, Roffler-Tarlov, Schachner, & Freedman, 1978). Although this is a common model used in neuroscience research, this undifferentiated cell line does not have typical neuronal characteristics, making this model unrepresentative towards PD research. They are immature sympathetic neuroblasts that express the non-differentiated

cell marker nestin (Lopes et al., 2010). However, this cell line can be induced to have a mature neuronal phenotype and the necessary genes through differentiation. This induction can occur by using all-trans-retinoic acid (RA) to achieve the inhibition of cell proliferation. RA is a biologically active form of vitamin A, and it is associated with early embryonic development and helps to regulate the transition from proliferating precursor cells to postmitotic differentiated cells (Påhlman, Ruusala, Abrahamsson, Mattsson, & Esscher, 1984). RA activates the PI3K/Akt pathway to downregulate transcription factors that inhibit differentiation (López-Carballo, Moreno, Masiá, Pérez, & Barettino, 2002). These differentiated neurons have mature markers of GAP043, NeuN, synaptophysin, and synapse associated protein 97 (SAP97), as well as increased neurite outgrowth (**Figure 4**) (Shipley, Mangold, & Szpara, 2017; Teppola, Sarkanen, Jalonen, & Linne, 2016). Differentiated cells have also demonstrated high expression of genes that correspond to dopamine synthesis and degradation, as well as the dopamine transporter, confirming that this is an adequate model to mimic dopaminergic neurons for studying PD (Korecka et al., 2013; Lopes et al., 2017).

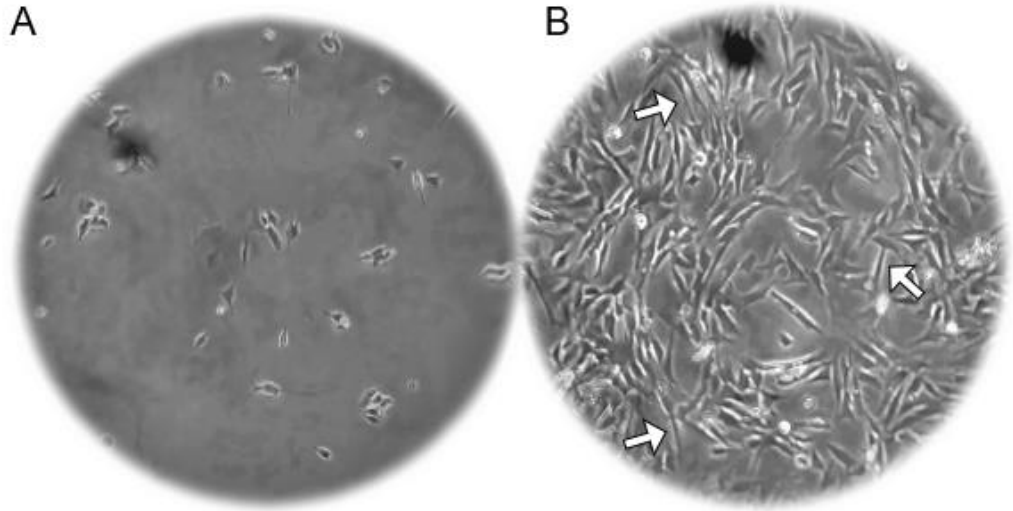

Figure 4. *SH-SY5Y* human neuroblastoma cells in an A) undifferentiated and B) differentiated state. Arrow indicate the increased neurite outgrowth in differentiated cells compared to undifferentiated cells.

1.7 Caenorhabditis elegans

Caenorhabditis elegans is a transparent nematode that can be used to study neurodegenerative diseases in a simple yet powerful model. Their genome is fully sequenced with high genetic and neurobiochemical conservation as with the human genome, and a short lifespan (Ma et al., 2018). In addition, there are several advantages of using this model to study PD such as the ease of genetic manipulation to express SNCA, LRRK2, and PINK-1 genes, the ability to complete these experiments quickly, and the low cost (Cooper & Van Raamsdonk, 2018). They contain 302 neurons, eight of which are dopaminergic neurons, with an additional six located at the tail of the male which

further establishes the value to examine *C. elegans* in PD research (Chase & Koelle, 2007). There are two pairs of cephalic neurons (CEP) and a pair of anterior deirid neurons (ADE) located in the head, along with another posterior deirid (PDE) pair found laterally in the posterior body, all acting as mechanosensory neurons (**Figure 5**) (Altun & Hall, 2011). These neurons can be assessed for neurodegeneration by expressing green fluorescent protein (GFP) under the dopamine transporter (*dat-1*) promoter to examine cell bodies and processes. Dopamine signaling in *C. elegans* is required for modulation of locomotor behaviour and learning (Chase & Koelle, 2007). In addition, they require their dopaminergic system to respond to environmental stimuli, such as reacting to the presence of bacteria which acts as a food source (Sawin, Ranganathan, & Horvitz, 2000). This is an adaptive mechanism known as the basal slowing response which allows well fed worms to slow their locomotor rate when they are in the presence of bacteria due to the mechanosensory properties from the dopaminergic neurons. This slow response enables the worms to respond to the bacteria and ensure they are aware of their surroundings. Another feature that allows *C. elegans* to model PD is the accumulation of ASN (Cooper & Van Raamsdonk, 2018). *C. elegans* do not carry ASN protein; however, they can be overexpressed with human SNCA to investigate how the aggregation of ASN can affect their dopaminergic morphology and locomotor behaviour. In addition, ASN can be linked to a fluorescent protein that can be visually expressed in the body.

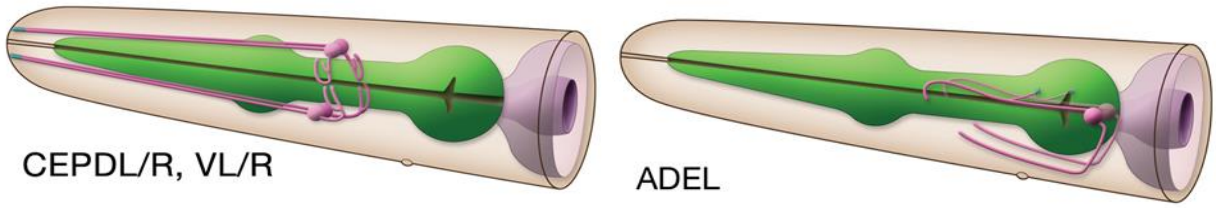

Figure 5. A schematic diagram of the dopaminergic neurons located in the head of *C. elegans*. Two pairs of cephalic neurons (CEP) on the left and a pair of anterior deirid neurons (ADE) on the right (purple) Adapted from (Altun & Hall, 2011).

1.8 Overview

Current treatments for Parkinson's Disease are not enough for individuals due to the temporary relief provided towards these clinical symptoms. These treatments do not target the pathway to prevent the progression of the disease or protect the neurons. With PD having a multifactorial etiology, it adds further complications towards treatment. However, most neurodegenerative diseases, including PD, have been associated with the hyperactivation of Cdk5 due to the binding of p25. We have a synthetic peptide that has demonstrated the potential to inhibit this hyperactivation and prevent the pathology from worsening thus protecting the neurons. Furthermore, we will explore the effects of TP5 in different models of PD to determine its efficacy as an inhibitor for the Cdk5/p25 complex. This will be done by exploring the effects of TP5, as well as its truncated fragment, in a cell line exposed to PQ to induce Parkinsonian-like dopaminergic neurons

through cell viability. Then the downstream effects of TP5 through the Cdk5 pathway will be investigated through the regulation of neurite outgrowth. Lastly, the therapeutic effects of TP5 are examined in an *in vivo* model, using *C. elegans* exposed to PQ to ensure pathological damage towards the dopaminergic neurons. These three studies will be divided into objectives, hypotheses, methods, results and discussion. We will conclude with the future steps that should be taken to explore TP5 as a potential therapeutic drug in neurodegenerative diseases such as Parkinson's Disease.

CHAPTER TWO

Investigation of TP5 and its fragment in an *in vitro* model of differentiated *SH-SY5Y* cells exposed to Paraquat

2.1 Objectives

Investigating the function of TP5 and its neuroprotective effect against PD in differentiated *SH-SY5Y* cells.

2.1.1 Summary

The purpose of the study is to determine TP5's downstream mechanism and its neuroprotective effect against PQ as a model of PD *in vitro*. Differentiated *SH-SY5Y* cells were exposed to TP5 and its truncated fragment, Peptide A, to determine if they have an influence on neurite outgrowth. Neurite outgrowth was determined to have significantly increased in differentiated *SH-SY5Y* cells as concentration of both TP5 and Peptide A increases. This demonstrates that as one of Cdk5's downstream effectors, neurite outgrowth, is affected by TP5 and Peptide A, TP5 is involved in the downstream mechanism of Cdk5. With this finding, the neuroprotective effect of TP5 against PQ was further explored in the same cell line. After treating cells with RA for six days to induce differentiation, TP5 was administered for 12 h and then PQ for 48 h. The live metabolic activity of the cells was then measured using an MTT assay to determine cell viability. Results indicated that TP5 increased cell viability for neuronal cells exposed to PQ, suggesting that TP5 has potential neuroprotective effects. This procedure was then replicated with Peptide A, the truncated fragment of TP5, to determine if this fragment was just as efficacious as TP5. However, Peptide A showed no neuroprotective effects for neuronal cells exposed to PQ. Future studies will examine Cdk5 activity to ensure TP5's neuroprotective effects act through the inhibition of Cdk5/p25 activity against PQ.

2.2 Hypotheses

The following were the study's hypotheses:

1) *SH-SY5Y* cells exposed to different concentrations of TP5 would demonstrate increased neurite length compared to cells that were not exposed with TP5.
2) *SH-SY5Y* cells exposed to different concentrations of Peptide A would demonstrate increased neurite length compared to cells that were not exposed with Peptide A.
3) *SH-SY5Y* cells treated with TP5 in a neuroprotective manner against PQ would demonstrate increased cell viability compared to the cells treated with PQ alone.
4) *SH-SY5Y* cells treated with Peptide A, the truncated fragment of TP5 in a neuroprotective manner against PQ would demonstrate increased cell viability compared to the cells treated with PQ alone.

2.3 Materials and Methodology

2.3.1 Cell Culture Conditions and Drug Treatments

SH-SY5Y cells were obtained from ATCC and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM: F12), supplemented with heat-inactivated fetal bovine serum (FBS) (10%v/v), L-glutamine (1%v/v), and pencillin-streptomycin (1%v/v) at 37°C in 5% CO_2.

2.3.2 Neurite Outgrowth

Cells were seeded at a density of $2.0x10^4$ cells/well in a 12 well plate and the experimental timeline is demonstrated in **Table 1**. The following day, the cells were differentiated with 10uM of RA and FBS (1%v/v). On the sixth day, different concentrations ranging from 0.01uM to 10uM of TP5, dissolved in ddH_2O, were added to the cells for 12 h. This experiment was replicated in the same manner except testing for Peptide A instead of TP5. Coverslips were maintained in poly-L-lysine for a minimum of 4 h at 4°C. The solution was removed, and the coverslips were washed three times with ddH_2O. After treating cells with TP5 or Peptide A, cells were fixed with 4% paraformaldehyde for 10 minutes. Cells were then washed three times with ddH_2O. Coverslips were removed with forceps and mounted onto glass slides using DAPI as a mounting agent. The glass slides were imaged using a Leica light fluorescence microscope at 10x magnification in phase contrast and DAPI staining. Images were overlaid to visualize DAPI stain for the nucleus and in phase contrast to depict clear processes of the neuronal cells. At least ten images were taken per treatment group, with approximately 30 neuronal cells in one image. Neurite length was measured using a plugin in ImageJ software called NeuronJ (NIH, USA). Neurites were only measured if they had clear extensions as demonstrated in Error! Reference source not found.. In all c onditions, the experimenter would be blind of each treatment group and a minimum of 100 measurements were required for each.

2.3.3 Drug Treatments

Cells were seeded at a density of $1.5x10^5$ cells/ml in a 96 well plate and the experimental timeline is demonstrated in **Table 1.** The following day, the cells were differentiated with 10uM of RA and FBS (1%v/v). On the sixth day, 12.5uM of TP5, dissolved in ddH_2O, was added to the cells for 12 h. 250uM of PQ, dissolved in differentiated media, was then added to the cells for 48 h. Other groups in the 96 well plate was also either exposed to only PQ or received no treatment. This experiment was replicated in the same manner except testing for 12.5uM of Peptide A instead of TP5. PQ and TP5's concentrations were determined based on performing a dose response curve of PQ and TP5 separately. These optimal concentrations ensure that they will not be lethal to cells and that TP5 is effective against PQ. TP5 peptide was synthesized by GenScript (Piscataway, NJ). Peptide A was created in collaboration with Patrick Gunning's lab from University of Toronto – Mississauga.

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Seed cells	Add differentiation media w/ 10uM of RA	Observe cells	Change ½ differentiation media	Observe cells	Treat cells (12.5uM TP5/ Peptide A for 12 hr)	Treat cells (250uM PQ for 48 hr)
Day 8	**Day 9**					
	MTT assay					

Table 1. Timeline of experimental procedure for *SH-SY5Y* cells.

2.3.4 Cell Viability Assay

Cell viability was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. On day nine, 20 uL of MTT powder, dissolved in differentiation media, was added into each well and incubated at 37°C for 3-4 h. MTT is metabolically converted into formazan, a precipitate, by the mitochondrial dehydrogenases of living cells. The solution is replaced with dimethyl sulfoxide (DMSO) to dissolve the precipitate and absorbance value was measured at 570nm with a microplate reader (Spectromax).

2.3.5 Statistical Analysis

Statistical analyses were performed using GraphPad. Any significant outliers were detected prior to analyses using GraphPad Outlier Tool. Comparison of groups were analyzed using one-way analysis of variance (ANOVA) and Tukey's post hoc tests, if groups were compared amongst each other, or Dunnett's post hoc tests, if groups were only compared to controls. P values smaller than 0.05 are considered statistically significant.

2.4 Results

2.4.1 Effect of TP5 on neurite length in differentiated *SH-SY5Y* cells

Cell bodies were visualized using DAPI staining and in phase contrast to visualize their processes in **Figure 6**. Controls had shorter processes (**Figure 6 , left**) compared to neuronal cells exposed to 0.1uM of TP5 (**Figure 6**, **middle**). **Figure 7** demonstrated TP5

having an effect on neurite length ($p<0.0001$). All TP5 concentrations were found to increase neurite length compared to cells that had no exposure to TP5 ($p<0.0001$). Cells exposed to the highest concentration of TP5, which was 10uM, was also significant against controls ($p<0.01$).

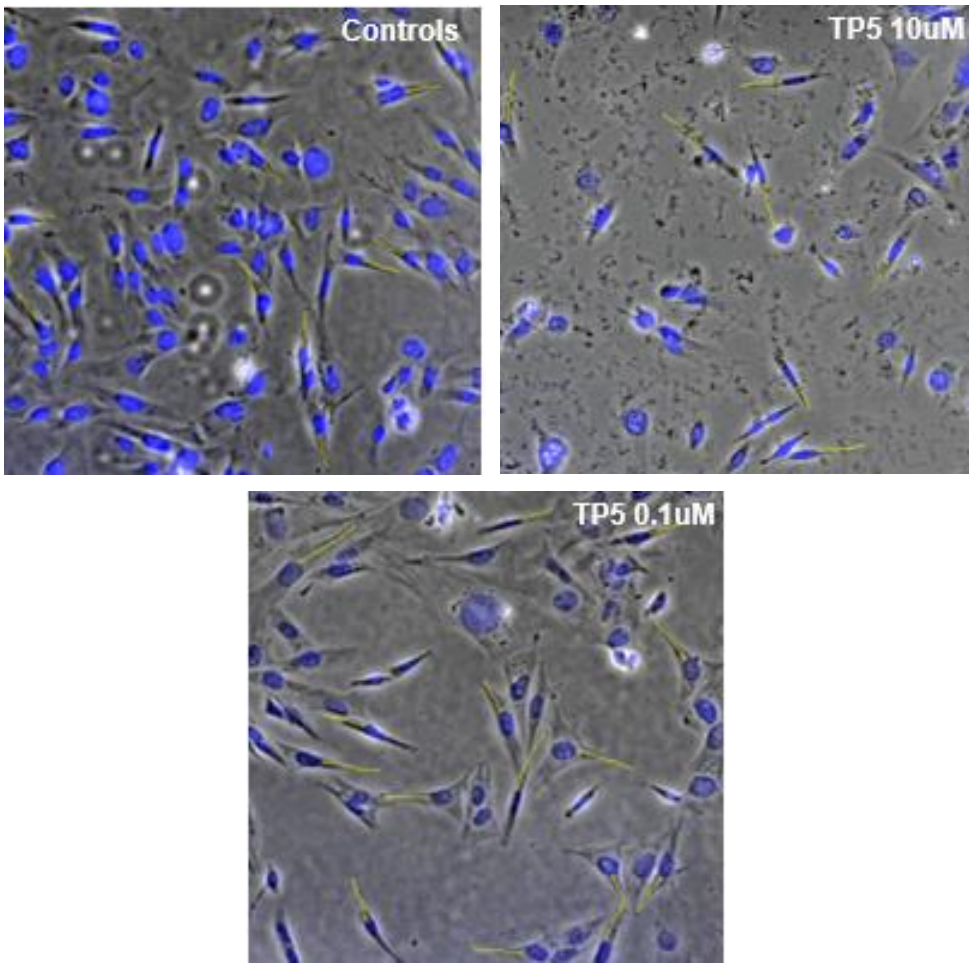

Figure 6. Effect of TP5 on Neurite Length in Differentiated *SH-SY5Y* cells. Phase contrast images are overlaid with DAPI. Visualization of the nucleus from the DAPI staining (blue) and the neurites (yellow) recorded from ImageJ.

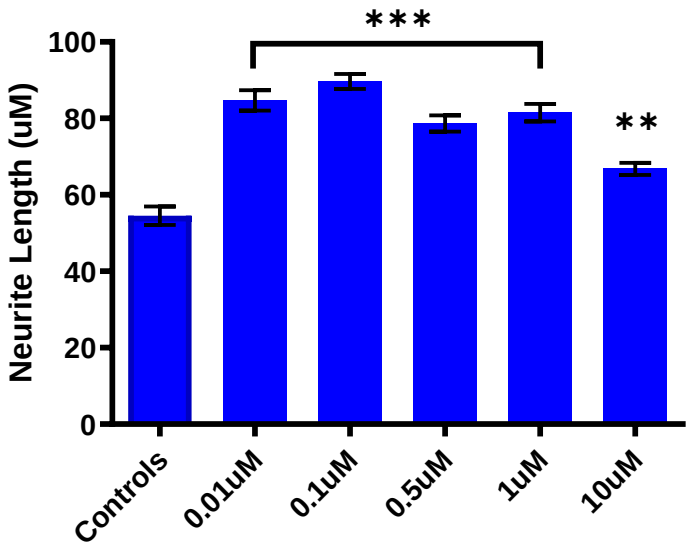

Figure 7. Effect of TP5 on Neurite Length in Differentiated *SH-SY5Y* cells. Concentrations starting from 0.01uM to 10uM of TP5 were tested against controls. One-way ANOVA revealed significant differences between treatments ($F=34.53$, $p<0.0001$). Dunnett's multiple comparison tests found neuronal cells treated with TP5 had increased neurite length than controls ($p<0.01$). Data represents mean ± standard error of mean.

2.4.2 Effect of Peptide A on neurite length in differentiated *SH-SY5Y* cells

Cell bodies were also visualized using DAPI staining and images are taken in phase contrast to visualize their processes in **Figure 8**. **Figure 9** demonstrated Peptide A having an effect on neurite length. All concentrations of Peptide A were found to increase neurite length ($p<0.0001$).

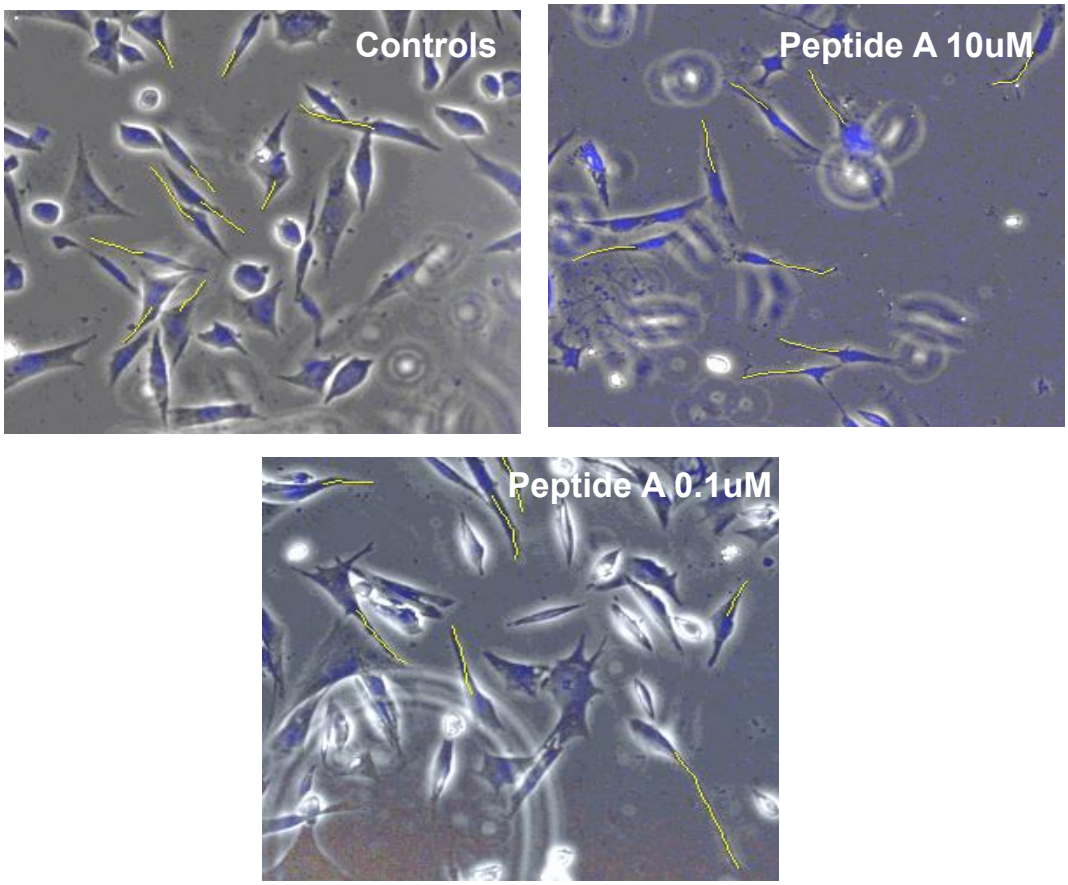

Figure 8. Effect of Peptide A on Neurite Length in Differentiated *SH-SY5Y* cells. Phase contrast images are overlaid with DAPI. Visualization of the nucleus from the DAPI staining (blue) and the neurites (yellow) recorded from ImageJ.

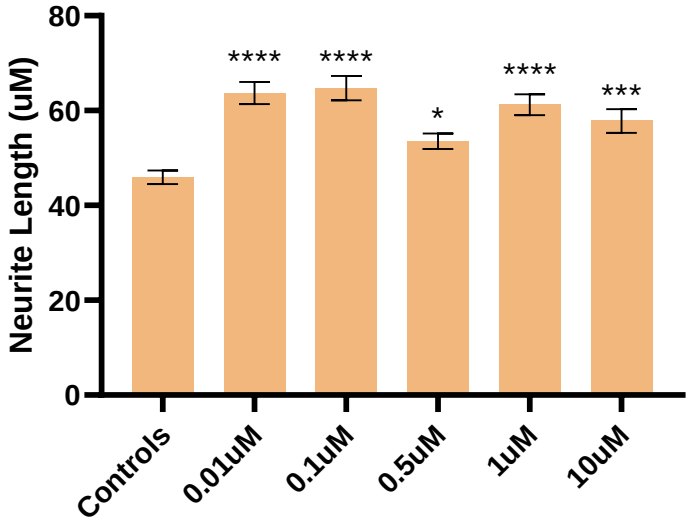

Figure 9. Effect of Peptide A on Neurite Length in Differentiated *SH-SY5Y* cells. Concentrations starting from 0.01uM to 10uM of Peptide A were tested against controls. One-way ANOVA revealed significant differences between treatments ($F=12.03$, $p<0.0001$). Dunnett's multiple comparison tests revealed that cells treated with Peptide A had increased neurite length than controls ($p<0.05$). Data represents mean ± standard error of mean.

2.4.3 Effect of TP5 on cell viability in differentiated *SH-SY5Y* cells exposed to PQ

PQ at 250uM was determined to be the most effective to induce cell toxicity in the *SH-SY5Y* cells ($p<0.05$) (**Figure 10**). PQ at 250uM with approximately 70% cell viability ensures that cells will not undergo too much cell death where the treatment cannot protect the high cell toxicity. 12.5uM of TP5 was determined to be the most optimal concentration to test against 250uM of PQ (**Figure 11**). This was due to observing very small effects of cell death against controls as TP5 should not be creating a high cell toxicity. To investigate whether TP5 can protect cells against PQ, the cells were treated with TP5 first, then later exposed to PQ. Differentiated *SH-SY5Y* cells exposed to TP5 in

a neuroprotective manner against PQ had increased cell viability of 76.4% compared to the cells that were exposed to PQ at 65.8% ($p<0.05$) (**Figure 12**).

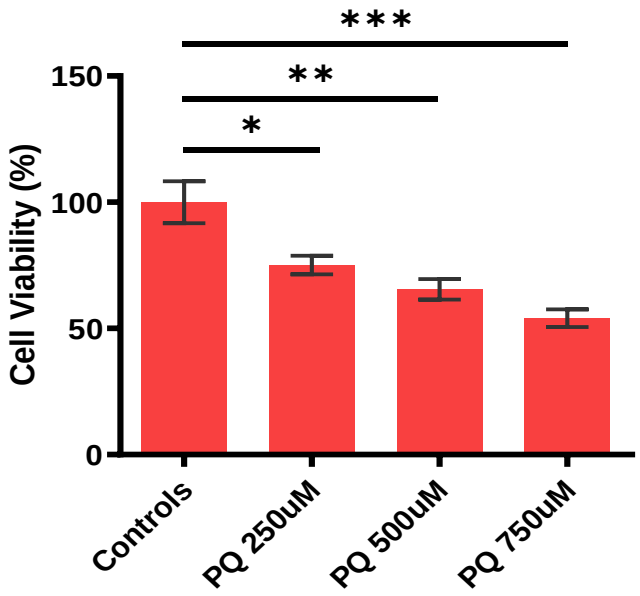

Figure 10. Effect of PQ on Cell Viability in Differentiated *SH-SY5Y* cells. MTT assay was performed to measure cell viability. Samples were normalized to controls. Concentrations at 250uM, 500uM, and 750uM of PQ were tested against cells not exposed to PQ. One-way ANOVA indicated a significant difference between treatments ($F=13.67$, $p=0.0001$). Dunnett's multiple comparison tests revealed that 250uMof PQ treated cells had decreased cell viability compared to controls ($p<0.05$). Furthermore, cells treated to PQ at 500uM ($p<0.01$) and 750uM ($p<0.001$) also has significant decreased cell viability compared to controls. N=2 (biological replicates). Analyzed with at least 5 triplicates per group. Data represents mean ± standard error of mean.

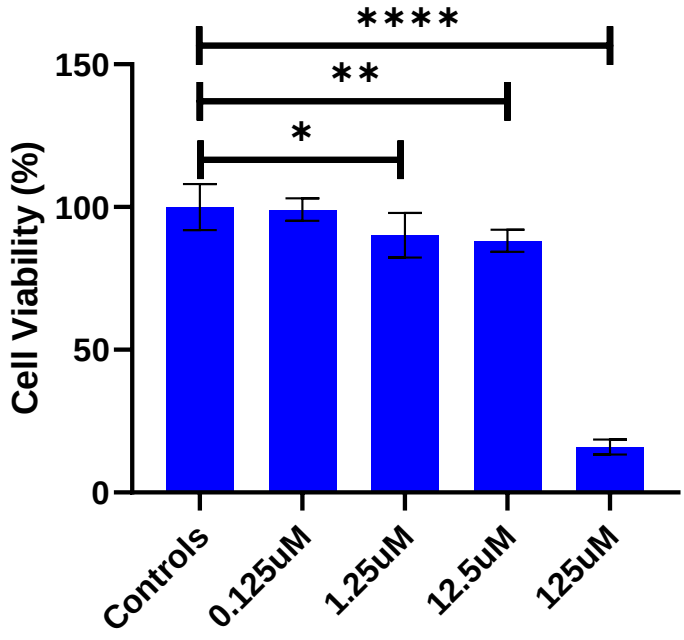

Figure 11. Effect of TP5 on Cell Viability in Differentiated *SH-SY5Y* cells. MTT assay was performed to measure cell viability. Samples were normalized to controls. 10-fold serial dilutions of TP5 starting at 0.125uM to 125uM were tested against cells not exposed to TP5. One-way ANOVA indicated a significant difference between treatments ($F=271.4$, $p<0.0001$). Dunnett's multiple comparison tests revealed that 125uM of TP5 treated cells had decreased cell viability compared to controls ($p<0.0001$). Cells treated with 12.5uM had 88% cell viability, demonstrating a significant decrease in cell viability ($p<0.01$). N=2 (biological replicates). Analyzed with at least 5 triplicates per group. Data represents mean ± standard error of mean.

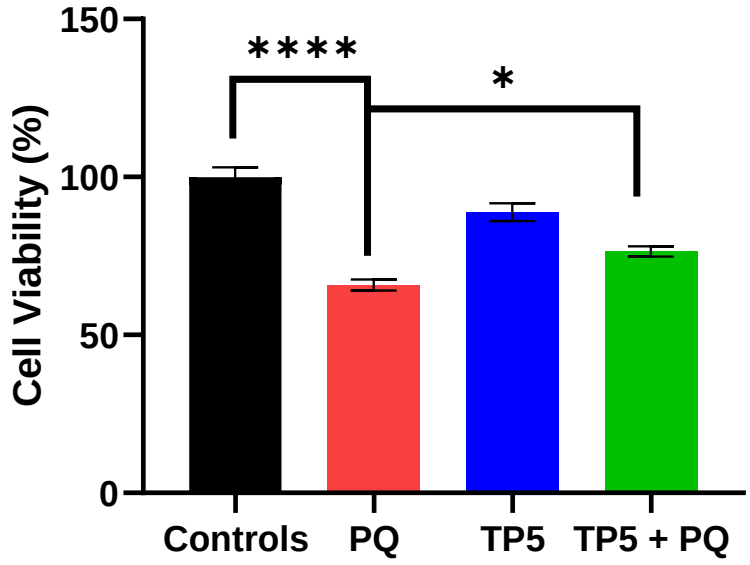

Figure 12. Neuroprotective Effect of TP5 on Cell Viability in Differentiated *SH-SY5Y* cells treated with PQ. MTT assay was performed to measure cell viability. Samples were normalized to controls. One-way ANOVA indicated a significant difference between treatments ($F=36.37$, $p<0.0001$). Tukey's multiple comparison tests revealed that cells exposed to 12.5uM of TP5 and PQ 250uM had significant increased cell viability than cells exposed to PQ alone ($p<0.05$). N=2 (biological replicates). Analyzed with at least 5 triplicates per group. Data represents mean ± standard error of mean.

2.4.4 Effect of Peptide A on cell viability in differentiated *SH-SY5Y* cells exposed to PQ

Examining Peptide A, the truncated fragment of TP5, is to determine if this fragment is as efficacious as TP5. Cells were first exposed to 12.5uM of Peptide A, then shortly after cells were treated to 250uM of PQ, as demonstrated in the timeline of **Table *1***. *SH-SY5Y* cells exposed to Peptide A in a neuroprotective manner against PQ did not demonstrate any significant differences compared to cells exposed to PQ ($p=0.9293$) (**Figure 13**).

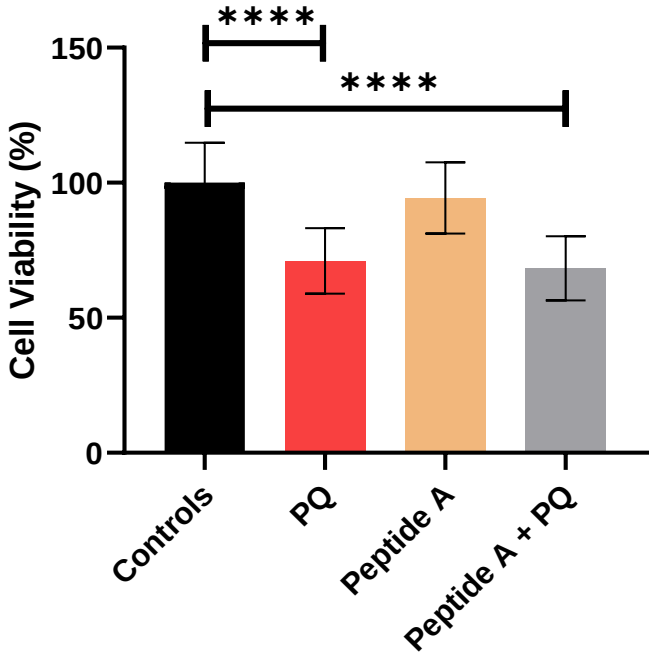

Figure 13. Neuroprotective Effect of Peptide A on Cell Viability in Differentiated *SH-SY5Y* cells treated with PQ. MTT assay was performed to measure cell viability. Samples were normalized to controls. One-way ANOVA revealed significant differences between groups ($F=36.37$, $p<0.0001$). However, Tukey's multiple comparison tests revealed that cells exposed to 12.5uM of Peptide A and PQ 250uM had no significant differences compared to cells exposed to PQ alone ($p=0.9293$). N=2 (biological replicates). Analyzed with at least 5 triplicates per group. Data represents mean ± standard error of mean.

2.5 Discussion

The Cdk5/p35 and Cdk5/p25 complex are continuously co-existing in the cellular system. However, there are higher levels of Cdk5/p35 activity than Cdk5/p25 activity under normal physiological conditions to allow functions such as neurite outgrowth to occur. When the cellular system is under stressful conditions, this balance between these two complexes will become disrupted, leading to pathological consequences due to aberrant Cdk5/p25 activity. However, if there are no stressful conditions present, such as the influence of PQ, there should not be any activation of the abnormal Cdk5/p25 complex. With the addition of TP5, acting as an inhibitor towards Cdk5/p25, that balance between these two complexes should be skewed with considerably less activity from Cdk5/p25. This enables the Cdk5/p35 complex to enhance its downstream effects. Normal Cdk5/p25 activity may prevent Cdk5/p35 from acting on its maximum potential to exert its physiological conditions, such as neurite outgrowth. Based on **Figure 6** and **Figure 7**, TP5 has demonstrated its effects towards potentiating neuronal outgrowth due to the increased significant neurite length in all concentrations compared to cells that were not exposed.

TP5 was originally derived from a 125 amino acid residue (CIP), a peptide that was inhibiting the Cdk5/p25 complex. We wanted to determine if TP5 being cleaved into smaller fragments, such as Peptide A, can produce a similar effect towards neurite outgrowth. This will further aid in clinical treatment purposes as TP5 is currently unable to cross the BBB without the HIV-TAT tag. Since Peptide A is only eight amino acids

long, this fragment can cross the BBB easily. Peptide A has also demonstrated an effect towards neurite outgrowth, based on **Figure 8** and **Figure 9**.

Due to Cdk5's physiological involvement with multiple mechanisms, Cdk5 has many substrates, one of them being synapsin (Shah & Rossie, 2018). Synapsins are a group of phosphoproteins that regulate neurotransmission and neuronal plasticity (Mirza & Zahid, 2018). There are three different classes of synapsins: Synapsin I, II, and III. Synapsin I (SynI) plays an important role in axon elongation and the regulation of synaptic vesicle fusion. Synapsin III (SynIII) is involved in the regulation of the early phase of neuronal development and known to be associated with neurite outgrowth (Porton, Wetsel, & Kao, 2011). This class is predominantly found in extra synaptic sites and growth cones. SynI and SynIII are regulated by the Cdk5 pathway. Once the Cdk5/p35 complex is activated, several substrates can be activated through phosphorylation, including SynI and SynIII (Perlini et al., 2015; Shah & Rossie, 2018). Once SynI is phosphorylated by Cdk5/p35, there is an increase in binding to F-actin, which provides support to the cytoskeleton at the growth cone, resulting in an increased neurite outgrowth. SynIII has also been known to be involved with the increased binding of F-actin, due to its localization present in the cell body and growth cones of developing neurites (Perlini et al., 2015; Piccini, Perlini, Cancedda, Benfenati, & Giovedì, 2015).

Based on **Figures 6-9**, TP5 and Peptide A enables the Cdk5/p35 activity to further allow expression of SynI and SynIII for enhanced neurite outgrowth. The presence of Cdk5/p25 may prevent the optimal expression of neurite outgrowth as aberrant Cdk5/p25 activity leads to the hyperphosphorylation of tau, disrupting the cytoskeleton in extreme

stressful conditions (Chen et al., 2008). However, since normal conditions are not stressful, these cells may be experiencing some mild disruption of the cytoskeleton regulation but not to the extent seen in pathological conditions. TP5 can further stop this cytoskeleton disruption for enhanced neurite outgrowth by further reducing those typical Cdk5/p25 levels. In addition to TP5's truncated fragment towards neurite outgrowth, Peptide A could potentially have the same effect as TP5 where it can inhibit the presence of Cdk5/p25 activity to prevent the cytoskeleton from being disrupted and allow enhanced neurite outgrowth for proper neurotransmission. Now that TP5 and Peptide A has been established in physiological conditions with neurite outgrowth, pathological conditions are necessary to determine how TP5 and Peptide A could have potential therapeutic effects.

Paraquat as a model of Parkinson's Disease has been commonly used to investigate the pathology of the disease and to test potential therapeutic drugs. PQ has demonstrated the two main hallmarks of PD as well as the motor dysfunctional symptoms *in vivo* (Brooks et al., 1999; Fernagut et al., 2007). Although the toxicity mechanism of PQ has been established, there has been no research on whether PQ leads to the hyperactivation of Cdk5. Many neurodegenerative diseases, including PD, had shown that abnormal activation of Cdk5 will lead to its pathological features (Lopes & Agostinho, 2011). Determining whether PQ can elevate the activity of Cdk5/p25 will confirm that PQ can be representative of this neurodegenerative disease; future studies to examine PQ's effect on Cdk5/p25 activity are required. However, MPTP, which has a very similar structure to PQ, exhibited increased levels of Cdk5/p25 activity. MPTP has been found to

cause PD-like symptoms *in vivo* and in individuals who were previously exposed to this toxin (Binukumar et al., 2015; Langston et al., 1999). Based on MPTP's similar structure to PQ, it is likely that PQ is involved in the hyperactivation of Cdk5/p25 due to PQ's main effect of increased oxidative stress having the ability to activate this complex.

TP5 has been tested in AD research and proven to be effective in a neuroprotective manner *in vitro* and *in vivo* (Shukla et al., 2013; Zheng et al., 2010b). Furthermore, TP5 has demonstrated neuroprotective effects against the MPTP model of PD (Binukumar et al., 2015). Although MPTP has some features that are characteristic of PD, such as impaired dopaminergic function, post mortem samples of individuals who were exposed to this drug did not express Lewy bodies which reveals an incomplete representation of this disease; whereas PQ has led to the increased aggregation of ASN (Langston et al., 1999; Manning-Bog et al., 2002). Therefore, TP5 is investigated in this study to determine if there are neuroprotective effects against PQ. In **Figure 12**, cells treated with TP5, were found protected against the toxin, compared to cells only exposed to PQ. However, these cells treated with TP5 and PQ are not completely protected to the extent that its cell viability can be considered similar to controls at 100%. PQ's high concentration makes cell viability difficult to completely be protected against cell induced toxicity. As mentioned before, PQ's primary toxicity mechanisms are mitochondria dysfunction and increased oxidative stress. PQ should theoretically increase Cdk5/p25 activity with the presence of its toxicity effects, by activating calpain to cleave p35 into p25 and p10. Since TP5's main action is inhibiting Cdk5/p25 activity, TP5 cannot protect the primary toxicity mechanism of PQ from occurring. TP5 can only prevent some level

of cell death by inhibiting Cdk5/p25 activity, confirming the neuronal cells cannot be fully protected against PQ. Based on the results, since TP5 has some capacity to protect cells as well as prevent any further neurodegeneration in the presence of PQ, TP5 can be a potential therapeutic drug in PD.

However, to ensure that TP5 is protecting the neuronal cells against PQ as a Cdk5/p25 inhibitor, future studies require examining TP5's mechanism of action against PQ. The treatment groups demonstrated in **Figure 12** will analyzed for their Cdk5/p25 activity, where we expect PQ to have elevated levels of Cdk5/p25 activity and TP5 inhibiting this elevated activity. TP5 has been confirmed through numerous studies that this peptide can inhibit the Cdk5/p25 activity (Binukumar et al., 2015, 2014; Zheng et al., 2010b). However, further testing is required to solidify TP5's effect against PQ in differentiated *SH-SY5Y* cells.

Based on **Figure 9**, Peptide A has an effect on neurite outgrowth in differentiated *SH-SY5Y* cells; therefore, there should be a potential neuroprotective effect against PQ. However, it is shown in **Figure 13**, Peptide A was unable to protect the neuronal cells against PQ. Due to Peptide A's eight amino acid length, compared to TP5's 24 amino acid length, this fragment may be unable to withstand against PQ's high concentration. Since Peptide A was tested in 12.5uM, this concentration may not be effective against PQ's high concentration compared to TP5. A dose response curve needs to be performed to determine the optimal concentration for Peptide A as it may be higher than TP5. In addition, it is also possible that all 24 amino acids are required to be effective to inhibit the aberrant activity of Cdk5/p25. Lastly, although Peptide A did not have

neuroprotective effects on cell viability against PQ, this truncated fragment may be effective in non-toxic conditions. Further testing in Cdk5 activity of Peptide A with PQ will confirm if Peptide A is efficacious against PQ.

In the future, research should examine the protein expression of SynI and SynIII in *SH-SY5Y* cells exposed to TP5 alone to correlate with the results found in **Figure 7**. Once confirmed that TP5's downstream effect leads to the activation of these neurite outgrowth regulators, we can further explore the mechanism of PQ's involvement with Cdk5. PQ has demonstrated decrease neurite outgrowth in *SH-SY5Y* cells (Alural et al., 2015); Once replicated in our model, we can test TP5 against PQ on neurite outgrowth. Lastly, the protein expression of SynI and SynIII can also be investigated with the treatment of TP5 and PQ combined to determine if TP5 can block the Cdk5/p25 activity that should be elevated from PQ, resulting in increased expression of SynI and SynIII.

CHAPTER THREE

Investigation of TP5 in an *in vivo* model of *Caenorhabditis elegans* exposed to Paraquat

3.1 Objectives

Investigating TP5's Therapeutic Effects in a *C. elegans* model exposed to PQ

3.1.1 Summary

The purpose of this study is to confirm TP5's therapeutic effects in an *in vivo* model of *C. elegans* exposed to PQ. The worms were first treated with TP5 in a neuroprotective manner, then exposed to PQ to induce PD-like motor symptoms. A dopamine dependent locomotor assay was performed to determine TP5 and PQ's effect in the basal slowing response. Worms that were treated with TP5 in a neuroprotective manner against PQ, performed similar to healthy controls compared to worms only exposed to PQ. These conditions were later visualized with dopaminergic neurons to investigate TP5 and PQ's effect in neurodegeneration. TP5 pretreated worms that were exposed to PQ demonstrated a rescued neuroprotective effect in dopaminergic neurons compared to worms exposed to PQ alone. Lastly, to determine if TP5 has a neurorestorative effect for clinical application, worms were first exposed to PQ, then administered TP5. Worms exposed to PQ, then TP5 in a neurorestorative manner also exhibited rescued dopaminergic morphology compared to worms treated to PQ alone.

3.2 Hypotheses

The following were the study's hypotheses:

1) Worms pretreated with TP5 as a neuroprotective manner, then exposed to PQ, would have rescued locomotor movements compared to worms exposed to PQ.

2) Worms pretreated with TP5 as a neuroprotective manner, then exposed to PQ, would have less degeneration in dopaminergic morphology compared to worms exposed to PQ.
3) Worms exposed to PQ, then administered TP5 in a neurorestorative manner, would have less degeneration in dopaminergic morphology compared to worms exposed to PQ.

3.3 Materials and Methodology

3.3.1 Strain and Culture Conditions

Caenorhabditis elegans worms were cultured on standard Nematode Growth Medium (NGM) agar plates and using *Escherichia coli* strain OP50 as a food source at 20°C (Brenner, 1974). The strain that was used in the study was *DY328* unc-119; bhEx120[unc-119(+) + pGLC72(Cel-dat-1 5\'UTR::YFP)]. The dat-1p::YFP plasmid pGLC72 was made by amplifying a 710 bp fragment of dat-1 5′ genomic region using primers GL563 (5′-AGGAAGCTTCCAGTTTTCACTAAAACGACCTCATACACTTCTC-3′) and GL564 (5′-ATGGGTACCGGCACCAACTGCATGGCTAAAAATTGTTGAG-3′). The resulting PCR product was digested with HindIII and KpnI and subcloned into pPD136.64 (Fire lab vector, www.addgene.com). pGLC72 was injected into unc-119(ed4) animals to generate stable transgenic lines. Age synchronized cultures were obtained by treating the strain with sodium hypochlorite and sodium hydroxide (3:2 ratio of NaOcl:NaoH). These cultures were maintained until they became Day 1 aged worms to proceed with the drug treatments detailed below.

3.3.2 Drug Treatments

Experimental timelines of the neuroprotective and neurorestorative conditions can be seen in **Table 2**. For the neuroprotective experiment, Day 1 age synchronized worms were treated with 10uM of TP5 then placed into an agar plate that contained 250uM of PQ for 48 h. There were three different techniques of administering TP5: 1) picolitres of TP5 at 10uM was microinjected into the distal gonads of the worm; 2) worms were suspended in 500uL of TP5 at 10uM inside an Eppendorf tube and placed in a rotator for 1 h; 3) worms were placed in an agar plate that contained 10uM of TP5 for 6 h. Different routes of administration for TP5 was tested to determine which route was most effective and non-lethal for worms. After exposure to PQ for 48 h, worms were tested for dopamine-dependent locomotor behaviour and imaged for neurodegeneration. For the TP5 injected group, a control was made with ddH_2O microinjected into the worm.

For the neurorestorative experiment, Day 1 age synchronized worms were exposed to an agar plate that contained 250uM of PQ for 48 h. Day 3 worms were then exposed to 1mM of TP5. 1mM was used in the neurorestorative group due to worms originally responding to the concentration very well after recovery. The neuroprotective group was not recovering well after 1mM of exposure, so it was diluted to 10uM. There were two different techniques of administering TP5: 1) picolitres of TP5 at 1mM was microinjected into the distal gonads of the worm; 2) worms were suspended in 500uL of TP5 at 1mM inside an Eppendorf tube and placed in a rotator for 1 h. Different routes of administration for TP5 was tested to determine which route was most effective and non-lethal for worms. After TP5 administration, worms were transferred to a standard NGM

agar plate to allow for recovery for two days. Day 5 worms were imaged for neurodegeneration. PQ exposed worms were exposed to PQ from Day 1 to Day 3, then transferred onto a standard seeded agar plate for two days without any injections. For the TP5 injected group, a negative control was made with ddH2O microinjected into the worm.

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Bleach worms	Plate L1 synchronized worms			Day 1 worms exposed to 10uM TP5 then exposed PQ plate 1) TP5 injections 2) TP5 in tube (1 h) 3) TP5 on plate (6 h)		Day 3 worms - Image - Test locomotor behaviour

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Bleach worms	Plate L1 synchronized worms			Day 1 worms exposed to PQ plate		Day 3 worms 1mM TP5 exposure 1) Injections 2) TP5 in tube (1 h)
Day 8	**Day 9**					
	Day 5 worms - Image					

Table 2. Experimental Timeline for neuroprotective (top) and neurorestorative (bottom) in *DY328* worms.

3.3.3 Dopamine Dependent Locomotor Assay

Assay plates were prepared as described in Sawin et al., 2000. The treatments groups in the neuroprotective experiment were tested in this assay (**Table 2, top**). One condition consisted of seeded plate of HB101 as food source for the worms (bacterial lawn), and another condition without any food source in the plate. Worms were removed from their original seeded plate and then transferred to the plate with bacteria directly in

the center with one worm at a time. After five minutes of recovery, sinusoidal body bends are counted for 20 seconds for three trials. The same protocol would apply for the absence of bacteria in the plate. In all conditions, the experimenter would be blind of each treatment group while counting the body bends. Sample size: 48-60 worms per condition.

3.3.4 Microscopy

Nematodes were mounted on 2% agar pad with a glass coverslip and anesthetized using 30mM sodium azide. GFP fluorescence was visualized using a Zeiss Observer Z1 microscope equipped with an Apotome 2 and X-Cite R 120LED fluorescence illuminator. Neurodegeneration was manually scored by determining if worms had defective dopaminergic cell bodies and processes. A typical perfect worm consists of four CEPS, at least one ADE, and no missing or punctuated axons. If a worm had any indication of defected cell bodies or punctuated processes that it would be considered neurodegenerative.

3.3.5 Statistical Analysis

Statistical analyses were performed using Graphpad. Comparison of groups were analyzed by using the One-way ANOVA with Tukey Multiple Comparisons Test. All bars are considered to be SEM unless indicated. P values smaller than 0.05 are considered statistically significant.

3.4 Results

3.4.1 Neuroprotective Effect of TP5 on Locomotor Behaviour

Well fed control worms exposed to a plate of bacteria demonstrated a decrease in body bends compared to well fed worms exposed to a plate that lacked bacteria, which confirms the basal slowing response ($p<0.0001$) (**Figure 14**). To confirm PQ's effect in dopamine-dependent locomotor behaviour, ddH_2O injected worms ($p<0.05$) and worms that were not injected ($p<0.001$), were both exposed to PQ to demonstrate significantly less body bends compared to healthy worms in the presence of bacteria. Next, worms injected with TP5 in a neuroprotective manner, then exposed to PQ, demonstrated an increase in body bends compared to the ddH_2O injected worms ($p<0.05$) and worms not injected ($p<0.001$) that were both exposed to PQ in the presence of bacteria. TP5 injected worms exposed to PQ did not reveal any differences between the healthy worms in the presence of bacteria.

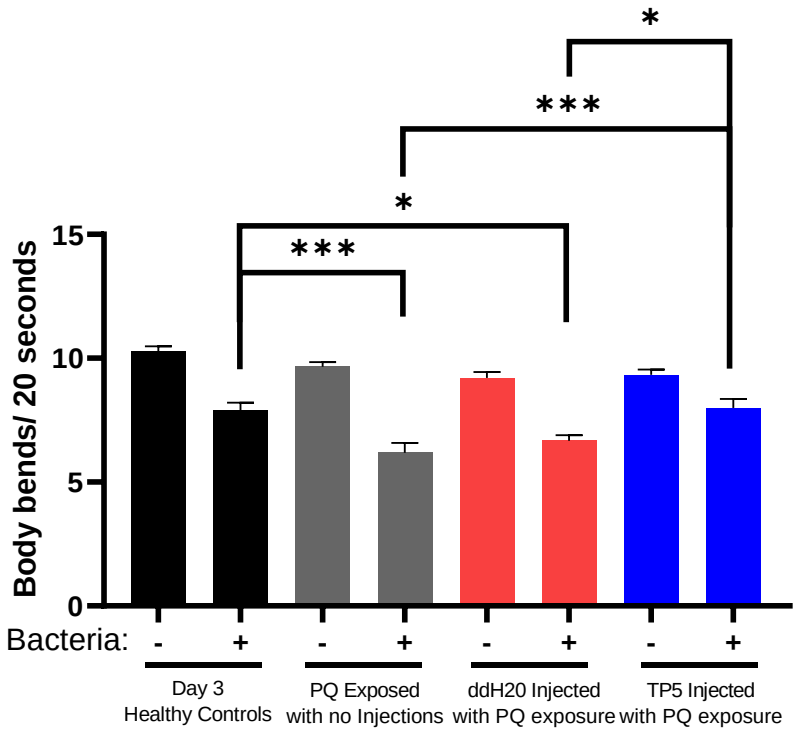

Figure 14. Effect of TP5 on Locomotor Behaviour in *DY328* worms exposed to PQ. One-way ANOVA indicated a significant difference between treatments ($F=28.18$, $p<0.0001$). Tukey's multiple comparison tests revealed that PQ exposed worms (grey) ($p<0.001$) and ddH_2O injected worms (red) ($p<0.05$) demonstrated decreased body bends compared to healthy controls (black) in a bacterial lawn. TP5 injected worms exposed to PQ (blue) had a significant increase in body bends compared to PQ exposed ($p<0.001$) and ddH_2O worms ($p<0.05$) when exposed to bacteria. Sample size ranged from 48 to 60. Data represents mean ± standard error of mean.

3.4.2 Neuroprotective Effect of TP5 on Neurodegeneration

The same worms, demonstrated in **Figure 14**, were investigated for neurodegeneration by visualizing their dopaminergic neurons in the head. **Figure 15 (Top left)** demonstrates control worms with three distinct CEPs and one ADE, with two axons. Worms injected with ddH_2O and exposed to PQ (**Top right**) shows four CEPs, with a faint ADE and a missing axon. TP5 injected worms that were exposed to PQ (**Bottom**), demonstrate four distinct CEPs, one clear ADE, and two axons. **Figure 16** revealed TP5 injected worms exposed to PQ had significantly decreased neurodegeneration compared to ddH_2O worms ($p<0.01$). This experiment was then replicated using different routes of administration and exposure times of TP5 due to the worms' unsuccessful recovery from injections in **Figure 16**. **Figure 17** demonstrates with the same results as seen in **Figure 16**. Worms exposed to TP5 on a plate for 6 h ($p<0.0001$) or suspended in TP5 for 1 h ($p<0.001$), which were both exposed to PQ afterwards, demonstrated significantly less neurodegeneration compared to worms exposed to PQ (**Figure 17**).

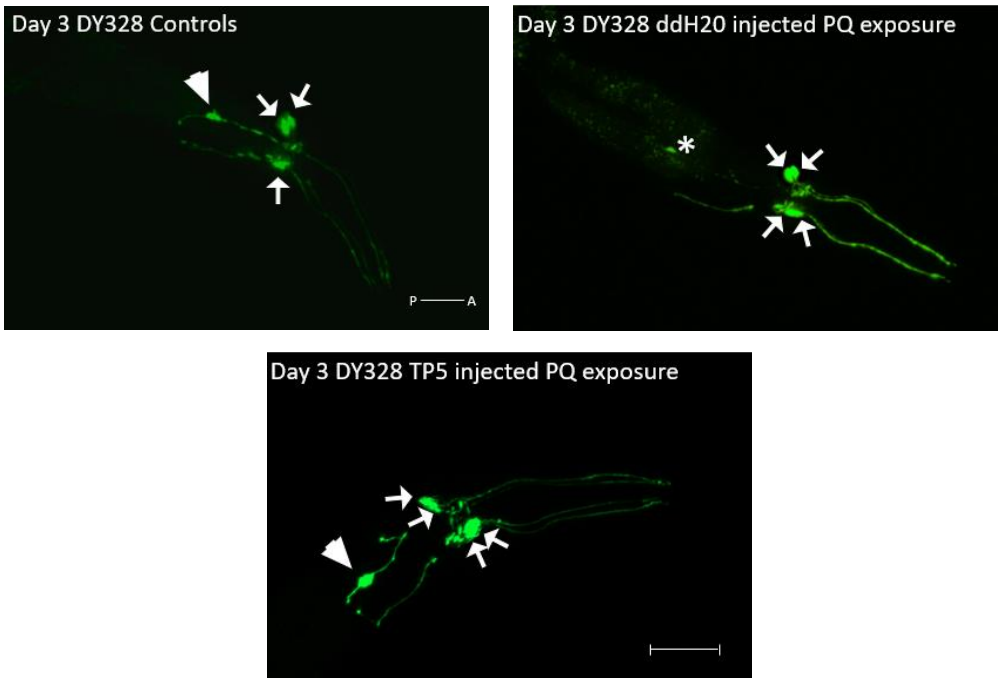

Figure 15. Neuroprotective Effect of TP5 on Neurodegeneration in *DY328* worms against PQ. Visualization of dopaminergic neurons in the head of *DY328* Control worms (Top Left), ddH_2O injected worms exposed to PQ (Top Right), and TP5 injected worms exposed to PQ (Bottom). The posterior pair of ADEs is indicated by the arrow heads, and four anterior CEPs are indicated by arrows. Asterisks demonstrate the missing axon and faint cell body (Top Right). Scale bar = 50μM

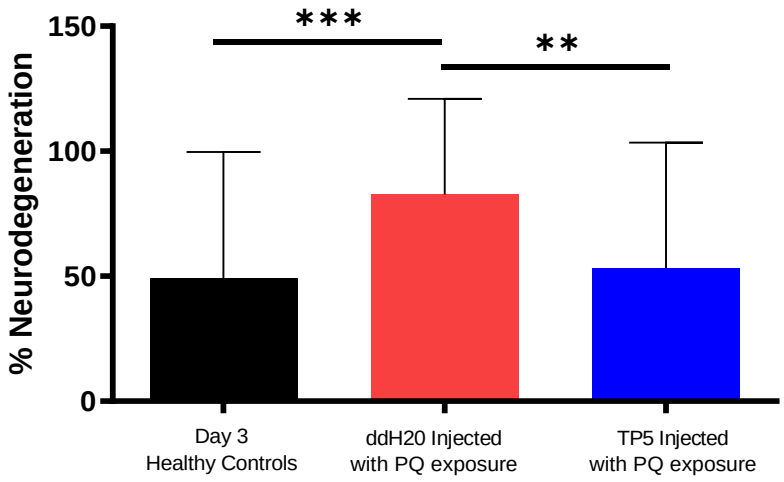

Figure 16. Neuroprotective Effect of TP5 Injections on Neurodegeneration in *DY328* worms exposed to PQ. One-way ANOVA indicated a significant difference between treatments ($F=8.453$, $p<0.001$). Tukey's multiple comparison tests revealed that ddH_2O injected worms (red) demonstrated increased neurodegeneration compared to healthy controls (black) ($p<0.001$). TP5 injected worms exposed to PQ (blue) had significant decreased in neurodegeneration compared to ddH_2O worms ($p<0.01$). Sample size: 69, 52, 64 (Healthy Controls, ddH2O injected with PQ exposure, TP5 injected with PQ exposure). Data represents mean ± standard deviation due to unequal number in batch sizes.

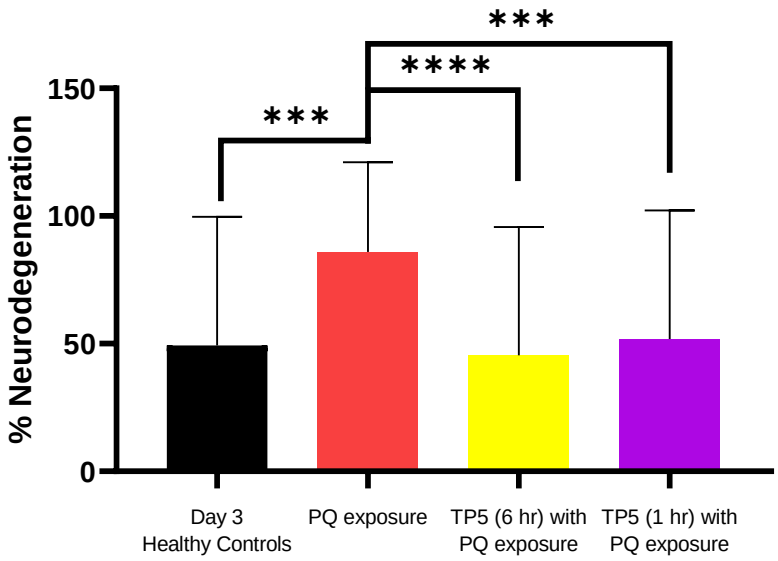

Figure 17. Neuroprotective Effect of TP5's Routes of Administration and Exposure Time on Neurodegeneration in *DY328* worms exposed to PQ. One-way ANOVA indicated a significant difference between treatments ($F=9.294$, $p<0.0001$). Tukey's multiple comparison tests revealed that PQ exposed worms (red) demonstrated increased neurodegeneration compared to healthy controls (black) ($p<0.001$). Worms exposed to TP5 for 6 h (yellow) and 1 h (purple) first, then PQ afterwards, both demonstrated significant decreased in neurodegeneration compared to PQ exposed worms ($p<0.001$). Sample size: 69, 57, 66, 56 (Healthy Controls, exposure, TP5 (6 h) with PQ exposure, TP5 (1 h) with PQ exposure) Data represents mean ± standard deviation due to unequal number in batch sizes.

3.4.3 Neurorestorative Effect of TP5 on Neurodegeneration

Worms were investigated for neurodegeneration by visualizing their dopaminergic neurons in the head in **Figure 18**. Controls worms were found with four CEPs and one ADE in the posterior end of the head (**Figure 18, top left**). ddH_2O injected worms exposed to PQ demonstrated four CEPs with missing axons and ADEs (**Figure 18, top right**). Lastly, worms exposed to PQ that are injected with TP5 revealed four CEPs, one ADE, and two axons **(Figure 18, bottom).** Worms were analyzed in **Figure 19** did not find significant differences with TP5 in a neurorestorative manner. TP5 injected worms exposed to PQ in a neurorestorative manner did not demonstrate significant decreased neurodegeneration compared to $dddH_2O$ worms exposed to PQ due to a small sample size ($p=0.0604$). Therefore, this experiment was repeated with different route of administration of TP5 in a neurorestorative manner to achieve a high sample size (**Figure 20**). Worms exposed to PQ before being suspended in TP5 for 1 h demonstrated significantly decreased neurodegeneration compared to worms exposed to PQ, further confirming that TP5's neurorestorative effects.

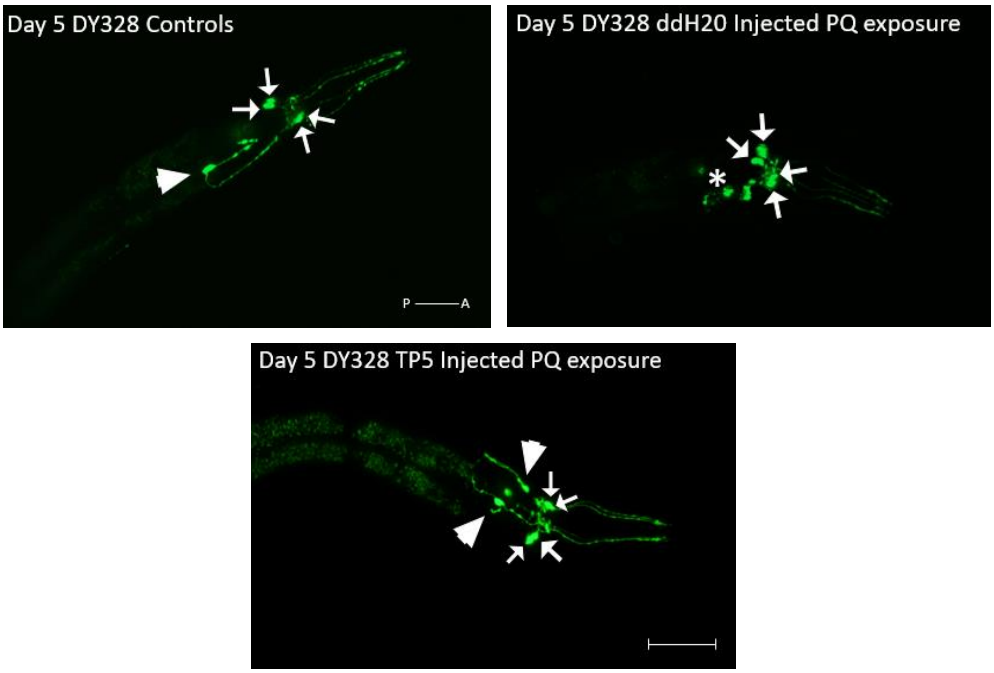

Figure 18. Neurorestorative Effect of TP5 on Neurodegeneration in *DY328* worms against PQ. Visualization of dopaminergic neurons in the head of *DY328* control worms (Top Left), ddH_2O injected worms exposed to PQ (Top Right), and TP5 injected worms exposed to PQ (Bottom). The posterior pair of ADEs is indicated by the arrow heads, and four anterior CEPs are indicated by arrows. Asterisks demonstrate the missing axon and faint cell body (Middle). Scale bar = 50μM

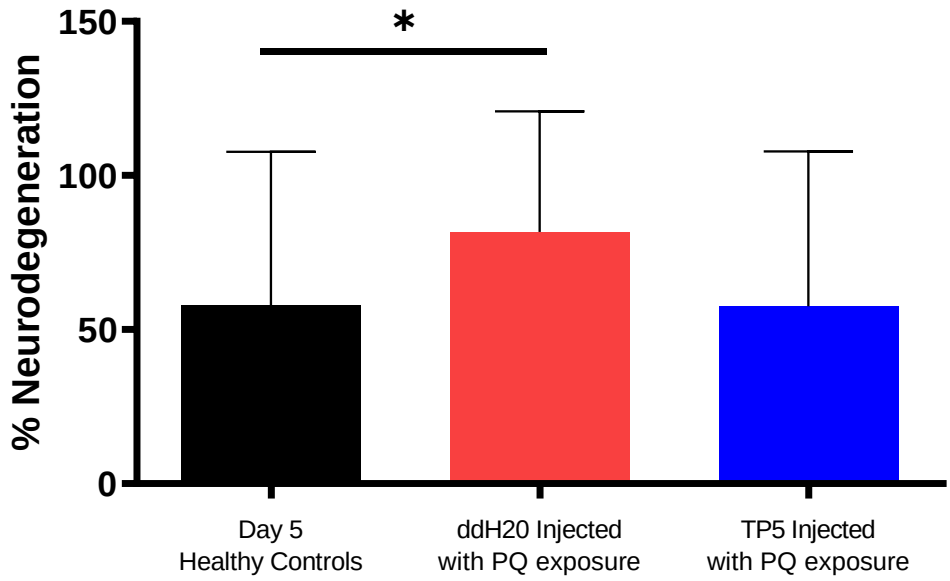

Figure 19. Neurorestorative Effect of TP5 Injections on Neurodegeneration in *DY328* worms exposed to PQ. One-way ANOVA indicated a significant difference between groups ($F=4.303$, $p<0.05$). Tukey's multiple comparison tests revealed that ddH_2O injected worms (red) demonstrated increased neurodegeneration compared to healthy controls (black) ($p<0.05$). TP5 injected worms exposed to PQ (blue) did not have any significant differences in neurodegeneration compared to ddH_2O worms ($p=0.0604$). Sample size: 69, 49, 33 (Healthy Controls, ddH_2O injected with PQ exposure, TP5 injected with PQ exposure) Data represents mean ± standard deviation due to unequal number in batch sizes.

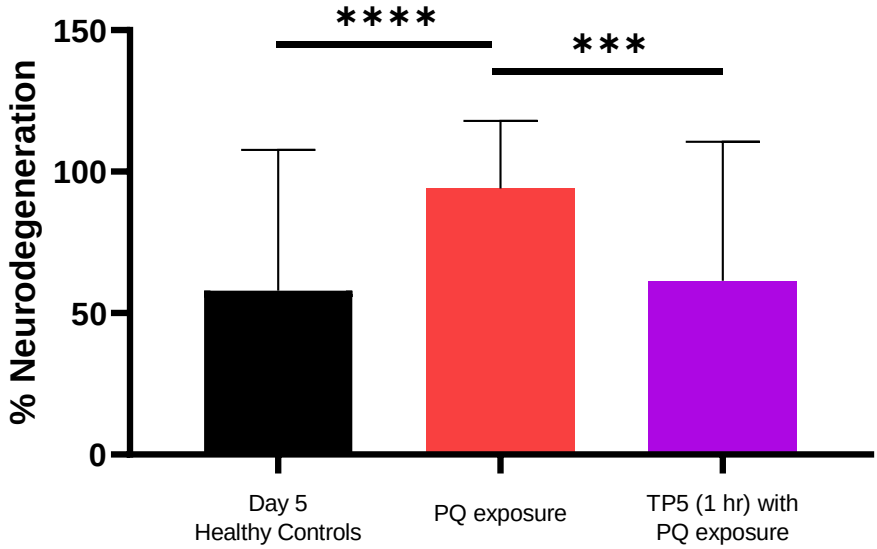

Figure 20. Neurorestorative Effect of TP5 for 1 h on Neurodegeneration in *DY328* worms exposed to PQ. One-way ANOVA indicated a significant difference between treatments ($F=11.20$, $p<0.0001$). Tukey's multiple comparison tests revealed that PQ exposed worms (red) demonstrated increased neurodegeneration compared to Healthy controls (black) ($p<0.0001$). Worms exposed to PQ before being suspended in TP5 for 1 h (purple) demonstrated significantly decreased neurodegeneration compared to PQ exposed worms ($p<0.001$). Sample size: 69, 50, 57 (Healthy controls, PQ exposure, TP5 (1 h) with PQ exposure) Data represents mean ± standard deviation due to unequal number in batch sizes.

3.5 Discussion

Although TP5 has been demonstrated to show neuroprotective effects in behaviour based on *in vivo* models (Binukumar et al., 2015), this is the first study that examines TP5's therapeutic effect in a *C. elegans* model. Since *C. elegans* are a simplistic model based on its eight dopaminergic neurons in its system, TP5's therapeutic effect can be correlated towards its dopamine-dependent locomotor behaviour based on the morphology and activity of the dopaminergic neurons. For the purposes of understanding the mechanism of the peptide, using a simple model will demonstrate the causal effects. Using a complex model, such as a mammal, will have confounding factors that is difficult to explain the reasoning behind the behaviour of a multifaceted organism. Using a *C. elegans* model will further confirm that TP5 has an effect towards dopaminergic neurons and the dopamine-dependent locomotor behaviour against PQ.

TP5 has established its neuroprotective effects in the dopamine-dependent locomotor behaviour of *C. elegans* exposed to PQ shown in **Figure 14**. Based on Sawin et al., 2000, the dopamine signaling pathway is required for the basal slowing response, where the locomotor behaviour is reduced when well fed worms are exposed to a bacterial lawn. With the ablation of dopaminergic neurons, an increased locomotor behaviour was observed when worms were exposed to the bacterial lawn. This indicates the mechanosensory properties of the dopaminergic neurons are impaired. When we exposed the worms to PQ for 48 h, we did not see the expected results demonstrated with Sawin et al., 2000. Instead, a significant decrease in locomotor behaviour compared to healthy control worms was observed. A couple elucidations can explain the unexpected

effects. Sawin et al., 2000, demonstrated the increased locomotor response was only present when they ablated all three classes of dopaminergic neurons. When they only ablate CEPs and ADEs, there was no effect of increased body bends compared to controls. Based on the visualization of the dopaminergic neurons in **Figure 15**, majority of the CEPs and ADEs are present. PDEs cannot be imaged and analyzed due to its unstable expression in the posterior body. In addition, to completely disrupt dopaminergic signalling in all three classes would require a higher concentration and longer exposure to PQ that can potentially be lethal to worms. Another explanation to why the worms move slower is due to PQ's main mechanism of toxicity. Increased oxidative stress and mitochondrial dysfunction can interfere with the locomotor behaviour; worms exposed to PQ demonstrated reduced velocity compared to controls (Gourgou & Chronis, 2016). Lastly, although PQ was unable to demolish the function of dopaminergic neurons, the purpose of the assay was to determine whether TP5 can protect neurons against PQ. Worms injected with TP5 in a neuroprotective manner against PQ, has shown to perform similar to healthy control worms based on **Figure 14**. This confirms that TP5 has neuroprotective effects in dopamine-dependent locomotor behaviour against a PD-like model in *C. elegans*.

Once the neuroprotective effect of TP5 on dopamine-dependent locomotor behaviour of the *C. elegans* against PQ has been established, dopaminergic morphology must be investigated to confirm whether TP5's protective effect in locomotor behaviour correlates with dopaminergic morphology in *C. elegans*. PQ has been demonstrated to have an effect on dopaminergic neurons in mice models (Brooks et al., 1999), this notion

can also be confirmed in *C. elegans* as well. Exposure to PQ for 48 h led to an increase in neurodegeneration compared to the healthy controls based on **Figure 16**. With TP5 supplemented prior to the exposure of PQ, worms treated with the combination of TP5 and PQ demonstrated less defected dopaminergic neurons compared to worms exposed to PQ. PQ can damage the dopaminergic neurons and TP5 has shown to rescue this effect. These neurodegeneration results can explain why the dopamine-dependent locomotor behaviour in this group improved, indicating a direct relationship between the dopaminergic neurons and locomotor behaviour. This further confirms that TP5 has strong neuroprotective effects *in vitro* and *in vivo* models exposed to PQ; therefore, TP5 can be an effective drug towards models of PD.

Although TP5's neuroprotective effects in a PQ model has been demonstrated *in vitro* and *in vivo*, it has yet to be studied through a neurorestorative manner. This is the first study that has examined whether TP5 can restore the dopaminergic morphology in a *C. elegans* model. However, when worms were exposed to PQ and later injected with TP5 in a neurorestorative manner, this treatment had no differences between worms exposed to PQ based on **Figure 19**. These results are potentially due to the small sample size of the TP5 injected worms as injections are stressful towards the worms; many worms would have an unsuccessful recovery. In addition, the combination of exposing worms to PQ then receiving injections could weaken the worm even more. Therefore, this experiment was replicated with a different route of administration to achieve a higher sample size and expose worms to TP5 in a non-invasive manner. **Figure 20** demonstrates that worms exposed to PQ first, then suspended in 1mM of TP5 for an hour had

significantly less neurodegeneration compared to the PQ treatment. These results confirm that TP5 can rescue the dopaminergic degeneration that occurs in *C. elegans*. The mechanism of how TP5 can restore dopaminergic morphology has yet to been established; however, as demonstrated in **Chapter Two**, where TP5 has shown to be involved in neurite outgrowth, it is possible that TP5 can also affect other physiological functions of Cdk5 such as synaptic function and neuronal survival. Further studies can examine whether the dopamine-dependent locomotor behaviour can be rescued by TP5 in a neurorestorative manner against PQ. This study will help identify whether TP5 is a potential therapeutic clinical drug towards individuals who have PD symptoms. In addition to confirming TP5 as a potential therapeutic drug for PD, TP5 can also examine its effect on the aggregation of ASN in *C. elegans* when exposed to PQ. Aggregation of ASN has been demonstrated in a Mesencephalic astrocyte-derived neurotrophic factor (MANF) mutant model in *C. elegans* (Richman et al., 2018). MANF is a neurotrophic factor that has demonstrated to protect dopaminergic neurons. Therefore, we can examine PQ's effect in the aggregation of ASN and determine whether TP5 can protect or rescue the dysfunctional protein. This exposure of PQ can lead to the dopaminergic loss and aggregation of ASN, two hallmarks that are identified in PD and TP5 has the potential to protect this pathology from worsening. These studies will further validate TP5's therapeutic effects in Parkinson's Disease.

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusion

These two studies investigated TP5's therapeutic effects *in vitro* and *in vivo* models, which were treated with PQ to represent pathological and phenotypical features of Parkinson's Disease. *In vitro* with *SH-SY5Y* cells, which are known to be representative of dopaminergic neurons, demonstrated TP5's potential to enhance neurite outgrowth and protect these neuronal cells against PQ. This further confirms TP5's neuroprotective effect *in vitro*, as this was first demonstrated in a different study with primary mesencephalic cultures that used MPTP as a toxin-induced model of PD (Binukumar et al., 2015). Due to PQ and MPTP having similar cellular toxicity and TP5 protecting against both toxins, this asserts that PQ has the potential to be an accurate representation of PD. Although directly measuring Cdk5/p25 levels were not performed in this study, we can hypothesize that based on PQ's similarity to MPTP, PQ should lead to a hyperactivation of Cdk5/p25 and TP5's ability to inhibit this pathway. Furthermore, investigating TP5's effects in neurite outgrowth enables us to correlate TP5's potential effects in restoration against cell toxicity. With the addition of PQ that should lead to the decrease in neurite outgrowth, TP5 is expected to protect neurite outgrowth impairment, which is analogous to lithium being used to protect neurite outgrowth from PQ (Alural et al., 2015). Another study examined a variety of procognitive compounds that are used for Alzheimer's disease and discovered that they were able to increase neurite outgrowth in primary culture (Page et al., 2015). This further confirms that compounds which enhances neurite outgrowth, like TP5, can provide symptomatic relief to individuals with neurodegenerative diseases such as AD and PD.

Although TP5 was found to show promising results towards *in vitro* models of PD, one glaring issue is that TP5 cannot cross the BBB due to its long amino acid length. However, a shorter fragment of TP5, known as Peptide A, was investigated to determine if it is just as efficacious as TP5, due to the ability of shorter peptides being able to pass through the BBB with more accessibility (Oller-Salvia, Sánchez-Navarro, Giralt, & Teixidó, 2016). Peptide A was determined to have an effect in neurite outgrowth but did not protect the neuronal cells against PQ. Future experiments will be necessary to confirm if this segment is efficacious as TP5.

Investigating TP5 *in vitro* demonstrates neuroprotective effects in a simple cellular level and has the ability to measure a large number of neurites quickly and efficiently. However, testing *in vitro* is limited due to the inability to examine TP5 in an organism, such as how TP5 affects behaviour and other classes of neurons. That is why we transitioned into investigating *C. elegans in vivo*; we can explore TP5's effect through behaviour, a diverse subtype of neurons, and eventually use transgenics models for further investigation of PD. Specifically, using *C. elegans* as an *in vivo* model to explore TP5's therapeutic effects provide the analysis of the basal slowing response, a locomotor behaviour that is dependent on dopamine, and the visualization of dopaminergic degeneration. TP5 demonstrated neuroprotective effects through the *C. elegans'* basal slowing response, and the prevention of dopaminergic degeneration when *C. elegans* were exposed to PQ. TP5 has proven to demonstrate neuroprotective effects *in vitro* and *in vivo* using PQ as the toxin-induced model of PD. Determining the neuroprotective

effects are beneficial to find the root cause of the pathophysiology of the disease, especially when PD is from a complex multifactorial etiology

However, current treatments in PD struggle to relieve symptoms of patients due to their temporary effects and associated long-term side effects. Therefore, establishing that TP5 has neurorestorative effects against dopamine degeneration due to PQ in the *C. elegans* implicates the potential this peptide has in the clinical setting. This can also be correlated with TP5's effect in neurite outgrowth to further demonstrate that TP5 has a role in neurorestoration. These findings implicate that TP5 has the potential to be a therapeutic drug as a preventative and restorative treatment towards PD. Nevertheless, TP5 has a long way to go before ever being approved for clinical trials. The mechanism of TP5 needs to be further explored, such as solely focusing on its effect without pathological conditions as its been shown to be involved in neurite outgrowth. Future studies will need to be conducted to ensure TP5 is safe and effective for its potential to be a therapeutic drug in neurodegenerative diseases such as Parkinson's Disease.

4.2 Future Directions

An important concept in this study is TP5's mechanistic pathway towards the treatment of PQ as a model of PD; specifically, TP5's role to inhibit the hyperactivation of Cdk5/p25 levels. Although TP5 has been proven to inhibit these levels through MPTP, it has yet to be proven in PQ. A future study is required to investigate the Cdk5 activity with exposure to PQ and TP5's capacity to inhibit the expected aberrant levels of Cdk5

from PQ. This is likely to be performed *in vitro* and *in vivo* to correspond with our current findings. Specifically, another way to determine if TP5 is directly affecting the Cdk5 pathway is through RNAi *in vivo*. By using *C. elegans*, RNAi can be used to knock down the Cdk5 homolog, leading to TP5 having no effect in the knock down model due to the absence of Cdk5. This will confirm that Cdk5 is required for TP5 to bind and inhibit the hyperactivation of the Cdk5/p25 complex.

Another study that needs to be explored is the amount of TP5 that enters the cavity of the worm. Although we microinjected the worms with the peptide, another method of exposing the worms to TP5 was by placing them in a suspended solution of TP5 and on the mixed agar plate to be absorbed. Using high performance liquid chromatography (HPLC), we can quantify the amount of TP5 in the worm to determine how much was absorbed to determine the dosage of TP5.

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