Nitric oxide-mediated neurite retraction is induced by aberrant α-synuclein deposition in Parkinson's disease

by

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ABSTRACT

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Parkinson's Disease (PD) is a neurodegenerative disorder associated with pathological deposits of aggregated α-synuclein in multiple brain regions. While motor dysfunction is a primary phenotype of PD, many patients develop non-motor symptoms such as cognitive impairment and dementia. Furthermore, PD dementia is highly associated with pathological deposits of aggregated α-synuclein in neurites. In addition, an early marker of neurodegeneration is the retraction of synaptic terminals and distal axons of neurons, which usually precede cell death. However, due to the lack of appropriate human *in vitro* model systems, it is unclear if neurite retraction in PD is an early marker of pathology, or is consequence to global neuronal cell death. Moreover, no mechanistic evidence has provided a link between α-synuclein aggregation and neurite retraction.

To investigate these questions, a human embryonic stem cell (hESC) model of PD was utilized in which the A53T-*SNCA* mutation was introduced by zinc-finger nuclease genome editing. This system allows for comparison of A53T α-synuclein mutant cells against isogenic controls. Following differentiation to dopaminergic (DA) neurons, it was shown that A53T DA neurons display

morphological deficits in neurite complexity relative to wild-type (WT) control. Interestingly, the addition of preformed α-synuclein fibrils (PFFs) to both WT DA neurons and WT primary mouse cortical neurons (mNs) evoked neurite degeneration and reduced branching. Treatment with Nω-Nitro-L-arginine methyl ester hydrochloride ($_{L}$-NAME), a nitric oxide synthase (NOS) inhibitor, was able to rescue neurite morphology in A53T DA neurons, suggesting a causal link to nitrosative stress. To further understand this pathology using a simpler model system, the SH-SY5Y neuroblastoma cell line was utilized in the presence or absence of PFFs. It was shown that PFF exposure was able to induce neurite retraction, and $_{L}$-NAME treatment rescued neurite length in SH-SY5Y cells. Furthermore, activation of the NRF2-mediated anti-oxidant response was investigated for a more appropriate therapeutic approach for neurite rescue. Treatment with a known NRF2 activator dimethyl fumurate (DMF), was able to rescue neurite retraction in SH-SY5Y cells. Lastly, these results were translated to A53T DA neurons, where DMF treatment was able to therapeutically rescue neurite retraction. The results presented here reveal innate differences in neurite morphology between A53T DA neurons and isogenic controls. More importantly, this phenotype could be rescued by alleviation of redox stress with DMF. As such, detoxifying neurons of reactive oxygen/nitrogen (ROS/RNS) through forced activation of NRF2 may provide a new preventive therapeutic avenue against PD and associated cognitive impairment.

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AUTHOR'S DECLARATION OF COMPLETED WORK

I declare that all work presented in this thesis is my own, with the following exceptions:

Primary culture of mouse cortical neurons and generation of preformed α-synuclein fibrils was completed by Carla Coackley and Kayla Humphries.

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GLOSSARY OF ABBREVIATIONS

3'NT	3'-nitrotyrosine
β III-Tub	beta 3 tubulin
ARE	anti-oxidant response element
BDNF	brain derived neurotrophic factor
DA	dopaminergic
DMF	dimethyl fumarate
DIV	days *in vitro*
ECL	enhanced chemiluminescence
ETC	electron transport chain
FGF8	fibroblast growth factor 8
FRET	frequency resonance energy transfer
GCLC	glutamate-cysteine ligase catalytic subunit
GNDF	glial derived neurotrophic factor
GSH	glutathione
hESC	human embryonic stem cell
hNs	human neurons
IMM	inner mitochondrial membrane
KSR	knockout serum replacement
$_{L}$NAME	N ω -Nitro-L-arginine methyl ester hydrochloride
MAPs	microtubule-associated protein
MEFs	mouse embryonic fibroblasts
mNs	primary cortical mouse neurons
NO	nitric oxide

NOS	nitric oxide synthase
NQO1	NAD(P)H:quinone acceptor oxidoreductase 1
NRF2	nuclear factor E2-related factor 2
PBS	phosphate buffered saline
PD	Parkinson’s disease
PDI	protein-disulphide isomerase
PFA	paraformaldehyde
PFF	preformed fibrils
PS129	phosphorylated serine 129
RA	retinoic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SHH	sonic hedgehog
sMAFs	small musculoaponeurotic fibrosarcoma proteins
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
TGF β 3	transforming growth factor beta 3
TH	tyrosine hydroxylase
Tx-100	triton X-100
TyTub	tyrosinated tubulin
Ubq	ubiquitin
VTA	ventral tegmental area
WT	wild type

CHAPTER 1: INTRODUCTION

(1.1) Overview of Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disease characterized by a progressive decline in voluntary movement. It is the most prevalent movement disorder worldwide, affecting approximately 100,000 Canadians over the age of 65[1]. About 95% of all PD cases are sporadic, where patients have no apparent hereditary history of the disease with immediate family (Dauer and Przedborski, 2003). The remaining PD cases are caused by the inheritance of a mutation in one of several causal genes, referred to as familial PD. The three main clinical features of PD are resting tremors, rigidity (stiffness) and bradykinesia (slowness of movement). However, non-motor features such as autonomic dysfunction, cognitive impairment, psychiatric changes and sleep disturbances can also manifest (Dauer and Przedborski, 2003; Shulman et al., 2011). As the course of this disease worsens, motor function eventually dissipates and patients lose the ability to both move and speak. Currently, there is no existing therapeutic treatment capable of stopping or reversing this disease pathology (Dias et al., 2013).

There are two distinct pathological hallmarks of PD that contribute to parkinsonism symptoms in patients. The first is the selective loss of nigrostriatal dopaminergic (DA) neurons in the Substantia Nigra pars compacta (SNpc) of the

[1] Parkinson's Disease: Social and Economical Impact. 2003. Health Canada; [accessed 2015 Jan 23]. http://parkinson.ca/Parkinsons-Disease-Fact-Sheet

brain (Dauer and Przedborski, 2003; Schapira and Jenner, 2011). The onset of symptoms in the majority of PD patients is not apparent until approximately 60-90% of these neurons are degenerated and the reason for this susceptible loss is fundamentally unclear (Samii et al., 2004). The second pathological hallmark of PD is the accumulation of intracellular protein aggregates, which primarily consist of α-synuclein (Baba et al., 1998; Giasson et al., 2002). It is commonly thought that neurodegeneration in PD is exclusive to the loss of nigrostriatal DA neurons, however neurodegeneration and α-synuclein aggregation have also been identified in both hippocampal and cortical structures (Hurtig et al., 2000; Irizarry et al., 1998; Mattila et al., 2000). This evidence could explain the occurrence of non-motor symptoms that also are correlated with the onset of PD. Nonetheless, with several fundamental questions still unanswered, it is imperative to develop new and more appropriate model systems to further understand neurodegenerative mechanisms in PD pathogenesis.

(1.2) DA Pathways in the Brain

The human brain consists of three major DA pathways, nigrostriatal, mesocortical and mesolimbic, each responsible for separate neurological functions (Figure 1). The nigrostriatal pathway consists of 80% of the brains DA neurons. Cell bodies of these neurons reside in the SNpc and primarily project to the putamen of the dorsal striatum (Kizer et al., 1976). This pathway partially makes up the basal ganglia motor loop, which is responsible for the initiation of

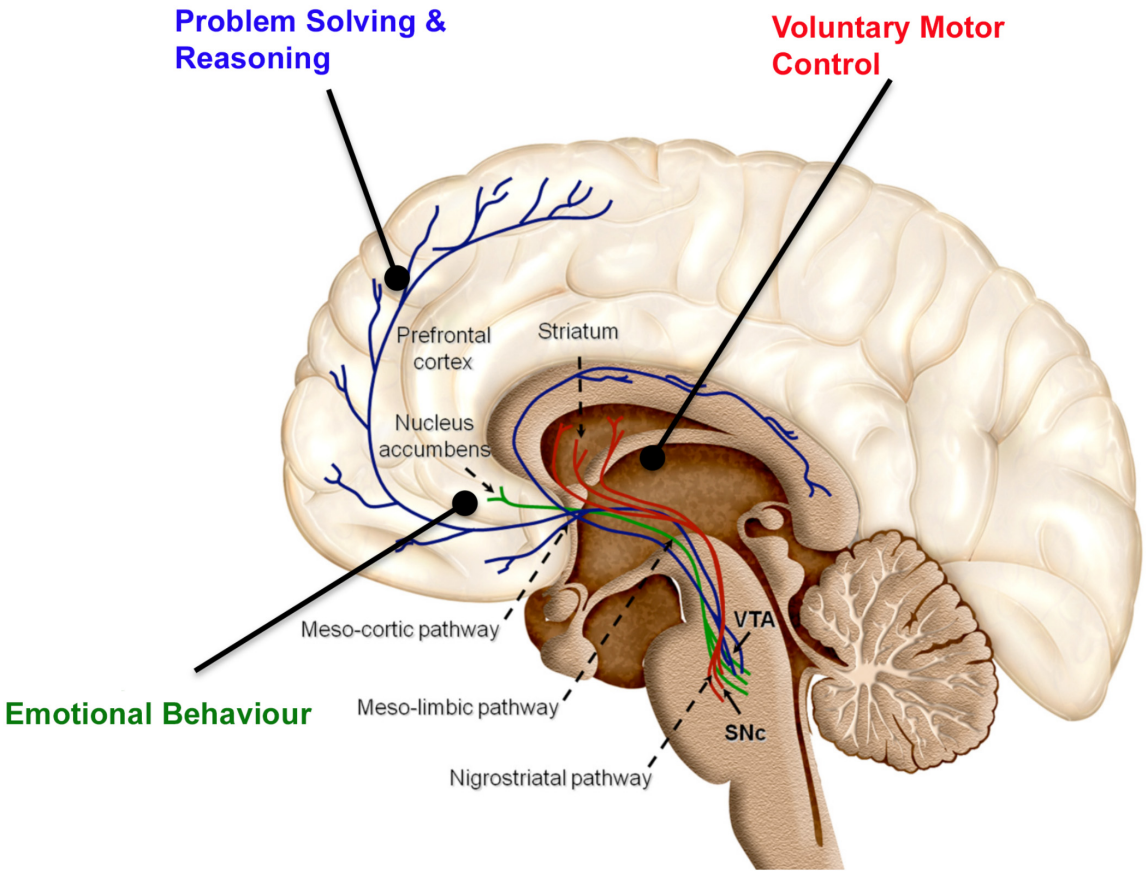

Problem Solving &
Reasoning
Voluntary Motor
Control
Prefrontal
cortex
Striatum
Nucleus
accumbens
Meso-cortic pathway
VTA
Emotional Behaviour
Meso-limbic pathway
SNc
Nigrostriatal pathway

Figure 1. Overview of dopaminergic pathways in the human brain. Cell bodies of dopaminergic (DA) neurons reside in the midbrain structures of the Substantia Nigra pars compacta (SNpc) and the ventral tegmental area (VTA). Several DA pathways exist in the brain and are responsible for specific neurological functions. The nigrostriatal DA pathway (red) projects out from the SNpc into the striatum and is responsible for initiating voluntary movement and motor control. The mesocortical DA pathway (blue) projects from the VTA into the prefrontal cortex with specific neurological functions involving cognitive control. The mesolimbic DA pathway (green) also projects from the VTA but synapses with the nucleus accumbens with specific functions regarding emotional behaviour. Neurodegeneration of these DA pathways would impede on any of these respective brain functions (Modified from Carrion et al. 2010).

voluntary movement and motor control. In the mesocortical pathway, DA neurons reside in the ventral tegmental area (VTA) located adjacent to the SNpc. These neurons project to and synapse with neurons in the dorsolateral prefrontal cortex of the brain that are essential for cognitive function. Also residing in the VTA but projecting to the nucleus accumbens are DA neurons that form the mesolimbic pathway, which is strongly correlated with emotional behavior and perceptional pleasure (Koob and Le Moal, 2001; Nieoullon and Coquerel, 2003; Wise, 1998; Yadid et al., 2001). Degeneration of any of these structural DA systems results in dopamine depletion and impairment of respective neurological function.

(1.3) Neurodegeneration in PD

PD often first presents clinically with symptoms of motor impairment including resting tremors, rigidity and bradykinesia. These symptoms can be attributed to the degeneration of DA neurons of the nigrostriatal pathway, which impede the motor loop of the basal ganglia (Perlow et al., 1979). In addition, the presence of debilitating non-motor manifestations can also occur in later stages of PD. These symptoms often include the decline in cognitive abilities such as problem solving and reasoning skills, sleep disturbances, depression and dementia (Bruck et al., 2004; Irwin et al., 2013; Ray and Strafella, 2012). Non-motor manifestations develop due to the degeneration of DA neurons in the mesocortical and mesolimbic pathways of the brain. As such, there appears to be a chronological order in which degeneration occurs throughout PD pathogenesis, which correlates to the onset of various symptoms seen in patients (Braak et al.,

2003). Although currently available therapies attempt to manage PD symptoms by increasing dopamine levels, they do not address the degeneration of DA neurons. Therefore, new research is trying to identify therapeutic targets that may be useful in hindering or reversing disease progression.

A common hallmark in neurodegenerative diseases is the retraction of synaptic terminals and distal axons of neurons, which usually precedes neuronal death (Luo and O'Leary, 2005; Yang et al., 2013). As such, the retraction of neurites has been used as an early pathological marker of neurodegeneration. Although the mechanism of neurite retraction in neurodegenerative disease is not fully understood, animal models of PD have shown that a delay in axon retraction can alleviate motor impairment (Coleman and Perry, 2002; Raff et al., 2002; Yang et al., 2013). These studies therefore suggest that the prevention of neurite retraction could be used as a therapeutic target for neurodegenerative disease. However, although these animal PD models have provided valuable insight, translational human model systems are still needed to further understand this disease pathology.

(1.4) Synucleinopathy in PD

Another early marker of neurodegeneration in PD is the presence and accumulation of α-synuclein inclusions, which arise from the aggregation of misfolded monomeric α-synuclein (Spillantini et al., 1997). These aggregates have been shown to deposit within the cell soma and neurites of neuronal

populations, most notably but not limited to DA neurons (Irizarry et al., 1998). The source of misfolded α-synuclein in PD has been linked to several direct and indirect mechanisms. Genetic mutations at the *SNCA* (α-synuclein gene) locus are known to directly induce improper folding of the gene product, leading to potentially toxic protein conformations (Narhi et al., 1999). To date, three distinct missense mutations have been identified (A53T, E46K and A30P), with the A53T mutation being the most frequent (Shulman et al., 2011). However, α-synuclein misfolding can also arise from indirect mechanisms consequent of post-translational modifications due to cellular stress and impairment of proteostasis pathways. As such, redox based protein modifications mediated through oxidative/nitrosative stress reactions have also been linked to misfolded protein accumulation (Hwang, 2013; Tsang and Chung, 2009). For example, chaperone proteins that promote normal protein folding, such as protein-disulphide isomerase (PDI), can be modified through the redox based nitrosylation modification (Uehara et al., 2006). Nitrosylation of PDI could result in an altered conformation, hindering its catalytic function. This would result in the inhibition of the unfolded protein response, allowing the accumulation of misfolded proteins and ultimately exacerbating PD pathology (Uehara et al., 2006).

Recent advances in PD modeling using synthetic preformed α-synuclein fibrils (PFFs) have yielded further insight in synucleinopathy mechanisms (Luk et al., 2009; Volpicelli-Daley et al., 2014; Volpicelli-Daley et al., 2011). Evidence suggests that the addition of PFFs to a healthy neuronal population can induce the recruitment of endogenously native α-synuclein and initiate the formation of

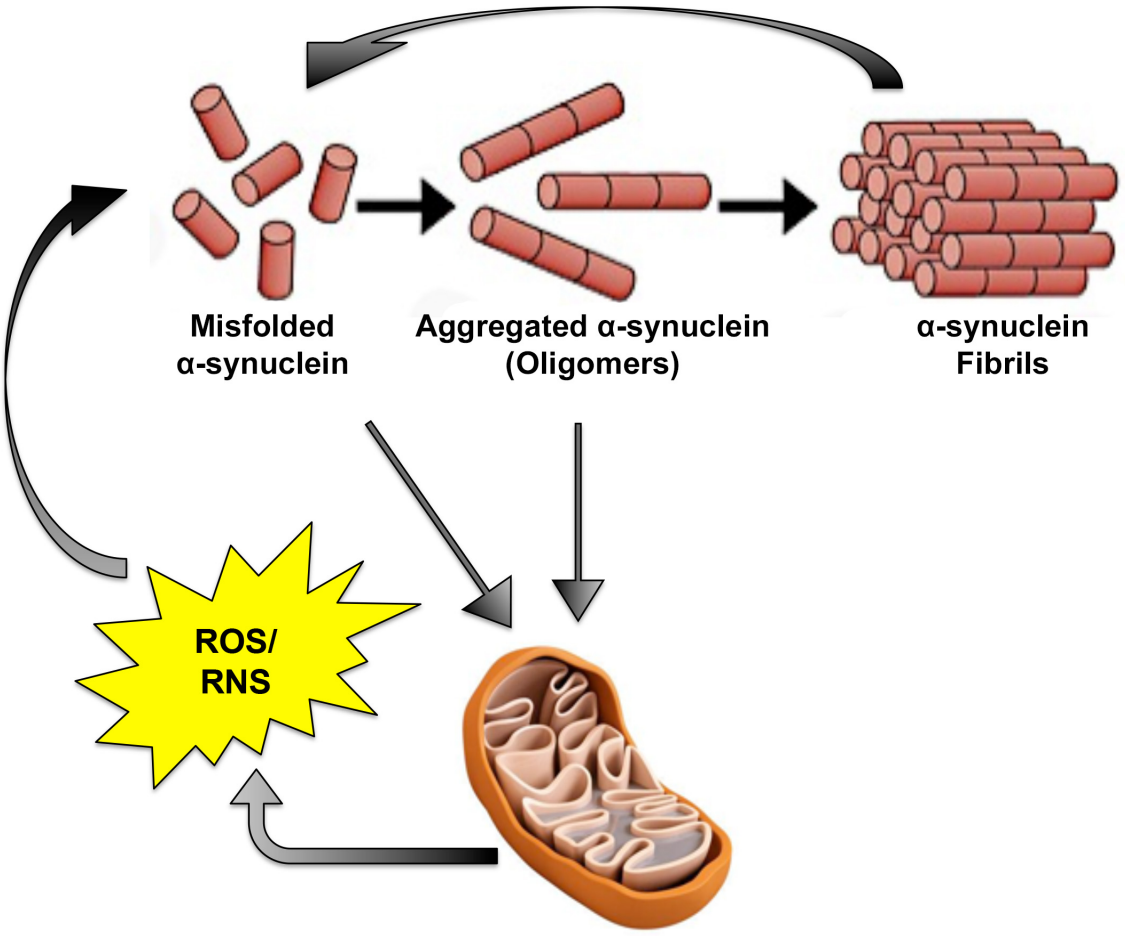

Misfolded
α-synuclein
Aggregated α-synuclein
(Oligomers)
α-synuclein
Fibrils
ROS/
RNS

Figure 2. Synucleinopathy in PD. Misfolded α-synuclein can aggregate into oligomers, which can further propagate the formation of fibrils. Recent studies have shown that α-synuclein fibrils can act in a positive feedback manner and propagate the misfolding of monomeric α-synuclein (Luk et al., 2009). Furthermore, aberrant α-synuclein can localize and disrupt the electron transport chain of the mitochondria resulting in excess production of ROS/RNS (Devi et al., 2008). Elevated levels of oxidative/nitrosative stress can evoke post-translational modifications of α-synuclein, which can further promote misfolding (Gaisson et al., 2000). Genetic mutations and environmental contaminants have also been shown to accelerate this pathology.

intracellular aggregated inclusions (Volpicelli-Daley et al., 2011). This research not only introduces a new system for modeling PD synucleinopathy, but also exposes a positive feedback mechanism in which higher forms of aggregated α-synuclein can further propagate synucleinopathy (Figure 2).

Interestingly, aberrant protein accumulation in the brain is not only a hallmark of PD, but of many other neurodegenerative diseases including Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Ross and Poirier, 2004). Although the characterization of protein aggregates differs between diseases, it suggests that aberrant protein accumulation is highly toxic to neurons (Ross and Poirier, 2004). However, despite the importance of protein accumulation in multiple diseases, the mechanisms through which these aggregates, including α-synuclein, trigger neurodegeneration are unclear. One plausible explanation could be that protein aggregates within neurons cause direct damage through the interference of intracellular trafficking and cell function (McNaught and Olanow, 2006; Philo and Arakawa, 2009; Tan et al., 2009). However, several lines of evidence suggest that pathogenic forms of α-synuclein, specifically misfolded monomeric and oligomeric α-synuclein, can localization to the inner mitochondrial membrane of the mitochondria and impair function of the electron transport chain (ETC) (Devi et al., 2008; Hsu et al., 2000; Nakamura et al., 2011; Nakamura et al., 2008; Sarafian et al., 2013). Disruption of the ETC would subsequently result in mitochondrial dysfunction and the increased production of reactive oxygen/nitrogen species (ROS/RNS). Despite previous

research in PD synucleinopathy, a large gap in the understanding of α-synuclein aggregation and mediated toxicity remains unknown. Therefore, much research is still needed in order for the development of novel therapeutics to combat PD pathology.

(1.5) Redox Stress in PD

In addition to synucleinopathy in PD, overwhelming evidence implicates the excessive formation of redox stress in PD pathogenesis (Giasson et al., 2000; Hwang, 2013; Tsang and Chung, 2009). As previously discussed, this can be primarily attributed to the excessive production of ROS/RNS due to the impairment of mitochondrial function (Keane et al., 2011; Palacino et al., 2004). The transport of negatively charged electrons through mitochondrial complexes in the ETC involves a series of redox reactions, creating a proton gradient across on the inner mitochondrial membrane (IMM) and the production of ATP. However, impairment of the ETC can result in a leakage of electrons from the IMM that can readily react with oxygen to produce the ROS superoxide (Keane et al., 2011). Subsequently, superoxide can react with nitric oxide (NO) to form the highly toxic RNS, peroxynitrite. This increased generation of ROS/RNS can significantly damage the functional integrity of the cell through the reaction of these molecules with lipids and nucleic acids, as well as post-translational modifications of proteins.

The most common mechanism of protein modification via ROS is the formation of carbonyl groups through the oxidation of specific amino acids (Ckless et al., 2007). Similarly, RNS have been shown to modify target proteins through the addition of NO-containing groups. As such, excessive levels of NO are capable of reacting with critical cysteine thiols (-SH) on target proteins to form S-nitrosothiol (-SNO) groups, termed as an S-nitrosylation reaction (Lipton et al., 1993). NO has been reported to inhibit mitochondrial complexes I and IV via S-nitrosylation, ultimately decreasing both electron transfer and ATP production (Clementi et al., 1998; Zhang et al., 2005). A reduction in cellular energy levels in DA neurons would compromise the membrane potentials in these cells, causing prolonged depolarization and potential apoptosis (Moon et al., 2005). In addition, the presence of the toxic RNS peroxynitrite can also lead to altered protein modification through nitration of tyrosine residues, termed nitrotyrosination (Gow et al., 1996). Together, these oxidative/nitrosative modifications can disrupt the functional structure of affected proteins, ultimately impeding protein function. As mentioned above, α-synuclein aggregation has been implicated in the impairment of the ETC, and consequent increase in oxidative/nitrosative stress. It was previously reported that increased redox stress subsequently leads to nitrotyrosination and methionine oxidation of α-synuclein, which can propagate synucleinopathy in PD (Giasson et al., 2000; Schildknecht et al., 2013). This therefore suggests yet another positive feedback mechanism implicating a potential link between mitochondrial dysfunction and PD pathology (Figure 2).

(1.6) The NRF2-mediated Anti-oxidant Response

To combat cellular oxidative/nitrosative stress, the cell has several defensive counter measures to ensure both stability and survival. Nuclear factor E2-related factor 2 (NRF2) is a master transcriptional regulator of the anti-oxidant response pathway, responsible for inducing over 600 cytoprotective genes, including several anti-oxidant enzymes (Moi et al., 1994; Nioi et al., 2003; Rushmore et al., 1991). The induction of these genes is promoted through a DNA enhancer sequence known as the anti-oxidant response element (ARE) (Nguyen et al., 2009). This recognition site facilitates NRF2 binding and is generally located within the promoter region of target genes. The role of NRF2 in detoxifying the cell from excess ROS and RNS makes it an attractive candidate for therapeutic intervention against PD.

(1.6.1) NRF2 Regulation

Under basal conditions, NRF2 is primarily localized in the cytosol through the binding of KEAP1, a cytosolic substrate adapter for a Cul3-containing E3 ubiquitin ligase (Itoh et al., 1999). The NRF2-KEAP1 interaction functions to promote Cul3 polyubiquitination and subsequent degradation of NRF2 by the 26S proteasome (Kobayashi et al., 2004). This proteostatic turnover is the primary means of regulating NRF2 activity in the absence of oxidative stress (Figure 3A). An increase in ROS and RNS alters the structure of the NRF2/KEAP1/Cul3 complex through either oxidative or nitrosative modification of

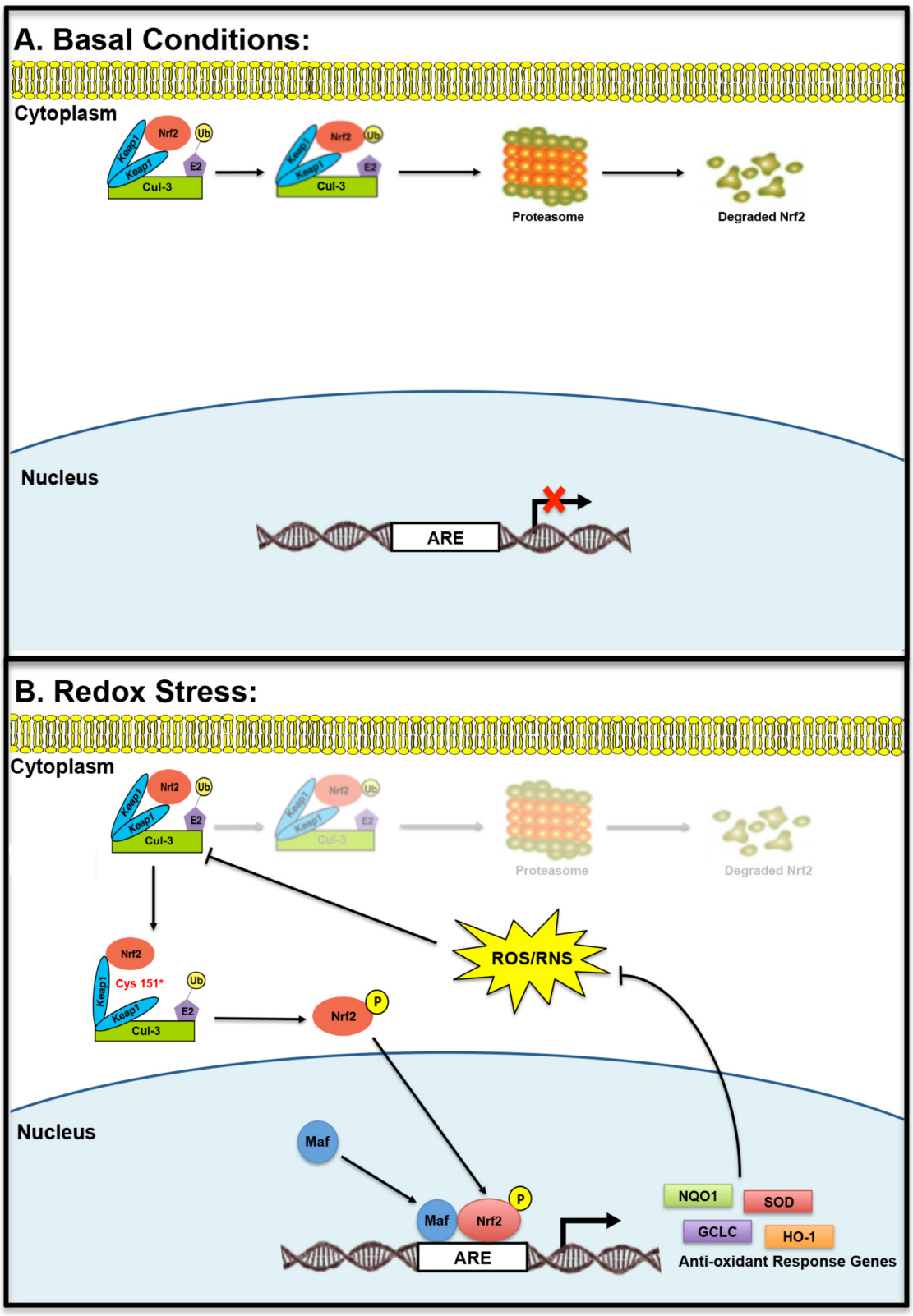

A. Basal Conditions:
Cytoplasm
Nrf2
Ub
Keap1
Keap1
E2
Cul-3
Proteasome
Degraded Nrf2
Nucleus
ARE
B. Redox Stress:
Cytoplasm
Nrf2
Ub
Keap1
Keap1
E2
Cul-3
Proteasome
Degraded Nrf2
ROS/RNS
Nrf2
Cys 151*
Ub
Keap1
E2
Cul-3
Nrf2
P
Nucleus
Maf
Maf
Nrf2
P
ARE
NQO1
SOD
GCLC
HO-1
Anti-oxidant Response Genes

Figure 3. Schematic overview of the NRF2-mediated anti-oxidant response.

(A) Under basal conditions, NRF2 is inhibited in the cytoplasm by the KEAP1 complex, where it is poly-ubiquitinated and subsequently degraded.

(B) Redox stress is able to modify key cysteine residues on KEAP1 resulting in a conformational change and release of NRF2. Once detached, NRF2 is phosphorylated and translocated to the nucleus, followed by the formation of a heterodimer complex with sMaf proteins. This enables NRF2 to bind to ARE promoter elements, ultimately increasing the transcription of anti-oxidant genes to combat redox stress.

critical cysteine thiols on KEAP1 (Kraft et al., 2004). This results in a conformational change of KEAP1 structure and release of NRF2. Upon dissociation, NRF2 can be phosphorylated at serine 40 by protein kinase C to further stabilize NRF2 activation (Huang et al., 2002; Jain et al., 2005). NRF2 then translocates to the nucleus where it forms a heterodimer with transcriptional co-activators known as small Maf (sMaf) proteins (Itoh et al., 1997). The NRF2/sMaf complex then binds to the ARE enhancer sequence presence within the promoting region of target genes (Hirotsu et al., 2012; Tanigawa et al., 2013). NRF2-ARE binding results in the increased transcription of specific anti-oxidant targets to combat and eliminate oxidative/nitrosative toxicity with the cell (Figure 3B).

(1.6.2) ARE Mediated Cellular Detoxification

NRF2 is responsible for the increased expression of an array of ARE-containing genes to facilitate the anti-oxidant response. Included in this response is the up-regulation of several enzymatic proteins involved in both the direct and indirect elimination of specific ROS/RNS to prevent further cellular damage. In humans, the multi-protective protein NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), is one of the most consistent NRF2-inducible genes of the anti-oxidant response pathway (Ahlgren-Beckendorf et al., 1999; van Muiswinkel et al., 2004). NQO1 functions as a catalytic enzyme to reduce several harmful molecules such as quinones and nitroaromatics (Nioi et al., 2003; van Muiswinkel et al., 2004).

The formation of DA quinones through the oxidation of dopamine can have highly toxic effects within the cell, ultimately exacerbating PD pathology. This further supports NRF2 as a potential therapeutic target against PD.

Members of the superoxide dismutase (SOD) family also have an imperative role in the anti-oxidant defence pathway (Kensler et al., 2007). These enzymatic proteins are responsible for the direct removal of superoxide anions, by catalyzing its reaction into molecular oxygen and hydrogen peroxide followed by further reduction to water by glutathione (GSH) peroxidase (Zelko et al., 2002). Under conditions of mitochondrial stress, superoxide levels accumulate, thus promoting NRF2 translocation and a subsequent increase in the expression of SOD via ARE activation (Ma, 2013).

GSH is a peptide that plays a fundamental role in several cellular defensive pathways, including the anti-oxidant response (McMahon et al., 2001). As such, GSH is an effective cellular scavenger for damaging free radicals and other ROS/RNS. The exposed sulfhydryl group on GSH serves as an oxidation target for several free radical molecules (Harvey et al., 2009). This protective reaction quickly reduces the reactivity of oxidative molecules, preventing aberrant modifications of fundamental cellular machinery. GSH synthesis is mediated by glutamate cysteine ligase catalytic subunit (GCLC), an NRF2 transcriptional target (Suh et al., 2004). As such, oxidative/nitrosative stress that induces NRF2-ARE activity, promotes GCLC-mediated GSH synthesis to combat their toxic effects.

Overall, the destructive positive feedback cycles generated by both α-synuclein fibrilization and mitochondrial dysfunction may act together to exacerbate neurodegeneration in PD. Therefore, identifying a molecular target that impacts on these mechanisms could yield a therapeutic alternative to halt or reverse PD pathology. As such, the major aim of this thesis is to utilize novel PD model systems to not only further understand cellular mechanisms that contribute to PD neurotoxicity, but also to investigate how they can be targeted for therapeutic benefit against neurodegeneration.

CHAPTER 2: RESEARCH PROPOSAL

(2.1) Rationale

Neurite retraction is a common morphological anomaly that is apparent in many neurodegenerative diseases, including PD. However, due to the severe global degeneration that is present in post-mortem PD brains studies, it is difficult to determine the sequence of neurodegenerative events that occur during the course of the disease. Although previous PD animal studies have shown promising rescue of motor function due to delayed axon retraction, translational human model systems are still needed to further understand this disease pathology. In addition, a link between neurite retraction and the accumulation of intracellular α-synuclein aggregates has yet to be elucidated.

Recently, an isogenetically matched PD stem cell model was developed in which the *SNCA*-A53T mutation was introduced into the BG01 human embryonic stem cell (hESC) line. This research resulted in a pair of isogenic cell lines that differ exclusively at a single nucleotide in the *SNCA* locus, thus eliminating phenotypic variability due to differences in genetic backgrounds (Soldner et al., 2011). In addition, advancements in differentiation protocols have made it possible to generate nearly pure populations of DA neurons from pluripotent stem cells (Kriks et al. 2011). Using this system my goal was to determine the earliest PD phenotypes that could be modeled *in vitro* to identify a target for early disease intervention. Through the use of a stem-cell line that incorporates the causal *SNCA*-A53T mutation and the appropriate genetically matched control, I

therefore sought to investigate a potential connection between neurite retraction and α-synuclein pathology.

(2.2) Hypothesis

It is postulated that α-synuclein fibrils trigger neurite retraction of both human DA neurons and mouse cortical neurons through an oxidative/nitrosative stress dependent mechanism.

(2.2) Research Objectives

1. To determine whether DA neurons harboring the *SNCA*-A53T mutation display morphological deficits in neurite branching relative to control.
2. To determine whether aberrant α-synuclein aggregation triggers neurite retraction in human DA and mouse cortical neurons.
3. To determine whether α-synuclein mediated neurite retraction is dependent on generation of RNS.
4. To determine whether activation of the NRF2-mediated anti-oxidant response can rescue α-synuclein induced neurite deficits in human DA and mouse cortical neurons.

CHAPTER 3: MATERIALS & METHODS

(3.1) hESC Culture

Human embryonic stem cells (hESC) were routinely cultured and maintained using a protocol described previously (Lin et al., 2009) with slight modifications. Briefly, pluripotent cells were culture in 6-well plates and seeded on γ-irradiated mouse embryonic fibroblasts (MEFs) using medium containing 20% KnockOut Serum replacement (KSR) and 20 ng/ml basic fibroblast growth factor (bFGF). Media was changed daily. The colonies were manually passaged every week.

(3.2) A9 DA Neuronal Differentiation

Differentiation of hESCs into DA neurons was performed as described with slight modifications (Ryan et al. 2013). Briefly, hESC colonies were dissociated with accutase to produce single cell suspension. To isolate hESCs from MEF feeders, medium containing dissociated fibroblasts and hESCs was added to gelatin-coated dishes. After seeding of MEFs, supernatant containing purified hESCs was collected and re-plated at $4x10^4$ cells/cm^2 on Matrigel-coated tissue culture dishes for differentiation. To carry out floor-plate induction, purified hESCs were treated with medium containing KSR, LDN193189 (100nM), SB431542 (10μM), Sonic hedgehog (SHH) C25II (100 ng/ml), purmorphamine (2μM), fibroblast growth factor 8 (FGF8; 100 ng/ml), and CHIR99021 (3μM). On day 5 of differentiation, KSR medium was transitioned every 2 days to N2

medium in 25%, 50%, and 75% increments respectively. On day 11, the medium was changed to Neurobasal/B27/Glutamax supplemented with CHIR99021. On day 13, CHIR99021 was replaced with Brain Derived Neurotrophic Factor (BDNF; 20 ng/ml), ascorbic acid (0.2mM), Glial Derived Neurotrophic Factor (GDNF; 20 ng/ml), transforming growth factor beta 3 (TGFβ3; 1 ng/ml), dibutyryl cAMP (db cAMP; 0.5 mM), and the Notch inhibitor DAPT (10μM) for 9 days. On day 20, cells were dissociated using accutase and re-plated under high cell density $4x10^5$ cells/cm^2 in terminal differentiation medium (NB/B27 + BDNF, ascorbic acid, GDNF, db cAMP, TGFβ3 and DAPT) or DAN Medium (DA Neuron) on dishes pre-coated with poly-ornithine (15 μg/ml)/laminin (1 μg/ml)/fibronectin (2 μg/ml). Medium was changed every 2 days and neurons were grown to 35 days *in vitro* (DIV) for experimental studies (Figure 4).

(3.3) Primary Neuronal Culture

Primary neuronal cells cultures were prepared from E18.5 CD1 mouse brains (Charles River). Cortical brain tissue was digested using filtered sterilized Papain solution. Dissociated cortical neurons were seeded at a density of $3x10^5$ cells/well in a 24-well plate containing poly-D Lysine coated coverslips. A 50% medium change was performed every 2 days with DMEM/F12 medium containing 0.1% β-mercaptoethanol, 1% antibiotic/antimycotic, 1% Glutagro, 2% B-27 and 0.7% BSA fraction V.

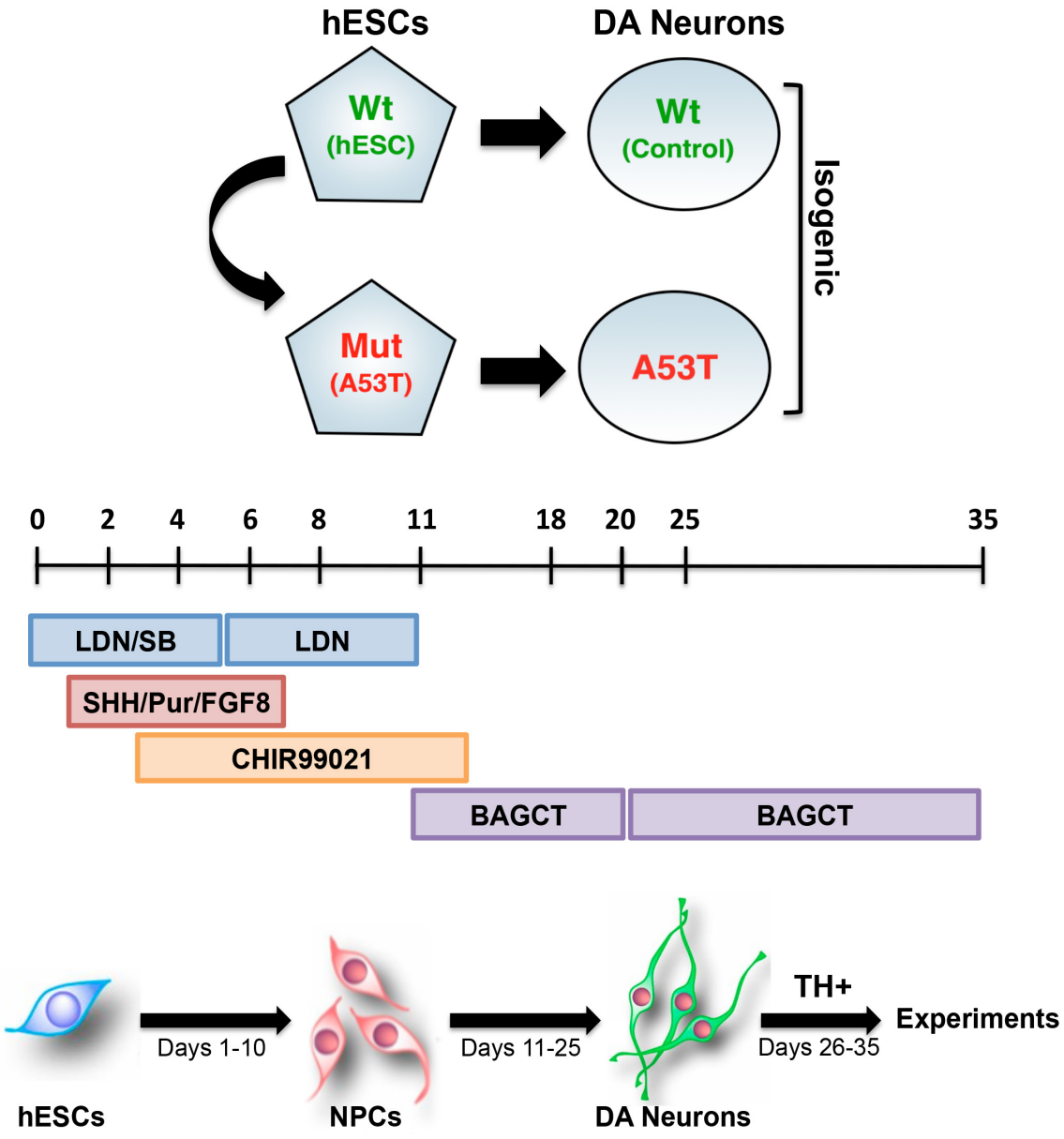

hESCs
DA Neurons
Wt
(hESC)
Wt
(Control)
Isogenic
Mut
(A53T)
A53T
0
2
4
6
8
11
18
20
25
35
LDN/SB
LDN
SHH/Pur/FGF8
CHIR99021
BAGCT
BAGCT
Days 1-10
Days 11-25
TH+
Days 26-35
Experiments
hESCs
NPCs
DA Neurons

Figure 4. DA neuron generation via floor plate induction. During the first 10 days of floor plate induction, small molecules LDN193189 and SB431542 are used to inhibit cell differentiation into trophectoderm/ectoderm and mesendoderm lineages respectively. Treatment with Wnt signaling agonist CHIR99021 in combination with purmorphamine, SHH and FGF8 are also required to drive NPC differentiation. On days 11-20, NPCs are supplemented with media containing BDNF, GDNF, ascorbic acid, TGFβ3 and dibutryl cAMP (BAGCT), which drives differentiation into DA neurons. By day 20, neurons are positive for the DA marker tyrosine hydroxylase, the rate-limiting enzyme required for dopamine synthesis. Experimental studies were done at DIV 35.

(3.4) SH-SY5Y Cell Culture

SH-SY5Y cells were grown in a 1:1 mixture of Ham's DMEM F-12 media (Gibco, USA), supplemented with 15% fetal bovine serum (Fisher Scientific, USA), 1% non-essential amino acids (HyClone, USA), 1mM sodium pyruvate (HyClone, USA), and 50 U/mL penicillin-streptomycin (Gibo, USA). Cells were maintained at 37°C in 5% CO2 and fed every other day. To differentiate, media was supplemented with 1μM retinoic acid (RA) (Sigma-Aldrich, USA) and changed every other day for 7 days.

(3.5) Immunofluorescence

Cells were fixed on 12-mm glass coverslips with 4% paraformaldehyde (PFA) for 15 min. For α-synuclein deposition staining, cells were fixed with 4% PFA and 1% Triton X-100 (Tx-100) to extract soluble proteins. Primary antibody was added to a buffer containing 3% BSA and 0.3% Tx-100 in PBS. Cells were incubated in primary antibody buffer overnight at 4°C. Primary antibodies and dilutions were used as described in Table 1. After 3x PBS washes, appropriate Alexa Fluor conjugated secondary antibodies were used at a dilution of 1:2000. Cells were counterstained with DAPI (1:1000). Fluorescent imaging of cells was analyzed using an Axio Observer Z1 laser-scanning microscope (Zeiss).

Table 1: List of used primary antibodies and respective dilutions

Primary Antibody	Species	Reactivity	Company	Product #	Dilution
alpha-Synuclein Phospho (Ser129)	Mouse	Human/Mouse	Biolegend/Covance	MMS-5091	1 in 1000
alpha-Tubulin 4a	Rabbit	Human	Thermo	PA5-29444	1 in 1000
beta3-Tubulin (TUJ1)	Mouse	Human	Biolegend	801201	1 in 2000
beta3-Tubulin (TUJ1)	Rabbit	Human	Biolegend	802001	1 in 2000
GAPDH	Mouse	Human	Sigma	G8795	1 in 1500
MAP2a	Mouse	Human/Mouse	Thermo	MS-249-S0	1 in 1000
Nitrotyrosine	Rabbit	Human	Themo	LF-PA0067	1 in 750
Nrf2 (H-300)	Rabbit	Human	Santa Cruz	sc-13032	1 in 250
Tyronsinated alpha-Tubulin	Rat	Human	Abcam	ab6160	1 in 1000
Tyrosine Hydroxylase	Rabbit	Human	Pel-Freez	p50101-0	1 in 1000
Ubiquitin	Rabbit	Human/Mouse	Biolegend	840501	1 in 1000

(3.6) Neurite Analysis

Neurons were antigenically labeled with MAP2 to visualize neurites. To analyze dendritic complexity, z-stacks of 5 randomly selected fields per coverslip were imaged via fluorescent microscopy and traced using Neurolucida software (MBF Bioscience). Sholl analysis was then performed on traced neurons using Neurolucida Explorer software. Statistical significance of Sholl analysis was conducted by area under the curve of respective sholl plots using GraphPad Prism. Data was analyzed using a one-way ANOVA, with a 95% confidence interval, and using Tukey's multiple comparison test to evaluate significance between neurite morphology.

For neurite analysis of differentiated SH-SY5Ys, cells were fixed in 4% PFA for 15 mins following a 1x wash in PBS. Neurons were then immunolabeled with βIII-tubulin to label neurites. Following fluorescent imaging of 5 randomly selected fields per coverslip, neurite length was quantified using ZEN2 software (Zeiss). Statistical significance of neurite retraction was performed using GraphPad Prism. Data was analyzed using an unpaired two-tailed t test with a 95% confidence interval.

(3.7) Formation of Synthetic α-synuclein PFFs

PFFs were generated as described by Volpicelli-Daley et al. (2011). Briefly, purified α-synuclein (5μg/mL in PBS) was incubated at 37°C with constant shaking for 7 days. PFFs were then aliquoted and stored at -80°C until needed for treatment. Prior to neuronal exposure, PFFs were sonicated at 10% amplitude for 30 secs (0.5 sec on, 0.5 sec off). PFFs were then added to the neuronal media (1μg/mL) and exposed to neurons for 7 days (Figure 5).

(3.8) Frequency Resonance Energy Transfer (FRET)

Following differentiation, DA neurons (DIV 35) were fixed with 4% PFA and stained for tyrosinated tubulin (donor) and 3'-nitrotyrosine (3'NT) (acceptor). Alexa Fluor 488 and 594 secondary probes were used for optimal resonance energy transfer. FRET images were captured using optimal configured parameters on an Axio Observer Z1 laser-scanning microscope. FRET analysis of 5 regions of interest was performed using Volocity software to obtain mean FRET fluorescent intensities. Statistical analysis of FRET data was conducted using GraphPad Prism. Data was analyzed using an unpaired two-tailed t test with a 95% confidence interval.

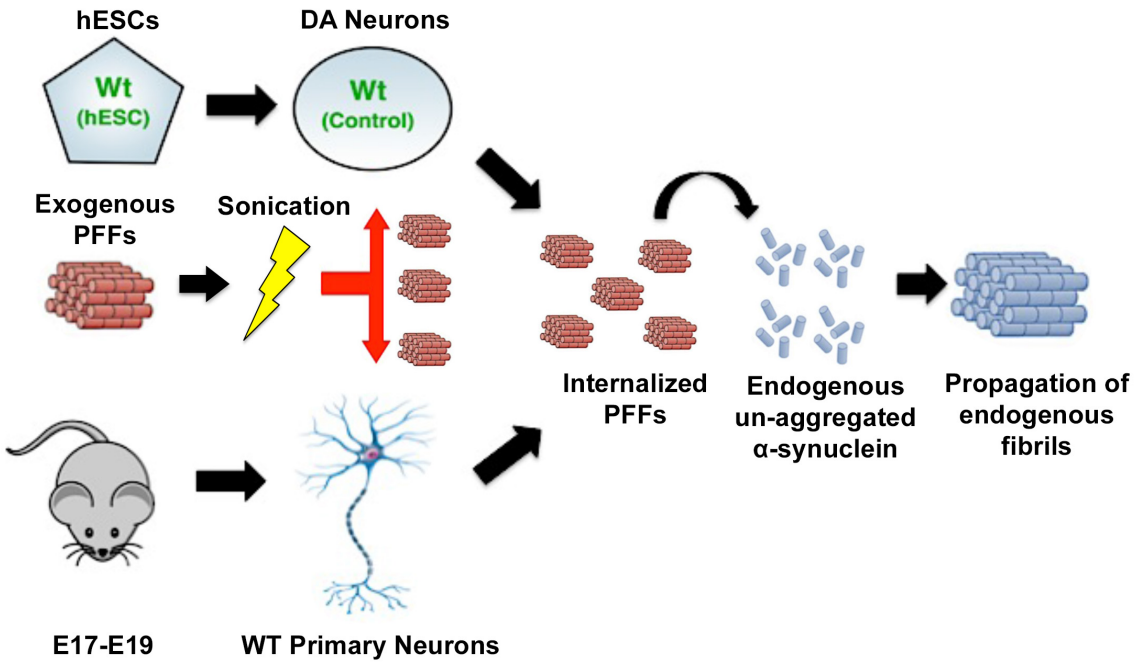

hESCs
DA Neurons
Wt
(hESC)
Wt
(Control)
Exogenous
PFFs
Sonication
Internalized
PFFs
Endogenous
un-aggregated
α-synuclein
Propagation of
endogenous
fibrils
E17-E19
WT Primary Neurons

Figure 5. Schematic outline of PFF model system. PFFs are supplemented to feeding media of both WT DA neurons and primary cortical mNs, and incubated for 7 days. During exposure, neurons internalize PFFs through an endocytosis-like mechanism. Once internalized, PFFs propagate the misfolding of endogenous monomeric α-synuclein. This ultimately initiates intracellular aggregation of α-synuclein and subsequent formation of fibrils.

(3.9) Western Blotting

RA differentiated SH-SY5Y cells were lysed in ice-cold radio immunoprecipitation assay (RIPA) buffer consisting of 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in phosphate buffered saline (PBS) supplemented with PMSF, aprotonin, sodium orthovanadate, and sodium fluoride. Samples were sonicated at 30% amplitude for 1 min (0.5 sec on, 0.5 sec off) and then centrifuged at 16,100g for 15min at 4°C. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher) as per manufacturer's protocol. Protein (35µg) was then loaded and separated via SDS-PAGE using a 7.5% gel at 90V. Proteins were transferred to nitrocellulose membranes overnight with a constant rate of 30V at 4°C. Membranes were subsequently blocked in blocking buffer (Tris-buffered saline with 0.01% Tween (TBST) supplemented with 5% nonfat dry milk) for 1 hr at room temperature with rocking. Primary antibodies were added to blocking buffer and incubated overnight at 4°C with rocking. The following day, blots were then washed in TBST followed by additional blocking in blocking buffer. Secondary horseradish peroxidase conjugated antibodies (1:2000) were added to blocking buffer and the blots were incubated for 1 hr at room temperature with rocking. Blots were then subjected to BioRad Clarity enhanced chemiluminescence (ECL) for 5 mins and visualized using photosensitive film. Quantitative densitometry was performed using ImageJ software and normalized to GAPDH loading control.

(3.10) Dual Luciferase Reporter Assay

SH-SY5Y cells were plated in 24-well plates at a density of $2x10^5$ and transfected at 60% confluency with Lipofectamine 2000 (Invitrogen) with both an ARE luciferase reporter and a Renilla luciferase control vector. For NRF2 overexpression, SH-SY5Y cells were also transfected with an NRF2 construct (pCDNA3-Myc3-Nrf2, Addgene). Cell lysate was harvested and analyzed using a Dual-Glo luciferase assay kit (Promega) according to manufacturer's protocol. Luminescence activity was measured using a LUMIstar Omega microplate reader (BMG Labtech). Fold change was calculated by normalizing ARE luciferase activity to Renilla luciferase activity.

CHAPTER 4: RESULTS

(4.1) A53T DA neurons display markers of PD pathology and early signs of neurodegeneration

Prior to assessing neurite morphology, the novel A53T DA model system was assessed for intracellular accumulation of α-synuclein deposits, a defining characteristic of PD pathology. Several markers of α-synuclein deposition have been previously reported, which include deposition of insoluble α-synuclein protein aggregates that are hyper-phosphorylated at serine-129 (PS129) and poly-ubiquitinated (Ubq) (Fujiwara et al., 2002; Luk et al., 2009; Spillantini et al., 1997). To assess this, Tx-100 was used to remove soluble proteins prior to labeling for PS129 and Ubq. Indeed, fixation in PFA containing 1% Tx-100 detergent revealed small insoluble puncta within A53T DA neurites that were positive for both PS129 and Ubq (Figure 6A). WT DA neurons did not display Tx-100-insoluble deposits. These results therefore provided visual confirmation that the A53T DA neurons exhibit early signs of synucleinopathy that correlates with known PD pathology.

The next question was to assess whether the accumulation of α-synuclein deposits correlate with a neurite phenotype in A53T DA neurons. Sholl analysis was performed to investigate if A53T DA neurons differ in neurite morphology relative to WT controls. To label neurites of DA neurons, I co-labeled with the dendrite marker MAP2 and DA neuronal marker tyrosine hydroxylase (TH) (Figure 6B). Sholl analysis of dendrite morphology revealed a significant global

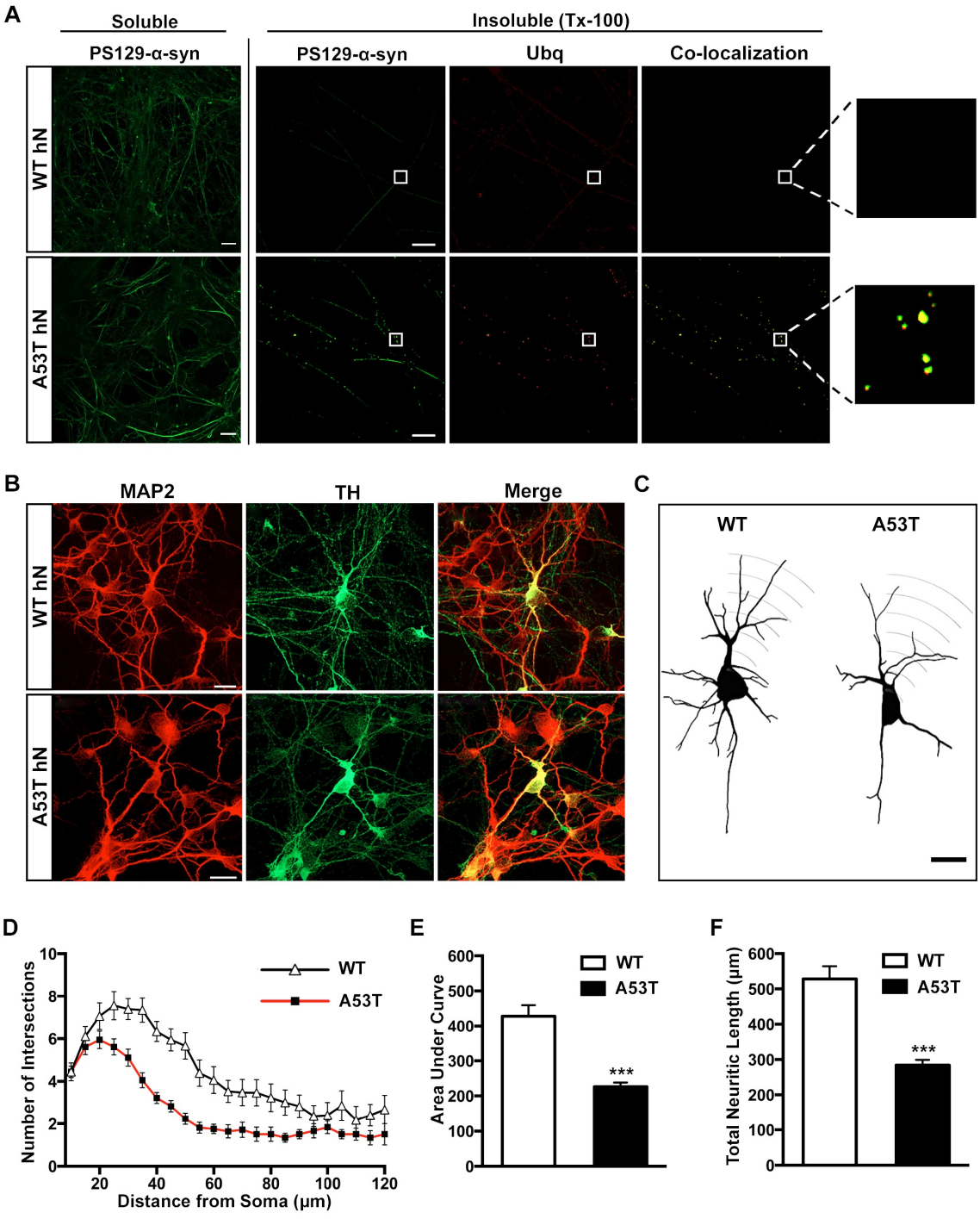

A
Soluble
Insoluble (Tx-100)
PS129-α-syn
PS129-α-syn
Ubq
Co-localization
WT hN
A53T hN
B
MAP2
TH
Merge
WT hN
A53T hN
C
WT
A53T
D
Number of Intersections
Distance from Soma (μm)
WT
A53T
E
Area Under Curve
WT
A53T
***
F
Total Neuritic Length (μm)
WT
A53T
***

Figure 6. A53T hNs display markers of PD pathology and early signs of degeneration.

(A) DA human neurons (hNs) fixed with 4% PFA and/or 1% Tx-100 to extract soluble proteins. Neurons were stained with PS129-α-syn and Ubq. A53T hNs displayed co-localized phosphorylated and ubquitinated α-synuclein insoluble deposits within neurites. Scale bar = 20μm.

(B) Neurons were fixed in 4% PFA and stained with MAP2 and tyrosine hydroxylase (TH). Scale bar = 20μm.

(C) Representative traces of WT (left) and A53T (right) hNs respectively using Neurolucida software.

(D,E) Sholl analysis displayed a significant reduction in dendritic branching in A53T mutant hNs relative to WT control. Statistics were calculated by area under the curve. Data displayed as Mean ± SEM. ***$p < 0.001$ by t test; n = 21.

(F) Total dendritic length of A53T mutant hNs is significantly less compared to WT control. Data displayed as Mean ± SEM. ***$p < 0.001$ by t test; n = 21. DIV 35; Scale bar = 20 μm.

decrease in dendritic complexity of A53T mutant neurons relative to WT control (Figure 6C-E). In addition, total dendritic length of A53T mutant DA neurons was significantly decreased compared to WT control (Figure 1F). Collectively, these results suggest that A53T DA neurons share hallmark features of PD pathology and are morphological different relative to WT control.

(4.2) Addition of PFFs evokes α-synuclein deposition and neurite retraction

Although A53T DA neurons display morphological deficits relative to WT, it was not possible to distinguish whether this is indicative of early degeneration, or if A53T DA neurons naturally undergo reduced neurite branching. To overcome this obstacle, healthy neurons were exposed to α-synuclein PFFs, which have been shown to evoke synucleinopathy in WT mice (Luk et al., 2009; Volpicelli-Daley et al., 2014). Ii was then determined whether PFF exposure evoked neurite retraction in healthy DA neurons. It was first confirmed that PFF exposure results in α-synuclein deposition in WT DA neurons. Following 7-day PFF exposure, WT DA neurons displayed positive PS129-α-syn and Ubq puncta within neurites that were detergent-insoluble (Figure 7A). α-Synuclein positive deposits were not present in PBS treated control neurons. To assess if PFF exposure evokes neurite retraction, Sholl analysis was performed on PFF and PBS exposed WT DA neurons. Sholl analysis revealed a significant reduction in dendritic complexity of PFF exposed WT DA neurons compared to PBS control

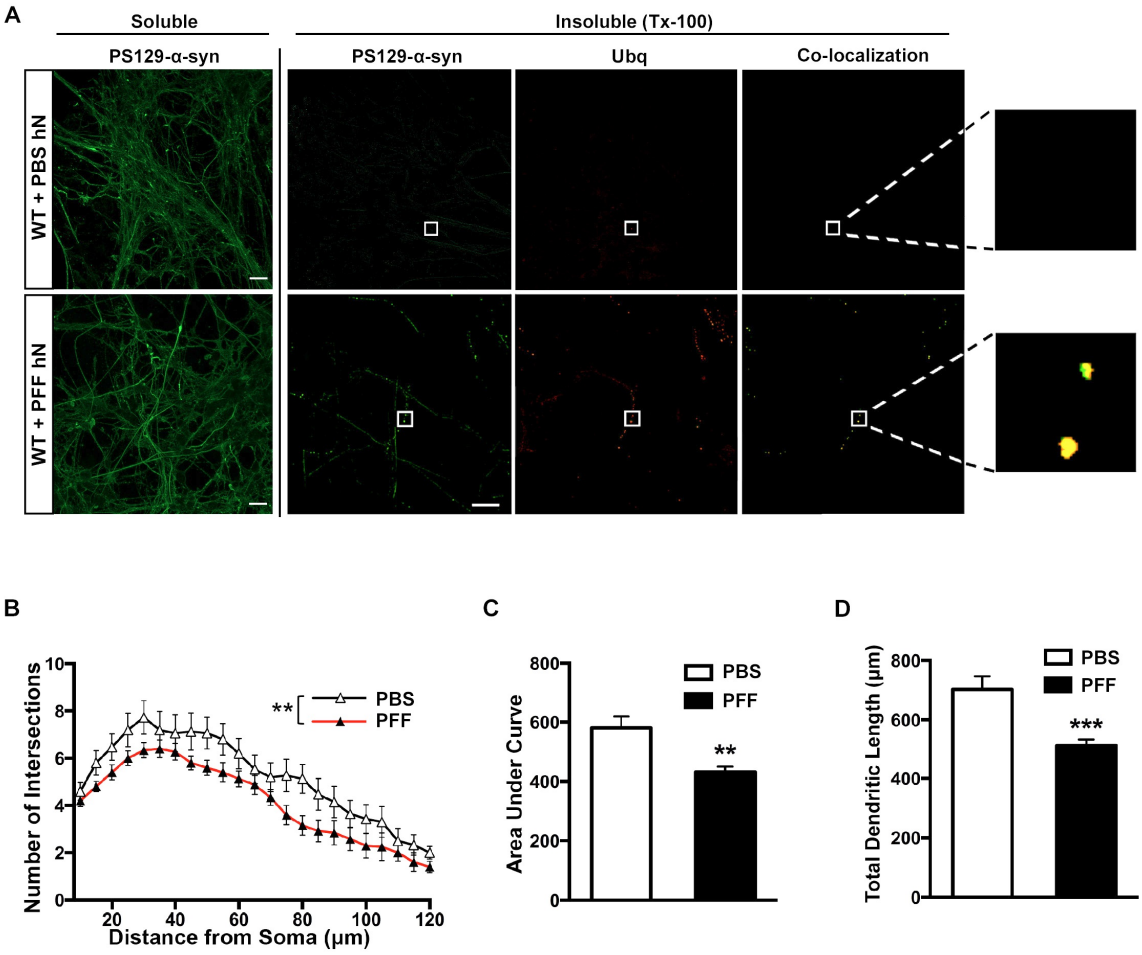

A
Soluble
Insoluble (Tx-100)
PS129-α-syn
PS129-α-syn
Ubq
Co-localization
WT + PBS hN
WT + PFF hN
B
Number of Intersections
Distance from Soma (μm)
**
PBS
PFF
C
Area Under Curve
PBS
PFF
**
D
Total Dendritic Length (μm)
PBS
PFF
***

Figure 7. Addition of PFFs to DA hNs evokes early α-synuclein deposition and neurite retraction.

(A) Following PFF exposure, DA hNs were fixed with 4% PFA and/or 1% Tx-100 to extract soluble proteins. Neurons were then stained with PS129-α-syn and Ubq. PFF exposed hNs displayed co-localized phosphorylated and ubquitinated α-synuclein insoluble deposits within neurites. PBS treated neurons did not show co-localization.

(B,C) WT DA hNs were exposed to PFFs, followed by fixation and staining. Neurons were stained with MAP2 to label dendrites and TH to label dopaminergic neurons. Sholl analysis displayed a significant reduction in dendritic branching in PFF treated WT hNs relative to PBS control. Statistics were calculated using area under the curve analysis. Data displayed as Mean ± SEM. **$p < 0.01$ by t test; n = 15.

(D) Total dendritic length of PFF exposed hNs was significantly reduced relative to PBS control. Data displayed as Mean ± SEM. ***$p < 0.001$ by t test; n = 15. DIV, 35; PFF exposure time, 7 days; Scale bar = 20 μm.

(Figure 7B,C). In addition, PFF exposure significantly reduced total dendritic length of WT DA neurons relative to PBS control (Figure 7D), consistent with findings from A53T neurons. Collectively, these data support the hypothesis that α-synuclein deposition contributes to neurite retraction in A53T mutant DA neurons. Moreover, this is the first evidence suggesting that synucleinopathy contributes to between neurite retraction in human PD neurons.

Although non-motor symptoms in PD are attributed to the loss of neuronal cell types other than DA neurons, it is likely that they share a common mechanism of neurodegeneration. To date, the highest correlate to PD dementia is the presence of aggregated α-synuclein deposits within neurites of cortical neurons (Hurtig et al., 2000; Irizarry et al., 1998; Irwin et al., 2013). As such, it was investigated whether the addition of PFFs to primary cortical mouse neurons (mNs) evoked aberrant α-synuclein deposition and neurite retraction. α-Synuclein deposition was first confirmed in PFF exposed primary cortical mNs. Imaging of PFF exposed mNs revealed small co-localized PS129-α-syn and Ubq puncta within neurites (Figure 8A), similar to those observed in DA neurons. These deposits were also detergent-insoluble in 1% Tx-100. Positive α-synuclein deposits were not present in PBS control neurons. To assess if PFF exposed mNs undergo neurite retraction, primary cortical mNs were exposed to PFFs for 7 days prior to imaging of MAP2 labeled dendrites. Sholl analysis revealed a

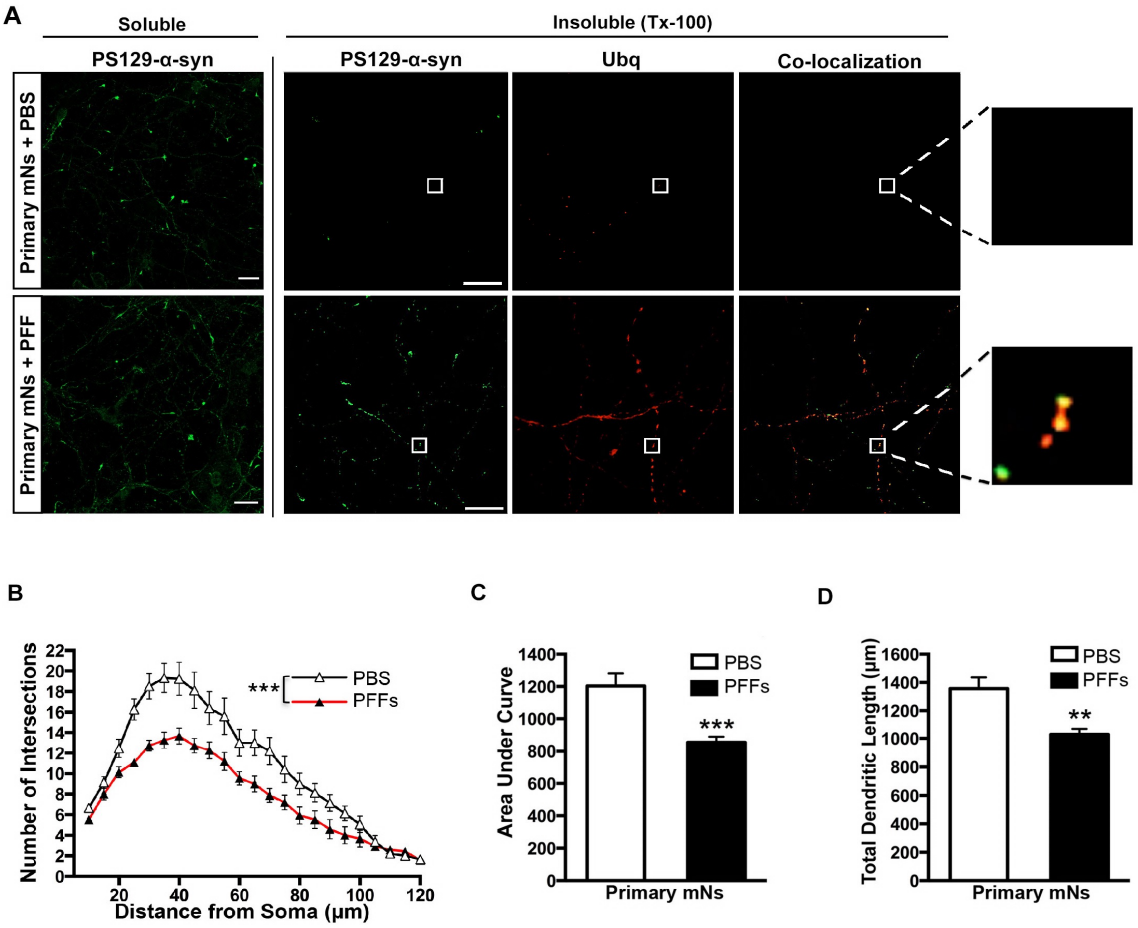

A
Soluble
Insoluble (Tx-100)
PS129-α-syn
PS129-α-syn
Ubq
Co-localization
Primary mNs + PBS
Primary mNs + PFF
B
Number of Intersections
Distance from Soma (μm)
***
PBS
PFFs
C
Area Under Curve
PBS
PFFs
***
Primary mNs
D
Total Dendritic Length (μm)
PBS
PFFs
**
Primary mNs

Figure 8. Addition of PFFs to primary cortical mNs evokes α-synuclein deposition and neurite retraction.

(A) Following PFF exposure, primary mNs were fixed with 4% PFA and 1% Tx-100 to extract soluble proteins. Neurons were then stained with PS129-α-syn and Ubq. PFF exposed mNs displayed co-localized phosphorylated and ubquitinated α-synuclein insoluble deposits within neurites. PBS treated neurons did not show co-localization.

(B,C) WT primary mNs were exposed to PFFs, followed by fixation and staining. Neurons were stained with MAP2 to label dendrites. Sholl analysis displayed a significant reduction in dendritic branching in PFF treated WT hNs relative to PBS control. Statistics were calculated using area under the curve analysis. Data displayed as Mean ± SEM. **$p < 0.01$ by t test; n = 15.

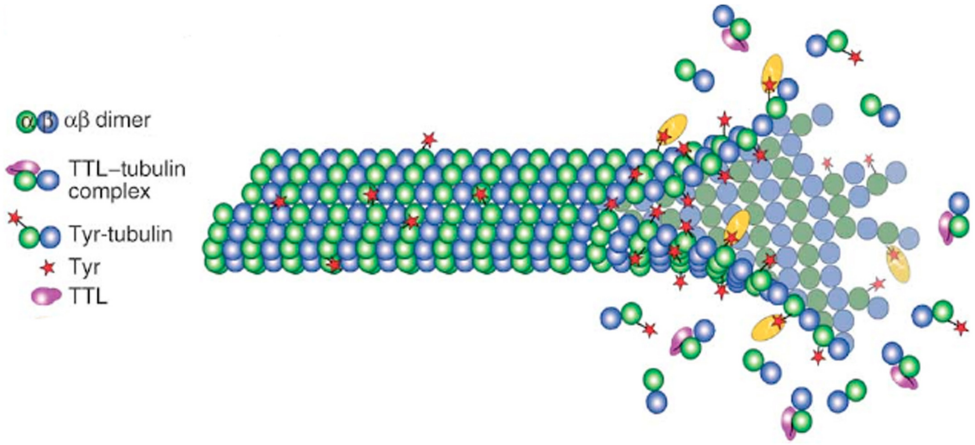
(D) Total dendritic length of PFF exposed mNs was significantly reduced relative to PBS control. Data displayed as Mean ± SEM. ***$p < 0.001$ by t test; n = 15. DIV, 14; PFF exposure time, 7 days; Scale bar = 40 μm.

significant reduction in dendritic complexity of PFF exposed mNs relative to PBS control (Figure 8B,C). In addition, there was also a significant reduction in total dendritic length of PFF exposed mNs relative to PBS control (Figure 8D) consistent with observations in DA neurons. This data collectively suggests that α-synuclein deposition and neurite retraction is not limited to DA neurons and can occur in other neuronal cell types.

(4.3) A53T mutant DA neurons display elevated levels of 3'NT modified tubulin

Microtubules are the major cytoskeletal structure present in all neurites and are essential for cell development and intracellular trafficking. The formation and dissociation of microtubules is a dynamic process that relies heavily on post-translational modifications of its tubulin subunits (Hammond et al., 2008). Tyrosination/detyrosination are among the modifications that are associated with microtubule assembly and stability, and impairment of this process could result in cellular dysfunction (Figure 9A). Indeed, the presence of the toxic RNS peroxynitrite can lead to altered protein modification through nitration of tyrosine residues, termed nitrotyrosination (3'NT) (Gow et al., 1996). In fact, 3'NT modification of tyrosinated α-tubulin (TyTub) heterodimers results in an irreversible modification, which impedes microtubule dynamics and consequent cytoskeletal stability (Figure 9B) (Eiserich et al., 1999). Previous work by Ryan et al. (2013), demonstrated that A53T mutant DA neurons display increased basal production of NO relative to control. Therefore it was then determined whether

A


αβ dimer
TTL–tubulin complex
Tyr-tubulin
Tyr
TTL

B

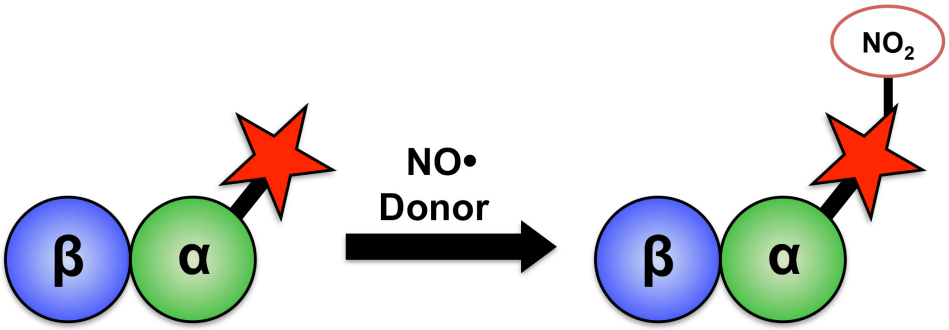

NO•
Donor
β
α
β
α
NO2

Figure 9. Elevated levels of nitrosative stress can induce microtubule dysfunction via nitrotyrosination of α-tubulin.

(A) The growing tip of microtubules are dynamic cytoskeletal structures that relies heavily on post-translational modifications of its tubulin subunits (Hammond et al., 2008). Tyrosination of α-tubulin by tyrosine tubulin ligase (TTL) results in the dissociation of tubulin heterodimers from the microtubule. This modification is reversible, as the removal of the tyrosine residue enables the tubulin heterodimer to be added back onto the growing tip of the microtubule (Hammond et al., 2008).

(B) In the presence of increased nitrosative stress, toxic RNS peroxynitrite can act as an NO donor leading to the nitrotyrosination of α-tubulin. This results in an irreversible modification, which is known to impede microtubule dynamics and cytoskeletal stability (Eiserich et al., 1999).

Figure modified from Szyk et al. 2011, Nature.

neurite retraction in A53T DA neurons is a consequence of increased 3'NT modification of TyTub due to elevated levels of nitrosative stress. However, due to the lack of an antibody able to specifically detect 3'NT α-tubulin, this hypothesis was investigated by means of FRET using antibodies against tyrosinated α-tubulin and 3'NT. FRET of DA neurons was imaged by exciting AlexaFluor 488-labeled TyTub, while recording 3'NT fluorescence using a 594 filter. Following FRET analysis, A53T DA neurons displayed a significant increase in mean FRET intensity relative to WT control (Figure 10A,B). Moreover, PFF exposed WT DA neurons also displayed an increase in FRET intensity relative to PBS control (Figure 10C,D). This data therefore suggests that nitrosative stress due to α-synuclein deposition results in a higher ratio of 3'NT modified α-tubulin relative to control, providing a potential mechanism for neurite retraction.

(4.4) Neurite retraction in A53T mutant hNs and PFF exposed neurons is mediated through elevated levels of nitrosative stress

Several studies have reported increased levels of nitrosative stress in PD (Chung, 2015; Fernandez et al., 2013; Gu et al., 2005; Tsang and Chung, 2009; Yao et al., 2004). Moreover, previous work from our lab determined that *SNCA*-A53T hESC exhibits elevated levels of NO upon differentiation to neurons (Ryan et al., 2013). Therefore, neurons were treated with a nitric oxide synthase (NOS)

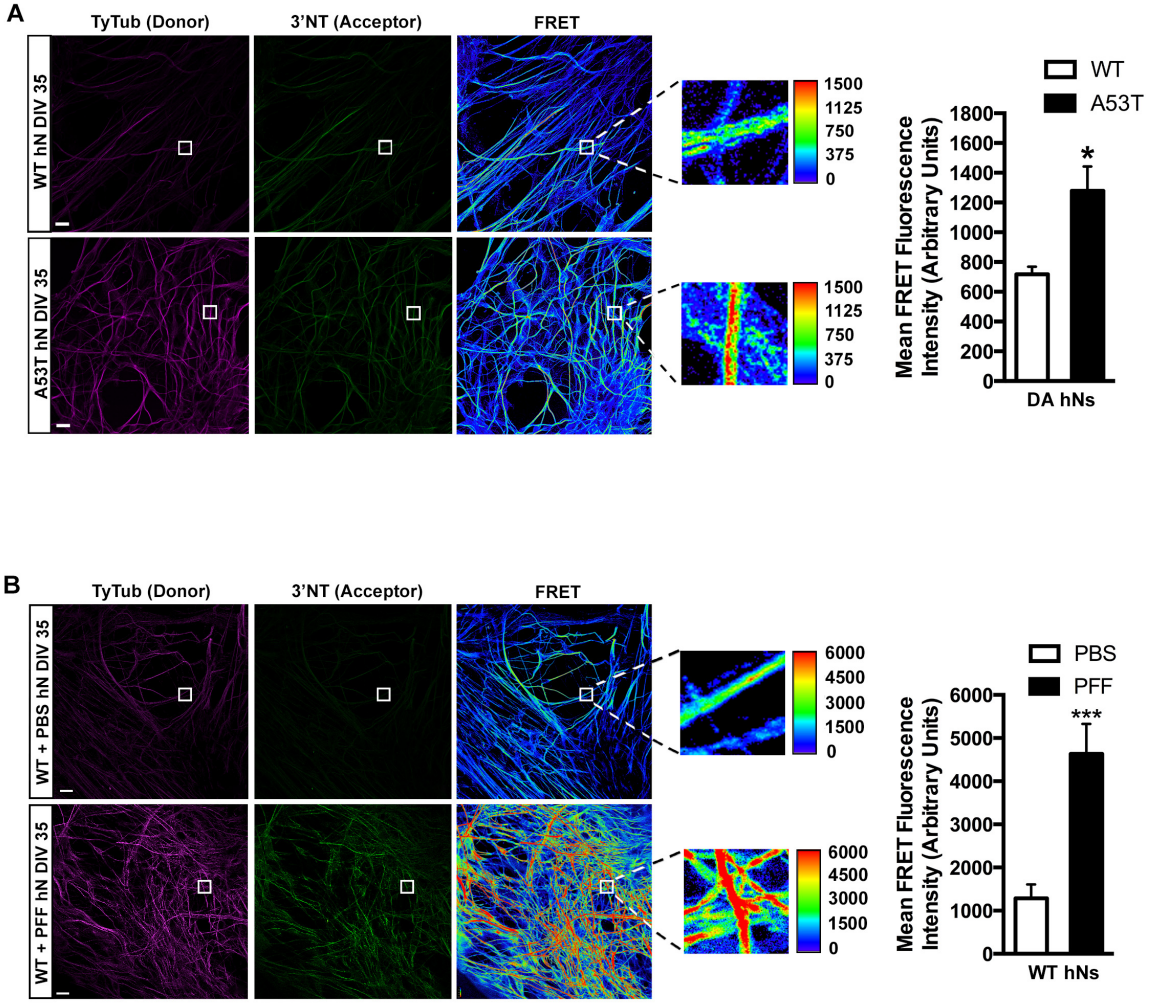

A
TyTub (Donor)
3'NT (Acceptor)
FRET
WT hN DIV 35
A53T hN DIV 35
1500
1125
750
375
0
WT
A53T
Mean FRET Fluorescence Intensity (Arbitrary Units)
1800
1600
1400
1200
1000
800
600
400
200
0
*
DA hNs
B
TyTub (Donor)
3'NT (Acceptor)
FRET
WT + PBS hN DIV 35
WT + PFF hN DIV 35
6000
4500
3000
1500
0
PBS
PFF
Mean FRET Fluorescence Intensity (Arbitrary Units)
6000
5000
4000
3000
2000
1000
0
***
WT hNs

Figure 10. A53T and PFF exposed DA hNs have higher levels of 3'-NT modified tubulin.

(A) WT and A53T DA hNs were fixed with 4% PFA and stained for tyrosinated tubulin (donor) and 3'-NT (acceptor). Appropriate fluorescent secondary probes were used for optimal resonance energy transfer. Neurons were imaged and FRET heat-map overlays were generated using Volocity software. A53T DA hNs displayed a significant increase in mean FRET intensity relative to WT control. Data displayed as Mean ± SEM. *$p < 0.05$ by t test; n = 10. DIV 35; Scale bar = 20 μm.

(B) WT DA hNs were exposed to PFFs for 7 days followed by fixation with 4% PFA and stained for tyrosinated tubulin (donor) and 3'-NT (acceptor). Appropriate fluorescent secondary probes were used for optimal resonance energy transfer. Neurons were imaged and FRET heat-map overlays were generated using Volocity software. PFF exposed WT DA hNs displayed a significant increase in mean FRET intensity relative to PBS control. Data displayed as Mean ± SEM. ***$p < 0.001$ by t test; n = 10. DIV 35; Scale bar = 20 μm.

inhibitor, Nω-Nitro-L-arginine methyl ester hydrochloride ($_L$-NAME), to investigate if the alleviation of nitrosative stress results in rescue of neurite retraction.

Following $_L$-NAME treatment, Sholl analysis of MAP2 labeled neurites revealed a significant rescue in dendritic complexity of A53T DA neurons relative to control levels (Figure 11A-B). In addition, $_L$-NAME treatment significantly restored dendritic length of A53T DA neurons to control levels (Figure 11C). Moreover, a significant rescue of dendritic branching and total dendritic length was also seen in both PFF exposed WT DA neurons (Figure 11D-F) and primary cortical mNs (Figure 11G-I) relative to control levels. Therefore, these results suggest that neurite retraction in A53T mutant neurons is mediated in part by increased levels of nitrosative stress. Blocking cellular NOS however, is not a viable therapeutic approach, as moderate levels of NO are essential for cell signaling and physiology, particularly cardiovascular function. Therefore, alternative targets to modulate NO for therapeutic application was further explored.

(4.4.1) Screening anti-oxidant response activators in SH-SY5Y neurons for therapeutic rescue of neurite retraction

In order to characterize alternative means of regulating NO levels, an immortalized neuroblastoma line was used as it more readily facilitates molecular biochemical approaches. Retinoic acid (RA) mediated differentiated SH-SY5Y neuroblastoma cells induce expression of DA neuron specific genes while promoting significant neurite outgrowth (Korecka et al., 2013; Krishna et al., 2014; Xie et al., 2010). This therefore allows the screening of NO modulators on

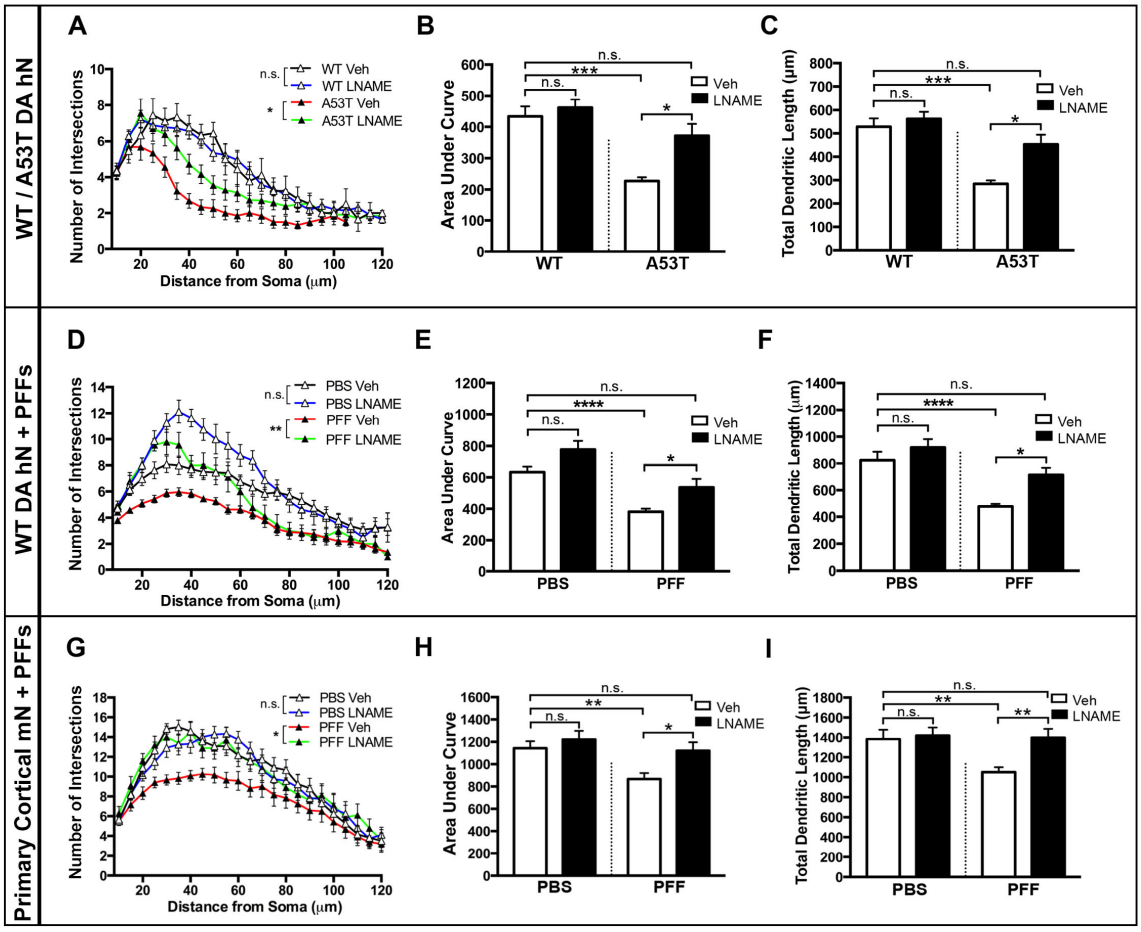

WT / A53T DA hN
WT DA hN + PFFs
Primary Cortical mN + PFFs
A
B
C
D
E
F
G
H
I
Number of Intersections
Distance from Soma (μm)
WT Veh
WT LNAME
A53T Veh
A53T LNAME
PBS Veh
PBS LNAME
PFF Veh
PFF LNAME
n.s.
*
**
Area Under Curve
Total Dendritic Length (μm)
Veh
LNAME
WT
A53T
PBS
PFF
***
****

Figure 11. Alleviation of NO-mediated toxicity rescues neuritic retraction in A53T mutant hNs and PFF exposed neurons.

(A,B) DA hNs were fixed in 4% PFA and stained with MAP2 to label dendrites and TH to label dopaminergic neurons. Following a 1mM $_L$NAME treatment, Sholl analysis displayed a significant rescue in dendritic branching in A53T mutant hNs relative to control levels. Statistics were calculated using area under the curve.

(C) $_L$NAME treated A53T mutant hNs displayed a significant rescue in total dendritic length relative to control levels. Data displayed as Mean ± SEM. **p < 0.01 by ANOVA with post-hoc Tukey; n = 15.

(D,E) WT DA neurons were exposed to PFFs for 7 days, followed by fixation and staining. Following 1mM $_L$NAME treatment, Sholl analysis of MAP2 labeled neurons displayed a significant rescue in dendritic branching in PFF exposed hNs relative to control levels.

(F) $_L$NAME treated PFF WT hNs displayed a significant rescue in total dendritic length relative to control levels. Data displayed as Mean ± SEM. **p < 0.01 by ANOVA with post-hoc Tukey; n = 15. $_L$NAME treatment time, 24 hr; hNs DIV, 35.

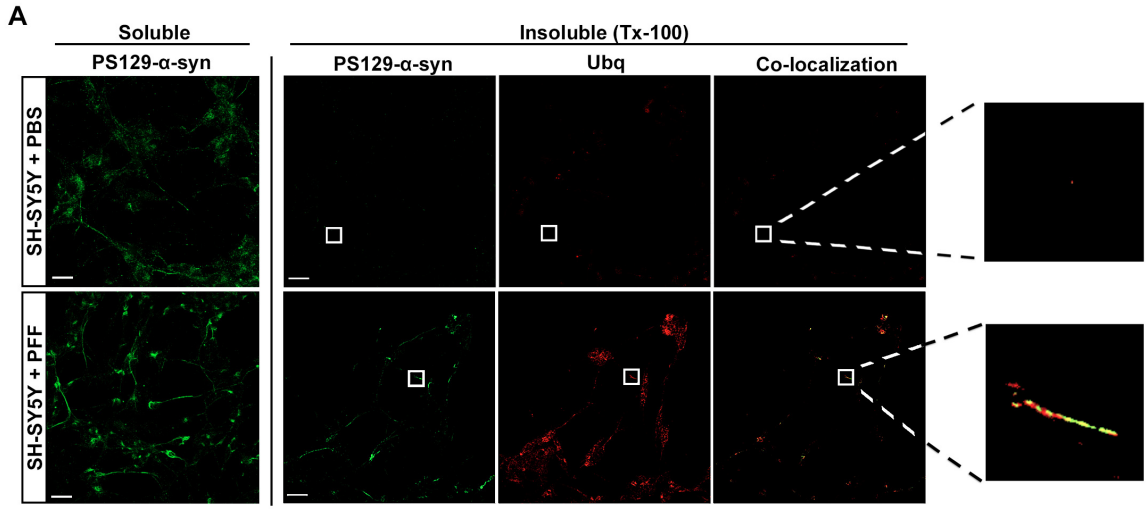
(G,H) Primary cortical mNs were exposed to PFFs for 7 days, followed by fixation and staining. Following 1mM $_L$NAME treatment, Sholl analysis of MAP2 labeled neurons displayed a significant rescue in dendritic branching in PFF exposed mNs relative to control levels.

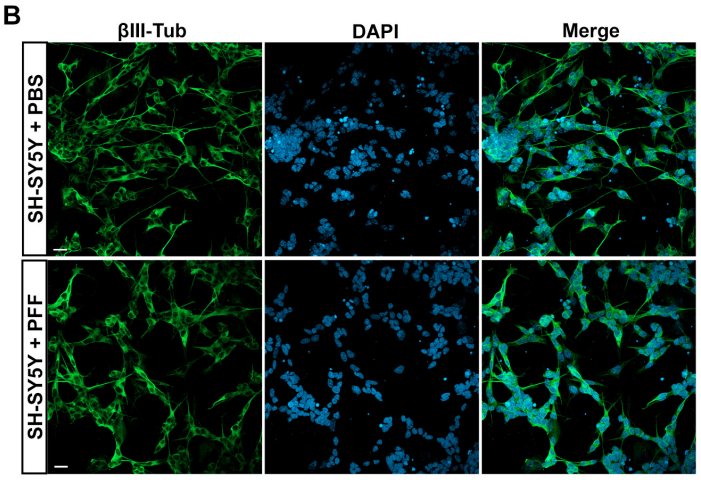
(I) $_L$NAME treated PFF mNs displayed a significant rescue in total dendritic length relative to control levels. Data displayed as Mean ± SEM. **p < 0.01 by ANOVA with post-hoc Tukey; n = 15. $_L$NAME treatment time, 24 hr; DIV, 14.

a much shorter time scale than required for differentiation of hESCs. As such, the SH-SY5Y neuroblastoma cell line was used to screen molecular targets for therapeutic rescue of neurite retraction.

Prior to exploring ways of modulating NO in SH-SY5Ys, it was first determined whether this system recapitulated earlier findings with respect to the effects of PFF exposure on α-synuclein deposition and neurite retraction. Following the paradigm described above, PFF treated SH-SY5Y cells were imaged and assessed for evidence of synucleinopathy following PS129-α-syn and Ubq staining. Immunofluorescent imaging of PFF exposed SH-SY5Ys revealed detergent-insoluble PS129-α-syn and Ubq positive deposits within cells, indicating the formation of intracellular α-synuclein inclusions (Figure 12A). Positive α-synuclein deposits were not presence in PBS control cells. To evaluate neurite retraction following 7-day PFF exposure, RA differentiated SH-SY5Y cells were stained with βIII-tubulin to label neurites. Upon analysis, PFF exposed SH-SY5Ys displayed significant retraction in neurite length relative to PBS control (Figure 12B-C).

An increase in redox stress has not only been strongly implicated in PD, but is also common in many neurodegenerative disorders (Uttara et al., 2009). As such, much attention has been focused on the NRF2-mediated anti-oxidant response as a potential target for therapeutic rescue. Several studies have shown the therapeutic potential of small molecules able to induce NRF2 activity and subsequent activation of the anti-oxidant response (Ahlgren-Beckendorf et al., 1999; Jing et al., 2015; Kraft et al., 2004; Satoh et al., 2008). Of particular


A
Soluble
Insoluble (Tx-100)
PS129-α-syn
PS129-α-syn
Ubq
Co-localization
SH-SY5Y + PBS
SH-SY5Y + PFF


B
βIII-Tub
DAPI
Merge
SH-SY5Y + PBS
SH-SY5Y + PFF

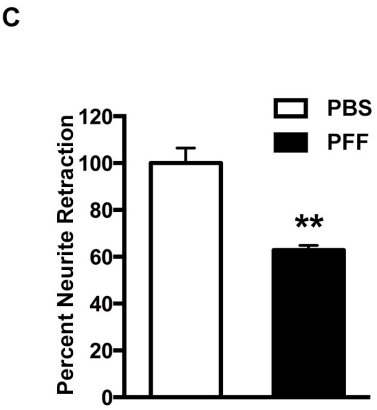

C
Percent Neurite Retraction
120
100
80
60
40
20
0
PBS
PFF
**

Figure 12. PFF exposed SH-SY5Y cells exhibit early signs of neurodegeneration.

(A) Following PFF exposure, RA differentiated SH-SY5Y cells were fixed in 4% PFA and/or 1% Tx-100 to extract soluble proteins. Neurons were stained with PS129-α-syn and Ubq. PFF exposed SH-SY5Ys displayed co-localized PS129-α-syn and Ubq α-synuclein insoluble deposits within neurites.

(B,C) SH-SY5Ys were exposed to PFFs, followed by fixation and staining. Neurons were stained with βIII-Tub to label neurites. PFF exposed SH-SY5Ys display significant neuritic retraction relative to PBS control. Data displayed as Mean ± SEM. **$p < 0.01$ by t test; n = 3. PFF exposure time, 7 days; DIV, 7.

interest, dimethyl fumarate (DMF) has recently passed clinical trials for MS treatment, with characteristic anti-oxidant properties via NRF2 activation. It was therefore determined whether DMF showed therapeutic potential in PD by preventing oxidative stress and subsequent neurite retraction.

DMF-mediated effects on NRF2 expression and activity were characterized in differentiated SH-SY5Y cells. Following 24 hr of DMF treatment, western blotting was used to quantify both total levels of NRF2 protein as well as phospho-NRF2, as an indication of activation. Quantitative densitometry revealed a significant increase in NRF2 protein levels of DMF treated cells relative to vehicle control (Figure 13A-B), suggesting that DMF inhibits KEAP1 mediated degradation of NRF2. To assess if this translated to increased NRF2 transcriptional activity in SH-SY5Ys, we performed a dual reporter assay using an NRF2-ARE promoter luciferase construct, normalized to Renilla. Luciferase analysis revealed a 3-fold induction of endogenous NRF2-ARE activity in DMF treated cells relative to control, indicating that DMF treatment induced NRF2 activity (Figure 13C).

To evaluate DMF as a potential therapeutic treatment for neurite rescue, its affect on neurite retraction was first assessed in PFF exposed SH-SY5Ys. Following 7-day PFF exposure, RA differentiated SH-SY5Ys were treated with DMF and stained with βIII-tubulin to measure neurites. Quantitative analysis of neurite length revealed that treatment with DMF significantly rescued neurite retraction in PFF exposed SH-SY5Ys (Figure 13D). Furthermore, to confirm that induction of the anti-oxidant response is responsible for neurite rescue, neurite

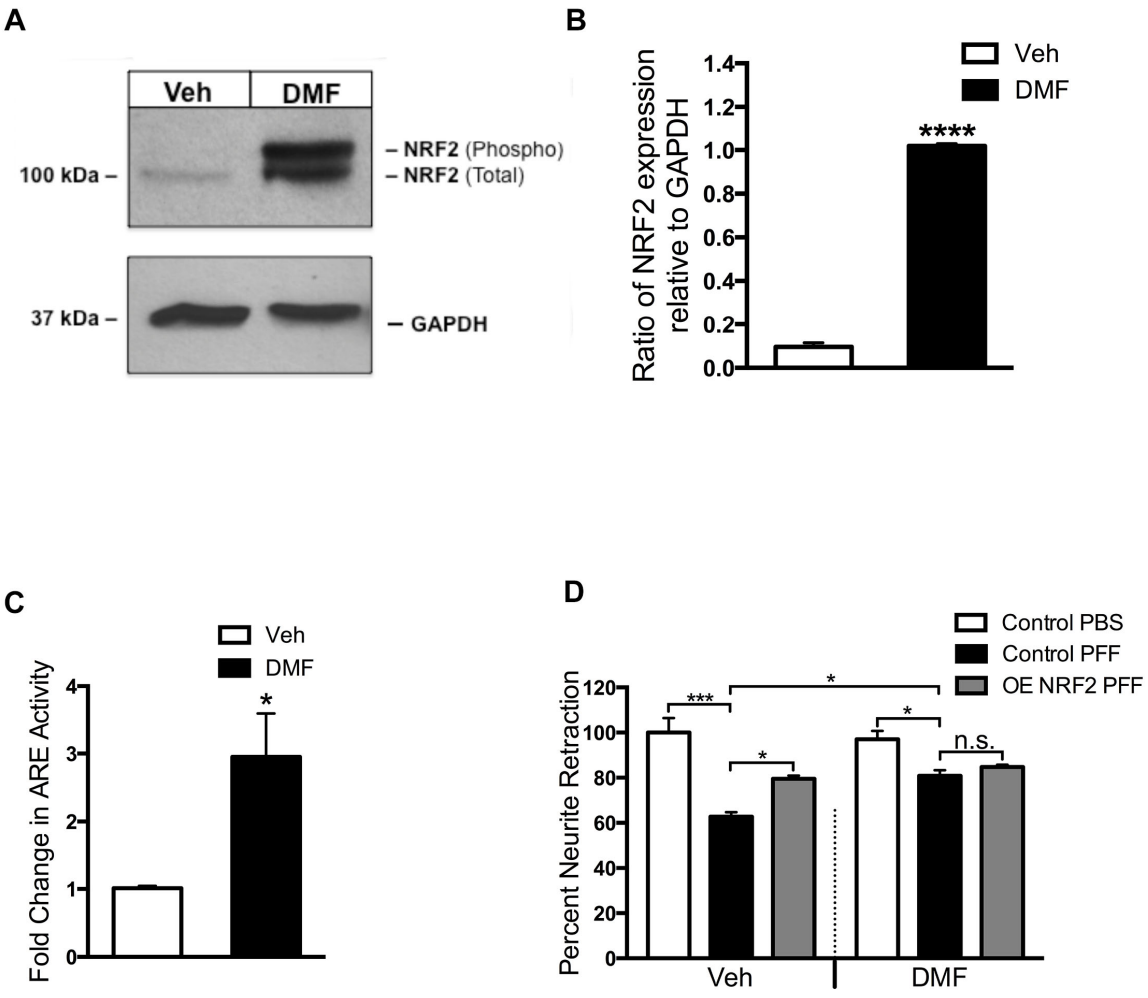

A
Veh
DMF
100 kDa –
– NRF2 (Phospho)
– NRF2 (Total)
37 kDa –
– GAPDH
B
Ratio of NRF2 expression relative to GAPDH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
****
Veh
DMF
C
Fold Change in ARE Activity
4
3
2
1
0
*
Veh
DMF
D
Percent Neurite Retraction
120
100
80
60
40
20
0
***
*
*
*
n.s.
Control PBS
Control PFF
OE NRF2 PFF
Veh
DMF

Figure 13. Forced activation of the NRF2-mediated anti-oxidant response via DMF treatment rescues neuritic retraction in PFF exposed SH-SY5Y cells.

(A,B) NRF2 protein expression and quantification in RA differentiated SH-SY5Y cells in the presence and absence of 10μM DMF. Quantitative densitometry of NRF2 expression normalized to GAPDH loading control. Data displayed as Mean ± SEM. ****$p < 0.0001$ by t test, n = 3.

(C) Dual-luciferase reported assay revealed a significant increase in NRF2-ARE activity in SH-SY5Y cells treated with 10μM DMF. Data represents fold change in NRF2-ARE luciferase activity normalized to Renilla luciferase and plotted as DMF relative to Veh control. Data displayed as Mean ± SEM. *$p < 0.05$ by t test, n = 3.

(D) On day 5 of PFF exposure, RA differentiated SH-SY5Y cells were transfected with NRF2 plasmid and/or empty vector mCherry. At 48 hr post transfection, cells were fixed in 4% PFA and stained with Tuj1 and DAPI. PFF exposed SH-SY5Ys displayed significant retraction in neurite length relative to PBS control. Both NRF2 overexpressed SH-SY5Ys and 10μM DMF significantly rescued neurite length of PFF-exposed neurons. Data displayed as Mean ± SEM. *$p < 0.05$ by ANOVA with post-hoc Tukey; n = 3. PFF exposure time, 7 days; DMF exposure time, 6 hr , DIV, 7.

length quantification was performed in PFF exposed SH-SY5Ys that overexpressed NRF2. Neurite analysis showed a significant retraction of empty vector control (EV) cells that were exposed to PFFs relative to PBS (Figure 13D). In addition, overexpression of NRF2 significantly attenuated neurite retractions in PFF exposed cells relative to EV control (Figure 13D). Interestingly, DMF treatment did not result in further rescue of PFF exposed cells that overexpressed NRF2. Collectively, these results show that DMF treatment can be used as a potential therapeutic to protect against neurite retraction in PFF exposed SH-SY5Y cells via NRF2-mediated activation of the anti-oxidant response.

(4.5) Forced activation of the anti-oxidant response via DMF treatment rescues neurite retraction in human DA and mouse cortical neurons

Since DMF treatment rescued neurite retraction in PFF exposed SH-SY5Y cells by activating the NRF2-mediated anti-oxidant response, it was of interest to determine whether DMF would similarly rescue both A53T mutant and PFF exposed WT DA neurons from neurite retraction. Sholl analysis revealed a significant rescue in dendritic complexity of DMF treated A53T mutant DA neurons relative to WT control (Figure 14A-B). In addition, DMF treatment significantly rescued total dendritic length of A53T mutant DA neurons compared to control (Figure 14C). Moreover, DMF treatment resulted in a significant rescue in dendritic branching and total dendritic length in both PFF exposed WT DA

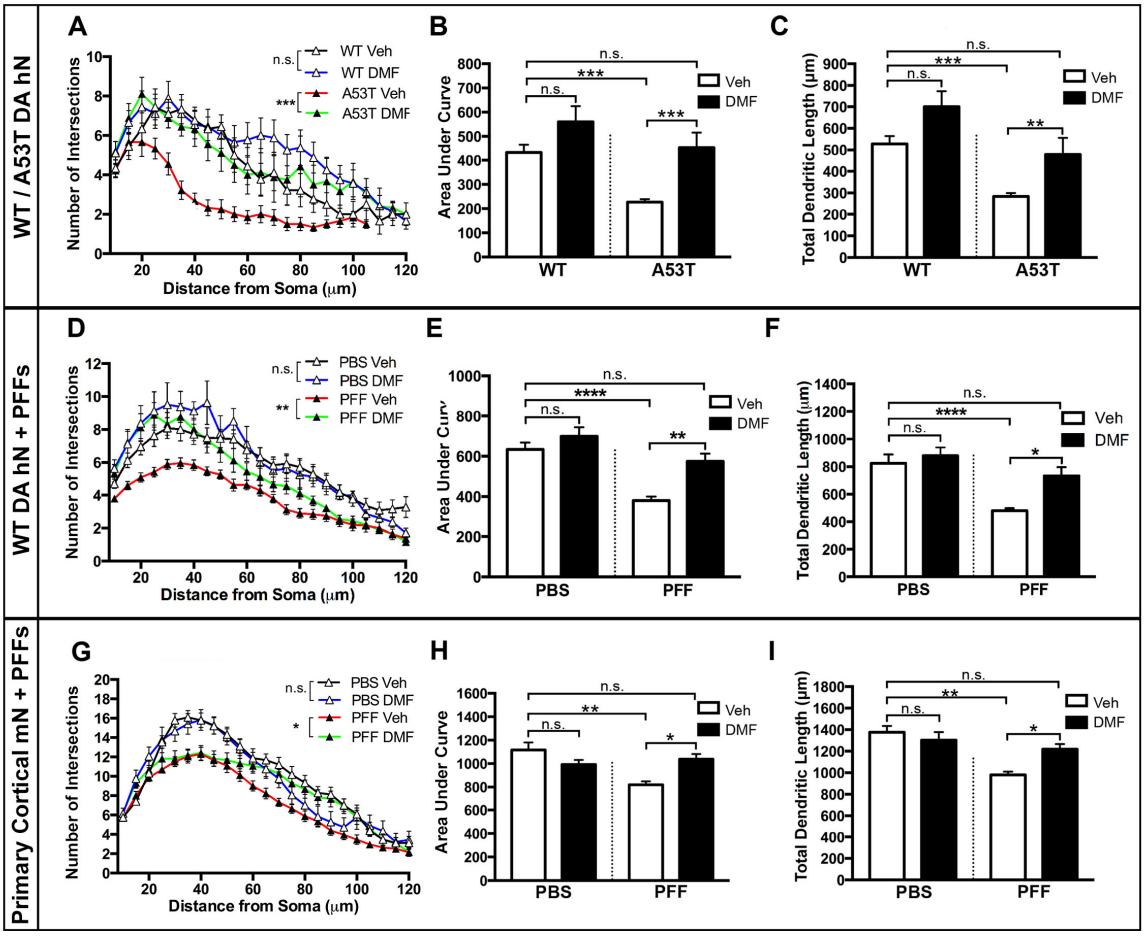

WT / A53T DA hN
WT DA hN + PFFs
Primary Cortical mN + PFFs
A
B
C
D
E
F
G
H
I
Number of Intersections
Distance from Soma (μm)
WT Veh
WT DMF
A53T Veh
A53T DMF
PBS Veh
PBS DMF
PFF Veh
PFF DMF
n.s.
***
**
*
****
Area Under Curve
Total Dendritic Length (μm)
Veh
DMF
WT
A53T
PBS
PFF

Figure 14. Forced activation of the anti-oxidant response via DMF treatment rescues neuritic retraction in A53T hNs and PFF exposed neurons.

(A,B) DA hNs were fixed in 4% PFA and stained with MAP2 to label dendrites and TH to label DA positive neurons. Following a 10μM DMF treatment, Sholl analysis displayed a significant rescue in dendritic branching in A53T mutant hNs relative to control levels. Statistics were calculated using area under the curve.

(C) DMF treated A53T mutant hNs displayed a significant rescue in total dendritic length relative to control levels. Data displayed as Mean ± SEM. **p < 0.01 by ANOVA with post-hoc Tukey; n = 15. DMF treatment time, 24 hr; DIV, 35.

(D,E) WT DA neurons were exposed to PFFs for 7 days, followed by fixation and staining. Following 10μM DMF treatment, Sholl analysis of MAP2 labeled neurons displayed a significant rescue in dendritic branching in PFF exposed hNs relative to control levels.

(F) DMF treated PFF WT hNs displayed a significant rescue in total dendritic length relative to control levels. Data displayed as Mean ± SEM. **p < 0.01 by ANOVA with post-hoc Tukey; n = 15. DMF treatment time, 24 hr; DIV, 35.

(G,H) Primary cortical mNs were exposed to PFFs for 7 days, followed by fixation and staining. Following 10μM DMF treatment, Sholl analysis revealed a significant rescue in dendritic branching in PFF exposed mNs relative to control levels.

(I) DMF treated PFF mNs displayed a significant rescue in total dendritic length relative to control levels. Data displayed as Mean ± SEM. **p < 0.01 by ANOVA with post-hoc Tukey; n = 15. DMF treatment time, 24 hr; DIV, 14.

neurons (Figure 14D-F) and primary cortical mNs (Figure 14G-I) relative to control levels. Overall, these data suggest DMF may have strong therapeutic potential for PD patients through forced activation of the NRF2-mediated anti-oxidant response.

CHAPTER 5: DISCUSSION

Several studies have reported that neurite retraction occurs early in neurodegeneration of PD (Kordower et al., 2013; MacLeod et al., 2006; Nordstrom et al., 2015; Tagliaferro et al., 2015). However, the mechanism underlying neurite retraction and how this deficit is connected to pathological hallmarks of PD has not yet been elucidated. In the work presented here, I show that hESC-derived DA neurons expressing the A53T-*SNCA* mutation exhibit deficits in neurite morphology, which is mediated in part through the accumulation of α-synuclein deposits within neurites. Moreover, I show that α-synuclein deposition increases NO-mediated modification of α-tubulin, which could potentially explain a contributing mechanism of neurite retraction seen in our PD neurons. Furthermore, alleviation of redox stress via DMF treatment significantly rescues neurite morphology, suggesting a new therapeutic alternative for PD neurodegeneration. Collectively, this thesis provides the first evidence that mechanistically links endogenous aggregation of α-synuclein and neurite retraction in an isogenic human stem-cell model of PD.

As demonstrated here, NO-mediated neurite retraction resulting from α-synuclein deposition was not limited to DA neurons as it also occurred in primary cortical mNs. Although PD pathology could be present in both cell types concurrently, there are several unique characteristics of nigrostriatal DA neurons that increase their susceptibility to cellular stress. This would also explain why motor dysfunction usually precedes the onset of non-motor cognitive impairment PD pathogenesis. DA neurons undergo fast spiking Ca^{2+} transients that result in

rapid and almost continuous Ca^{2+} influx (Dias et al., 2013). Mitochondrial buffering in this level of Ca^{2+} requires a considerable amount of energy, making these neurons highly sensitive to mitochondrial dysfunction (Dias et al., 2013). In addition, this high ATP demand also results in the increased production of superoxide as a natural byproduct of cellular respiration. Furthermore, dopamine itself can act as a source of oxidative stress, where it is easily oxidized by ROS to produce DA quinone (Asanuma et al., 2003). The generation of this reactive molecule has been linked to the covalent modification of various proteins associated with PD (Asanuma et al., 2003; Dias et al., 2013). As such, DA quinone modification of α-synuclein monomers have been reported to increase its propensity to aggregate within the cell (Conway et al., 2001). In addition, DA quinone has also been shown to modify parkin, causing inhibition of its E3 ubiquitin ligase activity, thus impeding on sufficient clearance of dysfunction mitochondria (LaVoie et al., 2005). Therefore, although this thesis demonstrated that other neuronal subtypes also exhibit early signs of neurodegeneration due to α-synuclein deposition, the previous findings discussed here suggest that DA neurons are more susceptible to pathological events that occur in PD. Furthermore, this susceptibility is correlated with the clinical progression of PD symptoms, where motor dysfunction usually precedes the onset of non-motor cognitive impairment.

In addition to the intracellular accumulation of α-synuclein aggregates, the presence of excessive ROS/RNS is another prominent feature of PD. However, despite substantial evidence implicating redox stress in PD, it is still unclear

whether it is a primary event in PD pathogenesis, or a consequence due to the impairment of other cellular processes. The work presented here argues that α-synuclein aggregation in A53T DA neurons is the initial insult of cellular injury, and generation of redox stress is consequent of this impairment. Previous studies demonstrating that oligomeric α-synuclein can indirectly increase levels of ROS/RNS through interference of the ETC would support this notion (Devi et al., 2008; Hsu et al., 2000). This however may only be plausible in the small percentage of cases where mutations at the *SNCA* locus increase the propensity of α-synuclein to aggregate. In the majority of PD cases, it is likely that mitochondrial impairment is a primary event in pathological onset. This is supported in studies showing an increased risk of PD onset after exposure of certain agrochemical pesticides, specifically rotonone, paraquat and maneb (Freire and Koifman, 2012; Moretto and Colosio, 2013; Vieregge, 2002). Although these toxins have been reported to evoke cellular toxicity by different mechanisms, they all have been shown to increase redox stress through inhibition of mitochondrial function. Moreover, degeneration of DA neurons in mice has been demonstrated following the exposure to these agrochemicals (Betarbet et al., 2000; Jenner, 2001). To further support this argument, several causal mutations in genes encoding mitochondrial proteins have been linked to the onset of PD, including the mitophagy relative proteins PINK1 and parkin, which are critical for mitochondrial quality control (Deas et al., 2011; Piccoli et al., 2008; Rakovic et al., 2010). Failure to remove damaged mitochondria could consequently result in further production of redox stress. Ultimately, the

production of ROS/RNS from mitochondrial injury can inflict cellular damage through redox-mediated modifications of proteins including α-synuclein. Previous evidence has shown that oxidation and nitration of α-synuclein can significantly increase its tendency to aggregate (Tsang and Chung, 2009). These specific examples therefore argue that redox stress could be a primary event in PD pathogenesis, where synucleinopathy is consequence of mitochondrial dysfunction. However, regardless of the primary insult that triggers PD pathology, the evidence discussed here suggests that both aberrant α-synuclein aggregation and mitochondrial dysfunction can feedback on each other to further exacerbate PD pathology. More importantly, alleviation of redox stress through forced activation of the anti-oxidant response would ultimately mitigate the toxic effects of the above circumstances.

The work presented here also demonstrates that 3'NT modification of α-tubulin may contribute to neurite retraction in PD. However, due to the multitude of toxic manifestations that arise from redox stress, it is possible that the impairment of several other cellular processes also contribute to this morphology. As such, several studies have demonstrated various mechanisms in which redox stress plays a fundamental role in neurite retraction. Of these studies, many have implicated 4-hydroxy-2-nonenal (4-HNE), a toxic product of lipid peroxidation and marker of oxidative stress, as a mediator in the impairment of microtubule polymerization (Neely et al., 2005; Roediger and Armati, 2003; Stewart et al., 2007; Valen et al., 1999). It was suggested that 4-HNE can preferentially modify unpolymerized soluble tubulin preventing its ability to polymerize, thus impeding

microtubule assembly (Kokubo et al., 2008). In addition, a previous study utilizing an *in vitro* model of Alzheimer's disease (AD) demonstrated that Aβ-oligomers induce the degradation of microtubule-associated proteins (MAPs), specifically MAP1A, MAP1B and MAP2. These proteins play a fundamental role in polymerization and stabilization of the microtubule network, thus an impairment of these proteins would impede on neuronal cytoskeletal structure (Fifre et al., 2006). Interestingly, not only did these authors demonstrate that MAP degradation and consequent perturbation of the microtubule network precedes neuronal death, but they also provided evidence that increased levels of oxidative stress mediate this impairment. Therefore, since the results presented in this thesis also suggest that neuronal structure is impaired due to aberrant protein deposition and increased redox stress, it would be informative to determine if MAP levels are also affected. This would therefore provide yet another mechanism in which neurite retraction occurs in PD neurons. In a more recent study, MAP1B was also implicated in redox-mediated neurite retraction through a different cellular mechanism. Stroissnigg et al. (2007) demonstrated that increased S-nitrosylation of MAP1B due to excess generation of NO results in a signal-transduction response that initiates growth-cone collapse and neurite retraction. The constant stimulation of this S-nitrosylation-dependent pathway by excess NO could also suggest another potential mechanism in which neurite retraction occurs in PD. Furthermore, post-translational modifications of Tau protein have been implicated in neurodegenerative diseases, including AD and PD. Specifically, oxidative cross-linking and nitration via RNS peroxynitrite have

been reported to inhibit the ability of Tau to promote tubulin assembly (Reynolds et al., 2006). Lastly, oxidative protein modifications mediated by DA quinones have also been shown to impede Tau function (Santa-Maria et al., 2005). Taken together, the evidence discussed here suggests several potential neurite retraction mechanisms that could be implicated in PD pathology. However, it is likely that a combination of these proposed mechanisms act in tandem to exacerbate microtubule dysfunction (Figure 15).

In recent years, researchers have focused on targeting the NRF2-mediated anti-oxidant response for therapeutic benefit against neurodegenerative diseases (Ellrichmann et al., 2011; Kanninen et al., 2008; Rojo et al., 2010; Scannevin et al., 2012). As such, several NRF2 activators have been identified that disrupt binding and subsequent inhibition by KEAP1. However, only a select few of these compounds have consistently demonstrated adequate pharmological properties in the brain (Sandberg et al., 2014). These NRF2 activators include CDDO-methyl ester, sulforaphane and DMF, but the recent clinical approval of DMF as a suppressor of neuroinflammation in MS patients has made it an ideal candidate for therapeutic benefit in other neurodegenerative diseases (Gold et al., 2012; Innamorato et al., 2008; Wang et al., 2014). To date, only two studies have investigated DMF for therapeutic potential in PD (Jing et al., 2015; Lastres-Becker et al., 2016). Although informative, there are currently no translational studies that validate DMF as a therapeutic in appropriate human PD models. Therefore, the work provided here demonstrates the first supporting evidence of therapeutic benefit upon DMF

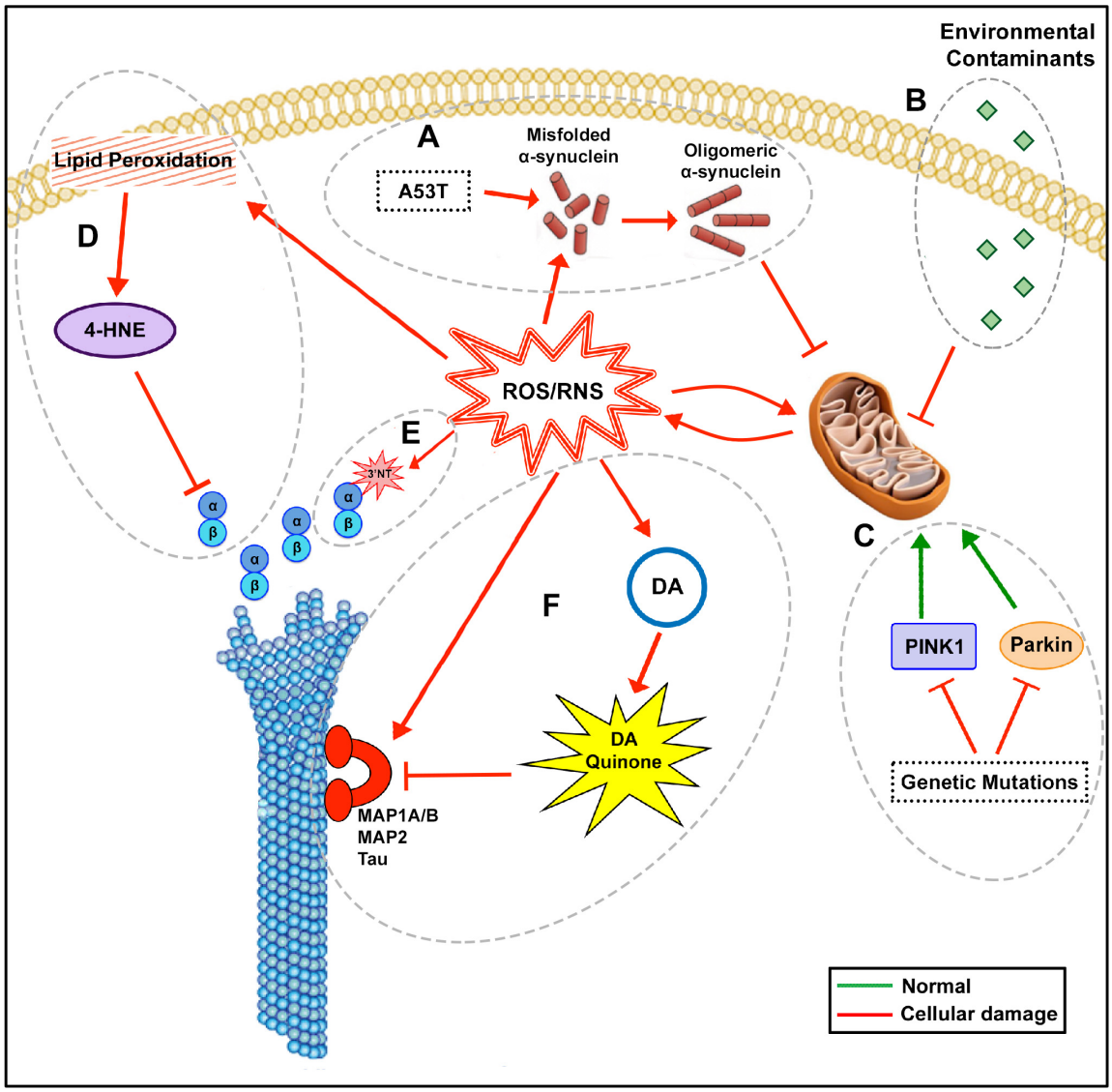

Environmental
Contaminants
B
A
Misfolded
α-synuclein
Oligomeric
α-synuclein
A53T
Lipid Peroxidation
D
4-HNE
ROS/RNS
E
3'NT
α
β
C
F
DA
PINK1
Parkin
DA
Quinone
Genetic Mutations
MAP1A/B
MAP2
Tau
Normal
Cellular damage

Figure 15. Proposed mechanisms of neurite retraction in PD.

(A) Genetic mutations and post-translational modifications can lead to misfolded conformational structure of α-synuclein. Misfolded α-synuclein can aggregate into oligomers and increase production of ROS/RNS through impairment of the ETC.

(B) Environmental contaminants such as the pesticides rotonone, paraquat and maneb can cause mitochondrial dysfunction through the inhibition of the ETC.

(C) Genetic mutations in PINK1 and parkin can impede sufficient clearance of damaged mitochondria, which would result in the accumulation of dysfunction mitochondria and continual generation of ROS/RNS.

(D) Lipid peroxidation of membranes due to ROS/RNS can produce reactive 4-HNE molecules, which has been shown to react with tubulin subunits and impair microtubule polymerization.

(E) Excess production of RNS results in 3'NT modification of α-tubulin, which could result in impaired stability of microtubule dynamics.

(F) Increased levels of NO have been shown to facilitate neurite retraction through the S-nitrosylation of MAP1B. Redox stress can also impede cytoskeletal structure by stimulating the proteolysis of MAP1A/B and MAP2. Furthermore, DA can be easily oxidized to form DA quinone, where it can cause oxidative modification of Tau protein and impair its function in promoting tubulin assembly.

treatment in a human model system of PD. Despite these novel findings however, it is important to note that DMF treatment of PFF exposed SH-SY5Y cells did not fully attenuate neurite retraction. This therefore suggests that other non-redox stress molecular mechanisms could also contribute to this morphology. Additionally, it is also possible that forced induction of the anti-oxidant response has maximized NRF2-ARE binding to target genes, in which further activation of NRF2 no longer increases transcriptional activity.

(5.5) Conclusion

In conclusion, despite previous research efforts to better understand the cellular basis of neurodegeneration in PD, no treatment options are available to delay or reverse this disease pathology. However, emerging evidence has suggested that therapeutic protection of neurite projections may provide an alternative therapy against PD. The novel findings presented in this thesis further suggest that prevention of neurite retraction may be of critical importance in rescuing neurodegeneration. However, due to the *in vitro* nature of this model system, further investigation is warranted not only to validate these results, but also to determine if DMF treatment has a therapeutic impact on physiological functions impaired in PD. Given the effectiveness of PFF exposure in the model systems presented here, it would be of interest to see if PFFs could recapitulate these neurite deficits in both *in vivo* and *ex vivo* models. Furthermore, DMF treatment and subsequent rescue of these new PFF models would strongly suggest a new therapeutic alternative against PD pathology.

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