MICROTUBULE DYNAMICS IN OXIDATIVELY-STRESSED NEURONAL CELLS

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Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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June 13th, 2012

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The microtubule (MT) system is important for many aspects of neuronal function, including motility, differentiation, and organelle trafficking. The dysregulation of this system can therefore have a significant impact on neuronal function and survival. Parkinson's disease (PD) is associated with alterations in integrity of the axon/dendrites as well as axonal transport, which is suggestive of altered MT function. In fact, recent studies using genetic and toxin models are beginning to implicate MT dysfunction as a key mechanism underlying neuronal degeneration in PD. To further study the role of MT dysfunction in PD neurodegeneration, the effects of oxidative stress, which plays a key role in PD pathogenesis, on MT function were examined using the commonly utilized PD toxin, 6-hydroxydopamine (6OHDA). In response to 6OHDA-induced oxidative stress in neuronal cells, significant alterations in MT function were observed with reductions in MT growth rate, increase in frequency of MT pauses/retractions, impaired end binding protein 1 (EB1) levels, and increase in levels of tubulin acetylation. Impaired function of tubulin deacetylases, specifically sirtuin 2 (SIRT2), was observed in oxidatively-stressed cells. Restoration of tubulin deacetylase function rescued MT function and the neuritic degeneration phenotype observed in response to 6OHDA, suggesting that oxidative stress impairs MT function by altering tubulin acetylation. In addition to its impact on the integrity of the neurite, oxidative disruption of MT function also affected MT-dependent nuclear transport, which could contribute to the selective declines in transcriptional responses of diseased dopaminergic neurons. In all, this study provides support for the role of MT dysfunction in the degenerative

processes seen in PD. Elucidating how oxidative stress-induced changes in MT function can lead to neuronal degeneration will provide key insight into the development of novel therapeutic strategies.

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ACKNOWLEDGMENTS

I would like to take this opportunity to thank the many people who have been instrumental in achieving this goal. First, I would like to thank Charleen Chu. Charleen has been a wonderful mentor and has provided key experimental and career guidance throughout my graduate studies. Even though at times my work seemed quite removed from the main focus of the lab, Charleen was just as enthusiastic and took just as much of an interest in the work. I thank you for everything.

I would also like to thank Donald DeFranco. Don, as a co-mentor and the committee chair, has provided excellent guidance and support throughout my graduate studies. Don has gone above and beyond in his role as a co-mentor and I sincerely thank you for your guidance.

I would like to thank the other members of my committee, Drs. Berman, Greenamyre, Monaghan, and Sheng, as they have provided key input on my project and have helped to mold it into the work that is presented here. I thank you for your guidance throughout my graduate studies.

To the past and current members of the Chu lab, I thank you for taking the time to provide feedback on my project as well as providing technical assistance. I also thank you for listening to my frustrations and offering encouragement. I deeply thank you for helping me to achieve this goal.

Finally, I would like to thank my family and friends. To my friends in the MSTP, thank you for guiding me, celebrating my accomplishments, understanding my frustrations, and encouraging me to push on. Having such close friends go through similar experiences has been vital to my scientific and personal development. To my family, I would not be here without your help and encouragement. Even though at times you may not have truly understood the reasons behind my highs and lows, it never limited you from sincerely sharing my excitement and sharing my pain. I cannot thank you enough for what you have done for me.

To everyone mentioned above, thank you!

ABBREVIATIONS

+TIP – plus-end tracking protein

6OHDA – 6-hydroxydopamine

8-oxodG – 8-oxodeoxyguanine

ARD1 – ADP-ribosylation factor domain protein 1

Cdk – cyclin-dependent kinase

EB – end binding protein

HAT – histone acetyltransferase

HDAC – histone deacetylase

HDAC6 – histone deacetylase 6

GCN5 – general control of amino acid synthesis 5

GDP – guanosine diphosphate

GFP – green fluorescent protein

GSH – glutathione

GSK3β – glycogen synthase kinase-3β

GTP – guanosine triphosphate

LDH – lactate dehydrogenase

LRRK2 – leucine-rich repeat kinase 2

MAP – microtubule-associated protein

MEF – mouse embryonic fibroblast

MPP+ – 1-methyl-4-pheylpyridinium

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MT(s) – microtubule(s)

mTOR – mammalian target of rapamycin

NAD+ – nicotinamide adenine dinucleotide

NADH – nicotinamide adenine dinucleotide, reduced

NAT1 – N-terminal acetyltransferase

NES – nuclear export signal

OAADPr – O-acetyl-ADP-ribose

OGG1 – 8-oxoguanine glycosylase

PARP – poly(ADP-ribose) polymerase

PD – Parkinson's disease

PINK1 – PTEN-induced kinase 1

PTM – post-translational modification

RA – retinoic acid

ROS – reactive oxygen species

RNS – reactive nitrogen species

SIRT2 – sirtuin 2

SOD – superoxide dismutase

SNpc – substantia nigra pars compacta

TH – tyrosine hydroxylase

TSA – trichostatin A

1.0 INTRODUCTION

Neurodegenerative diseases, such as Parkinson's disease (PD), are associated with alterations in the integrity of the axon/dendrites, axonal transport of organelles, and gene expression in affected neurons [1-4]. Defining the mechanisms that underlie such alterations is crucial to elucidating the pathogenesis of PD and to the development of novel therapeutic strategies. In the introduction that follows, I will lay the foundation for the studies that were carried out for this thesis with the goal of helping to advance our understanding of PD pathogenesis.

1.1 PARKINSON'S DISEASE

PD is the second most common neurodegenerative disease in the U.S. and the most common neurodegenerative movement disorder [5]. Its incidence increases with age, which is the primary risk factor, and affects more than 1 million Americans [5]. It is clinically characterized by resting tremor, bradykinesia (slowed movement), rigidity, and postural instability [6]. Pathologically, it is characterized by a predominant loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which project to the striatum where they release dopamine and regulate key circuits involved in movement control [4]. The presence of eosinophilic cytoplasmic inclusions, called Lewy bodies, is also a hallmark pathologic feature of the remaining dopaminergic neurons [4]. Although impaired nigro-striatal dopaminergic

projection accounts for the cardinal motor symptoms of the disease, it does not explain the many non-motor symptoms that are also found in patients with PD, such as cognitive impairment, autonomic dysfunction, sensory alterations, and psychiatric issues [6]. Lewy bodies have also been found in the peripheral nervous system of PD patients [7]. The presence of these symptoms and pathologic features suggests that neurons in other regions of the brain as well as outside the brain are also affected – making the proper treatment of this disease more difficult [4].

1.1.1 Role of Oxidative Stress in PD Pathogenesis

In addition to the study of human post-mortem PD brains, studies utilizing genetic and toxin models of PD have provided valuable insight into the pathogenesis of the disease, including the importance of oxidative stress in the development and/or progression of the disease. In the oxidative stress hypothesis, it is thought that an imbalance in cellular levels of reactive oxygen species (ROS) causes damage that ultimately leads to cell death. There is a fair amount of support for the oxidative stress hypothesis, including evidence from post-mortem brains as well as animal and cell culture models.

The high level of aerobic respiration (~20% of the resting total body oxygen) combined with lower levels and/or activities of antioxidants, such as glutathione, superoxide dismutase (SOD), catalase, and vitamin E, in the brain make it prone to oxidative damage [8,9]. Furthermore, given its unique biology, the nigral dopaminergic neurons are even more susceptible to degeneration. Dopamine is chemically unstable and can undergo autoxidation to form ROS and quinones/semiquinones, which themselves can also act as oxidants – all of which can support ROS formation [8,10]. Monoamine oxidase-medicated catabolism of dopamine can also generate ROS [11,12]. In addition, elevated levels of iron in regions such as the substantia

nigra can contribute to hydroxyl radical (OH·) production via Fenton chemistry [8,13]. The antioxidant glutathione (GSH) is found in both mitochondrial and cytosolic compartments and plays a key role in scavenging ROS and reactive nitrogen species (RNS) as well as helping to recycle other antioxidants [14]. GSH levels in the substantia nigra are lower than other brain regions like the cortex, cerebellum, and hippocampus and these levels also decline (~30%) in an age-dependent manner [14]. In fact, a further depletion of total glutathione (reduced and oxidized forms) in the substantia nigra is an early biochemical hallmark of PD [15,16]. Although other antioxidants, such as catalase, are also reduced in PD, the degree of GSH depletion parallels the severity of the disease [14,17]. Additionally, mitochondria play a major role in producing cellular ROS and hence impairment in mitochondrial respiratory chain, such as decreased complex I activity as observed in substantia nigra of PD brains, can further increase ROS production [18,19].

Increases in cellular ROS can cause significant damage to macromolecules and, in fact, there is a significant amount of evidence of oxidative damage in PD brains. There are increases in markers of lipid oxidation, such as malondialdehyde and 4-hydroxy-2-nonenal, compared to age-matched controls [20,21]. Interestingly, 4-hydroxy-2-nonenal can cause irreversible changes to α-synuclein, a protein that is implicated in the pathogenesis of PD, such that it may be prone to adopt more of a pathological (protofibril) confirmation that can cause neurotoxicity [22]. In addition, there is an increase in oxidative damage of proteins, such as carbonyl modifications as well as cross-linking and fragmentation [23,24]. Oxidative stress has been shown to cause many different types of DNA lesions as well – a very common and extensively studied lesion is formation of 8-oxodeoxyguanine (8-oxodG) [25,26]. Oxidative damage to DNA is observed in the substantia nigra from PD brains as evidenced by an increase in 8-oxodG levels in nuclear and

mitochondrial DNA compared to age-matched controls [27-29]. The cell has a few mechanisms to remove these DNA lesions and the primary enzyme responsible for this is 8-oxoguanine glycosylase (OGG1). Recent studies have shown that there is an up-regulation of a mitochondrial OGG1 in the substantia nigra of PD brains, likely as a compensatory response to oxidative damage [30]. Such alterations in the integrity of DNA can have significant impact on mitochondrial function and on gene transcription.

Genetic and toxin models of PD provide further support for the oxidative stress hypothesis in PD pathogenesis [31-35]. Mutations or deficiency of the proteins associated with familial PD, e.g. α-synuclein, Parkin, PINK1 (PTEN-induced kinase 1), DJ-1, and LRRK2 (leucine-rich repeat kinase 2), increase susceptibility of cells to oxidative stress-mediated cell death [32,36,37]. Parkinsonian mimetics such as, 6-hydroxydopamine (6OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat, are well-utilized toxin models of PD that generate ROS and can lead to neuronal death [31,34,38]. Hence, studies in various models of PD suggest that oxidative stress has an important role in the development and/or progression of the disease.

1.1.2 6-hydroxydopamine – Model of PD-Relevant Oxidative Injury

6OHDA has been widely used, *in vivo* and *in vitro*, to study neuronal injury responses in PD [12,39]. Isolated in 1959, it is a hydroxylated analog of catecholamine neurotransmitters that is taken up into cells by dopamine and norepinephrine reuptake transporters [12,39]. Early studies with systemic administration of the toxin showed depletion of noradrenaline in the nerves to the heart as well as damage to nerve endings of sympathetic neurons [40-45]. However, with a systemic application of the toxin, no CNS lesion is observed since 6OHDA cannot cross the

blood-brain barrier. In order to achieve pathology similar to what is seen in PD patients, 6OHDA is administered intracerebrally, typically in the striatum, substantia nigra, or the ascending medial forebrain bundle that contains the nigrostriatal tract [12]. With such 6OHDA applications, striatal dopamine is depleted and nigral dopaminergic neurons are damaged. The time course of injury does vary depending on where 6OHDA was injected, with a substantia nigra or medial forebrain bundle injection showing evidence of degeneration sooner than injection into the striatum [46-49]. Behaviorally, many tests to model PD clinical features, e.g. postural and gait abnormalities, dyskinesias, rigidity, resting tremors, and responsiveness to L-DOPA, have been developed and validated with the 6OHDA injury model [39,50].

The primary mode of 6OHDA-induced neurodegeneration is through the generation of ROS and quinones [12,51]. Quinones could contribute to toxicity via a ROS-independent mechanism (e.g. reacting with various amino acid residues). However, ROS are thought to be the primary toxic mediators as recycling of the quinones by ascorbic acid into 6OHDA with a net formation of hydrogen peroxide (H_2O_2) enhances neurotoxicity [38]. The formation of ROS by 6OHDA is likely to occur via multiple mechanisms, such as auto-oxidation, catalysis by monoamine oxidase, and its effects on mitochondrial function. Molecular oxygen and a redox-capable transitional metal are necessary for the chemical reaction to convert 6OHDA into quinones plus H_2O_2 [38]. Figure 1 shows the overall reaction for 6OHDA oxidation; however this reaction is more complicated as it proceeds through various intermediate steps involving formation of semiquinones and superoxide [38,52].

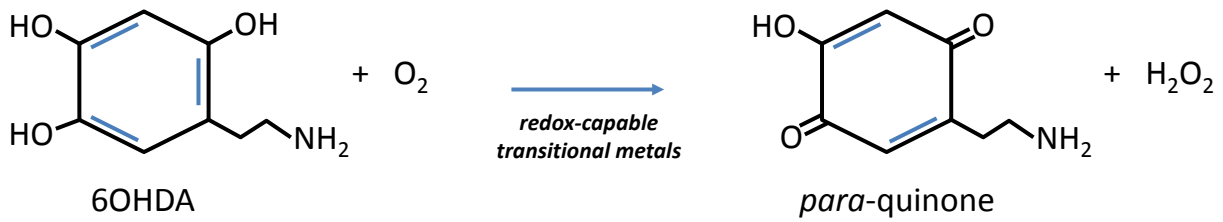

Figure 1: 6OHDA Oxidation

In the presence of molecular oxygen and transitional metals, such as iron or copper, 6OHDA is oxidized into a *para*-quinone and H_2O_2 through a series of intermediate steps. Adapted from [38].

Iron, which is elevated in the substantia nigra of PD brains, can react with H_2O_2 via Fenton chemistry to produce the very reactive hydroxyl radical [8,13]. Therefore, iron chelating agents have been shown to be protective against 6OHDA toxicity [53,54]. Furthermore, as is seen with dopamine, monoamine oxidase can catalyze 6OHDA to produce H_2O_2 as one of the side products [55,56]. The formation of ROS, e.g. H_2O_2, superoxide, and hydroxyl radical, has been shown to occur *in vivo* as well as *in vitro* and antioxidants, e.g. catalase, glutathione peroxidase, manganese superoxide, and metalloporphyrins, have been shown to protect against 6OHDA – all of which strongly support a ROS-mediated 6OHDA toxicity mechanism [57-72].

The oxidative imbalance that is created in cells exposed to 6OHDA impacts many aspects of cellular function. A key effect is on mitochondria as their dysfunction is thought to be an important factor in neuronal degeneration seen in PD [71,73,74]. 6OHDA has been shown to inhibit complexes I and IV of isolated mitochondria, uncouple oxidative phosphorylation, and collapse the mitochondrial membrane potential [63,73,75]. Mitochondrial dysfunction can increase the production of ROS [18,19]. Interestingly, in addition to the early autoxidation phase of ROS production, there is evidence of a delayed phase of mitochondrial ROS production in 6OHDA-treated cells [71]. Hence, mitochondrial dysfunction represents yet another way in which 6OHDA increases cellular ROS levels.

Although used as an exogenous neurotoxin, several studies have provided evidence that 6OHDA could also be a physiological endogenous neurotoxin [12]. 6OHDA has been observed in the brains of humans and rats and has also been detected in urine of patients treated with L-DOPA [12,76,77]. Furthermore, dopamine reactions with free iron and its oxidization in the presence of various agents, such as nitrite ions or manganese, can generate 6OHDA [78-82].

Given the possibility of endogenous production of 6OHDA and its ability to create pathological lesions, molecular alterations, and behavioral symptoms similar to those seen in PD, 6OHDA continues to be a very meaningful model for studying neuronal degeneration seen in PD and in testing new neuroprotective strategies.

1.2 THE MICROTUBULE SYSTEM

Microtubules (MTs) are one of the principle components of the cellular cytoskeleton and play an important role in many cellular processes, such as cell division, organelle trafficking, cell motility, and neuronal differentiation. MTs are hollow fibers with a diameter of approximately 25nm that are assembled from heterodimers of α-tubulin and β-tubulin [83]. These heterodimers polymerize from head-to-tail to form protofilaments and 13 of these protofilaments arrange in parallel to form a cylindrical microtubule lattice [83]. The arrangement of the tubulin dimers creates a polarity with a minus-end and a plus-end. The minus-end is anchored by the centrosome, which is the major microtubule-organizing center in animal cells [83]. Hence, MT assembly initiates at the centrosome (which is typically near the nucleus) and MTs elongate from their plus-ends (growing away from the nucleus and towards the periphery). γ-tubulin ring

complexes are the key components in the centrosome that are responsible for the nucleation of MTs [83].

1.2.1 Dynamic Instability

Microtubules switch between phases of growth (polymerization) and shrinkage (depolymerization) – a property known as dynamic instability [83,84]. The shift from growth to shrinkage is termed "catastrophe" and the shift from shrinkage to growth is termed "rescue". This process is driven by the hydrolysis of GTP (guanosine triphosphate) on β-tubulin. Although GTP also binds to α-tubulin, this pool of GTP is never hydrolyzed [83]. Soon after incorporation into the growing MT, GTP bound to β-tubulin is hydrolyzed. Hence, along the length of the MT, β-tubulin is bound to GDP (guanosine diphosphate). The conformation of the tubulin dimer depends on whether β-tubulin is bound to GTP or GDP. GTP-bound tubulin dimer has a relatively straight conformation while GDP-bound tubulin dimer has a more curved conformation [84]. While part of the MT wall, GDP-bound tubulin dimer is prevented from adopting the curved conformation by bonds (lateral and longitudinal) with adjacent tubulin dimers [84]. At the plus-end of the MT, a 'cap' of GTP-bound β-tubulin is found and serves to stabilize, by lateral bonds, the final layer of GDP-bound β-tubulin [84]. If the GDP-bound tubulin dimer is allowed to take its curved conformation, as is the case when the 'cap' is removed, MT depolymerization occurs. Depolymerized tubulin exchanges GDP for GTP and the cycle begins once again. The rate of tubulin addition compared to rate of GTP hydrolysis plays an important role in determining whether a MT grows or shrinks: growth if the rate of tubulin incorporation is greater than hydrolysis of GTP and shrinkage if rate of GTP hydrolysis is greater than the rate of tubulin incorporation [83].

1.2.2 Regulation of MT Dynamics

MT dynamics is regulated by a large network of proteins that interact with one another in a complex manner [83-86]. These proteins, referred to as MT-associated proteins (MAPs), serve to stabilize or destabilize the MT structure spatially and temporally [86]. There are many categories, with some overlap, of MAPs in eukaryotes, such as structural MAPs, MAPs with enzymatic activity, motor proteins, centrosome-associated proteins, and MT plus-end tracking proteins [84,86-89]. Two of the most studied MAPs, particularly with regards to neurons, are tau and MAP2. They have been traditionally used to highlight different regions of a neuron, specifically in identifying axons (tau) versus dendrites (MAP2) [87]. These MAPs stabilize the MT lattice and regulate many aspects of neuronal function, such as neurite initiation, signal transduction, MT spatial organization, and MT-dependent transport [87].

A major group of MAPs are the plus-end tracking proteins (+TIPs), which are conserved in all eukaryotes [84,85]. +TIPs, of which more than 20 have been described thus far, share the ability to bind to the plus-ends (growing ends) of MTs, where they play a significant role in regulating MT dynamics [84-86]. Although they belong to many structurally unrelated families, +TIPs share some evolutionarily conserved repeat sequences, domains, and motifs [85]. In addition to regulating MT dynamics, +TIPs also play an important role in MT nucleation and linking MTs to vesicles and macromolecules, cell membrane, actin cytoskeleton, kinetochores, and focal adhesions [84,85]. The end binding (EB) protein family of +TIPs are a particularly important group of proteins in the regulation of MT dynamics. There are three EBs in mammals, EB1-3, and they can track MT plus-ends autonomously, which is in contrast to most other +TIPs that require other factors for their MT binding [90,91]. EBs possess an EB homology domain that facilitates interactions with other +TIPs, which serves as one of the main mechanisms by

which other +TIPs interact with MT plus-ends [84]. EBs also contain domains for interactions with MTs at their plus-ends (calponin homology domain) and for homo- and heterodimerization (coiled-coil domain) [84,85]. In terms of MT dynamics, EBs promote MT growth by mechanisms such as increasing the frequency of MT rescue and reducing catastrophes and time spend pausing [91-94]. In neurons, EBs have also been shown to be important for axonal transport and establishing neuronal polarity [95,96]. Importantly, EBs are central players in regulating MT function since most +TIPs have been shown to interact with and be regulated by EBs [85,89].

1.2.3 Regulation of MT Function by Post-Translational Modifications

MT function is also regulated by post-translational modifications (PTMs) of the tubulin subunit, such as acetylation, tyrosination and detyrosination, polyglutamylation, palmitoylation, polyglycylation, and phosphorylation [97,98]. Different regions of a neuron show different patterns of tubulin PTMs – e.g. tyrosination is mostly seen in the growth cone and dendrites (more dynamic MTs), detyrosination and acetylation is mostly seen along the axon (more stable MTs), and polyglutamylation is seen throughout the neuron [97,98]. It is thought that MTs with a greater half-life accumulate specific modifications, such as acetylation and detyrosination. Hence, different tubulin PTMs help identify different pools of MTs – long-lived (stable) MTs are enriched in detyrosinated and acetylated tubulin whereas more dynamic MTs are enriched in tyrosinated tubulin [97,98]. Table 1 summarizes the subcellular distribution of the four major tubulin PTMs in neurons.

Table 1: Tubulin PTMs in Neurons

Modification:	Marker:	Neuronal Distribution (Predominant):
Tyrosination	Dynamic MTs	Distal axon and growth cone
Detyrosination	Stable MTs	Proximal axon
Acetylation	Stable MTs	Proximal axon
Polyglutamylation	Not a MT marker	Found on all MTs but precise localization of subtypes is unclear

Furthermore, in addition to serving as markers, tubulin PTMs have important functions in regulating MT-dependent processes. Several studies have recently shown that tubulin PTMs can regulate the binding of MAPs to MTs. For example, binding of kinesin and various +TIPs is regulated by the tyrosination status of tubulin, kinesin-1 and dynein are recruited by acetylated MTs, and polyglutamylation of tubulin regulates binding of kinesin and dynein motors along with numerous +TIPs [97-100]. Furthermore, given that different PTMs can coexist on the same tubulin molecule, the regulation of MAPs could be mediated by a combination of such PTMs in neurons [101-103]. In short, post-translational modification of MTs likely plays an important role in many aspects of neuronal function, such as differentiation, transport of cargo, axon guidance, and synapse formation.

Acetylation of tubulin occurs on the Lysine-40 residue of α-tubulin, which is predicted to face the MT lumen [97,104,105]. Recently, a novel acetylation site on β-tubulin (Lys-252) has been identified along with proteomic identification of numerous possible acetylation sites on α-tubulin and β-tubulin [106,107]. Many of these sites are predicted to be on the outside surface of the MT lattice – allowing for more straightforward mechanisms to regulate binding of MAPs.

Acetylation is a marker of stable MTs but whether it causes MTs to become stable and affects its dynamics is not certain as there are conflicting reports. These studies have approached this question primarily by regulating the function of one of the two known tubulin deacetylases, HDAC6 (histone deacetylase 6; discussed in detail later).

Matsuyama *et al.*, utilizing NIH 3T3 cells, showed that using trichostatin A (TSA), which inhibits class I and II histone deacetylases (HDACs) including HDAC6, increased tubulin acetylation levels, increased MT polymer content, and made the MTs more resistant to early depolymerization by the MT-depolymerizing agent demecolcine (colcemid; 1μM; up to 30min; no protection by 1hr) [108]. Treatment with trapoxin (TPX), which inhibits class I and II HDACs but not HDAC6, did not reproduce the results seen with TSA. Overexpression of HDAC6 was shown to accelerate demecolcine-induced MT depolymerization [108]. These results suggested that inhibition of HDAC6 and subsequent increase in tubulin acetylation could lead to MT stability. However, a couple of studies that were published in 2003 contradicted the conclusion that increased acetylation affects MT stability [109,110]. The study by Haggarty *et al.* utilizing A549 lung carcinoma cells showed that treatment with tubacin, a HDAC6-specific inhibitor, did not change the sensitivity to cold depolymerization or to nocodazole (332nM; 2hrs), a depolymerizing agent [110]. Palazzo *et al.* showed that NIH 3T3 cells treated with TSA have increased tubulin acetylation levels but do not show any changes to nocodazole-induced depolymerization (10μM; 30min) or changes in the amount of detyrosinated tubulin, a marker of stable MTs [109]. These studies suggest that an increase in MT acetylation by HDAC6 inhibition does not affect MT stability. A study by Dong-Anh Tran *et al.* in 2007 utilizing various cell types supported and expanded on the initial study by Matsuyama and coworkers, emphasizing the importance of tubulin acetylation in regulating the dynamic properties of MTs

[111]. These authors showed that treatment with TSA reduced rates and percentage of time spent depolymerizing / polymerizing. Reduction in dynamicity (measured as total distance of polymerized and depolymerized MTs divided by total elapsed time) was also observed. However, frequency of rescue and catastrophe was not affected. HDAC6 KO mouse embryonic fibroblast (MEF) cells showed an increase in the time spent pausing and this effect was rescued by overexpression of HDAC6. HDAC6 KO MEFs did not show any changes in the rate of depolymerization / polymerization or in the frequency of rescue and catastrophe. However, resistance to nocodazole treatment (10μM; protection up to 30min-1hr) was observed in TSA-treated NIH-3T3 cells, HDAC6 KO MEFs, and HDAC6 knock-down A549 cells. Finally, TSA treatment also protected against end-mediated depolymerization induced by ATP [111]. Hence, as can be noted from the above studies, there is some disagreement when it comes to the question of whether increased acetylation affects MT function. However, it is likely that differences in sensitivity of the assays, method used to increase acetylation levels, variable drug concentrations, and variable treatment times combine to explain why the results are contradictory. Supporting this is the study by Dong-Anh Tran *et al.*, which showed differences in MT dynamics with tubulin acetylation. When the authors used less sensitive assays, such as the ones used by the groups that showed no differences, they also did not observe any differences in cells with hyperacetylated MTs [111]. Hence, the assays used to study MT dynamics are an important consideration for these studies. In addition, through manipulation of tubulin acetylation via taxol and HDAC6 in fibroblast and neurons, Sudo *et al.* found that acetylation of tubulin leads to severing of MTs by katanin, which could be a way to destabilize acetylated MTs [112]. In conclusion, more studies using assays with greater sensitivities and examining more direct

effects of tubulin acetylation are required to fully elucidate the impact of acetylation on MT dynamics.

1.2.4 Tubulin Acetylases

Tubulin acetylases have only been recently identified but have provided insight into the role of tubulin acetylation for neuronal function. Ohkawa *et al.* showed that ARD1 (ADP-ribosylation factor domain protein 1) in complex with NAT1 (N-terminal acetyltransferase) can acetylate α-tubulin [113]. Mutant ARD1 with reduced acetyltransferase activity or knock-down of ARD1 reduced dendritic extensions in cultured Purkinje cells. Interestingly, the overexpression of ARD1 was able to rescue HDAC6-mediated reduction in dendritic arborization, suggesting a common substrate (e.g. α-tubulin) may be involved [113].

Another tubulin acetylase was also recently identified – the Elongator complex [114]. The authors showed that key subunits, Elp1 and Elp3, of the complex interact with MTs and regulate the acetylation of α-tubulin in various cell types, including cortical projection neurons. Overexpression of a dominant-negative α-tubulin that cannot be acetylated on Lys-40 (K40A) impaired neuronal migration and branching, a phenotype that was also seen after Elp1/Elp3 knock-down. Inhibition of HDAC6 by the specific inhibitor tubacin restored tubulin acetylation levels in the Elp1/Elp3 knock-down cells and also rescued the branching defects in Elp3-depleted cortical neurons. These data suggest that tubulin acetylation is directly involved in regulating neuronal differentiation.

MEC-17 and its homolog αTAT1 have also been recently identified as an α-tubulin acetylase at Lys-40 in *Tetrahymena thermophila*, C. *elegans*, and in zebrafish, where they may play an important role in nervous system development and touch sensation [115,116]. The

acetylation of other substrates in regulating these processes is yet to be determined. San, an acetyltransferase, has also been recently identified to acetylate a site in β-tubulin, Lysine-252, which affects its incorporation into growing MTs [106]. In addition, general control of amino acid synthesis 5 (GCN5) has been shown to be recruited to MTs during muscle cell differentiation via a cytoplasmic cleavage produce of the Myc transcription factor and leads to Lys-40 acetylation of α-tubulin [117]. Hence, there are many tubulin acetylases, depending on the tissue and organism examined, and it is likely that others are yet to be identified.

1.2.5 Tubulin Deacetylases

Most studies examining the effects of tubulin acetylation on MT function have focused on manipulating acetylation levels via regulation of the two tubulin deacetylases – HDAC6 (histone deacetylase 6) and SIRT2 (sirtuin 2). There are four categories of HDACs based on their protein sequence and structure: (1) class I includes HDAC1, HDAC2, HDAC3, and HDAC8, (2) class II includes HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10, (3) class III includes the silent information regulators (sirtuins or SIRTs) from 1 to 7, and (4) class IV consists of HDAC11 [118,119]. The subcellular localization of these proteins varies depending on the specific deacetylase; however, in general, class I members are mostly in the nucleus, class II and IV members are found both in the nucleus as well as the cytoplasm and can undergo nucleocytoplasmic trafficking, and class III members can be found in the nucleus, cytoplasm, or mitochondria [119,120]. Hence, despite possessing the name “histone deacetylase”, many of the HDACs also deacetylate substrates other than histones [119,120].

HDAC6 is a class II HDAC that is unique amongst other HDACs in that it possesses two catalytic domains [121]. The deacetylase domain is conserved among the other class I and II

HDACs and is formed by a stretch of approximately 390 amino acids [119]. Key histidine, aspartic, and tyrosine residues along with a Zn^{2+} ion are involved in a charge-relay system to remove the acetyl group [122,123]. Whether both catalytic domains are important for the full deacetylase activity of HDAC6 is uncertain as there are conflicting reports [118]. HDAC6 also contains a Ser-Glu-containing tetradecapeptide (SE14) repeat domain that is important for cytoplasmic anchoring, a nuclear export signal (NES) that is important for its export to the cytoplasm, and a zinc finger domain that is important for binding to ubiquitin [121,124,125].

HDAC6 has numerous substrates, including α-tubulin, cortactin, and heat shock protein 90 (Hsp90) *in vivo* and histones *in vitro* [118,126]. Given the various substrates and other interacting proteins, HDAC6 regulates many cellular systems, such as the MT and actin cytoskeletons and the protein chaperone system [118,126]. Some MT-dependent functions of HDAC6 have been discussed in previous sections, but a key function is in the recruitment of motor proteins, such as dynein and kinesin-1, to MTs to regulate processes such as cargo trafficking, e.g. mitochondria, brain-derived neurotrophic factor (BDNF), and polyubiquitinated protein aggregates for autophagic clearance [127-130]. In neurons, the polyubiquitination function of HDAC6 plays an important role in dendritic differentiation by regulating the Cdc20-anaphase-promoting complex (APC) ubiquitin signaling pathway [131]. Furthermore, HDAC6 has been shown to be important for the formation of the axon initial segment and for axonal growth likely by regulating tubulin acetylation [132].

It is not certain if several of the MT-dependent processes regulated by HDAC6 are only due to its effect on tubulin acetylation. Interestingly, HDAC6 has been shown to interact with and regulate MAPs, such as tau and EB1 [133-136]. Tau is an important MT stabilizing MAP and EB1 is a key regulator of the dynamic properties of MTs [87,89]. Work by Zilberman *et al.*

in non-neuronal cells showed that inhibition of HDAC6 activity, either via the specific inhibitor tubacin or mutation of one the deacetylase domains but not both domains, reduced MT growth velocities [136]. However, knock-down of HDAC6 did not show such results, leading the authors to conclude that an inhibited HDAC6 is needed to negatively affect MT dynamics. They further propose a model where binding of EB1 to MT plus-ends is altered by a defective HDAC6, however further studies are required to support this hypothesis. Nonetheless, HDAC6-mediated effects on MAPs along with its effects on MT acetylation likely combine to regulate MT-dependent processes in neurons.

SIRT2 is a class III HDAC and needs nicotinamide adenine dinucleotide (NAD+) as a cofactor for its deacetylase activity [120]. The catalytic domain is conserved in all SIRTs and is a stretch of 275 amino acids [120]. Key histidine, glutamine, asparagine, and aspartic residues have been shown to be important for its enzymatic activity [137]. SIRT2 also contains a canonical NES to export it out of the nucleus [138]. The detailed chemical mechanism of the SIRT2 deacetylase reaction needs to be clarified, however it is known that the reaction produces nicotinamide, an endogenous SIRT inhibitor, and O-acetyl-ADP-ribose (OAADPr) as side products [139,140]. SIRT2 has numerous known substrates, including α-tubulin, p53, FOXOs, and histones [141,142]. SIRT2 has been shown to co-localize and co-immunoprecipitate with HDAC6 and *in vivo* both proteins may be present in a complex that regulates MT deacetylation [143]. There are very few studies examining the role of SIRT2 in neurons but it has been shown to negatively regulate, in a deacetylase-dependent manner, neurite outgrowth and growth cone dynamics in hippocampal neurons [141,144]. SIRT2 has also been shown to promote axonal degeneration in a Wallerian degeneration model [145]. Given the importance of MTs in these

processes, these effects of SIRT2 may result from MT alterations; however, this has not been established and further studies are required to determine the specific substrates involved.

1.2.6 MT Dysfunction in PD

Axonal degeneration is one of the earliest features of neurodegenerative diseases, such as PD [4,146]. The MT system is crucial to the proper development and maintenance of axons, and hence axonal retraction is indicative of MT dysfunction [147]. Furthermore, axonal transport of cargo, such as organelle and protein complexes, is essential for proper neuronal biology [3,148]. MTs, which are nearly uniform in polarity as the plus-ends face the synapse and the minus-ends face the cell body, along with motor proteins, which show preferences for plus- and minus-ends, are the key elements of axonal transport [2]. Interestingly, there is an extensive amount of evidence of altered axonal transport of cargo in neurodegenerative diseases [2,148]. In PD, evidence of altered axonal transport comes from both genetic and toxin models. Mutations in α-synuclein, which is associated with familial PD, show reduced transport along axons and abnormal protein accumulation compared to the wild-type protein [149]. Aggregation of α-synuclein can impair MT-dependent transport while not affecting MT-independent trafficking [150]. In an α-synucleinopathy rat model, reductions in various anterograde motor proteins and an increase in retrograde motor proteins were found in the striatum [151]. In *Drosophila* larval motor neurons, PINK1 overexpression reduced mitochondrial transport (anterograde and retrograde) and knock-down of PINK1 increased anterograde transport [152]. MPP+ has been shown to impair movement of membrane-bound organelles, such as mitochondria, along axons / neurites [153-155]. In fibroblasts and SH-SY5Y neuroblastoma cells, rotenone treatment was shown to reduce mitochondrial motility [156,157]. However, chronic rotenone exposure in

neurons has been shown to increase mitochondrial retrograde transport [158]. Dopamine neurons with deficient respiratory chain function (MitoPark mouse), which is a key hallmark of PD, have reduced anterograde axonal transport of mitochondria [159].

The alterations in axonal integrity and transport are highly suggestive of MT dysfunction. In fact, in various models of PD, MT alterations are beginning to be identified. Parkin, mutations in which are associated with familial PD, has been shown to interact with tubulin heterodimers and MTs and reduces colchicine-induced depolymerization of MTs [160]. α-synuclein co-localizes with tubulin in Lewy bodies as well as other α-synuclein-positive lesions [161]. Tubulin can also promote α-synuclein fibril formation *in vitro* [161]. α-synuclein binds tubulin and the wild-type (WT) protein, but not mutants, can promote its polymerization into MTs [162]. Overexpression of α-synuclein and subsequent aggregation led to the disorganization of the MT network in cells and also causes co-aggregation of tubulin but not β-actin [150]. In the striatum of an α-synucleinopathy rat model, reduction in α- and γ-tubulin levels is observed [151]. Hence, α-synuclein and tubulin biology may be closely linked. LRRK2, mutations in which are also associated with familial PD, has been strongly linked to MTs as well [163]. LRRK2 binds tubulin and regulates phosphorylation of β-tubulin that is enhanced by the PD mutant G2019S, causing stability of MTs in the presence of MAPs [164,165]. LRRK2 was shown to increase tubulin polymerization and this has been linked to aggregation of α-synuclein and subsequent neuropathology in A53T α-synuclein mice [166]. Furthermore, WT and mutant LRRK2 proteins also play an important role in regulating interaction of tau with MTs as well as tau phosphorylation levels [167-169]. These data suggest that LRRK2 plays an important role in regulating MT function. Toxin models of PD, such as rotenone and MPP+, also provide further support of MT dysfunction. Rotenone causes

depolymerization of MTs in TH (tyrosine hydroxylase)-positive neurons, which was blocked by taxol treatment [170]. In addition, rotenone can bind tubulin and inhibit its polymerization *in vitro* [170]. MPP+ reduces tubulin polymerization *in vitro* and *in vivo* [171,172]. MPP+ has also recently been shown to reduce MT growth rates in differentiated PC12 cells along with causing alterations in tubulin PTMs, such as reducing tyrosinated tubulin and increasing acetylated tubulin [154]. As can be noted from these studies, there is growing evidence from various models that MT dysfunction may play an important role in the neuronal degeneration that is seen in PD.

1.2.7 Oxidative Stress & MT Dysfunction

A few studies have provided evidence of MT dysfunction in models of oxidative stress. In human osteosarcoma 143B cells, H_2O_2 treatment (200 – 400μM; 48hrs) resulted in increased levels of monomeric tubulin and decreased levels of polymerized tubulin, suggesting increased depolymerization and/or reduced polymerization [173]. In HeLa cells, H_2O_2 treatment (200μM; 4hrs) causes reduction in EB1 levels as well as EB1 "comet" length, which is correlated with decreased MT growth velocities [174-177]. Treatment for 45min with 200uM H_2O_2 caused alterations in MT dynamics, such as reduced growth rate, increased shortening rate, and increased time spent pausing [174]. Cysteine oxidation has been shown to reduce polymerization of tubulin into MTs [178-183]. Quinones (e.g. benzoquinone), which are linked to oxidative stress, can disrupt cellular MT organization in NIH 3T3 cells and reduce polymerization of tubulin *in vitro* [184]. Hence, the MT system is thought to be sensitive to oxidative stress. However, these studies have been done in non-neuronal cells and hence not much is known with regards to the effects of oxidative stress on MT function in neuronal cells.

2.0 RATIONALE AND HYPOTHESIS

The MT system is important for many aspects of neuronal function and the dysregulation of this system may play a key role in neuronal degeneration. Oxidative stress, which is implicated in the pathogenesis of many neurodegenerative diseases such as PD, has been linked to MT dysfunction; however these studies utilize high doses of oxidative stressors and have been carried out in non-neuronal cells. Therefore, utilization of a PD-relevant sub-lethal oxidative injury model can provide valuable insight into the neuronal degeneration observed in this disease. The overall goal of this study was to examine the effects of oxidative stress, as induced by the commonly utilized PD toxin 6OHDA, on MT function in neuronal cells. It is hypothesized that 6OHDA-induced oxidative stress alters MT dynamics and results in impairment in MT-dependent processes, such as neuritic growth / maintenance. The restoration of the dynamic properties of MTs is further hypothesized to reverse the effects on neuritic integrity and provide key insight into the mechanisms involved in the degeneration of neurons in PD as well as into potential therapeutic strategies.

3.0 MATERIALS AND METHODS

3.1 TISSUE CULTURE

Human SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA) were grown on 10cm cell culture dishes containing Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (BioWhittaker, Walkersville, MD), 2mM L-glutamine (BioWhittaker, Walkersville, MD), and 10mM HEPES (BioWhittaker, Walkersville, MD). For retinoic acid (RA) differentiation (Sigma-Aldrich, St. Louis, MO), cells were plated in 10μM RA for 3 days prior to any experimental manipulations, such as transfections or drug treatments, and then maintained in RA-containing media for the duration of the experiment. Cells were maintained at 37^{o}C in a humidified 5% CO_2 incubator.

3.2 PLASMIDS & TRANSFECTIONS

SH-SY5Y cells were transfected with Lipofectamine^{TM} 2000 reagent (Invitrogen, Carlsbad, CA) utilizing Opti-MEM® I reduced serum media (Invitrogen, Carlsbad, CA) according to the manufacture's protocol at a final Lipofectamine concentration of 0.1%. Undifferentiated SH-SY5Y cells were grown for at least 2 days before transfection and then allowed to express the protein of interest for another 48hr before treatment. Differentiated SH-SY5Y cells were grown

for 3 days before transfection and then allowed to express the protein of interest for another 24hr before start of treatment. The media was refreshed the day after transfection.

All plasmids were prepared with QIAGEN Plasmid Maxi Kit (Valencia, CA) as per the manufacturer's protocol and their sequences were confirmed (Sequencing Core Facility, Genomics and Proteomics Core Laboratories, University of Pittsburgh, Pittsburgh, PA). eGFP-C1 (green fluorescent protein) plasmid was purchased from Clontech (Mountain View, CA). pcDNA 3.1 was purchased from Invitrogen (Grand Island, NY). EB3-GFP, HDAC6-GFP, and SIRT2-GFP expression plasmids were kindly provided by Dr. Niels Galjart (Erasmus University, Rotterdam, The Netherlands), Dr. Francisco Sanchez-Madrid (Hospital Universitario de la Princesa, Instituto de Investigación Sanitaria Princesa, Madrid, Spain), and Dr. Eric Verdin (UCSF, San Francisco, CA), respectively. HDAC6-FLAG and SIRT2-FLAG were purchased from Addgene (Cambridge, MA). EB3-mCherry was produced via standard subcloning techniques. Briefly, EB3-GFP and mCherry-N1 (Clontech, Mountain View, CA) were cleaved with EcoRI (New England BioLabs, Ipswich, MA) and BamHI (New England BioLabs, Ipswich, MA) for 1hr at 37^{o}C and the cleaved products were separated on a 2.5% agarose (Invitrogen, Carlsbad, CA) gel. The EB3-GFP fragment and cleaved mCherry-N1 were cut from the gel and extracted with QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) as per the manufacturer's protocol. Extracted DNAs were then ligated overnight with the Fast-Link^{TM} DNA Ligation Kit (Epicentre Biotechnologies, Madison, WI) as per the manufacturer's protocol. The sequence of the ligated product, EB3-mCherry, was then confirmed (Sequencing Core Facility, Genomics and Proteomics Core Laboratories, University of Pittsburgh, Pittsburgh, PA).

3.3 PHARMACOLOGICAL TREATMENTS

Retinoic acid was prepared in dimethyl sulfoxide (DMSO) at a stock concentration of 10mM and stored at -20^{o}C. 6OHDA (Sigma-Aldrich, St. Louis, MO) was prepared in cold sterile water immediately before each use. NAD+ (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water at a stock concentration of 50mM and stored at -80^{o}C. Colchicine (Sigma-Aldrich, St. Louis, MO) was prepared in sterile water at a stock concentration of 1mM and stored at 4^{o}C. All drugs were used at concentrations as indicated in the text and handled while minimizing light exposure.

3.4 CELL TOXICITY ASSAYS

3.4.1 Lactate Dehydrogenase (LDH) Release Assay

Cells were plated in a black, clear-bottom 96-well plate and treated with 6OHDA for varying dosage and time. LDH (lactate dehydrogenase) release was measured using the CytoTox-ONE Homogeneous Membrane Integrity Assay per the protocol provided by the manufacturer (Promega, Madison, WI). Dead or dying cells with damaged membranes release LDH into the culture medium, which is involved in a series of enzymatic reactions to produce a fluorescent product that is proportional to the amount of LDH present. Spectramax M2 plate reader was utilized to read the fluorescent signal (Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was calculated by the following formula: 100 x [(Experimental – Medium Background) /

(Maximum LDH release – Medium Background)]. Maximum LDH release was determined by treatment of cells with lysis buffer as per manufacturer's protocol.

3.4.2 MTS Assay

Cells were plated in a clear 96-well plate and treated with 6OHDA for varying dosage and time. Cell toxicity resulting from 6OHDA was determined via the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) as per the manufacturer's protocol (Promega, Madison, WI). MTS is a salt that is reduced by dehydrogenase enzymes in metabolically active cells into a formazan product that has absorbance at 490nm. The amount of absorbance is directly proportional to the number of cells. Since 6OHDA oxidation itself can cause media color changes, a parallel 96-well plate with only media was similarly treated with 6OHDA and the absorbance readings were used to correct for any color changes resulting from 6OHDA itself. Spectramax M2 plate reader was utilized to read the colorimetric signal.

3.5 IMMUNOCYTOCHEMISTRY

For EB1 protein detection, media was removed from the cells, -20^{o}C methanol (Fisher Scientific, Pittsburgh, PA) was added, and cells were placed at -20^{o}C for 5min. For all other fixations, cells were first washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, Grand Island, NY) and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) prepared in phosphate buffered saline (PBS) for 30min at room temperature. After PBS washes, cells were permeabilized with 0.1% Triton X-100 (Fisher Scientific, Pittsburgh, PA) in PBS for 20min and

then blocked with a commercial blocking buffer (SuperBlock, Thermo Scientific, Rockford, IL) for 1hr at room temperature to decrease non-specific antibody binding. After PBS washes, cells were incubated in primary antibody overnight at 4^{o}C or 1hr at room temperature. Secondary antibody was used at a dilution of 1:1000 for 1hr at room temperature. Both primary and secondary antibodies were diluted in a commercial antibody diluent (BioGenex, Fremont, CA). After further PBS washes, cells were imaged on an Olympus IX71 inverted microscope. Primary antibodies used: α-tubulin (1:5000; Sigma-Aldrich, St. Louis, MO), acetylated-tubulin (1:1000; Sigma-Aldrich, St. Louis, MO), EB1 (1:500; BD Biosciences, San Jose, CA), FLAG (1:5000; Sigma-Aldrich, St. Louis, MO), HDAC6 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), and SIRT2 (1:1000; Sigma-Aldrich, St. Louis, MO). Secondary antibodies used: Alexa Fluor-488 (Invitrogen, Carlsbad, CA) and Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA).

3.6 IMMUNOBLOTTING

Cells were washed in DPBS and then collected via a cell scraper in scraping buffer: 25mM HEPES pH 7.5 (Research Organics, Cleveland, OH), 50mM NaCl (Fisher Scientific, Pittsburgh, PA), 5mM EDTA (Fisher Scientific, Pittsburgh, PA), 2mM PMSF (Acros Organics, Geel, Belgium), and 1mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO). Cells were centrifuged at 1000 *x g* for 5min and lysed in lysis buffer: 25mM HEPES pH 7.5, 150mM NaCl, 5mM EDTA, 10% glycerol (Acros Organics, Geel, Belgium), 1% Triton X-100, 1mM sodium orthovanadate, and 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

Cells were lysed on ice for at least 30min, centrifuged at 14,000 *x g* for 15min, and the supernatant was collected and stored at -80^{o}C.

Protein concentration was determined using the Coomassie Plus (Bradford) Protein Assay (Thermo Scientific, Rockford, IL). 25 – 30ug of total protein was separated by SDS-PAGE using the Bio-Rad (Hercules, CA) gel running system and transferred to a PVDF (Millipore, Billerica, MA) membrane using a Trans-Blot SD Semi-Dry Transfer Cell apparatus (Bio-Rad, Hercules, CA). The membrane was then blocked with 5% milk in PBST (PBS with 0.3% Tween 20 [Fisher Scientific, Pittsburgh, PA]) for 1hr at room temperature. After 3 PBST washes, the membrane was incubated in primary antibody (diluted in PBST with 1% bovine serum albumin and 0.05% sodium azide) at 4^{o}C overnight. After 3 PBST washes, the membrane was incubated in horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Piscataway, NJ) diluted (1:5000) in 5% milk for 1hr at room temperature. The membrane was washed 3 times in PBST and developed using SuperSignal West Pico (Thermo Scientific, Rockford, IL). Primary antibodies used: acetylated tubulin (1:10:000; Sigma-Aldrich, St. Louis, MO), α-tubulin (1:5:000; Sigma-Aldrich, St. Louis, MO), GAPDH (1:10:000; Abcam, Cambridge, MA), HDAC6 (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), and SIRT2 (1:500; Sigma-Aldrich, St. Louis, MO). ImageJ (NIH) was utilized for densitometry.

3.7 IMMUNOPRECIPITATION & DEACETYLASE ACTIVITY ASSAY

Cells were grown in 10cm dishes to at least 80% confluency, washed with DPBS, and then lysed directly on the plate with lysis buffer: 50mM Tris HCl pH 8.0, 137mM NaCl, 2.5mM EDTA pH 8.0, 1% Triton X-100, 1mM sodium orthovanadate, and 1:100 dilution of protease inhibitor

cocktail. Cells in lysis buffer were scraped and allowed to lyse for at least 30min at 4°C with constant agitation. Lysates were centrifuged at 14,000 *x g* for 15min. Supernatant was collected and protein concentration was determined via a Bradford Assay. Approximately 1mg of protein was added to 50uL of Protein A-Agarose beads (Sigma-Aldrich, St. Louis, MO) that had been previously washed 5 times in 500uL lysis buffer. 2 – 3ug of IgG (Vector Laboratories, Burlingame, CA) depending on if it's a control for HDAC6 or SIRT2, 2ug of HDAC6, or 3ug of SIRT2 antibodies were then added to the lysate/bead mixture. The immunoprecipitation was carried out overnight at 4°C with constant agitation. The following day, the beads were collected at 0.1 *x g* for 5min and washed 3 times in lysis buffer with protease inhibitors and twice in HDAC Assay Buffer that is provided with the kit. At each wash, beads were incubated in 500uL of buffer for 5min at 4°C with constant agitation. After the final wash and centrifugation, nearly all of the buffer was carefully removed.

To assess the activity of the pulled-down deacetylase, a Fluor-de-Lys-Green HDAC fluorometric activity assay kit was utilized (Enzo Life Sciences, Farmingdale, NY) as per the manufacturer's protocol. Briefly, 100μM of the Fluor-de-Lys-Green substrate that contains an acetylated lysine side chain was added to the bead/antibody/enzyme mixture and the reaction was carried out for 15min to 1hr at 37°C with constant agitation. For SIRT2 activity, 500μM NAD+ was also added to the reaction. Once the reaction was complete, 50uL of the reaction was added to an opaque, white, ½-volume 96-well plate and 50uL of a Fluor-de-Lys Developer solution containing 2μM Trichostatin A (for HDAC6 inhibition; final concentration after addition was 1μM) or 2μM nicotinamide (for SIRT2 inhibition; final concentration after addition was 1μM) was added. The plate was incubated at room temperature for at least 20min and the

fluorescent signal was read (excitation: 485nm; emission: 530nm) using a Spectramax M2 plate reader.

After completion of the deacetylase reaction, beads were washed 3 times in 500uL lysis buffer as before. After the final wash and centrifugation, nearly all of the lysis buffer was carefully removed and 40uL of SDS-PAGE sample buffer was added (66mM Tris-HCL pH 6.8 [Fisher Scientific, Pittsburgh, PA], 26.3% glycerol, 2.1% SDS [Bio-Rad, Hercules, CA], 0.01% bromophenol blue [Fisher Scientific, Pittsburgh, PA], and 50mM DTT [Sigma-Aldrich, St. Louis, MO]). The beads in sample buffer were boiled for 5min, centrifuged, and the supernatant was collected and stored at -80^{o}C.

3.8 NAD+ / NADH QUANTIFICATION

To determine levels of NAD+ in cells, a NAD+/NADH Quantitation Kit was utilized (BioVision, Milpitas, CA). Cells were washed in DPBS, trypsinized (BioWhittaker, Walkersville, MD), and collected in media. Cell number was determined using trypan blue exclusion (Thermo Scientific, Rockford, IL). 2 million cells were pelleted at 1000 *x g* for 5min and the pellet was washed twice with DPBS. 400uL of Extraction Buffer was added and two freeze/thaw cycles (20min on dry-ice and 15min at room temperature) were carried out to extract NAD+ and NADH. The sample was vortexed for 10sec and centrifuged at 14,000 rpm for 5min. 30uL of the sample was removed at this point for protein assay. The remaining sample was filtered through a 10kD molecular weight cut off filter (Abcam, Cambridge, MA) to remove any enzymes that could consume NAD+ or NADH. The sample was stored frozen at -80^{o}C until use. For determination of NAD+ and NADH (NADt), 50uL of the extracted sample was transferred to a 96-well plate in

duplicates. To determine NADH, the extracted sample was incubated at 60^{o}C for 30min to decompose NAD+ but leaving NADH intact. 50uL of the decomposed sample was then transferred to the 96-well plate in duplicates. To these samples, 100uL of buffer containing enzymes that carry out the cycling reaction and 10uL of developer solution were added per the manufacturer's protocol. The reaction was incubated at room temperature from 1hr to 4hr and the absorbance was read at 450nm using a Spectramax M2 plate reader. To calculate NAD+, the value for NADH was subtracted from the value from NADt. A blank that does not contain any NAD+ or NADH was used for background subtraction. A BCA (bicinchoninic acid) protein assay (Thermo Scientific, Rockford, IL) was carried out on the samples saved prior to filtration per the manufacturer's protocol. Absorbance values of NAD+ and NADH were normalized to protein concentration.

3.9 LIVE CELL IMAGING OF MT DYNAMICS

To study MT dynamics, media on cells expressing fluorescently-tagged EB3 was removed and 37^{o}C DPBS, which contains glucose, was added. Cells were then transferred to an Olympus IX71 inverted microscope with a 60X oil objective and a heated stage set to 37^{o}C (Warner Instruments, Hamden, CT). Cells with low expression of EB3-GFP / EB3-mCherry, e.g. showing a clear comet-like pattern, were followed since it has been shown that low-level expression of such proteins does not affect the dynamic behavior of MTs [185]. Images were taken every 5sec at an exposure time of 500ms (EB3-GFP) or 1sec (EB3-mCherry; less bright than GFP). A neutral density filter that reduces light intensity by 50% was used for all live cell imaging to minimize any potential damage of excessive ultraviolet light exposure.

3.10 IMAGE ANALYSIS

Neurite length was measured in GFP expressing cells via the ImageJ software (NIH). At least 50 neurites were quantified for each group of an experiment (per replicate). EB comet length was measured in cells that were imaged at the same exposure time and, using ImageJ, thresholded with the same parameters to minimize the background noise and highlight the EB comets. The longest distance between two points (Feret's diameter) was determined for each comet. At least 10 comets per cell and at least 5 cells were analyzed for each group of an experiment (per replicate). For MT growth rate measurements, EB3 comets that could be followed for at least three consecutive frames were measured using the MTrackJ plugin for ImageJ [186]. For measurements of pauses/retractions, the number of comet dimming events, time elapsed in each dimming event, and time between two dimming events was determined. For measurements of growth rate and pauses/retractions, at least 5 comets per cell and at least 5 cells were analyzed for each group of an experiment (per replicate).

3.11 STATISTICAL ANALYSIS

Student's *t* test was used for comparison of means between two groups. One-way analysis of variance (ANOVA) followed by post-hoc Tukey HSD test was used for multiple comparisons. A p-value < 0.05 was considered statistically significant.

4.0 RESULTS

4.1 6OHDA MODEL OF OXIDATIVE STRESS

For this study, human SH-SY5Y cells were utilized. These cells were originally derived from SK-N-SH cells that were recovered from a human metastatic neuroblastoma tissue [187]. SH-SY5Y cells have been widely used in the field of PD given that they possess many dopaminergic characteristics [188,189]. These cells express tyrosine hydroxylase, dopamine-β-hydroxylase, and the dopamine transporter [188,190,191]. Furthermore, SH-SY5Y cells can be differentiated, e.g. with retinoic acid, to a more mature neuronal phenotype, biochemically as well as morphologically. Upon differentiation, cells stop proliferation, various neuronal markers are expressed, and long neurites are formed [188,189,192]. Hence, given these properties of SH-SY5Y cells, they have served as an important model of dopaminergic neurons in PD.

It was first important to carry out cell viability assays to determine the concentration and length of 6OHDA treatment that do not cause cellular toxicity. Previous work from the lab as well as my studies with nuclear transport (see Appendix) had suggested that 150μM of 6OHDA was an effective pre-lethal dose. Therefore, a time course of 150μM 6OHDA in undifferentiated SH-SY5Y cells was carried out to determine if there was cellular toxicity (Fig. 2). Since 6OHDA, particularly at high concentrations, can change the color of the media, there were concerns of interference with colorometric assays such as the MTS assay. Hence, a

fluorescence-based LDH release assay was performed. As seen in Figure 2, no significant toxicity was observed until 6hr after 150μM 6OHDA treatment. For subsequent experiments utilizing this pre-lethal dose, treatment times of ≤ 4hr were used.

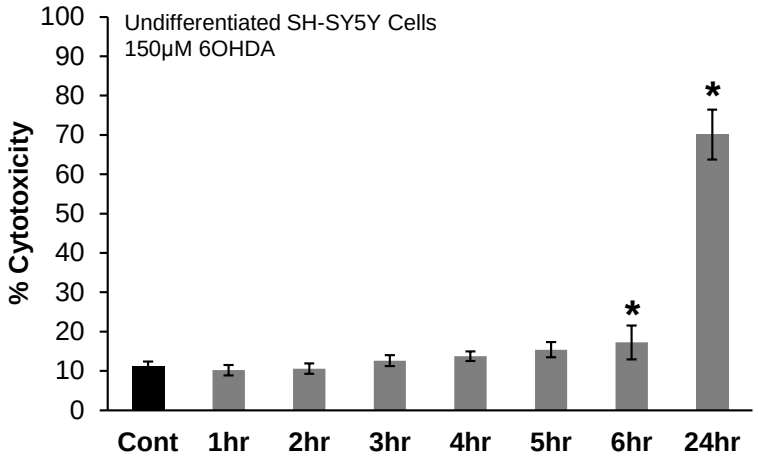

Figure 2: Cell Viability in Response to Pre-lethal 6OHDA

LDH release assay showed no evidence of toxicity until 6hr of 150μM 6OHDA treatment in undifferentiated SH-SY5Y cells. Mean +/- SEM, *$p < 0.05$ vs. Cont. Compiled from 3 independent experiments.

It was also of great interest to develop a sub-lethal oxidative stress injury model where the effects of more long-term oxidative stress, as is the case in aging and neurodegenerative diseases such as PD, can be studied. For these studies, examining neurite integrity was of particular interest and hence SH-SY5Y cells were differentiated with retinoic acid (RA) as described in the Methods Section. Viability assays were carried out in differentiated cells treated with varying concentrations of 6OHDA over a time of 24hr – 72hr. First, over the course of 24hr, the effects of various concentrations of 6OHDA were examined via both LDH release and MTS assays (Fig. 3A & 3B). Concentrations ≤ 30μM did not cause cell death, which was a consistent observation in both toxicity assays. Allowing cells to grow for another 48hr after a 24hr 6OHDA treatment also did not lead to cell death for doses ≤ 30μM, suggesting that the cells were not irreversibly damaged (Fig. 3C). Furthermore, cells were treated every 24hr for a total

of 72hr with various concentrations of 6OHDA and again doses ≤ 30μM did not cause cell death (Fig. 3D). Only the MTS assay was carried out for Fig. 3C & 3D because media change was required for these treatment paradigms, making the LDH release assay incompatible since any LDH released into the media would have been washed away with the media changes.

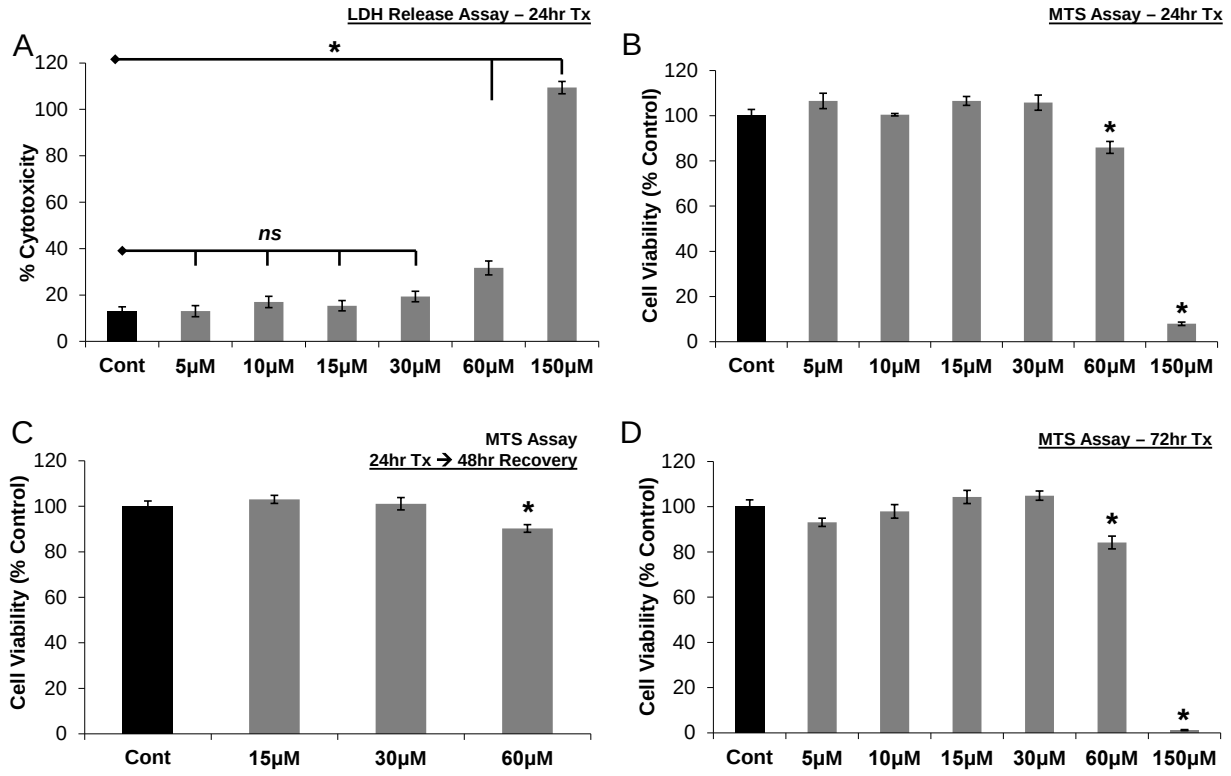

Figure 3: Cell Viability in Response to Sub-lethal 6OHDA

Differentiated SH-SY5Y cells were treated with various concentration of 6OHDA from 24hr to 72hr. (A & B) LDH release and MTS assays – no evidence of toxicity for 6OHDA concentrations ≤ 30μM (24hrs) was observed. (C) MTS assay – cells were treated for 24hr with 15μM to 60μM 6OHDA and then allowed to recover for another 48hr. No significant cell loss was observed for concentrations ≤ 30μM. (D) MTS assay – cells were treated three times (every 24hr) with varying concentrations of 6OHDA and no significant cell loss was observed for concentrations ≤ 30μM. Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ vs. Cont. Compiled from 3 independent experiments.

In summary, for studying the effects of pre-lethal 6OHDA, undifferentiated SH-SY5Y cells and 150μM 6OHDA with treatment times less than 4hr were used. For studying the effects of sub-lethal 6OHDA, RA-differentiated SH-SY5Y cells and 30μM 6OHDA every 24hr for a

total of 72hr (3 total treatments) were used. Figure 4 summarizes the two models and also shows the morphological change that occurs upon differentiation.

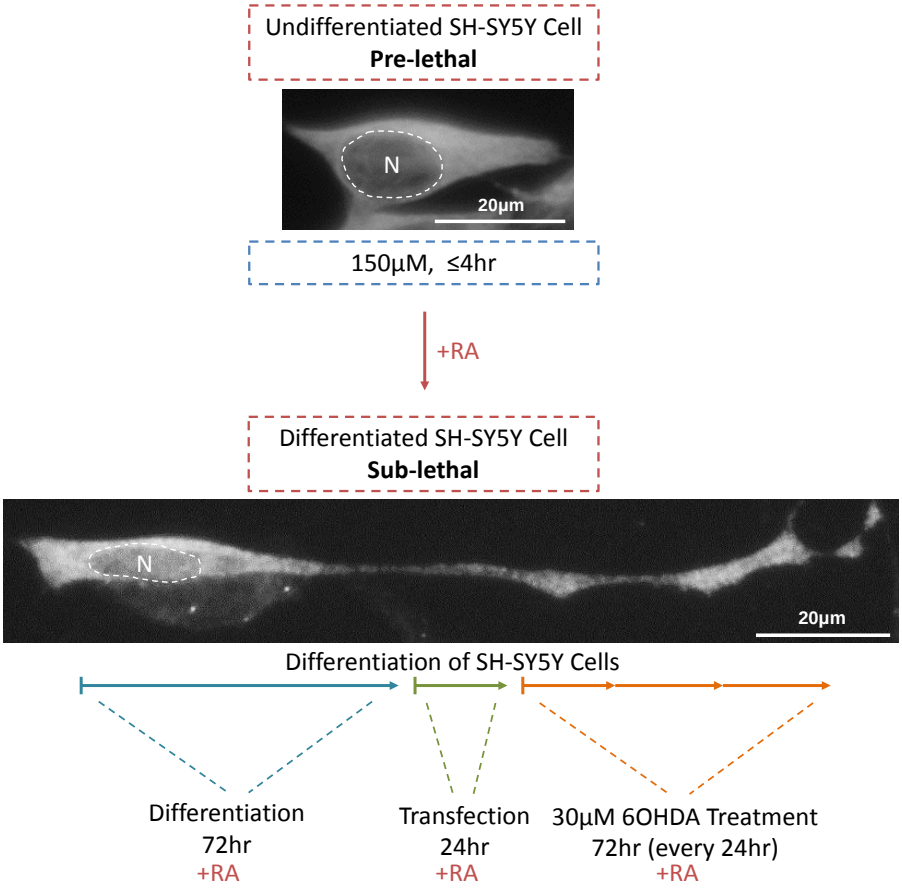

Figure 4: Pre-lethal and Sub-lethal 6OHDA Models

Two 6OHDA injury models are used in this study. Undifferentiated cells are used for pre-lethal treatments (150µM; ≤ 4hr) and retinoic acid (RA)-differentiated cells are used for sub-lethal treatments (30µM; 72hr total treatment time; every 24hr). Upon differentiation, a prominent neuritic process, sometimes two, extends from the cell body. N = nucleus.

4.2 EFFECT OF 6OHDA ON MT DYNAMICS

A couple of studies have suggested that 6OHDA could disrupt MT function. In 1979, Tomlinson *et al.* showed that noradrenergic preterminal fibers of 6OHDA treated rats show disorganized or absent MT staining [193]. In 1986, Davison *et al.* showed that high (millimolar) concentrations

of 6OHDA reduced the polymerization rate of tubulin *in vitro* [64]. These studies involved high concentrations of 6OHDA and did not study MT function in detail. With advancement in molecular biology and imaging technology since the publication of these initial reports, one can now examine detailed MT function in individual cells.

4.2.1 EB Comets

A major method to examine MT function in cells is through the study of +TIPs. When +TIPs, such as EBs, are visualized via transfection of a fluorescently-tagged protein or immunochemical detection of an endogenous protein, a "comet" like pattern is observed (Fig. 5). In Figure 5, endogenous EB1 comets are visualized by immunofluorescence. This unique pattern is thought to be due to disappearance of +TIP binding sites as the MT grows – leading to regions of low (comet tail) and high (comet head) densities of these proteins [84].

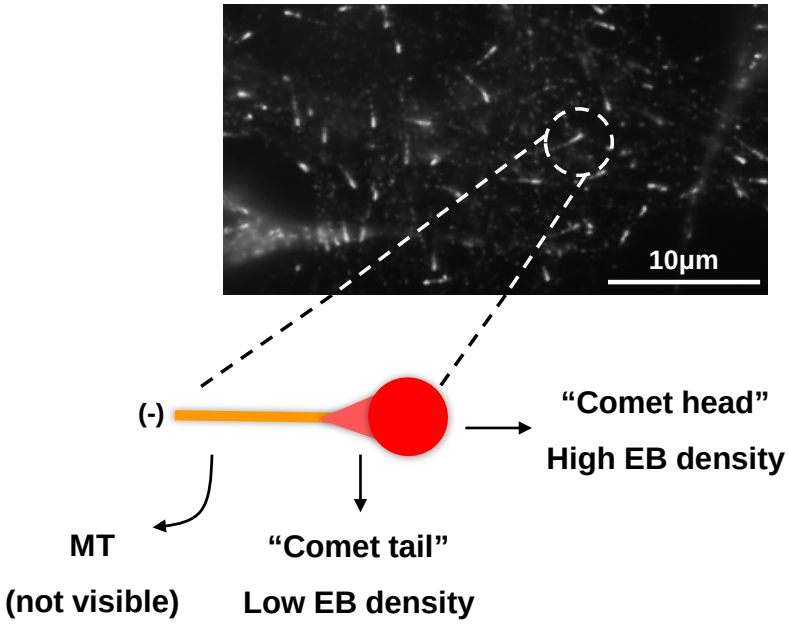

Figure 5: Anatomy of an EB Comet

Methanol-fixed SH-SY5Y cells were immunostained for endogenous EB1. Comet-like pattern is observed with a head (high EB density) and a tail (low EB density). The rest of the MT lattice is not visible. (-) indicates the minus-end of the MT.

4.2.2 Tracking MTs in Live Cells

The effects of pre-lethal and sub-lethal 6OHDA injury on MT dynamics were examined using fluorescently-tagged EB3. The use of fluorescently-tagged +TIPs, such as EBs, have been extensively characterized for the study of MT dynamics in living cells [177,185,194]. Since these proteins bind to the growing (plus) ends of MTs, they allow one to determine the rate of MT growth in cells. Figure 6A shows the comet-like fluorescence for EB3-GFP in a neurite of a differentiated SH-SY5Y cell. A few growing MTs (two are indicated by the arrows) can be seen moving to the right, which is towards the growth cone (not seen in the images).

EBs, along with other +TIPs, only bind to the MTs when they are growing. Therefore, during MT pauses or retractions, the MT ends are not as bright or invisible when using EB3-GFP to visualize them. As seen in Figure 6B, when the MTs pause or retract, the intensity of the comet-like fluorescence of EB3-GFP decreases. If the MT in this example had paused for a longer period of time, all of the EB3-GFP at the MT plus-end would have been lost and the MT would no longer be visible. As the MT beings to grow again, the fluorescent intensity of the EB3-GFP comet is again increased. Given this feature of +TIPs, one can study other important aspects of MT dynamics besides growth rate, such frequency and length of MT pauses/retractions. Since it is difficult at times to differentiate between a pause and a retraction, each “dimming” event will be referred to as a pause and/or retraction event, or a “P/R” event, in subsequent studies.

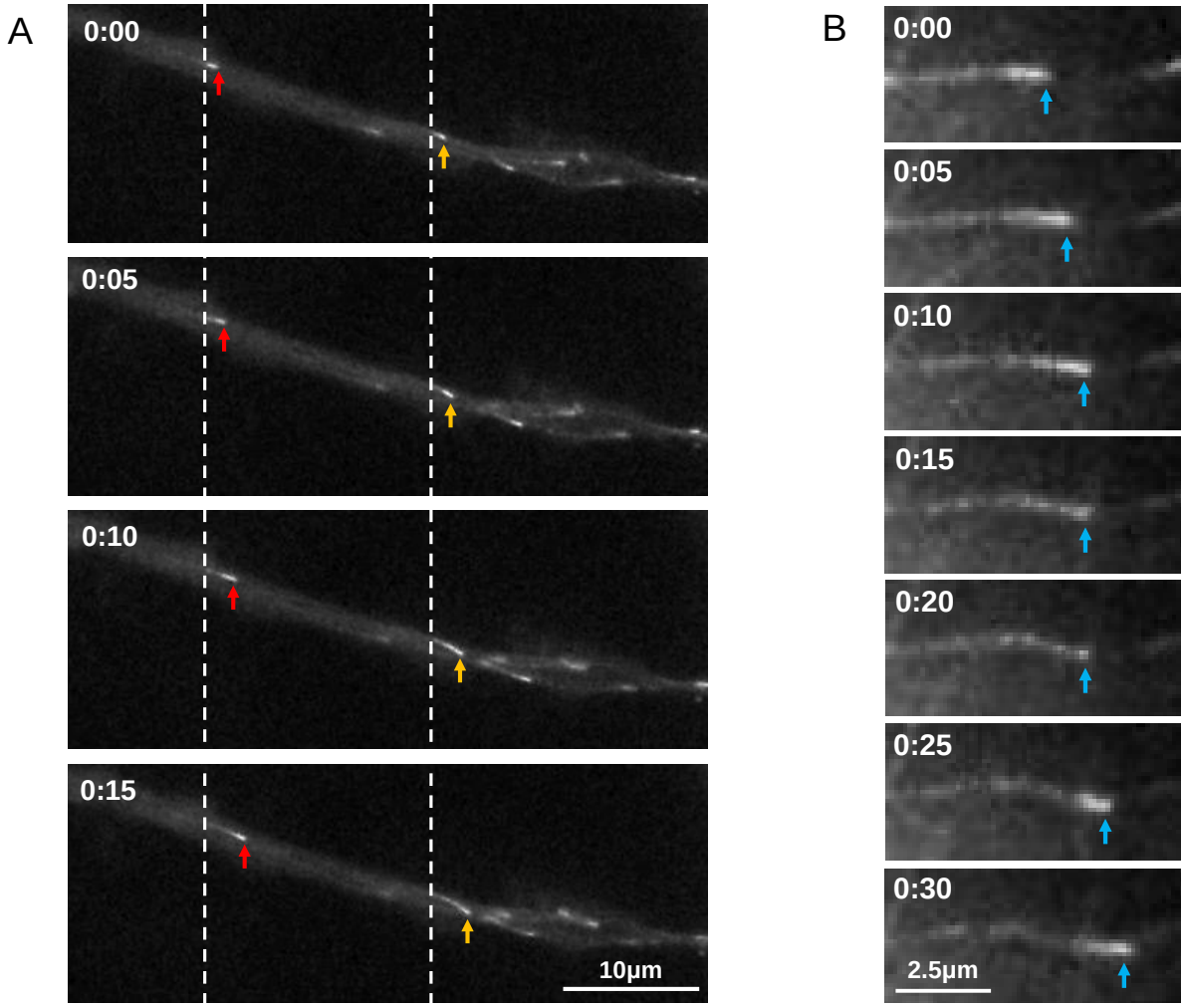

Figure 6: Studying MT Dynamics in Live Cells

SH-SY5Y cells were transfected with an expression plasmid for EB3-GFP. (A) A comet-like pattern of EB3-GFP is seen and the comets are shown (arrows) moving to the right (towards growth cone; not seen in images). (B) An example of a "dimming" event where the EB3-GFP comet loses its fluorescent intensity (0:15 to 0:20), suggesting MT pausing or retraction. Return to the growth phase is noted by the increase in the fluorescent intensity and the forward movement of the comet. Images were taken at 5sec intervals.

4.2.3 Effect of 6OHDA on MT Growth Rate

Using EB3-GFP as a tool to study MT dynamics in live cells, the effects of 6OHDA-induced oxidative stress on MT growth rate was examined. In the pre-lethal 6OHDA model, a significant decrease in MT growth rate was observed (Fig. 7A). With a low-dose and long-term exposure to

6OHDA (sub-lethal), a less dramatic but still statistically significant decrease in MT growth rate was observed (Fig. 7B). For the sub-lethal injury model, MTs along the neurite, not the cell body, were examined since neuritic biology is of particular interest in this study.

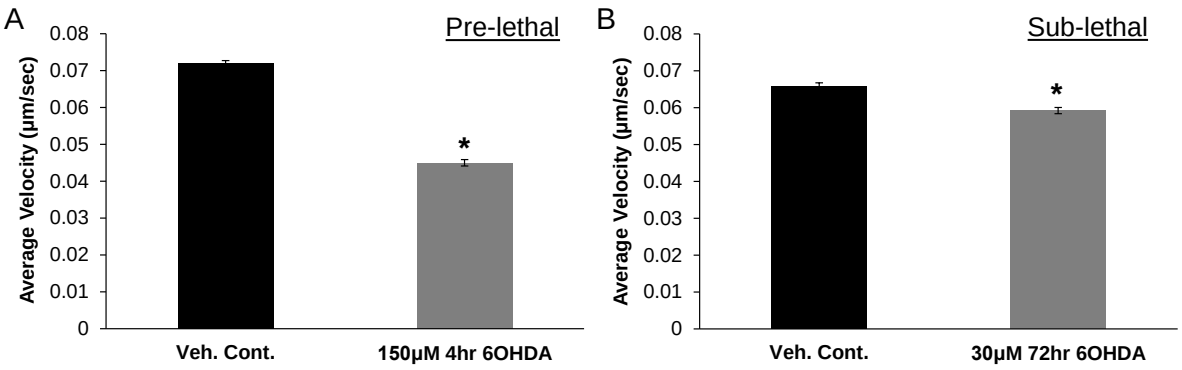

Figure 7: 6OHDA Reduces MT Growth Rate

(A) Undifferentiated SH-SY5Y cells expressing EB3-GFP were treated with 150µM 6OHDA for 4hr (Pre-lethal). Oxidatively-stressed cells show reduced MT growth rates. (B) Differentiated SH-SY5Y cells expressing EB3-GFP were treated with 30µM 6OHDA for 72hr (treatment every 24hr) (Sub-lethal). MTs along the length of the neurite in 6OHDA-treated cells show reduced growth rates. Mean +/- SEM, $*p < 0.05$ vs. Veh. Cont. $n = 150 – 200$ EB3-GFP comets per group compiled from 3 independent experiments.

4.2.4 Effect of 6OHDA on MT Pauses / Retractions

Although a reduction in MT growth rate was observed in cells treated with sub-lethal doses of 6OHDA, the effect was more subtle than that observed in the pre-lethal condition. As previously mentioned, there are other aspects of MT dynamics that are just as important, such as MT pauses and retractions. Hence, in the sub-lethal oxidative stress model, MTs along the length of the neurite were also analyzed for parameters such as frequency of MT pauses/retractions (P/R event), time spent pausing/retracting, and time elapsed between pauses/retractions. As mentioned above, an EB3-GFP "dimming" event is referred to as a "P/R" event. With sub-lethal 6OHDA stress, an increase in the frequency of P/R events is observed (Fig. 8A). 6OHDA treatment caused individual MTs to pause/retract more over a given period of time as seen in

Figure 8B – a lower percentage of 0 and 1 P/R events and a greater percentage of 2 and 3+ P/R events is observed. The amount of time that elapses between two P/R events was reduced in 6OHDA treated cells (Fig. 8C). If there is an increase in number of events per time, it would make sense that the time between each event would be reduced. However, this is the case only if the time elapsed in each P/R event is the same, which is in fact the case with sub-lethal 6OHDA injury (Fig. 8D). Hence, significant alterations in many aspects of MT dynamics, including MT growth rate and frequency of pauses/retractions, are observed with sub-lethal oxidative stress.

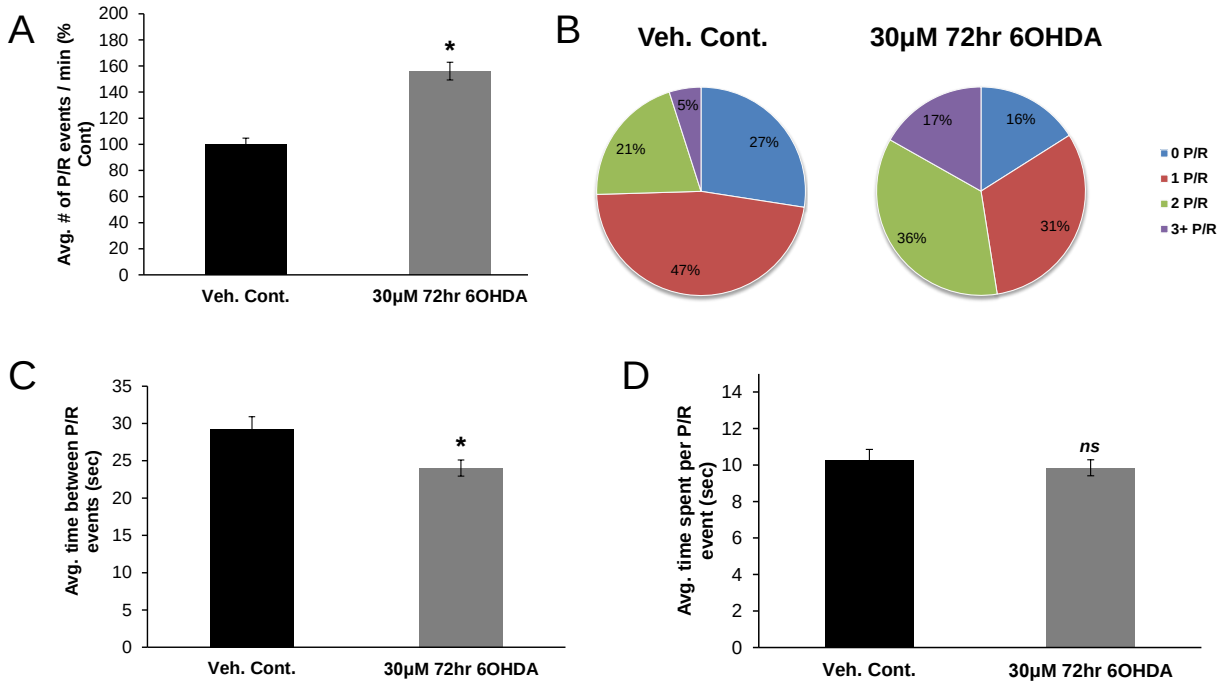

Figure 8: Sub-lethal 6OHDA Alters MT Pauses/Retractions

SH-SY5Y cells were treated with sub-lethal 6OHDA and MT dynamics was examined. (A & B) An increase in the frequency / number of MT pauses and retractions (P/R) is seen after treatment. (C) The average amount of time between two P/R events is reduced with treatment; however the amount of time spent in an individual P/R event is not altered (D). Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ vs. Veh. Cont. n = 100 – 200 EB3-GFP comets per group compiled from 3 independent experiments.

4.3 EFFECT OF 6OHDA ON END BINDING PROTEINS

4.3.1 Effect of Pre-lethal 6OHDA on EB1 Comet Length

In addition to studying MT dynamics in live cells expressing EB3-GFP, the effects of 6OHDA on endogenous EBs were also studies. As discussed previously, EBs are an important group of +TIPs as they play a key role in the regulation of MT dynamics. Interestingly, the length of the EB comet is positively correlated with MT growth rate [175-177]. Hence, this feature allows one to indirectly determine MT growth rate in cells based on the EB comet length. With pre-lethal 6OHDA injury, cells showed decreased EB1 comet length (Fig. 9A & 9B). This result is in agreement with reduced MT growth velocities seen in live cells and serves as another way to confirm that 6OHDA has a significant effect on MT dynamics.

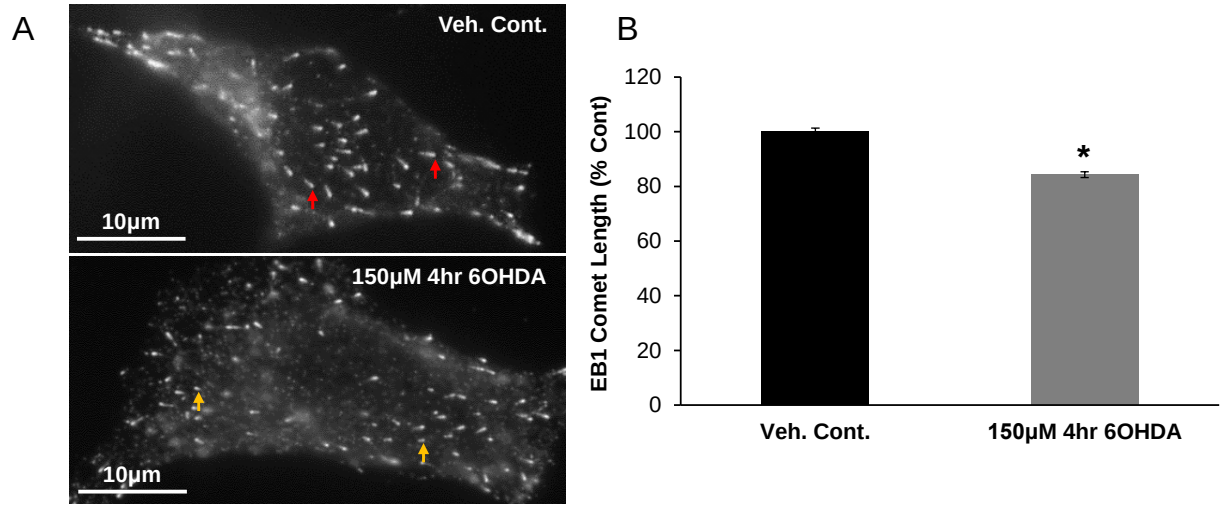

Figure 9: Pre-lethal 6OHDA Reduces EB1 Comet Length

SH-SY5Y cells treated with a pre-lethal dose of 6OHDA were fixed and immunostained for endogenous EB1. (A) Representative images of control and 6OHDA treated cells showing reduction in comet length. (B) Quantification of comet length shows a reduction with 6OHDA treatment. Mean +/- SEM, *$p < 0.05$ vs. Veh. Cont. $n = 200 – 300$ EB1 comets per group compiled from 3 independent experiments.

4.3.2 Effect of Sub-lethal 6OHDA on EB1 Levels

The effects of sub-lethal 6OHDA injury on EB1 along the length of the neurite was also examined (Fig. 10). A more punctate pattern of EB1 fluorescence is observed along the neurite in control cells, making comet length analysis more difficult and possibly less meaningful (Fig. 10A). However, the levels of EB1 can be accurately assessed and can also provide very valuable information about MT dynamics. As can be seen in the representative images in Figure 10A, sub-lethal 6OHDA decreased EB1 staining along the length of the neurite. Western blot analysis of total cellular EB1 levels showed a reduction with 6OHDA treatment, which confirms the immunocytochemical analysis (Fig. 10B).

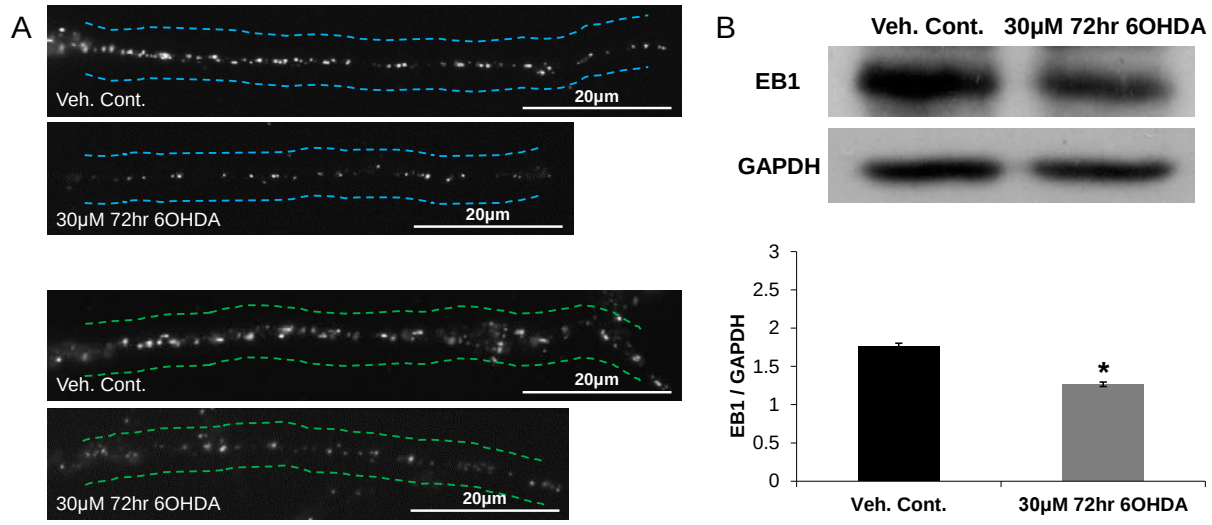

Figure 10: Sub-lethal 6OHDA Reduces EB1 Levels

SH-SY5Y cells were treated with sub-lethal 6OHDA and EB1 levels were analyzed via immunocytochemistry and Western blotting. (A) Two sets of representative images are shown where reduced EB1 staining is noted along the length of the neurite in 6OHDA treated cells. (B) Western blot of total cellular EB1 levels shows a reduction with 6OHDA treatment. Mean +/- SEM, *$p < 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

4.4 EFFECT OF 6OHDA ON THE TOTAL MT NETWORK

Given the significant impact of 6OHDA-induced oxidative stress on MT dynamics, it was of interest to determine if the overall MT network was affected in treated cells. Neither pre-lethal nor sub-lethal 6OHDA treatment altered the overall MT organization in cells as determined by immunostaining for α-tubulin (Fig. 11).

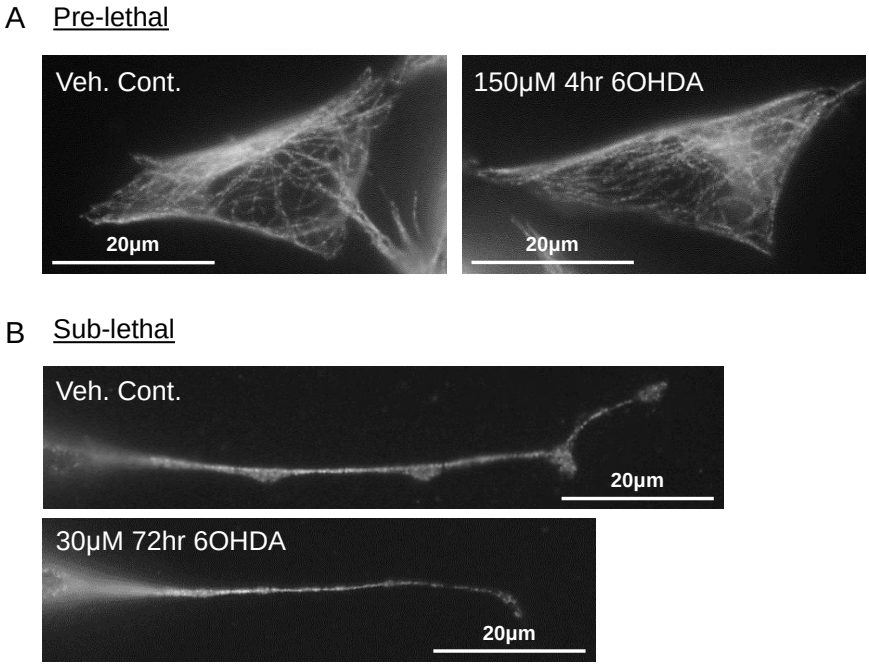

Figure 11: 6OHDA Does Not Alter Overall MT Organization

SH-SY5Y cells treated with pre-lethal (A) or sub-lethal (B) 6OHDA were fixed and immunostained for α-tubulin. No significant alterations in the overall organization of MTs were noted. Representative images of 3 independent experiments.

The levels of total α-tubulin were also examined in 6OHDA treated cells. Neither pre-lethal nor sub-lethal 6OHDA treatment caused a significant change in cellular α-tubulin levels (Fig. 12A & 12B). Together with the immunocytochemical data, the results suggest more specific alterations in MT dynamics as opposed to gross alterations in the MT network.

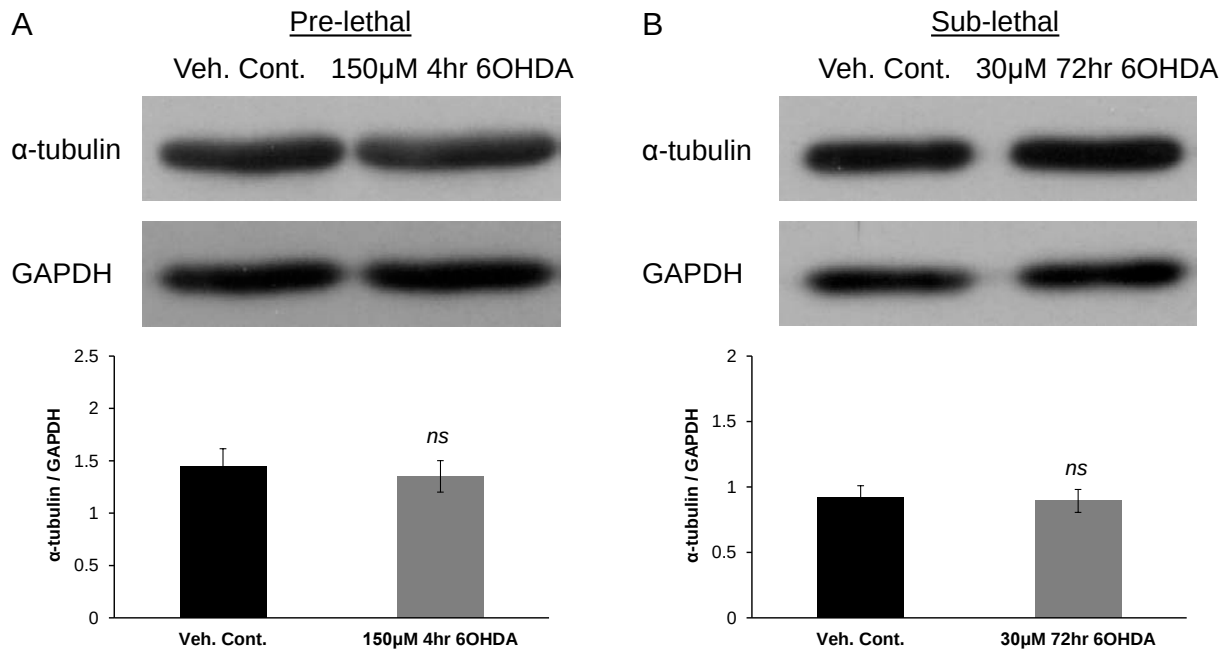

Figure 12: 6OHDA Does Not Alter α-Tubulin Levels

Total α-tubulin levels were examined in SH-SY5Y cells treated with pre-lethal (A) or sub-lethal (B) 6OHDA. 6OHDA did not cause any significant alterations in levels of α-tubulin. Mean +/- SEM, $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

4.5 EFFECT OF 6OHDA ON TUBULIN ACETYLATION

In addition to +TIPs, tubulin post-translational modifications are another major mechanism by which MT function is regulated. One such modification is tubulin acetylation, which not only serves as maker of the stable pool of MTs but has been shown to regulate binding of MAPs and MT dynamics. Furthermore, the PD toxin MPP+ has been shown alter the tubulin acetylation levels [154]. Hence, the effect of 6OHDA-induced oxidative stress on tubulin acetylation levels was also examined in this study.

4.5.1 Detection of Acetylated MTs

The acetylated tubulin antibody, which detects acetylation at Lys-40 of α-tubulin, used in this study has been extensively utilized and validated in the MT field. Nonetheless, this antibody was first validated for its ability to specifically detect tubulin acetylation for this study. SH-SY5Y cells were treated with colchicine (1μM; 1hr), a MT depolymerizing agent, and acetylated tubulin was detected by immunocytochemical (Fig. 13A) and Western blot analyses (Fig. 13B). As can be seen via immunocytochemistry, acetylated MTs are dense around the centrosome from which fiber-like processes emanate and minimal acetylated MTs are seen at the cell periphery, where more dynamic MTs are found (Fig. 13A). A reduction in tubulin acetylation levels was observed with colchicine treatment as expected – confirming that acetylated tubulin can be specifically detected.

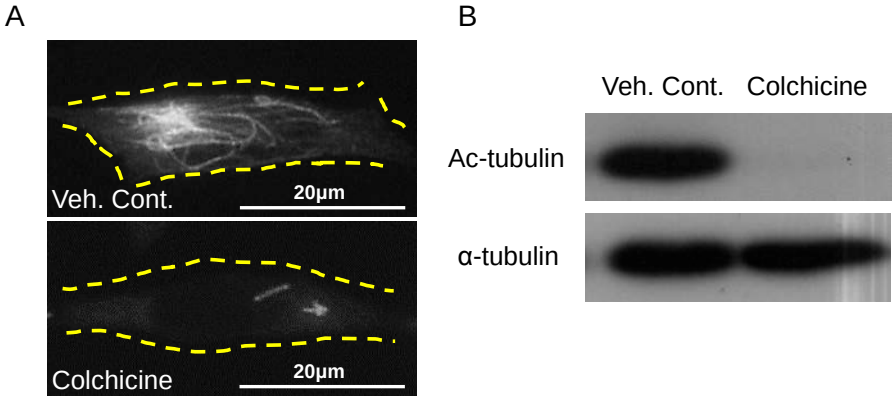

Figure 13: Detection of Acetylated MTs

SH-SY5Y cells were treated with the MT depolymerizing agent colchicine (1μM; 1hr) and immunostained (A) or Western blotted (B) for acetylated tubulin. A reduction in the level of acetylated tubulin was seen with colchicine treatment.

4.5.2 Effect of 6OHDA on Levels of Acetylated MTs

The effects of pre-lethal and sub-lethal doses of 6OHDA on acetylation of MTs were examined in SH-SY5Y cells via Western blot analysis. A significant increase in levels of acetylated tubulin was observed after 6OHDA treatment (Fig. 14). The effect was more prominent after a pre-lethal injury (Fig. 14A) as compared to a sub-lethal injury (Fig. 14B).

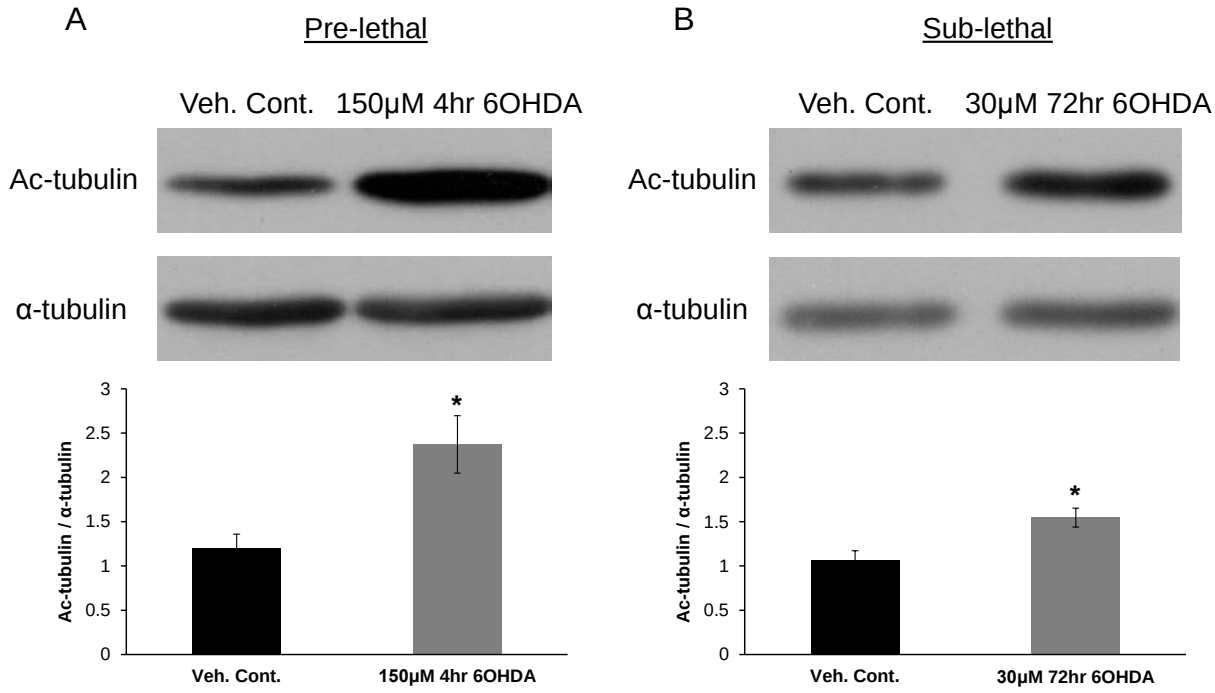

Figure 14: 6OHDA Increases MT Acetylation

SH-SY5Y cells were treated with pre-lethal (A) or sub-lethal (B) 6OHDA and levels of tubulin acetylation were examined via Western blot analysis. A significant increase in tubulin acetylation was observed in treated cells. Mean +/- SEM, $*p < 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

4.6 EFFECT OF 6OHDA ON NEURITE LENGTH

One of the earliest changes seen in neurodegenerative diseases, such as PD, is the degeneration of axons [4]. MTs play a key role in the development and maintenance of axons / neuritic

processes and 6OHDA-induced oxidative stress significantly affects MT function. Hence, the effect of sub-lethal oxidative stress, which more closely mimics the injury that occurs in PD, on neurite integrity was examined. Differentiated SH-SY5Y cells expressing GFP were treated with sub-lethal 6OHDA, which resulted in a reduction of neurite length (Fig. 15). Hence, sub-lethal 6OHDA injury was able to reproduce a very important aspect of neuronal degeneration that is seen in PD.

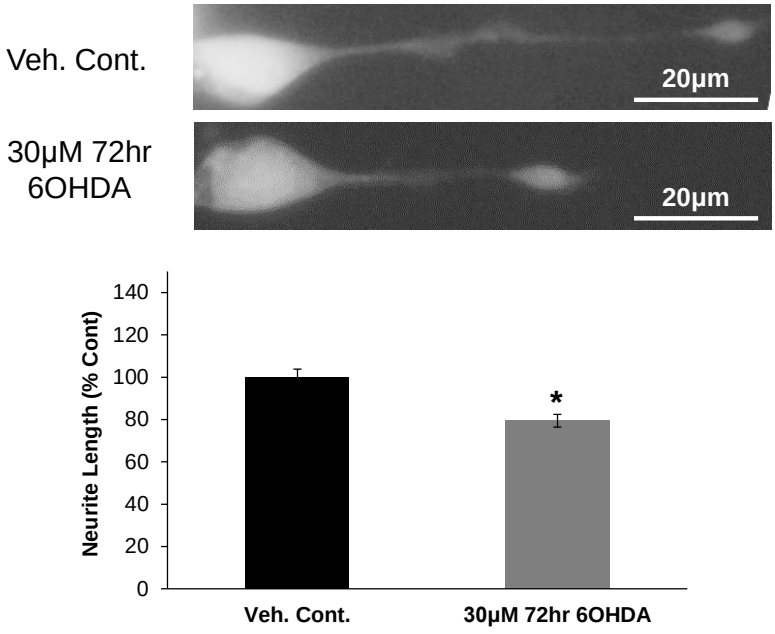

Figure 15: Sub-lethal 6OHDA Reduces Neurite Length

Differentiated SH-SY5Y cells were transfected with GFP and treated with sub-lethal 6OHDA. A significant reduction in neurite length was observed with 6OHDA treatment. Mean +/- SEM, *$p < 0.05$ vs. Veh. Cont. $n = 150 – 200$ cells per group compiled from 3 independent experiments.

4.7 EFFECT OF 6OHDA ON TUBULIN DEACETYLASES

Given that tubulin PTMs can affect many aspects of MT function, the mechanism(s) by which such a significant change in tubulin acetylation was occurring in 6OHDA treated cells was then examined. Alteration in tubulin acetylation levels suggests the involvement of tubulin acetylases

and/or deacetylases. As previously discussed, tubulin acetylases have only been recently identified and not much is known about their regulation. However, many studies have examined in detail the tubulin deacetylases, HDAC6 and SIRT2. Interestingly, oxidative stressors, e.g. H_2O_2 and cigarette smoke, has been linked to altered localization, levels, and/or activity of various HDACs, e.g. HDAC1, HDAC2, HDAC4, and SIRT1, in a variety of models [195-201]. Given the structural similarities of the deacetylase domain between the various classes of HDACs, it is possible that 6OHDA-induced oxidative stress may affect HDAC6 and/or SIRT2 function. Hence, the effect of 6OHDA on tubulin deacetylases was studied by examining their subcellular localization, protein levels, and enzymatic activity.

4.7.1 Effect of 6OHDA on HDAC6 and SIRT2 Subcellular Localization

HDAC6 and SIRT2 are cytoplasmic proteins but have been shown under certain conditions to be present in the nucleus. Upon differentiation of B16 mouse melanoma cells by butyrate where cell cycle is arrested, a small amount of HDAC6 can be found in the nuclear fraction [124]. For SIRT2, a very small fraction is also found in the nucleus in HeLa and 293T cells [138,143]. Both proteins contain a nuclear export signal that helps maintain their predominant cytoplasmic localization. Given the possibility that HDAC6 or SIRT2 can be found in the nucleus, the effect of 6OHDA on their subcellular localization was examined.

SH-SY5Y cells were treated with a pre-lethal dose of 6OHDA and endogenous HDAC6 was visualized via immunocytochemistry (Fig. 16A). A complete cytoplasmic localization of HDAC6 was observed and treatment with pre-lethal 6OHDA did not cause a change in its subcellular distribution (Fig. 16A). In addition, to confirm the results of endogenous HDAC6, a FLAG-tagged HDAC6 was also transfected in cells and detected via anti-FLAG antibody (Fig.

16A; lower panel). Overexpressed HDAC6 also showed a cytoplasmic localization which did not change with pre-lethal 6OHDA treatment. Endogenous HDAC6 localization was then examined in differentiated SH-SY5Y cells treated with sub-lethal 6OHDA (Fig. 16B). As with pre-lethal 6OHDA, a cytoplasmic localization of HDAC6 was seen in control cells, which did not change with 6OHDA treatment. Hence, no significant alteration in the subcellular localization of HDAC6 was seen in either 6OHDA injury models.

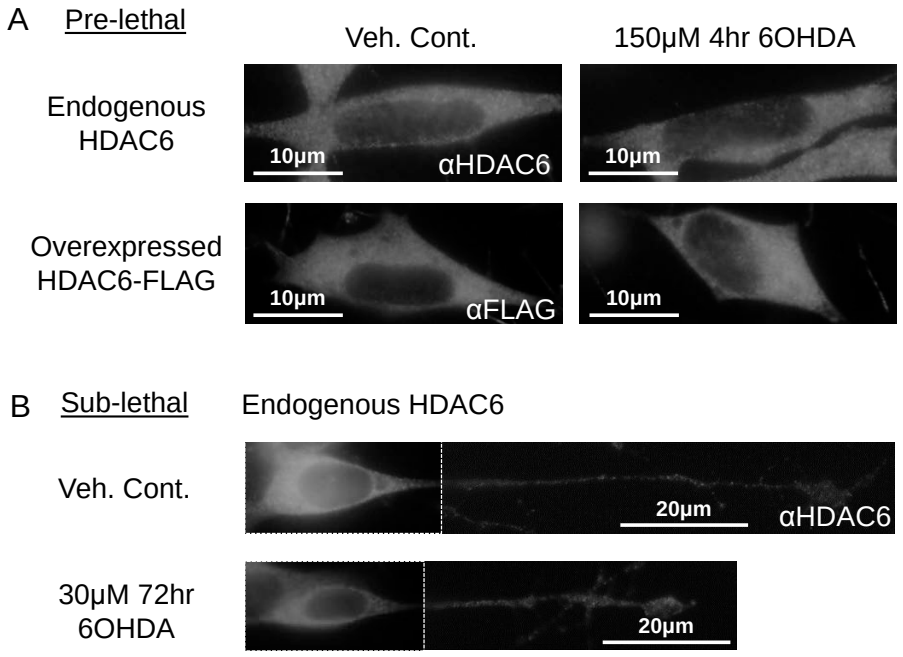

Figure 16: 6OHDA Does Not Alter the Subcellular Localization of HDAC6

(A) Undifferentiated SH-SY5Y cells were treated with a pre-lethal dose of 6OHDA. Endogenous (αHDAC6) and overexpressed HDAC6 (αFLAG) were detected via immunocytochemistry. No significant change in the subcellular localization of HDAC6 was observed. (B) Endogenous HDAC6 was detected in differentiated SH-SY5Y cells and no change in its subcellular localization was observed after sub-lethal 6OHDA. Two separate images are overlaid (marked by the dotted white line), one with the cell body in focus and one with the neurite in focus. Representative images of 3 independent experiments are shown.

Similar studies were also carried out for SIRT2 (Fig. 17). SH-SY5Y cells were treated with a pre-lethal dose of 6OHDA and endogenous SIRT2 was visualized via immunocytochemistry (Fig. 17A). A cytoplasmic localization of SIRT2 was observed (no nuclear SIRT2 was ever seen in these cells) and treatment with pre-lethal 6OHDA did not cause

a change in its subcellular localization (Fig. 17A). Furthermore, overexpressed SIRT2 also showed a cytoplasmic localization which did not change with pre-lethal 6OHDA treatment (Fig. 17A; lower panel). Endogenous SIRT2 localization was also examined in differentiated SH-SY5Y cells treated with sub-lethal 6OHDA (Fig. 17B). As with pre-lethal 6OHDA, a cytoplasmic localization of SIRT2 was seen in control cells, which did not change with 6OHDA treatment. Hence, as seen with HDAC6, no significant alteration in the subcellular localization of SIRT2 was observed after 6OHDA-induced oxidative stress.

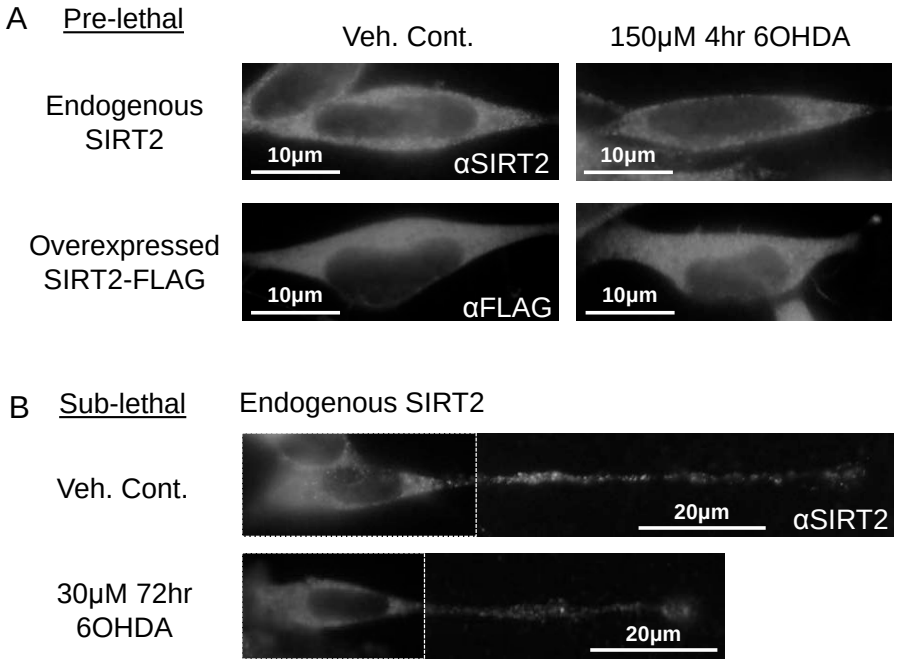

Figure 17: 6OHDA Does Not Alter the Subcellular Localization of SIRT2

(A) Undifferentiated SH-SY5Y cells were treated with a pre-lethal dose of 6OHDA. Endogenous (αSIRT2) and overexpressed SIRT2 (αFLAG) were detected via immunocytochemistry. No significant change in the subcellular localization of SIRT2 was observed. (B) Endogenous SIRT2 was detected in differentiated SH-SY5Y cells and no change in its subcellular localization was observed after sub-lethal 6OHDA. Two separate images are overlaid (marked by the dotted white line), one with the cell body in focus and one with the neurite in focus. Representative images of 3 independent experiments are shown.

4.7.2 Effect of 6OHDA on HDAC6 and SIRT2 Protein Levels

Endogenous protein levels of HDAC6 and SIRT2 in 6OHDA treated cells were also examined. Levels of HDAC6 were not altered in response to either pre-lethal or sub-lethal 6OHDA (Fig. 18A & 18B). Hence, changes in HDAC6 protein levels do not contribute to the increased tubulin acetylation that is observed.

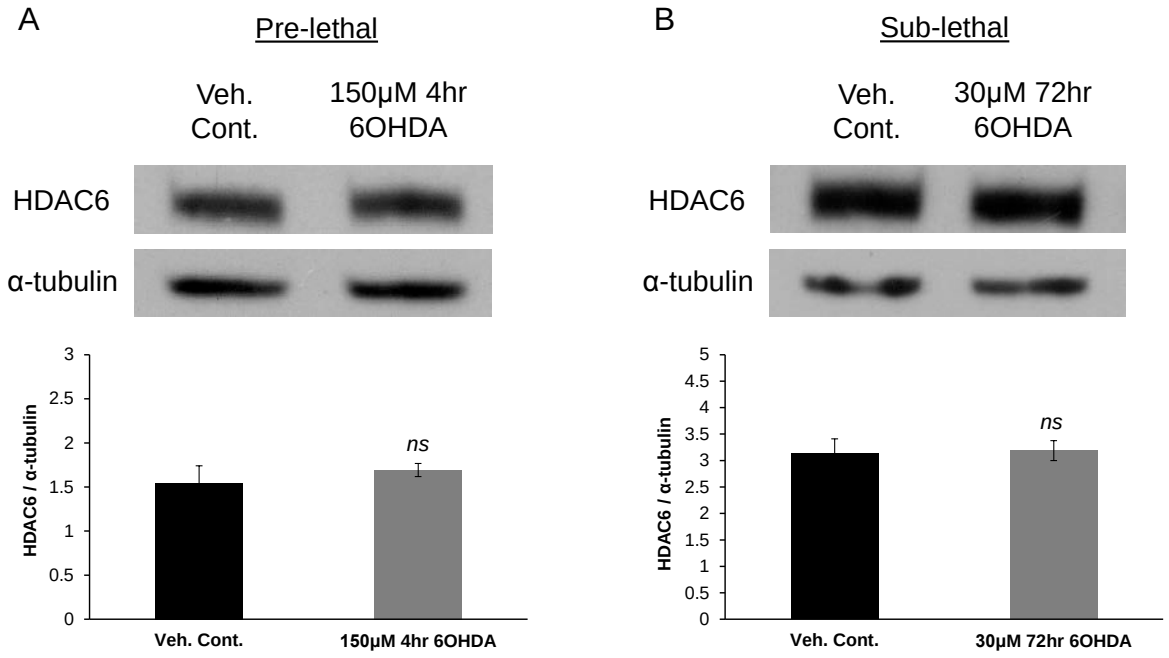

Figure 18: 6OHDA Does Not Alter HDAC6 Protein Levels

SH-SY5Y cells were treated with pre-lethal (A) or sub-lethal (B) 6OHDA and HDAC6 protein levels were examined via Western blot analysis. No significant changes in protein levels were detected. Mean +/- SEM, $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

For SIRT2, although not statistically significant, pre-lethal dose of 6OHDA caused a trend towards a reduction in protein levels (Fig. 19A). This trend was consistently observed in the majority of the replicates. However, sub-lethal 6OHDA did not significantly alter SIRT2 protein levels and no consistent pattern was observed despite the quantified results showing a trend towards an increase (Fig. 19B).

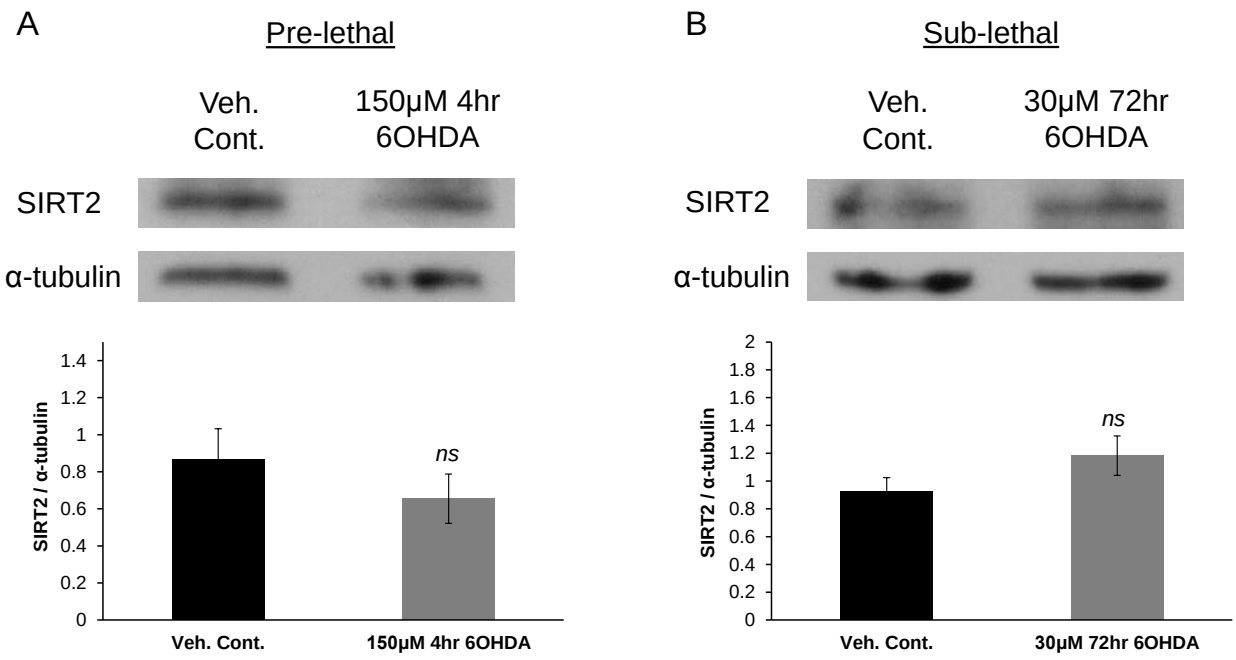

Figure 19: 6OHDA Does Not Alter SIRT2 Protein Levels

SH-SY5Y cells were treated with pre-lethal (A) or sub-lethal (B) 6OHDA and SIRT2 protein levels were examined via Western blot analysis. No statistically significant changes in protein levels were detected although a consistent trend towards reduced SIRT2 levels was seen after pre-lethal treatment. Mean +/- SEM, $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

Hence, in the pre-lethal injury model, it is possible that reduced SIRT2 levels contribute to an increase in tubulin acetylation. However, this effect is not statistically significant and hence other mechanisms that could explain increased tubulin acetylation levels in both the pre-lethal as well as the sub-lethal models were explored.

4.7.3 Effect of 6OHDA on HDAC6 and SIRT2 Deacetylase Activity

Deacetylase activity of various HDACs has been shown to be sensitive to oxidative stress. Hence, the effect of 6OHDA-induced oxidative stress on the deacetylase activity of HDAC6 and SIRT2 was examined. First, immunoprecipitation of endogenous HDAC6 and deacetylase activity of the pulled-down protein was examined (Fig. 20). Figure 20A shows the strong pull-

down of HDAC6. Various substrate and developer incubation times were also examined to determine the linear range of the enzymatic reaction, which was used for all subsequent experiments looking at HDAC6 activity (Fig. 20B). Pulled-down HDAC6 showed significant activity above IgG control (Fig. 20C).

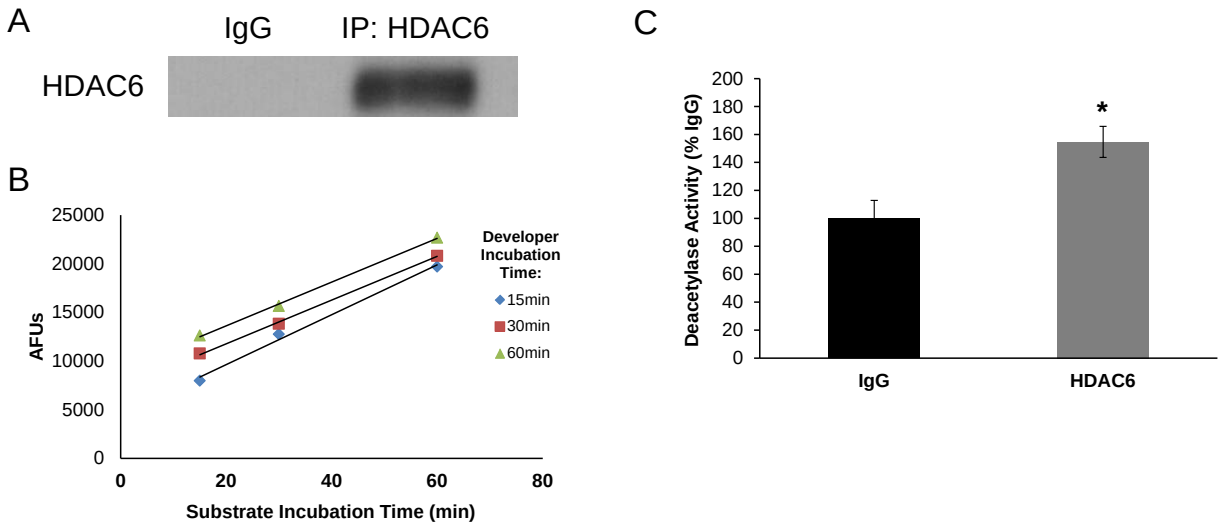

Figure 20: HDAC6 Deacetylase Activity

(A) Endogenous HDAC6 was pulled-down in SH-SY5Y cells. (B) Activity of HDAC6 was examined at various substrate incubation times and with varying developer incubation times. Times in the linear range were chosen for subsequent experiments. R^2 values > 0.99. AFUs = Artificial Fluorescence Units. (C) Significant HDAC6 activity was seen above IgG control. Mean +/- SEM, *$p < 0.05$ vs. IgG. Compiled from 3 independent experiments.

HDAC6 was pulled-down from equivalent amount of lysates collected from SH-SY5Y cells treated with either pre-lethal or sub-lethal 6OHDA and the deacetylase activity was examined. Fluorescence values of IgG control were subtracted from the HDAC6 activity values and percent change was determined. No significant change in the deacetylase activity of the pulled-down enzyme was detected in either of the 6OHDA-induced oxidative stress models (Fig. 21). Hence, altered deacetylase activity of HDAC6 does not contribute to the increased level of tubulin acetylation that is observed.

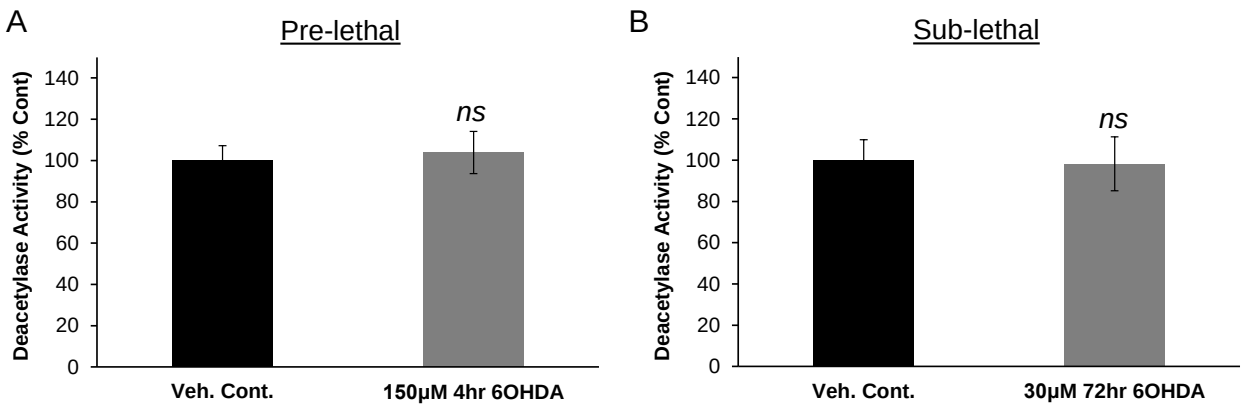

Figure 21: 6OHDA Does Not Alter HDAC6 Deacetylase Activity

SH-SY5Y cells were treated with either pre-lethal (A) or sub-lethal (B) 6OHDA and the deacetylase activity of pulled-down HDAC6 was examined. No significant change in enzymatic activity was observed. Mean +/- SEM, $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

The deacetylase activity of SIRT2 was then examined. Again, endogenous SIRT2 pull-down was first confirmed and a linear range of substrate and developer incubation times was determined for subsequent studies (Fig. 22A & 22B). The arrow in Fig. 22A points to a non-specific band seen only in the IgG lane (likely an IgG heavy chain fragment). SIRT2 deacetylase activity was seen above IgG control (Fig. 22C). A significant portion of this activity was dependent on NAD+ as would be expected for a sirtuin (Fig. 22D). Although SIRT2 pull-down is not as strong (addressed in the Discussion section) as that seen for HDAC6, it does appear to be enough to see significant activity above IgG control. The SIRT2 antibody strongly detects SIRT2 at the correct molecular weight via Western blotting and has been confirmed for immunoprecipitation by the manufacturer. However, in the immunoprecipitations done in this study, an unknown band in addition to SIRT2 is also observed. This band is stronger than the SIRT2 band that is pulled-down and hence requires follow-up. A detailed discussion of this matter is provided later in the Discussion section.

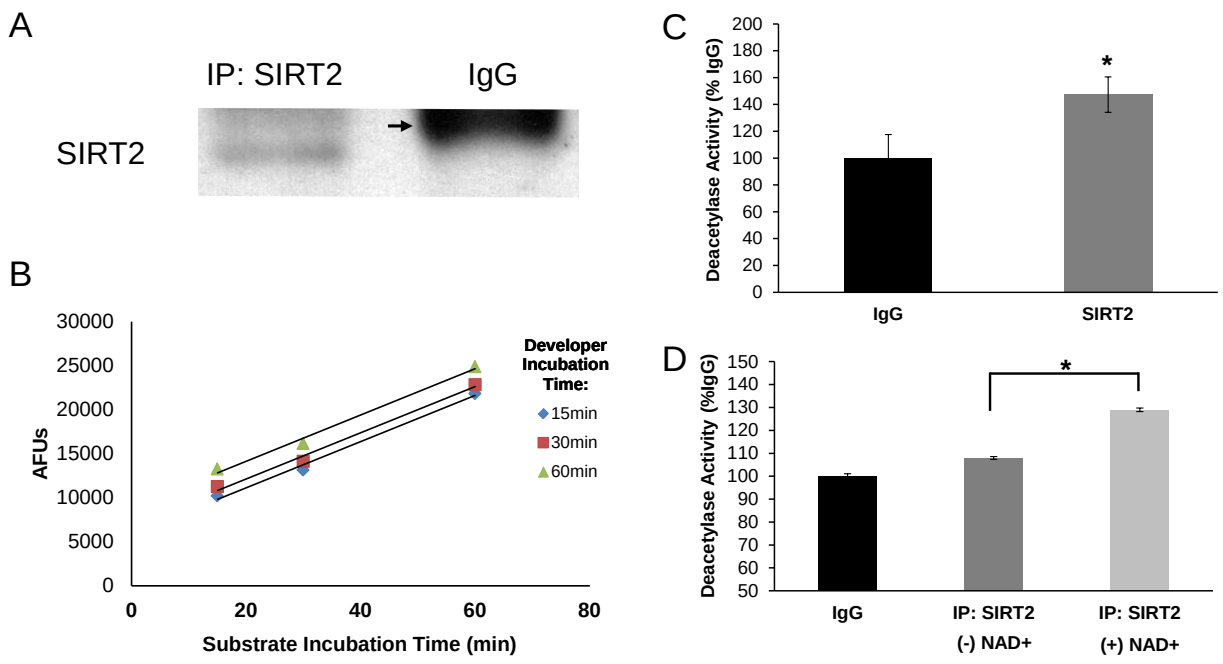

Figure 22: SIRT2 Deacetylase Activity

(A) Endogenous SIRT2 was pulled-down in SH-SY5Y cells. Arrow points to a non-specific band in the IgG lane. (B) Activity of SIRT2 was examined at various substrate incubation times and with varying developer incubation times. Times in the linear range were chosen for subsequent experiments. R^2 values > 0.99. AFUs = Artificial Fluorescence Units. (C) Significant SIRT2 activity was seen above IgG control. Mean +/- SEM, $*p < 0.05$ vs. IgG. Compiled from 3 independent experiments. (D) Deacetylase activity of the pulled-down enzyme is dependent on NAD+. Mean +/- SD, $*p < 0.05$ as indicated.

SIRT2 was pulled-down from equivalent amount of lysates collected from SH-SY5Y cells treated with either pre-lethal or sub-lethal 6OHDA and the deacetylase activity was examined. As with HDAC6, fluorescence values of IgG control were subtracted from the SIRT2 activity values and percent change was determined. For pre-lethal dose of 6OHDA, no significant change in the deacetylase activity of the pulled-down enzyme was detected (Fig. 23A). However, a significant reduction in the deacetylase activity of SIRT2 after sub-lethal injury was observed (Fig. 23B). Hence, reduced SIRT2 activity could account for the increased levels of tubulin acetylation in the sub-lethal oxidative stress injury model.

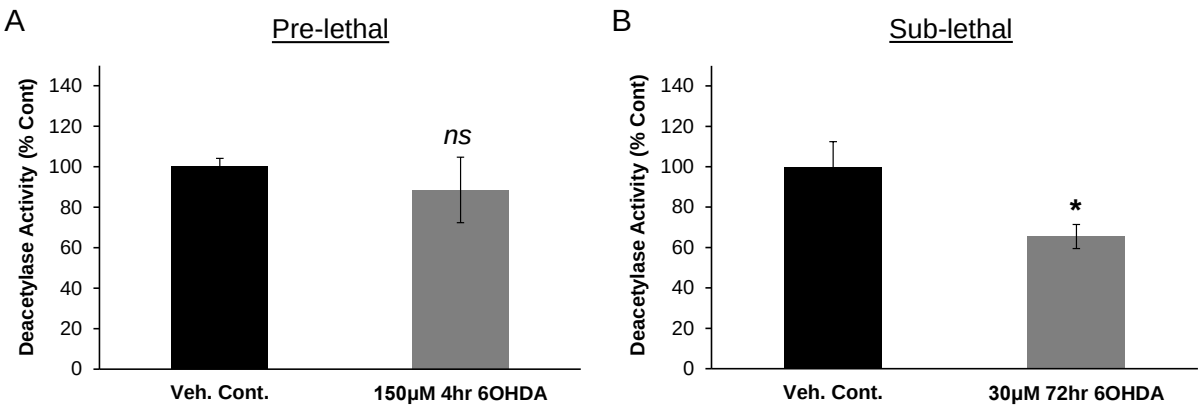

Figure 23: Sub-lethal 6OHDA Reduces SIRT2 Deacetylase Activity

SH-SY5Y cells were treated with either pre-lethal (A) or sub-lethal (B) 6OHDA and the deacetylase activity of pulled-down SIRT2 was examined. (A) For pre-lethal 6OHDA, no significant change in enzymatic activity was observed. (B) Significant reduction in activity was observed for SIRT2 from cells treated with sub-lethal 6OHDA. Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

4.8 EFFECT OF 6OHDA ON NAD+ / NADH LEVELS

Results thus far suggest that reduction in SIRT2 deacetylase activity in the sub-lethal 6ODHA injury model could explain the observed increase in levels of tubulin acetylation. However, only a trend towards a reduction in SIRT2 protein level was seen with pre-lethal 6OHDA injury, where a very robust increase in levels of tubulin acetylation is observed. NAD+ is an essential co-factor for SIRT2 deacetylase activity and is an important determinant of the redox state of the cell. If 6OHDA-induced oxidative stress alters the levels of NAD+ in the pre-lethal model, it could explain how SIRT2 function could be decreased in cells without seeing a decrease in the deacetylase activity assay, which is under optimal conditions where NAD+ is provided. Using an enzyme cycling reaction, the levels of NAD+ and NADH were examined in cells treated with either pre-lethal or sub-lethal 6OHDA. First, the linear range of the assay was determined by varying the number of cells used for extraction and the reaction incubation time (Fig. 24A).

Based on the results, 1 – 2 million cells and a reaction time of 2hr was used for subsequent studies. In addition, cells showed a much higher level of NAD+ than NADH, which is biologically valid (Fig. 24B) [202].

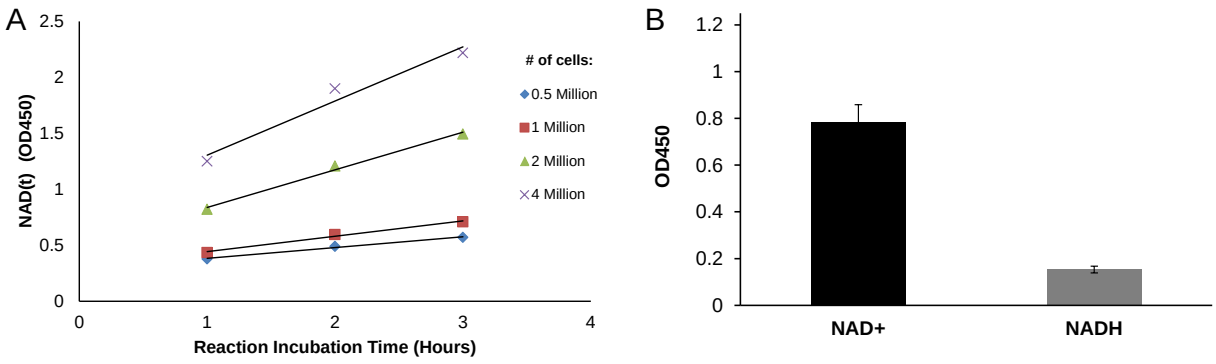

Figure 24: Detection of NAD+ and NADH in Cells

(A) Linear range of the assay was determined using NAD(t) (combined NAD+ and NADH levels) and 1 – 2 million cells and a reaction time of 2hr was used for subsequent studies. $R^2 > 0.99$ for all conditions except for 4 million cells ($R^2 = 0.96$). (B) NAD+ levels were significantly higher in cells than NADH levels. OD450 = Absorbance at 450nm. Compiled from 3 independent experiments.

The effect of 6OHDA-induced oxidative stress on NAD+ and NADH levels was then examined. Pre-lethal 6OHDA treatment caused a significant reduction in the levels of both NAD+ and NADH (Fig. 25A & 25B). However, no such reduction was observed after sub-lethal injury (Fig. 25C & 25D). Hence, in the pre-lethal model, reduction in the levels of the essential co-factor NAD+ can lead to reduced SIRT2 function in cells, causing an increase in the levels of tubulin acetylation.

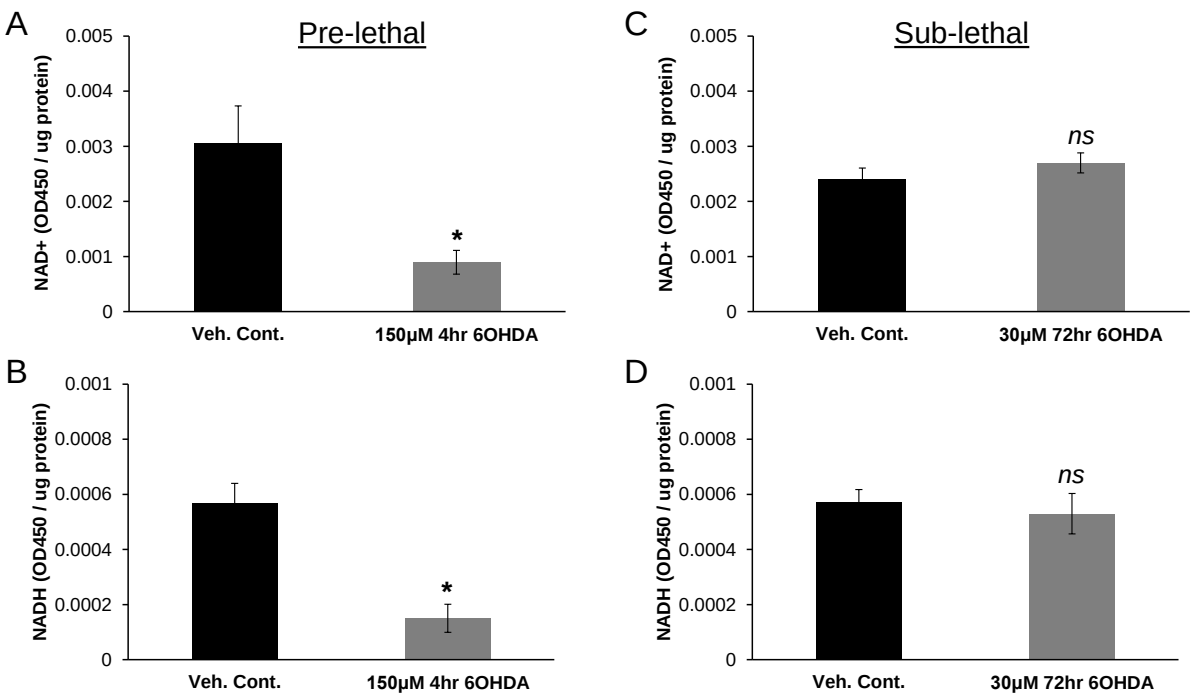

Figure 25: Pre-lethal 6OHDA Reduces NAD+ and NADH Levels

SH-SY5Y cells were treated with either pre-lethal (A & B) or sub-lethal (C & D) 6OHDA. NAD+ and NADH levels were determined via an enzyme cycling reaction and the levels were normalized to total protein. (A & B) Significant reduction in both NAD+ and NADH levels was observed after pre-lethal injury. (C & D) No reduction in levels of either NAD+ or NADH was observed after sub-lethal injury. Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

The combined results suggest that different mechanisms are involved in the altered levels of tubulin acetylation that is observed. After pre-lethal injury, significant reduction in levels of the co-factor NAD+ leads to diminished SIRT2 function in cells. However, after sub-lethal injury, significant reduction in the intrinsic enzymatic activity of SIRT2 leads to diminished SIRT2 function in cells. The end result of these changes is the same – an increase in the levels of tubulin acetylation.

4.9 RESCUE OF MT FUNCTION

Significant changes are observed in the function of tubulin deacetylases, particularly SIRT2, after 6OHDA injury. It is possible that altered function of tubulin deacetylases can, via deacetylase-dependent and/or -independent mechanisms, affect MT dynamics and neurite integrity in 6OHDA-treated cells. Hence, the effects of rescuing impaired deacetylase function on MT dynamics and neurite integrity was examined.

4.9.1 Rescue of MT Dynamics by Increasing NAD+ Levels

Given the reduction in NAD+ levels in the pre-lethal injury model, the effects of rescuing cellular NAD+ levels on MT dynamics was examined. The addition of NAD+ directly to the cellular medium has been shown to increase intracellular NAD+ levels in a variety of cell lines, including SH-SY5Y cells [203-205]. As consistent with the literature, the addition of NAD+ (1mM) for 6hr to the culture medium caused a significant increase in the levels of intracellular NAD+ (Fig. 26A). To examine the effects of NAD+ restoration on 6OHDA-mediated increase in tubulin acetylation levels, cells were treated with vehicle or NAD+ for 6hrs before treating with a pre-lethal dose of 6OHDA (added directly to the medium containing NAD+). 6OHDA-mediated increase in tubulin acetylation was significantly reduced in cells that were pre-treated with NAD+ (Fig. 26B & 26C). To study the effects of NAD+ restoration on MT growth rate, cells expressing fluorescently-tagged EB3 were treated with NAD+ and pre-lethal 6OHDA as mentioned above. MT growth rate was significantly reduced in response to 6OHDA treatment as before. However, in cells that were pre-treated with NAD+, a rescue in MT growth rate was

observed (Fig. 26D). These results suggest that restoration of NAD+ levels and hence SIRT2 function rescues tubulin acetylation levels and MT dynamics in 6OHDA-treated cells.

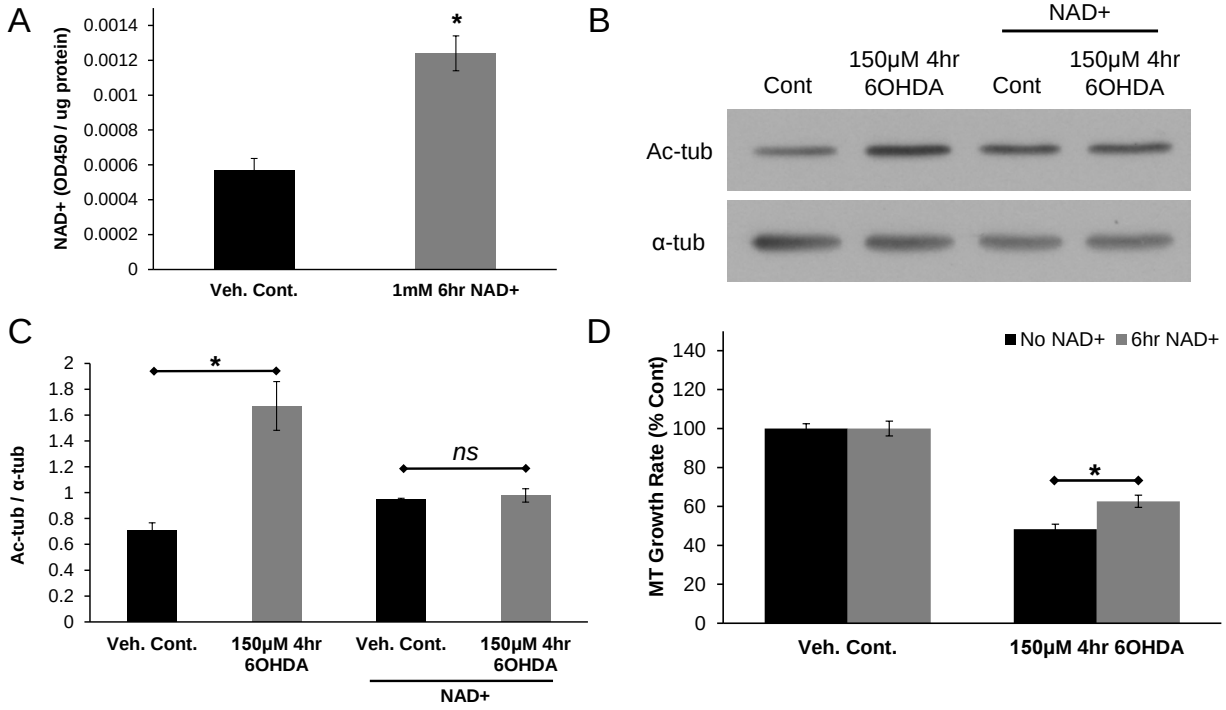

Figure 26: NAD+ Rescues Tubulin Acetylation Levels & MT Growth Rate

(A) Treatment of SH-SY5Y cells with 1mM NAD+ for 6hr leads to an increase in intracellular NAD+ levels. (B & C) Pre-treatment of cells with NAD+ (1mM; 6hr) protected against the 6OHDA-mediated increase in tubulin acetylation levels. (D) Pre-treatment with NAD+ reduced the impairment in MT growth rate caused by pre-lethal 6OHDA. Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ as indicated. Compiled from 3 independent experiments (for MT growth rate measurements, $n = 75 – 100$ EB3-mCherry comets per group).

4.9.2 Rescue of MT Dynamics by Increasing HDAC6 and SIRT2 Levels

The effect of directly increasing levels of the tubulin deacetylases on MT dynamics was also examined. GFP, HDAC6-GFP, or SIRT2-GFP was transfected along with EB3-mCherry into SH-SY5Y cells and MT dynamics was examined via live cell imaging as before. First, the effect of pre-lethal injury on MT growth rate was examined (Fig. 27A). Overexpression of either HDAC6-GFP or SIRT2-GFP resulted in a reduction in the impairment of MT growth rate from

6OHDA-induced oxidative stress (Fig. 27A). For sub-lethal injury, as shown earlier, the most significant alteration in MT dynamics was an increase in frequency of pauses / retractions. This effect was again confirmed in cells expressing just GFP (Fig. 27B). However, a significant rescue was observed in cells overexpressing HDAC6-GFP or SIRT2-GFP (Fig. 27B). Therefore, the overexpression of either HDAC6-GFP or SIRT2-GFP protects against 6OHDA-induced alteration in MT dynamics.

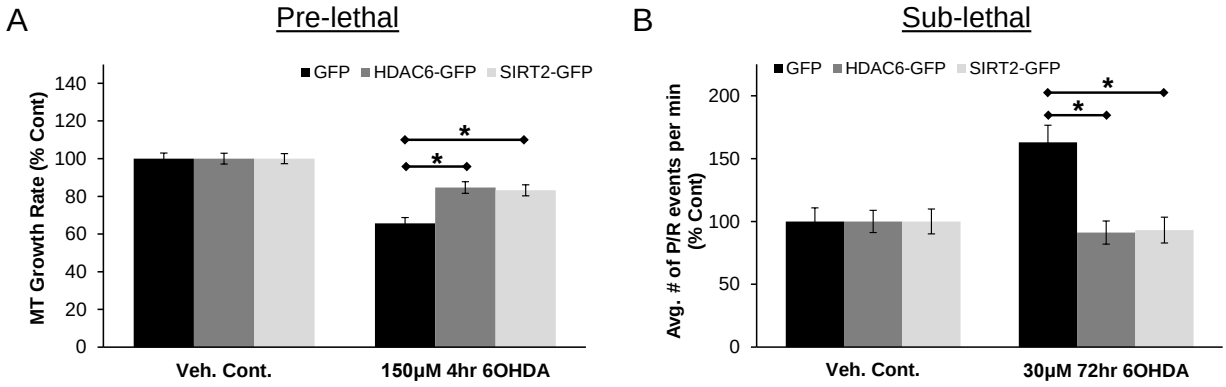

Figure 27: HDAC6 and SIRT2 Overexpression Rescues MT Dynamics

(A) SH-SY5Y cells expressing EB3-mCherry and either GFP, HDAC6-GFP, or SIRT2-GFP were treated with a pre-lethal dose of 6OHDA and MT growth rate was determined. Rescue was observed in cells overexpressing HDAC6-GFP or SIRT2-GFP. (B) Frequency of pauses / retractions was determined in cells treated with sub-lethal 6OHDA. Increase in the frequency of these events was diminished by the overexpression of either HDAC6-GFP or SIRT2-GFP. Mean +/- SEM, $*p < 0.05$ as indicated. $n = 75 – 100$ EB3-mCherry comets per group compiled from 3 independent experiments.

4.9.3 Rescue of Neurite Length

Given that modulation of tubulin deacetylases affects MT dynamics, it was of interest to examine if rescue of MT dynamics can also rescue neurite integrity. Differentiated SH-SY5Y cells were transfected with GFP and either pcDNA, HDAC6-FLAG, or SIRT2-FLAG and then treated with sub-lethal 6OHDA. Control cells expressing SIRT2-GFP showed slightly reduced neurite lengths compared to pcDNA control, as consistent with literature [144]. Nonetheless, the percent

reduction in neurite length in response to 6OHDA-induced oxidative stress was lower in cells expressing HDAC6-FLAG and SIRT2-FLAG compared to pcDNA control (Fig. 28). Hence, the rescue of MT dynamics also appears to rescue neurite length.

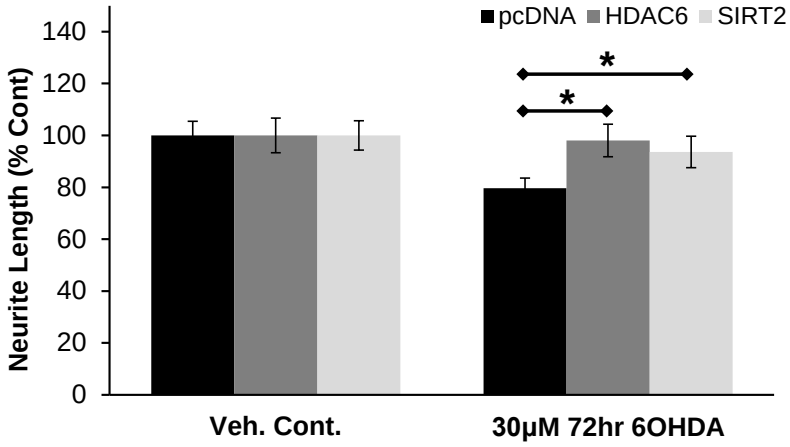

Figure 28: HDAC6 and SIRT2 Overexpression Rescues Neurite Length

SH-SY5Y cells expressing GFP and either pcDNA, HDAC6-FLAG, or SIRT2-FLAG were treated with sub-lethal 6OHDA. HDAC6 and SIRT2 were detected by FLAG immunofluorescence. Rescue in neurite length was observed with either HDAC6 or SIRT2 overexpression. Mean +/- SEM, $*p < 0.05$ as indicated. $n = 150 – 200$ cells per group compiled from 3 independent experiments.

5.0 DISCUSSION

5.1 6OHDA INJURY MODELS

Two different paradigms of a PD-relevant oxidative stress injury were utilized in this study – a pre-lethal and a sub-lethal 6OHDA treatment. The pre-lethal model involved a relatively higher dose and shorter time (hours) course of 6OHDA exposure. Such a model allows one to accelerate an injury that may occur over a long period of time. However, to better understand neurodegenerative processes, a more long-term oxidative injury would be needed. Hence, in addition to the pre-lethal 6OHDA model, a sub-lethal injury model involving a longer time course (days) was also developed. Furthermore, for the sub-lethal model, SH-SY5Y cells were differentiated to a more mature neuronal phenotype where long neuritic processes are present. This allowed for the study of neurite biology, which is very relevant to PD where axonal degeneration is one of the earliest events. Studying oxidative injury via a pre-lethal and a sub-lethal model can be very useful as similarities between the two models can help strengthen the observed findings whereas differences between the two models can help in identifying different mechanisms and in determining the relevance of the models to the study of PD.

5.2 OXIDATIVE STRESS AND MT DYNAMICS

5.2.1 Studying MT Dynamics in Live Cells

In this study, growing MTs were highlighted in living cells with the use of fluorescently-tagged end binding proteins. This technique has been extensively characterized and utilized for the study of MT dynamics in living cells [177,185,194]. However, it is of importance to briefly discuss why this particular technique was chosen over some of the other common techniques. One alternative is the transfection of fluorescently-tagged tubulin, which would then be incorporated into growing MTs [174,206]. However, there are a couple of key limitations to this particular technique that would have made the study of MT dynamics in this study difficult. First, there is a high background in transfected cells due to unpolymerized fluorescently-labeled tubulin which would make finding individual MTs difficult. Second, given that this technique strongly labels the entire MT lattice, it makes finding MT ends in cells with a dense network of MTs, such as along the neurites, very difficult. Therefore, the use of fluorescently-tagged tubulin would not have been ideal in the cell culture model used in this study.

Another alternative method to study MT dynamics in live cells is the use of Fluorescent Speckled Microscopy (FSM), which has also been well characterized [207,208]. In FSM, fluorescently-labeled tubulin is microinjected into cells where it mixes with the endogenous tubulin pool and incorporates into MTs, forming a “speckled” pattern of fluorescence along the MT lattice. The advantage of this technique over transfection of fluorescently-tagged tubulin is the lower background fluorescence and the ease of following movements of the “speckles” with time. However, the need for microinjection is a key technical limitation of this particular

approach and hence would make its use in this study, where extensive live cell imaging was carried out, suboptimal.

The use of fluorescently-tagged +TIPs provides a low-background and specifically highlights the plus-ends of MTs – making it quite easy to follow growing MTs, even in MT dense areas such as the neurite. However, there are a couple of limitations to this technique as well. First, there is variation in expression level of the transfected protein where in some cells the complete network of MTs is highlighted instead of showing the typical comet-like pattern. In these cells, it has been shown that the MT behavior is abnormal. This issue can be easily avoided by simply selecting cells where a comet-like pattern is observed [185,206]. Hence, one must be careful in selecting cells for MT dynamics analysis with this technique. The other limitation of this technique is in the analysis of other parameters of MT dynamics, such as MT retraction. During MT pauses or retractions, the MT ends are not as bright or invisible given that these proteins only bind to MTs during the growth phase. Hence, it makes specific determination of parameters such as MT retraction rate difficult. However, there are published algorithms that can be used to determine such aspects of MT dynamics [177]. Nonetheless, for the study of certain parameters of MT dynamics, such as growth rate and frequency of pauses, the use of fluorescently-tagged +TIPs is preferred over the other alternatives.

5.2.2 MT Dynamics in Oxidatively-Stressed Cells

In response to 6OHDA-induced oxidative stress, a significant alteration in MT dynamics was observed. Specifically, MT growth rates were reduced in 6OHDA treated cells. This effect was more robust after pre-lethal injury as compared to sub-lethal injury. Hence, other parameters of MT dynamics were also examined after sub-lethal injury, which showed increased frequency of

MT pauses/retractions. The observation of significant alterations in MT dynamics in both the pre-lethal and sub-lethal models strengthens the conclusion that oxidative stress impairs MT function in neuronal cells. Furthermore, utilization of the sub-lethal injury model makes the results more relevant to the neurodegenerative process seen in PD.

How could oxidative stress affect MT dynamics? One mechanism is through the direct oxidative modification of the tubulin heterodimer, specifically cysteine residues as there are 12 in α-tubulin and 8 in β-tubulin [105,181]. Oxidation of cysteine residues of tubulin by a variety of agents has been shown to reduce tubulin polymerization rate [178-183]. Along the same lines, the addition of various disulfide reducing agents restores a considerable portion of the polymerization activity [178-180]. Certain cysteine residues that are "assembly-critical" have been proposed, however whether these are specifically oxidized and involved in the reduction of polymerization activity after oxidative stress is still uncertain [180,181]. Furthermore, methionine oxidation (26 methionine residues in the tubulin heterodimer) is another modification seen after treatment with certain oxidants (e.g. hypochlorous acid), although whether such a modification affects tubulin polymerization is still uncertain [180]. Nonetheless, given its abundance in neurons, tubulin is a likely target for oxidative modification.

Another key mechanism by which MT dynamics is regulated is through MAPs. As discussed extensively in the introduction, these proteins regulate many aspects of MT function, such as polymerization rate and stability. Key members of the MAPs are the EBs as they regulate MT dynamics as well as the binding of other +TIPs to MTs. Altered binding of EBs to MTs, which is thought to occur primarily via electrostatic and hydrophobic interactions, can therefore significantly alter MT dynamics [84,85]. Interestingly, H_2O_2 treatment has been shown to alter EB1 function [174]. 6OHDA-induced oxidative stress was shown in this study to affect

EB1 levels and comet length without significantly affecting the overall MT organization. Reduced comet length is correlated with reduced MT growth rates and therefore is a nice confirmation of the live cell imaging results [175-177]. Given their important role, reduced EB1 levels would be expected to reduce MT growth rate and increase frequency of MT pauses and retractions, which is exactly what is observed in response to 6OHDA treatment in this study. Hence, altered function of EB1 and perhaps other +TIPs could explain the altered MT dynamics that is observed. It is currently unclear how oxidative stress could impact EB stability and interactions with MTs. Oxidative modification could directly modify EB proteins or could activate signaling cascades that lead to post-translational modifications, such as phosphorylation. Although not much is known about EB phosphorylation, there have been numerous studies examining the phosphorylation of other +TIPs and its impact on MT binding [209-214]. Phosphorylation of these +TIPs (e.g. by glycogen synthase kinase-3β [GSK3β], protein kinase A, and mammalian target of rapamycin [mTOR]) can reduce interactions with MTs as well as with other +TIPs. Interestingly, 6OHDA can regulate the activity of various kinases, including GSK3β and mTOR [215-217]. Hence, phosphorylation of +TIPs may be one mechanism by which 6OHDA can affect MT function.

5.3 OXIDATIVE STRESS AND TUBULIN ACETYLATION

5.3.1 Tubulin Acetylation in Oxidatively-Stressed Cells

Tubulin PTMs represent another way in which MT function is regulated and includes modifications such as acetylation, tyrosination and detyrosination, polyglutamylation,

palmitoylation, polyglycylation, and phosphorylation [97,98]. These modifications regulate the binding of MAPs to MTs and play an important role in neuronal differentiation, transport of cargo, axon guidance, and synapse formation. With both pre-lethal and sub-lethal 6OHDA injury, a significant increase in levels of tubulin acetylation was observed. A significant portion of this study was dedicated to determining the mechanism(s) by which levels of tubulin acetylation change after 6OHDA treatment. Given that oxidative stressors, e.g. H_2O_2 and cigarette smoke, has been linked to altered localization, levels, and/or activity of various HDACs, the effects of 6OHDA-injury on the two known tubulin deacetylases, HDAC6 and SIRT2, was examined.

It was found that 6OHDA did not significantly alter the subcellular localization of HDAC6 and SIRT2. This was expected since the both are cytoplasmic proteins. Although a few studies have shown that nuclear localization can occur in some cases in certain cells, no nuclear HDAC6 or SIRT2 was observed in control or treated SH-SY5Y cells [124,138,143]. The experiments in this study were carried out via immunofluorescence and hence very small changes in subcellular localization might be missed. Therefore, one cannot rule out that some alteration in HDAC6 and SIRT2 distribution is occurring with 6OHDA treatment. Although, given the nature of the acetylation changes that are observed, it would be safe to argue against small changes in subcellular localization as a possible mechanism.

The effect of 6OHDA injury on the protein levels of HDAC6 and SIRT2 was also examined. Levels of HDAC6 were not altered in response to either pre-lethal or sub-lethal 6OHDA. For SIRT2, although not statistically significant, pre-lethal dose of 6OHDA caused a trend towards a reduction in protein levels, which was a consistent observation in the majority of the replicates. No such consistent pattern was observed for sub-lethal injury despite the

quantified results showing a trend towards an increase. Hence, in the pre-lethal injury model, it is possible that reduced SIRT2 levels contribute to an increase in levels of tubulin acetylation.

However, since these trends were not statistically significant, other possible mechanisms were explored. Furthermore, tubulin acetylation level at earlier 6OHDA time points (e.g. 30min) is elevated but no reduction in SIRT2 protein levels is observed (data not shown). Therefore, after pre-lethal as well as sub-lethal injury, there must be another mechanism(s) that can explain the increased levels of tubulin acetylation.

The deacetylase activity of HDAC6 and SIRT2 was also examined in response to 6OHDA-induced oxidative stress. Utilizing an acetylated substrate, the deacetylase activity of immunoprecipitated endogenous HDAC6 and SIRT2 was studied. For HDAC6, no significant change in activity was observed after a pre-lethal or a sub-lethal 6OHDA injury. It is important to mention again that these assays were carried out with the use of a synthetic peptide that has been acetylated (as provided by the manufacturer). These substrates are designed based on either a standard ε-acetyl-lysine or based on acetylated sites/regions found in various proteins, including p53 and histones. Therefore, different substrates have preferences for different HDACs, which is confirmed by the manufacturer and recommendations are provided in selecting the ideal substrate. Although a couple of other substrates were also used for HDAC6 deacetylase activity assays, no significant alteration was observed. Nonetheless, it is still possible that HDAC6 in 6OHDA treated cells has an altered activity against tubulin *in vivo*. Therefore, one cannot rule out that HDAC6 activity against tubulin is not affected by oxidative stress.

SIRT2 deacetylase activity assays showed no change in activity in response to pre-lethal 6OHDA injury, however a significant reduction was observed after sub-lethal 6OHDA injury. Hence, at least in the sub-lethal case, diminished SIRT2 activity can explain the increased levels

of tubulin acetylation. With respect to the SIRT2 assays, it is important to discuss the antibody issues that were referred to earlier. The SIRT2 antibody strongly detects SIRT2 at the correct molecular weight via Western blotting and has been confirmed for immunoprecipitation by the manufacturer. However, in the immunoprecipitations done in this study, a strong band in addition to SIRT2 is also seen in the pulled-down sample. This band is stronger than the SIRT2 band that is pulled-down and hence is an issue to be addressed. Based on the optimization assays carried out with the SIRT2 antibody, the deacetylase activity that is observed is NAD+ dependent. Hence, it is safe to say that a NAD+-dependent deacetylase is being examined in the activity assays. However, given that the unknown band (above IgG heavy chain; around 55 – 60kDa) is much stronger than the SIRT2 that is pulled down, it is important to confirm that the activity observed is due to SIRT2 and not another sirtuin or an unknown deacetylase. First, given the conditions in which the deacetylase reaction takes place (37^{o}C with constant agitation), it is possible that much of the SIRT2 bound to the antibody is eluted and washed away in the subsequent wash steps. Although this would not affect the deacetylase reaction itself, it would show much reduced protein levels when the levels are checked via Western blotting. This could be one explanation for why such a small SIRT2 level is observed in the pulled-down samples. Furthermore, extensive analysis of the literature indicates that the molecular weights of the other six known sirtuins are not anywhere close to the size of the unknown band. This provides some support that the observed NAD+-dependent activity likely results from SIRT2. Nonetheless, deacetylase activity in SIRT2 knock-down samples will be examined, which should be able to confirm if the activity observed is in fact due to SIRT2.

How could the deacetylase activity of SIRT2 be modified by oxidative stress? Oxidative stress can cause reversible (e.g. disulfide bond formation and *S*-nitrosylation) and irreversible

(e.g. carbonylation and tyrosine nitration) modifications to proteins that can affect their stability and function [218-220]. Evidence of oxidative modification to SIRT1 exists in the literature, which can provide some valuable insight to how SIRT2 function may be similarly affected since the catalytic domain is conserved in all SIRTs [120,221,222]. H_2O_2 has been shown to increase carbonylation on SIRT1 in cell culture (bronchial epithelial cell line BEAS-2B) and animal models (mouse lung) [221]. This modification was observed prior to a reduction in SIRT1 deacetylase activity was seen and therefore could play a role in reducing SIRT1 activity [221]. H_2O_2 also caused cysteine oxidation in SIRT1 [221]. Although not directly involved in the enzymatic reaction, some cysteine residues in the sirtuin fold domain are thought to be important for binding to the substrate, NAD+, and Zn^{2+} [223-225]. In fact, S-glutathiolation of certain cysteine residues in this region of SIRT1 has been shown to reduce its activation by resveratrol [222]. Hence, cysteine modification by oxidative stress could also reduce SIRT2 function. Another mechanism by which SIRT2 function could be regulated is via phosphorylation. Various cyclin-dependent kinases (Cdks) and the CDC14B phosphatase can regulate the phosphorylation status of SIRT2 and regulate its protein stability and enzymatic activity [144,226,227]. For example, p35–Cdk5 can phosphorylate SIRT2 at Ser-331, leading to inhibition of catalytic activity [144]. Interestingly, 6OHDA may up-regulate Cdk5 function in cell culture (e.g. PC12 cells) as well as animal models (e.g. striatal injection in rats) [228,229]. Hence, there are a number of ways in which 6OHDA-induced oxidative stress can affect the enzymatic activity of SIRT2.

5.3.2 NAD+ Levels in Oxidatively-Stressed Cells

Reduction in SIRT2 deacetylase activity was only seen in the sub-lethal 6ODHA injury model; hence increase in acetylated tubulin cannot yet be explained after pre-lethal injury. Interestingly, NAD+ / NADH system is an important regulator of the redox state of the cell and their levels is found to be sensitive to oxidative stress [230]. Pre-lethal 6OHDA exposure caused a significant decline in intracellular NAD+ levels in this study. However, no such reduction was observed after sub-lethal 6OHDA exposure. Hence, reduction in the essential co-factor for SIRT2 can decrease its function in cells, leading to increased levels of tubulin acetylation.

How could NAD+ and NADH levels change with oxidative stress? One possibility is the overconsumption of NAD+. NAD+ is consumed primarily by sirtuins, poly(ADP-ribose) polymerases (PARPs), ADP-ribosyl cyclases, and mono(ADP-ribosyl)transferases [202]. PARPs are responsible for a significant portion of the NAD+ consumption in cells [202,204,231]. PARP-1 senses DNA damage and cleaves NAD+ into nicotinamide and ADP-ribose, the latter is then polymerized onto nuclear acceptor proteins – ultimately leading to DNA repair [232]. In the case of oxidative stress, reactive oxygen species such as H_2O_2 and hydroxyl radical cause significant DNA strand breakage, which results in the over-activation of PARP-1. Abnormal activation of PARP-1 then depletes the cell of NAD+ and subsequently ATP, leading to significant cellular dysfunction and death [232]. This process is thought to play a key pathogenic role in a vast number of disorders, such as stroke, diabetes, myocardial ischemia, hemorrhagic and septic shock, various forms of inflammation, traumatic brain injury, and MPTP-induced parkinsonism [232,233]. Furthermore, oxidative stress can shift the balance of reduced pyridine nucleotides away from NADH and towards NADPH, which helps in the defense against ROS, by the regulation of various NADPH and NADH forming dehydrogenases

and NAD kinases [234-239]. Therefore, the above mentioned processes are likely mechanisms by which 6OHDA-induced oxidative injury causes depletion of cellular NAD+ and NADH. Another mechanism by which NAD+ and NADH levels could be reduced is if oxidative stress affects their synthesis. There are two pathways for NAD+ synthesis, the *de novo* pathway that uses L-tryptophan as the precursor and the salvage pathway that uses nicotinamide and nicotinic acid as precursors [202]. There are numerous enzymes involved in the two pathways; however it is currently unclear if and how oxidative stress could affect their function.

NAD+ and NADH levels are reduced after pre-lethal injury but not after sub-lethal injury. A possible explanation for this difference is that after pre-lethal 6OHDA, there is a significantly higher degree of ROS exposure compared to sub-lethal 6OHDA– leading to greater consumption and reduced production by the mechanisms mentioned above. Furthermore, it is possible that there is a brief dip in NAD+ / NADH levels after a sub-lethal dose is administered but by the time the measurements are made (24hr after the last dose), the levels return to baseline. In this case, the temporary reductions in NAD+ levels could also contribute to elevated tubulin acetylation levels.

5.3.3 Involvement of Tubulin Acetylases?

Although the focus in this study was on the tubulin deacetylases, it is possible that the function of tubulin acetylases is modified after 6OHDA-induced oxidative stress. As previously mentioned, tubulin acetylases have only been recently identified and hence not much is known about their regulation under oxidative conditions. However, oxidative stress has been shown to increase histone acetyltransferase (HAT) activity [240-242]. A possible mechanism is through the activation of redox-sensitive signaling pathways, e.g. MAPK, that can regulate various

HATs, such as cAMP-response element binding protein (CBP), p300, and activating transcription factor-2 (ATF-2) [241]. It is possible that similar signaling pathways could activate tubulin acetylases as well and lead to increased levels of tubulin acetylation in 6OHDA treated cells.

5.4 RESCUE OF MT FUNCTION

5.4.1 Restoring Tubulin Deacetylase Function

To better understand the consequence of tubulin acetylation on MT dynamics, rescue studies were carried out. As discussed in detail in the introduction, evidence of acetylation regulating MT dynamics is a bit conflicting. This is due primarily to differences in the assays used to study MT dynamics. When more sensitive assays were used, such as time spent growing and pausing, modulation of tubulin acetylation resulted in altered MT dynamics. Given the significant alterations in tubulin acetylation and deacetylase function observed in response to 6OHDA-induced oxidative stress, rescue studies were performed where the effects of modulating deacetylase function on MT function was examined. Restoring NAD+ levels in the pre-lethal 6OHDA injury model where NAD+ depletion was observed resulted in rescue of tubulin acetylation levels as well as MT growth rate. Hence, increasing NAD+ levels restores SIRT2 function and subsequently reduces tubulin acetylation levels, which is thought to result in rescue of MT growth rate.

Another mechanism by tubulin acetylation levels can be modified is through the manipulation of the tubulin deacetylases themselves. Over-expression of HDAC6 and SIRT2

resulted in rescue of MT dynamics, e.g. growth rate and frequency of pauses/retractions. Seeing rescue with both HDAC6 and SIRT2 suggests that increased tubulin acetylation is responsible for a significant portion of the alteration in MT dynamics. Interestingly, in the pre-lethal injury model, overexpression of SIRT2 was able to rescue MT function similar to HDAC6 despite reduced NAD+ levels. How could overexpression of SIRT2 in these cells with reduced NAD+ levels lead to rescue of deacetylase activity? There are a number of enzymes that utilize NAD+ in their enzymatic reactions, with PARPs being the major consumers. Hence, under normal conditions, SIRT2 competes with numerous other enzymes for the available pool of NAD+. With further reductions in NAD+ levels after oxidative stress, even less is available for SIRT2 deacetylase activity. It is hypothesized that the overexpression of SIRT2 makes it more competitive for the available pool of NAD+ – leading to an overall increase in tubulin deacetylase activity. It is nonetheless interesting that the magnitude of rescue is similar to over-expression of HDAC6. It is possible that SIRT2 has a more robust deacetylase activity on tubulin than HDAC6 in this model. Unfortunately, given the low transfection efficiency of these plasmids, it is difficult to confirm whether over-expression of HDAC6 and SIRT2 leads to a similar reduction in tubulin acetylation levels.

5.4.2 Possible Mechanism(s) of Rescue

How could tubulin acetylation affect MT function? This question is of great interest in the MT field and the answer is yet to be determined. It is important to note that majority of the studies examining the role of tubulin acetylation on MT function have been primarily through the manipulation of its deacetylases and recently acetylases. Hence, it has been difficult to attribute the effects of manipulating these enzymes directly to the effects of acetylation of tubulin. Given

that Lys-40, the most well studied acetylation site, is predicted to face the luminal surface of the MT polymer, it is difficult to comprehend how it could influence binding of MAPs. However, these predictions were based on flat Zn sheets with random polarity, which is unlike MTs [105]. It is possible therefore that this acetylation site could be facing the outside wall in properly assembled MTs. Furthermore, it is also possible that acetylation at Lys-40 could cause conformational changes in the tubulin dimer that then is propagated to the surface of the MT polymer [243]. Acetylation of tubulin could also lead to severing of MTs by katanin, which would affect MT function [112]. Furthermore, the recently identified Lysine-252 acetylation site on β-tubulin by San (which modifies the tubulin heterodimers and not polymerized MTs) has been shown to reduce the incorporation of the acetylated tubulins into growing MTs [106]. In addition, some of the other recently identified acetylation sites are predicted to face the outside surface of the MT lattice and hence could regulate MT dynamics by affecting the binding of MAPs [107,243]. Further characterization of these sites and of the tubulin acetylases should provide key insight into the question of how tubulin acetylation can regulate MT function.

It is also possible that the rescue observed by restoring deacetylase function could be independent of tubulin acetylation. Interestingly, HDAC6 has been shown to interact with and regulate MAPs, such as tau and EB1 [133-136]. Tau is an important MT stabilizing MAP and EB1 is a key regulator of the dynamic properties of MTs [87,89]. SIRT2 has been shown to co-localize and co-immunoprecipitate with HDAC6 and *in vivo* both proteins may be present in a complex that regulates MT function [143]. Hence, it is possible that restoring deacetylase function also restores binding of MAPs, such as EB1, to MTs and subsequently restores MT dynamics.

5.4.3 Restoring Neurite Integrity

Given the significant alterations in MT function, MT-dependent processes would be expected to be affected. One of the earliest changes observed in neurodegenerative diseases is axonal degeneration [4]. MTs play a key role in the development and maintenance of axons / neuritic processes. Sub-lethal oxidative stress, which more closely mimics the injury that occurs in PD, causes reduction in neurite length. It is unclear whether 6OHDA-induced oxidative stress impairs neurite growth or induces neurite shortening. The neuritic process in differentiated cells attains the majority of its maximal length during the initial differentiation phase (before 6OHDA treatment). There is limited neurite growth (< 10μm) in the vehicle treated cells after the start of the 6OHDA treatment. Hence, 6OHDA-induced destabilization of MTs is likely causing neurite shortening but one cannot confidently rule out some contribution from impaired growth as well. Nonetheless, it is safe to say that neuritic integrity is compromised after 6OHDA-induced oxidative stress. Neurite length was rescued in cells where MT function was restored by the overexpression of HDAC6 or SIRT2. Given the importance of MT function in neurite growth and stability, it makes sense that restoring MT function also helps restore neuritic integrity.

6.0 CONCLUSION AND FUTURE PERSPECTIVES

In response to 6OHDA-induced oxidative stress in neuronal cells, significant alterations in MT function were observed. Specifically, oxidative stress reduced MT growth rate, increased frequency of MT pauses/retractions, altered EB1 levels, and increased levels of tubulin acetylation. Along with these MT alterations, degeneration of neuritic processes, which is an early event in PD, was also observed in oxidatively-stressed cells. Alterations in MT function were observed in both the pre-lethal as well as the sub-lethal oxidative stress models – helping to strengthen the conclusion of MT dysfunction after oxidative injury and also increasing the relevance to the degenerative process seen in PD. Alteration in levels of tubulin acetylation was a result of impaired function of tubulin deacetylases, specifically SIRT2. Furthermore, restoration of tubulin deacetylase function rescued MT function as well as the neuritic degeneration phenotype observed in response to 6OHDA, suggesting that oxidative stress impairs MT function, at least partially, by altering tubulin acetylation. In addition to its impact on the integrity of the axon/dendrites and axonal trafficking of organelles, oxidative disruption of MT function could also affect gene expression. In another set of experiments (see Appendix), oxidative stress disrupted MT-dependent nuclear transport, which could contribute to the selective declines in transcriptional responses of diseased dopaminergic neurons. In all, this study provides support for the role of MT dysfunction in the degenerative processes seen in PD.

Figure 29 summarizes these findings and proposes a model of how 6OHDA-induced oxidative stress can lead to impairment in MT-dependent processes.

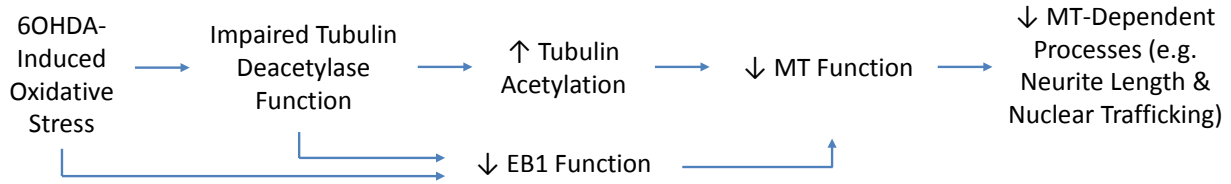

Figure 29: Proposed Model of 6OHDA-Induced MT Dysfunction

6OHDA-induced oxidative stress impairs tubulin deacetylase (specifically SIRT2) function, leading to increased levels of tubulin acetylation. Increased tubulin acetylation subsequently impairs MT dynamics. In addition, 6OHDA-induced oxidative stress could also impair EB1 function, either directly or through impaired deacetylase function, and lead to decreased MT dynamics. Decreased MT function subsequently affects MT-dependent processes, such as the integrity of the neurite and trafficking of transcription factors to the nucleus. Restoring deacetylase function rescues MT function by reducing levels of tubulin acetylation and also possibly restoring EB1 function – ultimately resulting in the restoration of key MT-dependent processes.

The study of MT function in degenerating neurons of PD is an exciting avenue for future studies as genetic and toxin-based models of PD are beginning to identify significant alterations in MT function (see section 1.2.6 for details). The current study utilizing an oxidative stressor adds to this growing evidence in the literature that MT dysfunction may play an important role in the neuronal degeneration that is seen in PD. In addition to the parameters of MT function examined in this study and the many future experiments mentioned in the Discussion section, there are other aspects of MT function that are also of great interest to examine in future studies.

Given the significant impact on tubulin acetylation in response to 6OHDA-induced oxidative stress, it would be very interesting to examine the effects of such stress on other tubulin PTMs. Not only do these PTMs serve as markers of dynamic and stable pools of MTs, they can also regulate binding of MAPs and hence likely play a role in many aspects of neuronal function, such as differentiation, transport of cargo, axon guidance, and synapse formation. Evidence of MT regulation by tubulin tyrosination / detyrosination and polyglutamylation exists

in the literature [243]. Tyrosinated tubulin can recruit MT-depolymerizing kinesins and subsequently promote MT depolymerization [244]. Detyrosinated tubulin, which is often found along with acetylated tubulin on stable MTs, may also regulate MT dynamics by altering the binding of +TIPs, such as EB1 and Clip170 [97,245,246]. Polyglutamylation can recruit MT-severing enzymes, e.g. katanin and spastin, and lead to MT depolymerization [247]. Hence, it is also possible that oxidative stress affects these tubulin PTMs and subsequently alters MT dynamics, which would be a very interesting direction for future studies.

One of the key functions of MTs is the trafficking of organelles and protein complexes along the axon. As mentioned in the introduction, there is growing evidence of altered axonal transport in various genetic and toxin models of PD. In addition, the study presented in the Appendix implicates a role for altered MT-dependent trafficking in the differential effects of 6OHDA on the nuclear transport of transcription factors. Motor proteins, such as dynein and kinesin, are a key component of MT-dependent trafficking and their recruitment to MTs is affected by tubulin PTMs [97,98,243]. Hence, with the 6OHDA-induced oxidative stress model, it would be very interesting to study the effects of altered MT PTMs on the binding of motor proteins. Furthermore, it would also be of interest to examine the impact of such alterations on the trafficking of organelles, such as mitochondria and autophagosomes. Given evidence of impaired axonal transport in PD, such studies would provide more evidence for the role of MT dysfunction in the neuronal degeneration that is seen in PD.

The study HDAC6 and SIRT2 function is also of great interest in the field of PD [118,141,142]. Given their regulation of processes such as neuronal motility and differentiation, heat-shock protein chaperone system, transport of ubiquitinated proteins to aggresomes, and autophagic removal of protein aggregates and impaired mitochondria, these enzymes are likely

to play an important role in the neurodegenerative process seen in PD as well as in the development of therapeutic strategies. Interestingly, SIRT2 inhibition has been shown to be protective against α-synuclein toxicity [248]. It is possible that increased tubulin acetylation leads to enhanced MT-dependent transport and clearance of the α-synuclein aggregates. However, the exact mechanism is still unknown and it is unclear if tubulin or another substrate is involved. Furthermore, imbalance in the levels of acetylation is thought to be a common theme in neurodegenerative diseases [249]. Although this imbalance refers primarily to histones and the subsequent impact on gene expression, given the non-histone targets of many of these HATs and HDACs, the acetylation imbalance may also apply to non-genomic processes. Balance in tubulin acetylation levels is important for proper MT function and hence imbalance towards either side would be expected to significantly affect MT function and have severe consequences for neuronal differentiation and survival. Hence, the study of how oxidative stress modulates the function of the tubulin deacetylases could be an important aspect to understanding the pathogenesis of PD.

To further implicate the role of MT dysfunction in neuronal degeneration seen in PD, it would be of great interest to study the effects of oxidative stress on MT dynamics and PTMs in dopaminergic neurons. Furthermore, in addition to the 6OHDA injury model, it would be very informative to study effects of various genetic models of PD on MT function, including expanding on existing studies with α-synuclein, Parkin, and LRRK2 and adding other important contributors, such as PINK1 and DJ-1. Doing so can help identify an underlying process that is involved in neuronal dysfunction and degeneration in PD, as is the case with mitochondrial biology and autophagy. Advancement in understanding the mechanisms involved in neuronal degeneration are key to the development of therapeutic strategies for PD. The question is what

should be the target of the therapeutic agent. It would be ideal to prevent the degeneration from ever initiating, however by the time symptoms clinically manifest, the degeneration process is long underway. In this case, it would be ideal to target the underlying degenerative event such that further degeneration can be prevented and some normal neuronal function can be restored. Antioxidant therapy could be promising if it can be successfully delivered early in the injury process and at a sufficient concentration to the midbrain dopaminergic neurons, which has thus far been difficult to do [71,250-254]. Additionally, if MTs play a key role in the degenerative process seen in PD, as the literature is beginning to suggest, then targeting MTs could provide a means by which neuronal function could be restored and further degeneration delayed or halted. Furthermore, an ideal therapeutic agent would be one that can affect the biology of multiple underlying events, such as the mitochondria and MTs. Interestingly, olesoxime (cholest-4-en-3-one, oxime; TRO19622), a molecule in the cholesterol-oxime compound family of mitochondrial pore modulators, has been recently shown to be protective in a wide-range of neurodegeneration models [255-258]. In addition to affecting the mitochondria, likely the mitochondrial permeability pore, it has also been shown to have significant MT dynamics promoting properties, such as enhancing EB1 accumulation at MT plus-ends and increasing MT growth rates [257]. Neurite degeneration and mitochondrial trafficking impairments induced by the MT-depolymerizing agent vincristine were prevented by olesoxime [257]. Given that this agent affects the biology of mitochondria and MTs, it is thought to be a promising therapeutic agent in numerous degenerative diseases. In fact, it is in either clinical or preclinical stages for a variety of neurodegenerative diseases, such as amyotrophic lateral sclerosis, spinal muscular atrophy, peripheral neuropathy, multiple sclerosis, and PD [255]. This compound serves as an example of an agent that is promising due to its ability to regulate numerous processes that may be involved

in neurodegeneration and therefore, for the development of future therapeutic strategies, it further emphasizes the need to understand the key underlying events that lead to neuronal degeneration in PD.

APPENDIX

ALTERED TRANSCRIPTION FACTOR TRAFFICKING IN OXIDATIVELY-STRESSED NEURONAL CELLS

The following work is a study of transcription factor trafficking in oxidatively-stressed neuronal cells. This project represents the other significant portion of my efforts during graduate study. Utilizing the pre-lethal model of 6OHDA, the effects of oxidative stress on the nuclear trafficking of select transcription factors was examined. The significance of the microtubule system for this process was also examined in this study. In addition to the main body of work presented above, the following study also contributes to understanding the mechanisms by which oxidative injury could impact neuronal function in PD.

ABSTRACT

Age-related neurodegenerative diseases are associated with alterations in gene expression in affected neurons. One of the mechanisms that could account for this is altered subcellular localization of transcription factors, which has been observed in human post-mortem brains of each of the major neurodegenerative diseases, including Parkinson's disease (PD). The specific mechanisms are yet to be elucidated; however a potential mechanism involves alterations in nuclear transport. In this study, we examined the nucleocytoplasmic trafficking of select transcription factors in response to PD-relevant oxidative injury, 6-hydroxydopamine (6OHDA). Utilizing a well-established model of ligand-regulated nucleocytoplasmic shuttling, the glucocorticoid receptor, we found that 6OHDA selectively impaired nuclear import through an oxidative mechanism without affecting nuclear export or nuclear retention. Interestingly, impaired nuclear import was selective as Nrf2 (nuclear factor E2-related factor 2) nuclear localization remained intact in 6OHDA-treated cells. Thus, oxidative stress specifically impacts the subcellular localization of some but not all transcription factors, which is consistent with observations in post-mortem PD brains. Our data further implicate a role for altered microtubule dependent trafficking in the differential effects of 6OHDA on transcription factor import. Oxidative disruption of microtubule-dependent nuclear transport may contribute to selective declines in transcriptional responses of aging or diseased dopaminergic cells.

1. INTRODUCTION

Age-related neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), are associated with alterations in expression of specific genes in affected neurons [1-3]. Transcription factors play a key role in the regulation of gene expression and the activity of many transcription factors is limited by their localization within the cytoplasm. A number of mechanisms are utilized to direct sequestered transcription factors into the nucleus in response to specific signals [4-11]. Since nucleocytoplasmic protein trafficking can be bidirectional [12], transcription factors exhibiting predominant nuclear localization may transit through the cytoplasm and also function in non-genomic signaling networks [13-16]. Interestingly, alterations in the subcellular localization of a number of transcription factors are documented in human post-mortem brains of each of the major neurodegenerative diseases [5]. Examples include cytoplasmic aggregation of phosphorylated (p)CREB (cAMP response element binding protein) and lack of nuclear pCREB in PD [17], reduced nuclear localization of Nrf2 (nuclear factor E2-related factor 2) in AD but not in PD [18], increased cytoplasmic ATF2 (activating transcription factor 2) levels in AD [19], and increased cytoplasmic:nuclear ratios of TDP-43 (TAR DNA-binding protein 43) in frontotemporal lobar dementias and ALS [20].

Extensive evidence of oxidative damage to proteins, lipids, and DNA is found upon analysis of post-mortem brains of various neurodegenerative diseases, including PD [21-31]. Furthermore, genetic and environmental models of various neurodegenerative diseases support the notion that oxidative stress plays a primary role in their pathogenesis [24-26]. In the case of PD, mutations or deficiency of proteins such as Parkin, PINK1 (PTEN-induced putative kinase

1), α-synuclein, DJ-1, and LRRK2 (leucine-rich repeat kinase 2), have been shown to increase susceptibility to oxidative stress-mediated cell death [32-34]. Parkinsonian mimetics such as, 6-hydroxydopamine (6OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat, generate reactive oxygen species (ROS) and cause neuronal death [35]. These studies suggest a key role for oxidative stress in the pathogenesis of these diseases.

6OHDA has been widely used to study neuronal injury responses in PD. It is an analog of catecholamine neurotransmitters that is taken up into cells by dopamine and norepinephrine reuptake transporters. It is commonly used to model PD-relevant oxidative stress as it demonstrates an early autoxidation phase of ROS production and also has a delayed phase of mitochondrial ROS production [36]. Although used as an exogenous neurotoxin, there is evidence of spontaneous ring hydroxylation of dopamine *in vivo*, with elevated body fluid levels of 6OHDA detected in patients treated with L-Dopa [37,38].

6OHDA treatment leads to increased cytoplasmic accumulation of (p)ERK (extracellular signal-regulated kinase) [39] and decreased nuclear levels of GFP (green fluorescent protein)-ERK2 [40] as well as decreasing the nuclear to cytoplasmic ratios of pCREB in SH-SY5Y cells and in primary midbrain neurons [17]. These findings are consistent with alterations observed in dopaminergic neurons in post-mortem PD brains [5]. CREB-regulated gene transcription, which is important for axon growth, mitochondrial biogenesis, and neuronal survival, was also repressed in 6OHDA treated cells. Given the dynamic shuttling of transcription factors between the nuclear and cytoplasmic compartments, there are a number of mechanisms that could result in altered distribution of transcription factors in degenerating neurons, including compartmentalization, altered expression levels, sequestration in protein aggregates, and nuclear transport deficits [5]. Interestingly, 6OHDA impairment of CREB-mediated transcription (e.g.

Bcl2 [B-cell lymphoma 2] and BDNF [brain-derived neurotrophic factor]) was reversed with cAMP treatment, which activates CREB through mechanisms not requiring active nuclear import [17,41] – suggesting a possible impairment in the nucleocytoplasmic trafficking of CREB. The observed steady state alterations in the distribution of transcription factors could result from altered nuclear import/export with significant consequences on neuronal survival. The main goal of this study was to directly examine, in a neuronal cell line, the impact of 6OHDA-induced oxidative stress on distinct steps in the regulated nucleocytoplasmic trafficking of select transcription factors.

2. MATERIALS AND METHODS

2.1. Cell Culture

SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA) were grown on 10cm cell culture dishes containing Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, and 10mM HEPES. Cells were maintained at 37^{o}C in a humidified 5% CO_2 incubator.

2.2. Plasmids and Transfections

SH-SY5Y cells were transfected with Lipofectamine^{TM} 2000 reagent (Invitrogen, Carlsbad, CA) utilizing Opti-MEM® I reduced serum media (Invitrogen, Carlsbad, CA) according to the manufacture's protocol at a final Lipofectamine concentration of 0.1%. Cells were grown for at least 48hr before transfection and then allowed to express the protein of interest for another 24hr - 48hr before treatment. GR-GFP and Nrf2-GFP plasmids were kindly provided by Dr. Ian Macara (University of Virginia) and Dr. Manabu Furukawa (University of Nebraska), respectively.

2.3. Drug Treatments

6OHDA (Sigma, St. Louis, MO) was prepared in cold sterile water immediately before use. Dexamethasone (Sigma), a synthetic glucocorticoid hormone, was prepared at a stock concentration of 10^{-3}M in 95% EtOH and used at a working concentration of 10^{-6}M unless otherwise stated. Cortisol (Sigma) was prepared at a stock concentration of 10^{-3}M to 10^{-4}M in 100% EtOH and used at a working concentration of 10^{-6}M to 10^{-7}M unless otherwise stated. For

antioxidant treatment, a metalloporphyrin antioxidant, MnTBAP (A.G. Scientific, San Diego, CA), was utilized at a final concentration of 150μM. Colchicine (Sigma) and Paclitaxel (Sigma) were prepared in dH_2O and DMSO, respectively, and used at the concentrations indicated in the text.

2.4. Monomeric Tubulin Extraction

Cells treated with either vehicle or the drug of interest were washed in 37°C DPBS (Dulbecco's Phosphate-Buffered Saline) and then incubated with tubulin extraction buffer (10mM PIPES, pH 6.8, 50mM KCl, 2mM EGTA, 1mM $MgCl_2$, 2M glycerol, 0.5% Triton X-100, 1mM Na_3VO_4, and protease inhibitor cocktail [Sigma]) for 15min at room temperature. The resulting lysate was then centrifuged at 37°C at 16,000 x g for 2min to pellet any polymerized microtubules. The resulting supernatant, containing soluble (monomeric) tubulin, was then subjected to Western blotting. The protocol was modified from [42].

2.5. Immunoblotting

Cell extracts were collected and immunoblots were performed as previously described [43] with the following exception: BioRad (Hercules, CA) gel running system with a 10% gel was used to separate proteins. Primary antibodies used: anti-α-tubulin (1:5000; Sigma) and anti-GAPDH (1:10000; Abcam). ImageJ was utilized for densitometry (from $N \geq 3$ experiments).

2.6. Cell Toxicity Assay

Cells were plated in 96-well plates and treated with 150μM 6OHDA for varying amounts of time. LDH (lactate dehydrogenase) release assay was carried out according to the protocol

provided by the manufacturer (Promega, Madison, WI). Spectramax M2 plate reader was utilized to read the fluorescent signal (Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was calculated by the following formula: 100 x [(Experimental – Medium Background) / (Maximum LDH release – Medium Background)]. Maximum LDH release was determined by treatment of cells with lysis buffer as per manufacturer's protocol.

2.7. Quantitative image analysis and statistics

An inverted epifluorescent microscope (Olympus IX71) was used for imaging and ImageJ (NIH) was utilized for quantification. Nuclear:Cytoplasmic ratios were calculated from at least 50 randomly imaged cells per replicate (and pooled from at least three replicates) by measuring the fluorescent intensity within a randomly distributed region of interest of fixed size within the nuclear and the cytoplasmic compartments. Raw fluorescent intensity is shown in the figures. Nrf2 distribution was also scored into two categories: (1) cytoplasmic and (2) cytoplasmic and nuclear. Example images of these categories are shown in the results section. Each experiment was replicated at least three times. Student's t test was used to compare means between two groups. One-way analysis of variance (ANOVA) followed by posthoc Tukey HSD test was used for multiple comparisons. A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Subcellular localization of GR in response to hormone

The glucocorticoid receptor (GR) was used to examine the effects of 6OHDA-induced oxidative stress on bidirectional nucleocytoplasmic trafficking. Transport of GR from the cytoplasm to the nucleus is hormone-dependent and bidirectional as hormone withdrawal triggers its export from the nucleus [12,44-46]. Therefore, both steps in the nucleocytoplasmic trafficking of this transcription factor are regulated and can be experimentally isolated. SH-SY5Y human neuroblastoma cells were transfected with an expression vector for GR-GFP and treated for 1hr with either Dexamethasone (Dex), a synthetic glucocorticoid, or Cortisol (Cort), the primary circulating glucocorticoid in humans. In the absence of Dex or Cort, GR-GFP is cytoplasmic in the majority of the cells (~60% on average) with the remainder of cells also showing some nuclear localization. The mean fluorescent intensity within equal size nuclear and cytoplasmic regions (nuclear:cytoplasmic ratio) for untreated cells is on average around 1, as shown by the dotted line (Fig. 1). A dose-dependent increase in nuclear transport of GR-GFP was observed with maximum nuclear localization attained at $\geq 10^{-7}$M Dex or Cort (Fig. 1).

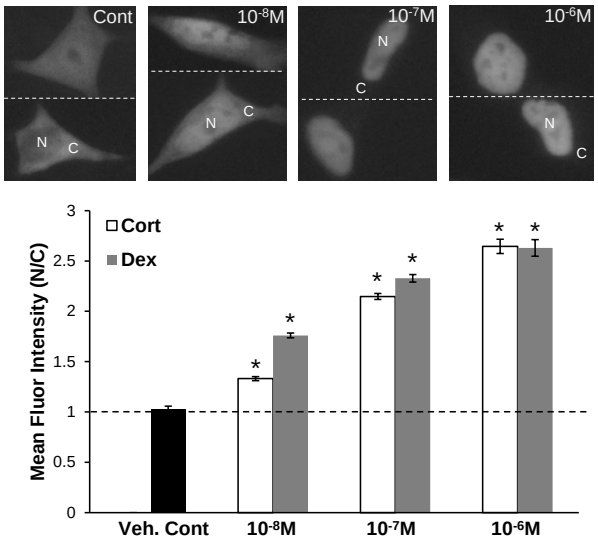

Figure 1: Dexamethasone / Cortisol Titration

SH-SY5Y cells expressing GR-GFP were treated for 1hr with Dex or Cort at the indicated concentrations. In the absence of either ligand, GR-GFP is cytoplasmic in the majority of the cells (~60%) with the remainder showing some nuclear localization as well. The raw mean fluorescent intensity within equal size nuclear and cytoplasmic regions (N/C ratio) for untreated cells is approximately 1. After hormone treatment, GR-GFP is primarily localized within the nucleus. Nuclear:Cytoplasmic GR-GFP ratios were determined as noted in the Materials and Methods section. Representative images from Dex treatment are shown. Mean +/- SEM, $*p < 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

3.2. 6OHDA treatment impairs nuclear import of GR

With 150μM 6OHDA (dose where impaired CREB/pCREB localization is observed [17]), treatment for less than 6hr did not cause cell death (Fig. 2A). To determine if 6OHDA treatment causes an impairment in nuclear import, GR-GFP expressing SH-SY5Y cells were first treated with 150μM 6OHDA for 1hr and 4hr and then treated with 1μM Dex for 1hr to induce the nuclear localization of GR-GFP. As seen in Figure 2B and 2C, pre-treatment with 6OHDA impaired the hormone-dependent nuclear import of GR-GFP. Significant reduction in the extent of nuclear import was also seen at doses varying from 90μM to 300μM and treatment times as early as 30min (data not shown).

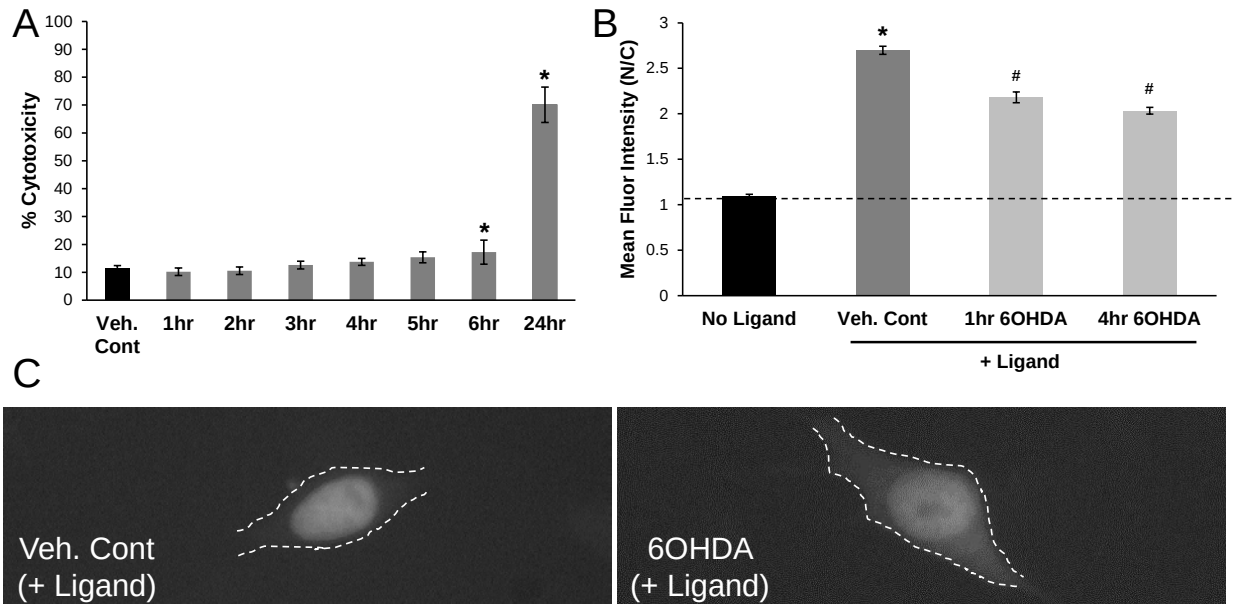

Figure 2: 6OHDA Reduces GR Nuclear Import

(A) LDH release assay showed no evidence of toxicity until 6hr of 6OHDA treatment (150μM). Mean +/- SEM, *$p < 0.05$ vs. Veh. Cont. (B & C) GR-GFP expressing SH-SY5Y cells were exposed to 150μM 6OHDA for 1hr and 4hr and then treated with 1μM Dex for an additional 1hr. Significant reduction in GR import was observed in 6OHDA-treated cells. Mean +/- SEM, *$p < 0.05$ vs. No Ligand; $^{\#}p < 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

3.3. Antioxidant treatment reverses 6OHDA-induced impairment in nuclear import

6OHDA has the propensity to produce free radicals [36,47]. To determine whether the nuclear import impairment observed in 6OHDA-treated cells involves oxidative stress, SH-SY5Y cells expressing GR-GFP were treated with MnTBAP, a cell permeable SOD mimetic, 30min before or 30min after the start of 6OHDA treatment (150μM; 4hr) (Fig. 3). When cells were treated with MnTBAP before being exposed to 6OHDA, a near complete rescue in GR-GFP nuclear import was observed (Fig. 3A). When cells were first treated with 6OHDA for 30 minutes before the addition of MnTBAP, a partial protection was observed (Fig. 3B). This is consistent with the observation that 6OHDA treatment as short as 30min is enough to cause some impairment in GR nuclear import (data not shown). The rescue observed with MnTBAP

treatment suggests an oxidative mechanism is responsible at least in part for the 6OHDA-mediated reduction in GR nuclear import.

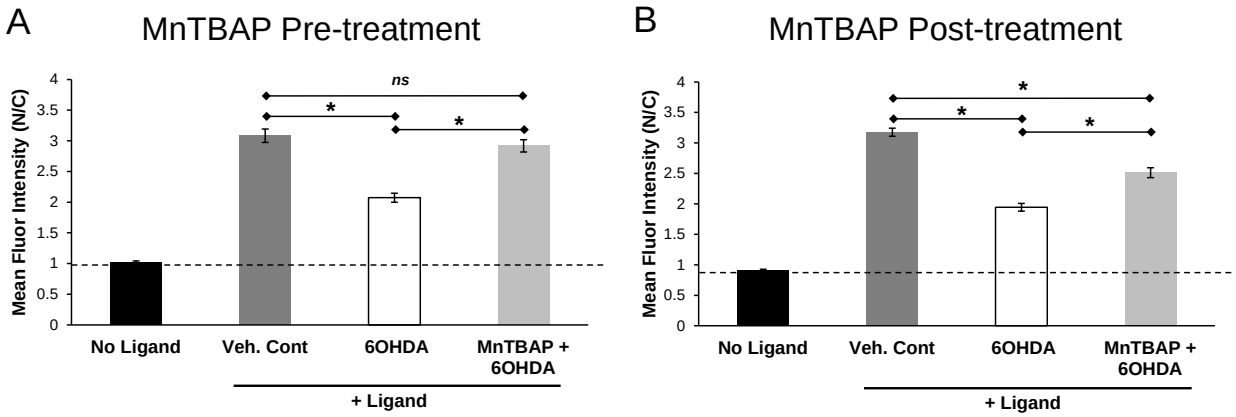

Figure 3: MnTBAP Rescues 6OHDA-Induced Impairment in GR Nuclear Import

SH-SY5Y cells expressing GR-GFP were treated with MnTBAP, a cell permeable SOD mimetic, 30min before (A) and 30min after (B) the start of 6OHDA treatment (150μM for 4hr). A complete and a partial protection was observed with pre- and post-6OHDA treatments with MnTBAP, respectively. Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ as indicated. Compiled from 3 independent experiments.

3.4. Nuclear export of GR is not altered in 6OHDA-treated cells

In addition to impaired nuclear import, enhanced nuclear export could also explain the altered transcription factor distribution observed in neurodegenerative diseases. To study if nuclear export is affected by 6OHDA-induced oxidative stress, a hormone withdrawal study was performed in SH-SY5Y cells expressing GR-GFP. Cort, the naturally occurring ligand for GR, dissociates rapidly from GR and is therefore better suited for studying the effects of hormone withdrawal [46,48]. SH-SY5Y cells expressing GR-GFP were first treated with 10^{-7}M Cort for 1hr to induce the nuclear localization of GR-GFP. The cells were then withdrawn from the ligand for up to 4hr in the presence of either vehicle or 150μM 6OHDA. No significant change in nuclear export of GR-GFP was observed in cells exposed to 6OHDA compared to vehicle control (Fig. 4).

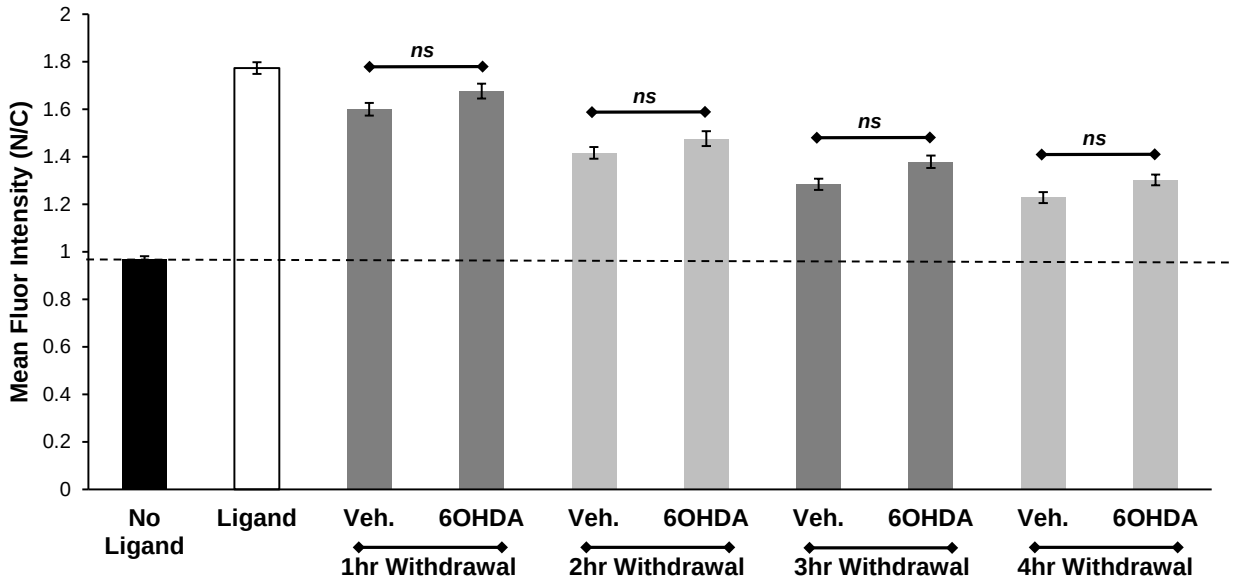

Figure 4: 6OHDA Does Not Alter GR Nuclear Export

SH-SY5Y cells expressing GR-GFP were treated with 10^{-7}M Cort for 1hr and then withdrawn from the ligand in the presence of vehicle or 6OHDA for up to 4hr. No significant impairment in nuclear export of GR-GFP was observed in 6OHDA treated cells. Mean +/- SEM, $^{ns}p > 0.05$ as indicated. Compiled from 3 independent experiments.

3.5. 6OHDA does not alter GR nuclear retention

6OHDA-induced oxidative stress could damage the nuclear envelope or chromatin binding sites and therefore alter nuclear retention of GR. To investigate this possibility, SH-SY5Y cells expressing GR-GFP were first treated with Dex for 1hr to fully induce the nuclear translocation of GR-GFP and then exposed to 150μM of 6OHDA from 2 to 6hr in the presence of Dex (Fig. 5). If 6OHDA-induced oxidative stress damaged the nuclear envelope or reduced GR binding to high affinity chromatin binding sites, an increase in ligand-bound cytoplasmic GR-GFP could occur. However, no significant change in subcellular localization was observed – suggesting that 6OHDA does not reduce GR-GFP nuclear retention or trigger its leakage from a "damaged" nucleus. In combination with the results of GR nuclear import and export assays, our data suggest that 6OHDA-induced oxidative stress impairs the hormone-dependent nuclear import of GR.

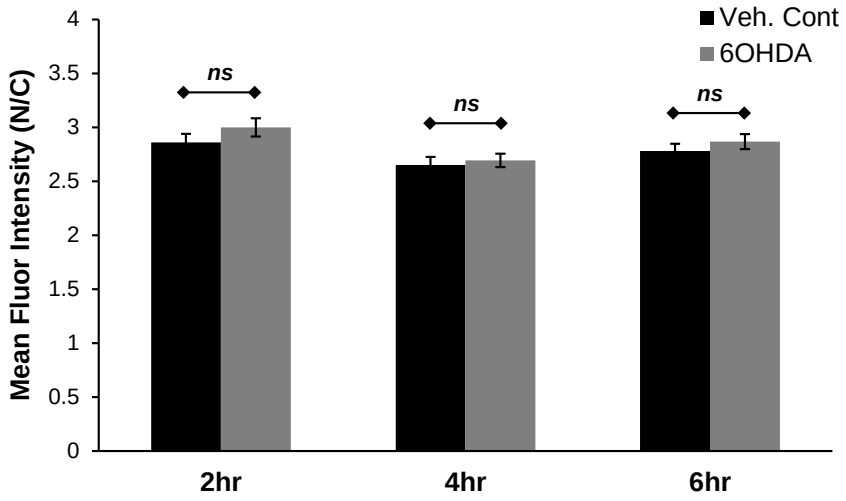

Figure 5: 6OHDA Does Not Alter GR Nuclear Retention

SH-SY5Y cells expressing GR-GFP were first treated with Dex for 1hr and then exposed to 150μM of 6OHDA from 2 to 6hr in the presence of Dex. No significant change in nuclear retention of GR-GFP was observed. Mean +/- SEM, $^{ns}p > 0.05$ as indicated. Compiled from 3 independent experiments.

3.6. Nrf2 translocates into the nucleus in response to 6OHDA

One of the transcription factors that did not show reduced nuclear localization in post-mortem human PD brains is Nrf2, a key protein in the defense against cellular stresses. Under basal metabolic conditions, Nrf2 is sequestered in the cytoplasm and targeted for proteasomal degradation via its association with Keap1 (kelch-like ECH-associated protein 1). Upon exposure to ROS, electrophiles, or serum factors, Nrf2 dissociates from the Keap1 inhibitory complex and translocates into the nucleus, where it upregulates stress response genes [49-54]. For this reason, studies of Nrf2 nuclear translocation typically involve pre-incubation under serum-deprived conditions to shift the basal distribution of Nrf2 towards the cytoplasm [54,55]. SH-SY5Y cells expressing Nrf2-GFP exhibited reduced nuclear:cytoplasmic ratios when grown in low-serum media (1% FBS) for 24hr (Fig. 6A). Serum deprivation also caused an increase in the percentage of cells with cytoplasmic localized Nrf2-GFP and a decrease in the percentage of cells showing both cytoplasmic and nuclear localization (Fig. 6B).

Nrf2 nuclear localization in response to 6OHDA-induced oxidative stress was then studied (Fig. 6C). Serum deprived SH-SY5Y cells expressing Nrf2-GFP were treated with increasing doses of 6OHDA (from 30μM to 150μM), which caused a progressive increase in the nuclear localization of Nrf2. The results from cells treated with 150μM 6OHDA are shown in Figure 6C. Quantitation of Nrf2-GFP localization revealed a decrease in the percentage of cells showing predominant cytoplasmic localization and an increase in the percentage of cells showing both cytoplasmic and nuclear Nrf2 localization. These results are similar to the studies examining Nrf2 nuclear translocation in response to H_2O_2 treatment [54]. Thus, inhibitory effects of 6OHDA on nuclear transport are selective and influence the active nuclear import of GR but not of Nrf2.

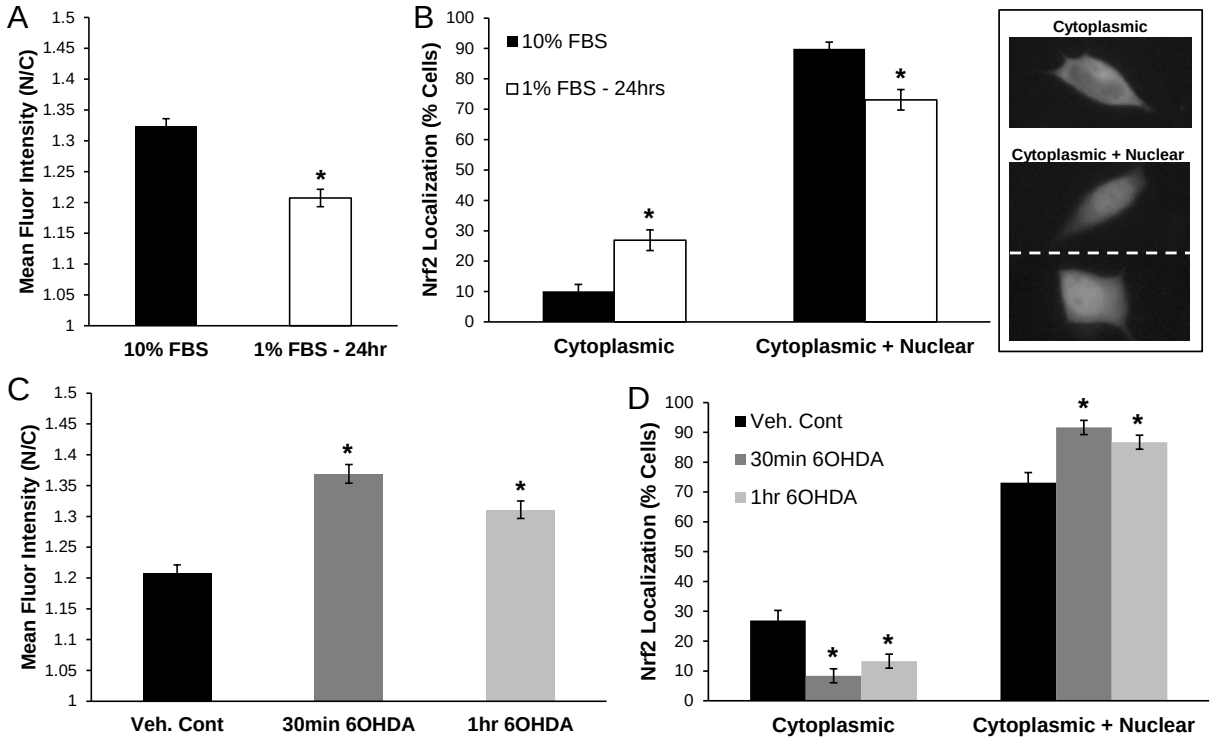

Figure 6: 6OHDA Induces Nrf2 Nuclear Localization

(A & B) Standard culture conditions elicit Nrf2 nuclear translocation. To desaturate the assay, cells were incubated in low serum media (1% FBS) for 24hr and nuclear localization was quantified by two methods as described in the Materials and Methods section. Example images of cells scored as cytoplasmic Nrf2-GFP or cytoplasmic + nuclear Nrf2-GFP are shown. Mean +/- SEM, *$p < 0.05$ vs. 10% FBS. (C & D) Treatment of serum deprived SH-SY5Y

cells with 150μM 6OHDA caused an increased nuclear and a decreased cytoplasmic localization of Nrf2-GFP. Mean +/- SEM, *$p < 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

3.7. 6OHDA increases levels of unpolymerized tubulin

The nucleocytoplasmic trafficking of some transcription factors, including CREB [56] and GR [57,58], is dependent on microtubules (MTs) in some cells. Hence, alterations in MT function due to 6OHDA-induced oxidative stress could help explain the selective trafficking impairments. This is of particular relevance given the alterations in MT function observed in several age-related neurodegenerative diseases [59,60]. In the case of PD, both toxin (e.g. MPP+ and rotenone) and genetic (e.g. α-synuclein and LRRK2) models implicate impaired MT function as a common pathway of neuronal degeneration [61-64].

We investigated the effects of 6OHDA-induced oxidative stress on polymerization of tubulin. We first determined the effects of MT-altering agents, colchicine and paclitaxel, on the levels of unpolymerized tubulin (Fig. 7A). SH-SY5Y cells were treated with either vehicle, 1μM colchicine (2hr), or 50nM paclitaxel (1hr) and then incubated with tubulin extraction buffer to extract monomeric tubulin. Colchicine increased and paclitaxel reduced the levels of monomeric tubulin, respectively, while not significantly affecting the levels of total tubulin (Fig. 7A & 7B). These concentrations of MT-altering agents did not cause cell death (data not shown). We then examined the effects of 6OHDA on the levels of unpolymerized tubulin. SH-SY5Y cells were treated with 150μM 6OHDA from 1hr to 4hr after which monomeric tubulin was extracted. Cells treated with 6OHDA showed increased levels of monomeric tubulin (Fig. 7C). Levels of total tubulin were not altered in 6OHDA-treated cells (Fig. 7D).

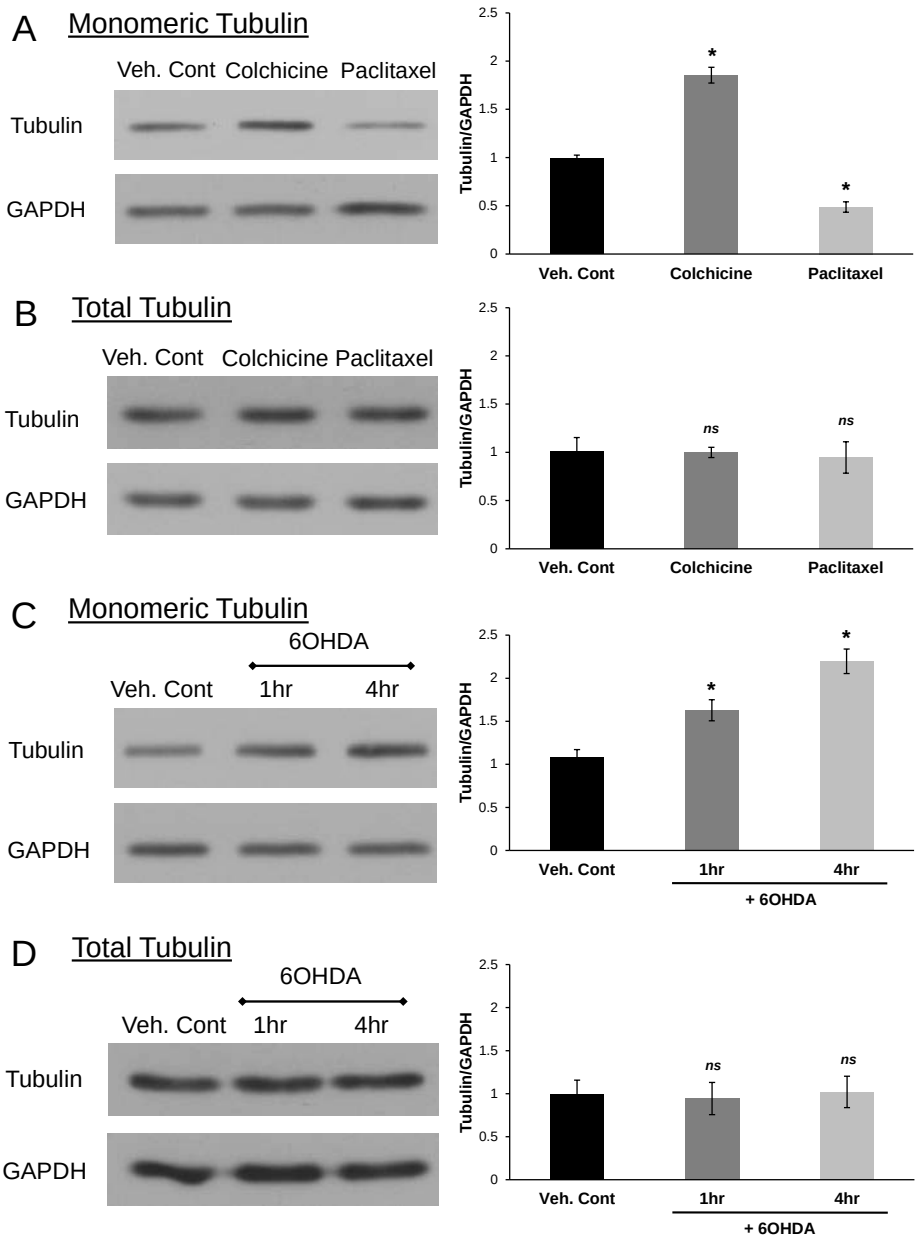

Figure 7: 6OHDA Increases Levels of Unpolymerized Tubulin

(A) SH-SY5Y cells were treated with either vehicle, 1μM colchicine (2hr), or 50nM paclitaxel (1hr) and then incubated with tubulin extraction buffer to extract monomeric tubulin and resolved by Western blotting. Colchicine increased and paclitaxel reduced the levels of monomeric tubulin, respectively. (B) Whole cell extracts of colchicine and paclitaxel treated cells were subjected to Western blotting, which showed no significant changes in total tubulin levels. (C) SH-SY5Y cells were treated with 150μM 6OHDA for 1hr and 4hr after which monomeric tubulin was extracted. 6OHDA-induced oxidative stress increased the levels of monomeric tubulin. (D) Whole cell extracts of 6OHDA-treated cells were subjected to Western blotting, which showed no changes in total tubulin levels. Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

3.8. MTs regulate GR but not Nrf2 nuclear trafficking

We then examined the effects of directly modulating MTs on the trafficking of GR and Nrf2. Colchicine and paclitaxel were utilized to disrupt or stabilize, respectively, the MT network in SH-SY5Y cells expressing GR-GFP and Nrf2-GFP (Fig. 8). SH-SY5Y cells expressing GR-GFP were exposed to these agents at doses and times indicated above and then treated with Cort to induce the nuclear translocation of GR-GFP. MT stabilization using paclitaxel did not alter the nuclear import of GR-GFP, however a decrease in nuclear import was observed in cells where the MT network was disrupted by colchicine treatment (Fig. 8A). Neither MT stabilization nor disruption significantly impaired Nrf2-GFP subcellular localization compared to vehicle in response to 6OHDA stress (Fig. 8B). Thus, the effect of 6OHDA on MT stability was similar to that of colchicine, and colchicine recapitulated the selective nuclear import impairment elicited by 6OHDA.

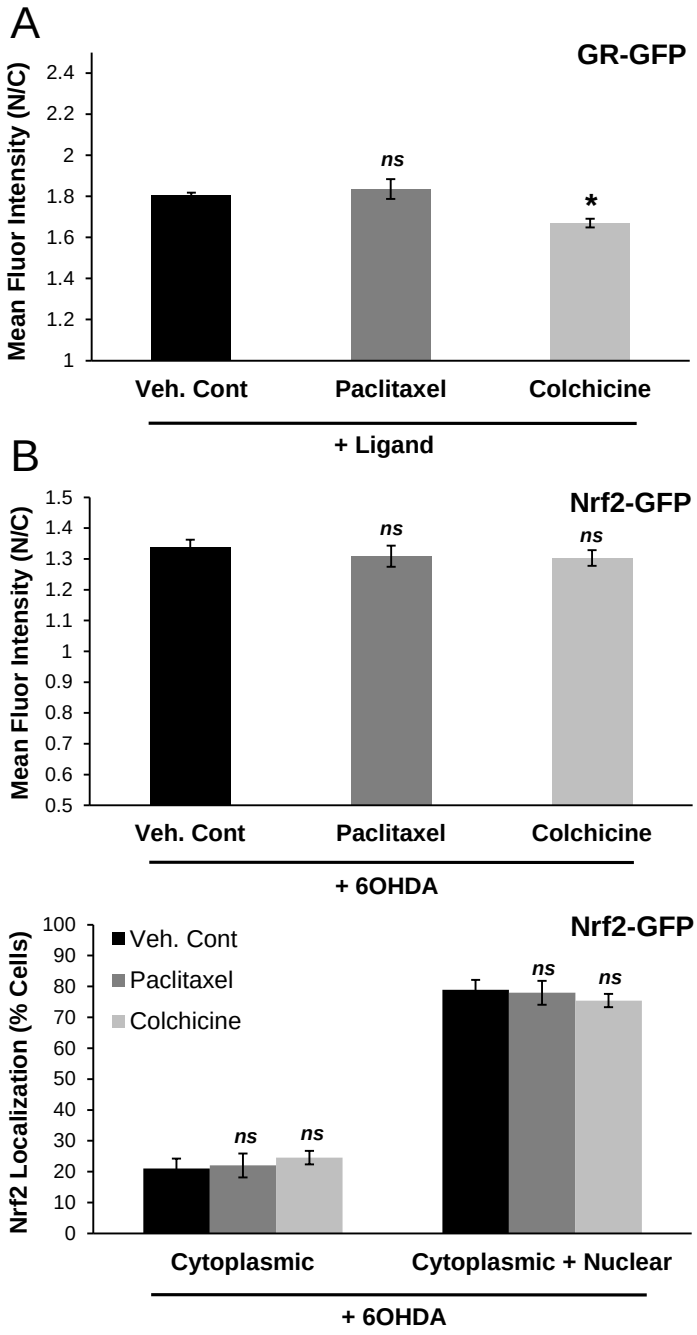

Figure 8: MTs Regulate GR but Not Nrf2 Nuclear Trafficking

(A) GR-GFP expressing SH-SY5Y cells were treated with either paclitaxel or colchicine and then treated with Cort for 30min to induce GR-GFP nuclear import. Reduced GR-GFP nuclear import was observed in colchicine treated cells but not in paclitaxel treated cells. (B) Nrf2-GFP expressing SH-SY5Y cells were treated with either paclitaxel or colchicine and then treated with 150μM 6OHDA for 30min to induce Nrf2-GFP nuclear localization. No significant impairment was observed with either MT-altering agent. Mean +/- SEM, $*p < 0.05$ or $^{ns}p > 0.05$ vs. Veh. Cont. Compiled from 3 independent experiments.

4. DISCUSSION

In this study, we examined the effects of 6OHDA on the nucleocytoplasmic trafficking of two transcription factors that are sequestered in the cytoplasm, but utilize distinct mechanisms for release from cytoplasmic anchors and import into the nucleus. Previously published work has demonstrated adverse functional effects of altered pCREB or pERK2 subcellular localization on midbrain dopaminergic neuron survival [17,39], but the mechanism underlying such localization changes remained unclear. Utilizing a well-established model of regulated nuclear transport, the glucocorticoid receptor, we found that 6OHDA treatment elicited early impairments in nuclear import without causing reduced nuclear retention or enhanced nuclear export. Treatment with an antioxidant rescued the defect in GR nuclear import, supporting an oxidative mechanism for the 6OHDA-induced effects.

While relatively high doses of H_2O_2 can affect nuclear transport through global mechanisms in non-neuronal cells [10], the effects of sublethal/prelethal oxidative stress has not been previously studied in neuronal cells. Interestingly, the impaired nuclear import observed in our system did not result from global nuclear import failure as Nrf2 nuclear localization remained intact in 6OHDA-treated cells. The selective nature of these effects suggests a specific alteration that impacts the subcellular localization of some but not all transcription factors. Our *in vitro* results are consistent with observations that Nrf2 shows clear nuclear localization in post-mortem PD substantia nigra neurons [18], while kinases and transcription factors activated by trophic signals show abnormal cytoplasmic accumulation [5].

Interestingly, GR has been linked to the regulation of the dopaminergic system, including through interactions with the nuclear receptor related 1 protein (Nurr1) – which is important for

dopaminergic differentiation and phenotype maintenance as well as being associated with PD [65-75]. Furthermore, glucocorticoids have been shown to play a protective role in models of PD, although the mechanisms may involve more than one cell type in the brain [66,69]. In any case, selective impairment of a subset of transcription factors in the aging or oxidatively stressed brain may account for downregulation of dopaminergic differentiation markers observed in PD models [76-82] and for alterations in neurotrophic gene expression observed in PD midbrain tissues [1,83-86].

The mechanism(s) underlying the selectivity in nuclear trafficking of proteins observed in this model of parkinsonian injury and in diseased PD brains may be of particular relevance to chronic neurodegeneration. Since not all transcription factors are affected, a global mechanism that would impair all nuclear transport, such as bioenergetic collapse, is unlikely. This is further supported by the early time points at which the trafficking impairment becomes apparent. The current data implicate a role for altered microtubule dependent trafficking. Some transcription factors utilize MTs for their transport. Indeed, a sequence within the parathyroid hormone related protein was recently found to efficiently transport fusion proteins into the nucleus via a MT facilitated "fast track" mechanism [87]. Oxidative stress (e.g. H_2O_2) has been linked to impaired MT function, such as decreased tubulin polymerization rate and MT growth rate [88-91]. In our model, we found that 6OHDA increased the levels of free monomeric tubulin similar to colchicine, suggesting enhanced depolymerization and/or reduced polymerization. Furthermore, colchicine-mediated disruption but not stabilization of MTs mimicked the effects of 6OHDA in reducing nuclear import of GR without affecting Nrf2 import. These results suggest that 6OHDA modulates transcription factor trafficking through effects on MT-dependent transport of transcription factors.

In addition to altered MT dynamics, there are other potential mechanisms that could contribute to selective impairments in nuclear transport. Oxidative injury, e.g. H_2O_2, has been shown to alter the distribution of the small G protein Ran, which is a key player in the classic import/export cycle [92-95]. Although such an alteration would be expected to affect nuclear transport of many proteins, it is possible that different transcription factors have differential sensitivity to the breakdown of the Ran gradient across the nucleus or utilize an alternative non-Ran dependent mechanism for their nuclear import [11,96]. Another possibility is the involvement of carrier proteins for nuclear import, importins. Different transcription factors utilize different importins for their nuclear import, as is the case for CREB, GR, and Nrf2 – hence oxidative stress-induced alterations in different importins could also help explain the observed selectivity [97-99]. Evidence of altered levels or localization of specific importins have been described in various neurodegenerative diseases [10].

In summary, we have found that 6OHDA selectively impairs nuclear import through an oxidative mechanism that may involve alterations in MT-dependent trafficking of transcription factors. Given the importance of transcription factors for regulating gene expression, alterations in their nucleocytoplasmic transport could have significant consequences for neuronal differentiation, function, and survival. For chronic diseases such as PD, oxidative stress related to aging, the cellular handling of the oxidative catecholamine neurotransmitters, or mitochondrial pathology could contribute to reduced trophic and reparative transcriptional responses through selective impairments in nuclear import.

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