A53T alpha-synuclein induces mitophagy through MAM-induced fission and reduced impairment of LC3B to promote alpha-synuclein degradation

by

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ABSTRACT

A53T alpha-synuclein induces mitophagy through MAM-induced fission and reduced impairment of LC3B to promote alpha-synuclein degradation

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University of Guelph, 2017

Advisor: Dr. Scott Ryan

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by mitochondrial dysfunction and aggregated α-synuclein (α-syn). Some cases of PD are caused by mutations in α-syn, resulting in an increased propensity for aggregation. To investigate the link between mitochondrial dysfunction and the α-syn mutation A53T, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), each consisting of an A53T line and a wildtype or corrected control line, were differentiated into dopaminergic human neurons (hNs). Transmission electron microscopy demonstrated that A53T cells had elongated mitochondrial-associated membranes (MAMs) at early timepoints, which were shortened to normal lengths by day 60 of differentiation, concurrent with mitochondrial fragmentation. Golgi dilation and vacuolar area were increased in A53T hNs, indicative of an increased autophagic load. Immunofluorescence showed that aggregated α-syn has a greater colocalization with lysosomes in A53T hNs, suggesting lysosomes degrade, or attempt to degrade, aggregated α-syn. This degradation could occur alongside mitophagy, as knockdown of the apoptotic protein beclin1 decreased cell survival in the A53T line, indicating mitophagy is protective. Further, in vitro experiments demonstrate that A53T α-syn has a reduced ability to impair binding of the mitophagy-initiating protein LC3B to mitochondria, suggesting that A53T cells would be more vulnerable to mitophagy. Potential therapies should focus on preventing MAM-induced fission and restoring the ability of A53T cells to impair LC3B-mediated mitophagy.
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AUTHOR’S DECLARATION OF COMPLETED WORK

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

Stem cell and neuronal culture and immunostaining was performed by Morgan Stykel and Dr. Scott Ryan.

Transmission electron microscopy was performed by Keith Sherriff.

Validation of BECN1 was performed by Carla Coackley and Keith Sherriff.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Glossary</th>
</tr>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>α-syn</td>
<td>Alpha-synuclein</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy-related protein</td>
</tr>
<tr>
<td>BECN1</td>
<td>Beclin1</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Casp3</td>
<td>Caspase-3</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenylhydrazone</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
</tr>
<tr>
<td>Corr</td>
<td>Corrected</td>
</tr>
<tr>
<td>DA</td>
<td>Dopaminergic</td>
</tr>
<tr>
<td>DAN</td>
<td>Dopaminergic neuron</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>Dibutyryl cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DRP1</td>
<td>Dynamin-related protein 1</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FGF8</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Fis1</td>
<td>Mitochondrial fission 1</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>hiPSC</td>
<td>Human induced pluripotent stem cell</td>
</tr>
<tr>
<td>hN</td>
<td>Human neuron</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>hPSC</td>
<td>Human pluripotent stem cell</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>KSR</td>
<td>Knockout serum replacement</td>
</tr>
<tr>
<td>LAMP1</td>
<td>Lysosome-associated membrane protein 1</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy body (or Lysogeny Broth)</td>
</tr>
<tr>
<td>LBD</td>
<td>Lewy body dementia</td>
</tr>
<tr>
<td>LN</td>
<td>Lewy neurite</td>
</tr>
<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>LRW</td>
<td>LR White</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>MAM</td>
<td>Mitochondria-associated ER membrane</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Mfn1</td>
<td>Mitofusin 1</td>
</tr>
<tr>
<td>Mfn2</td>
<td>Mitofusin 2</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPPP</td>
<td>1-methyl-4-phenyl-4-propionoxypiperidine</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NAC</td>
<td>Non-Aβ-amyloidogenic component</td>
</tr>
<tr>
<td>NB</td>
<td>Neurobasal</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline with 0.1% Tween</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFF</td>
<td>Pre-formed fibril</td>
</tr>
<tr>
<td>PINK1</td>
<td>P-TEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PS129</td>
<td>Phosphorylated serine 129</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering ribonucleic acid</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra <em>pars compacta</em></td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>Transforming growth factor beta-3</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
(1.1) Parkinson’s disease

Parkinson’s disease (PD) is a progressive neurodegenerative disorder and the most common movement disorder [1]. It is characterized, in part, by impaired motor function resulting from the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the appearance of protein aggregates known as Lewy bodies (LBs) and Lewy neurites (LNs; Figure 1.1) [1]. These motor impairments include tremor-at-rest, rigidity, bradykinesia (slowness of movement), poor balance, and loss of voluntary movement [1]. Over time, more DA neurons are lost and Lewy body pathology spreads to other parts of the brain, including the hippocampus and the cortex, which is associated with the onset of dementia (Figure 1.1) [2,3]. This neurodegeneration in the hippocampus and cortex could be responsible for non-motor PD symptoms including autonomic, cognitive and psychiatric impairments [1,2].

Age is known to be the biggest risk factor for PD, though there is no identifiable cause for over 90% of PD cases [1,2]. However, the remaining cases are caused by mutations in a variety of genes including LRRK2, PARK7, PARK2, PINK1, and SNCA (Table 1.1; reviewed in [4]). LRRK2 is the most commonly mutated gene associated with PD and it codes for leucine-rich repeat kinase 2 (LRRK2), also known as dardarin [4]. It is thought to have kinase activity, GTPase activity and act as a scaffold for protein interactions, but its specific function is unknown [4]. Mutations in this protein interfere with cellular signalling and cytoskeletal trafficking [5]. DJ-1, encoded by PARK7, is speculated to be a sensor for oxidative stress due to its sensitivity to H$_2$O$_2$ [4], and mutations cause mitochondrial dysfunction [5]. PARK2 transcribes the protein Parkin, an E3 ubiquitin ligase that polyubiquitinates proteins on dysfunctional mitochondria to
initiate mitophagy, selective autophagy of mitochondria (Figure 1.2A) [4]. Loss-of-function mutations in this gene impair mitophagy [4]. Likewise, loss-of-function mutations in PINK1, coding for the protein PINK1 (P-TEN induced putative kinase 1), also disrupts mitophagy, as PINK1 recruits Parkin to depolarized mitochondria (Figure 1.2A) [6]. Lastly, SNCA codes for α-synuclein (α-syn), the main protein component of Lewy bodies. Mutations in SNCA, as well as duplication or triplication events, are believed to promote α-syn aggregation [7,8]. As these disease-causing mutations suggest, both mitochondrial dysfunction and aggregation of α-syn have been identified as common cellular pathologies in the majority of PD cases [9]. Though these pathologies are thought to be connected, it has yet to be fully elucidated.

![Figure 1.1 – Progression of Parkinson’s disease.](image)

PD pathology begins with the loss of A9 dopaminergic neurons in the substantia nigra pars compacta (shown in red) and the formation of protein inclusions known as Lewy bodies (shown in dark pink). As the disease progresses more neurons are lost, and Lewy body pathology can spread to the cortex (in grey), associated with dementia. (Modified with permission from [3]).
Table 1.1 – Mutations in Parkinson’s disease.

Mutations of several genes involving a variety of proteins and cellular phenotypes are causal for Parkinson’s disease (Adapted from [5]).

<table>
<thead>
<tr>
<th>Mutated gene</th>
<th>Protein</th>
<th>Cellular phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNCA</td>
<td>α-synuclein</td>
<td>Protein aggregation leading to Lewy bodies</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkin</td>
<td>Impaired mitophagy leading to mitochondrial dysfunction</td>
</tr>
<tr>
<td>PARK6</td>
<td>PINK1</td>
<td>Impaired mitophagy leading to mitochondrial dysfunction</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>Oxidative stress; mitochondrial dysfunction</td>
</tr>
<tr>
<td>LRRK2</td>
<td>LRRK2</td>
<td>Lewy bodies; disruption of signalling pathways</td>
</tr>
</tbody>
</table>

(1.2) Mitochondrial regulation

Before understanding the role of mitochondrial dysfunction in PD, it is important to understand the physiological state of mitochondria in the cell. Mitochondria are dynamic organelles, regularly undergoing fusion and fission. Mitochondrial fusion is regulated by proteins that include mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atrophy 1 (OPA1) [10]. Specifically, Mfn1 and Mfn2 initiate fusion of the outer mitochondrial membrane (OMM), and optic atrophy 1 (OPA1) initiates fusion of the inner mitochondrial membrane (IMM) [10]. Conversely, mitochondrial fission is initiated by the GTPase dynamin-related protein 1 (DRP1) and its interactions with mitochondrial fission 1 (Fis1; Figure 1.2B) [10]. Fusion and fission are both important to cell health, with fusion suggested to allow mitochondria to share and redistribute proteins and metabolites, and fission promoting a better distribution of mitochondria throughout the cell [10]. Dysregulation of this balance can lead to cellular dysfunction. Specifically, a combination of reduced fusion or increased fission leads to mitochondrial fragmentation [10]. Mitochondrial fragmentation has been reported in many PD patients [11], indicating that the regular fusion and fission patterns are disrupted in PD.
As previously described, Parkin and PINK1 are also involved in mitochondrial quality control (Figure 1.2A). While historically Parkin was believed to be involved in maintaining mitochondrial function, the specific mechanism was elucidated more recently [12]. After verifying that Parkin was localized to the cytosol, coincident with small, fragmented mitochondria, Narendra and colleagues evaluated previous claims that Parkin promotes clearance of depolarized mitochondria [12]. Using carbonyl cyanide m-chlorophenylhydrazone (CCCP) as a mitochondrial uncoupling agent in HEK293 cells, they found that Parkin was recruited to the mitochondrial membrane, suggesting that Parkin translocates to mitochondria with low membrane potential [12]. To further support their findings, they expressed YFP-Parkin in mouse embryonic fibroblasts that had Mfn1 and Mfn2 knocked out. As outlined above, the mitofusins play a role in mitochondrial fusion, and their activity reduces fragmentation [10]. Further, it had been previously reported that some mitochondria of Mfn1−/Mfn2− double knock-out mice are physiologically normal and others have a low membrane potential [13]. By combining YFP-Parkin with MitoTracker red, a mitochondrial dye that is potential-dependent, they observed that YFP-Parkin colocalized with mitochondrial membranes that had low MitoTracker staining, indicative of depolarization [12]. They then confirmed that the cells expressing YFP-Parkin had decreased mitochondrial volume after 12 and 48 hours, suggesting that the depolarized mitochondria had been cleared from the cell through the involvement of Parkin [12]. Therefore, loss-of-function mutations in Parkin, such as those causal for PD, can prevent degradation of mitochondria, implicating mitochondrial function in PD.
A PINK1 recruits Parkin to depolarized mitochondrial membranes. Parkin then polyubiquitinates pro-fusion proteins on the outer membrane, targeting them for degradation. B Fission proteins, including DRP1 and Fis1 are recruited to the mitochondria and initiate fission, leading to mitochondrial fragmentation. Some cases of fission involve MAM interaction with DRP1. C Fragmented mitochondria are then engulfed in a double membrane to form an autophagosome and fuse with a lysosome, to be degraded by mitophagy.

Following this study, a second group confirmed previous reports that PINK1 and Parkin interact by measuring co-immunoprecipitation of the proteins after exposure to CCCP [6]. They also observed that knocking down PINK1 with short interfering RNA (siRNA) greatly reduced localization of Parkin to the mitochondria [6], confirming that PINK1 is involved in the recruitment of Parkin to mitochondria. It has since been determined that Parkin, as an ubiquitin kinase, polyubiquitinates proteins on the OMM, including mitofusins, targeting them for degradation [14]. Since mitofusins are required for mitochondrial fusion, their degradation
promotes fragmentation. The fission proteins DRP1 and Fis1 then interact to initiate fission (Figure 1.2B), further promoting fragmentation [10]. These findings further confirm the role of PINK1 and Parkin in mitochondrial clearance, and how their mutations in PD can lead to mitochondrial dysfunction.

(1.3) Mitophagy

Dysfunctional mitochondria are cleared from the cell by a process known as mitophagy, or specific autophagy of mitochondria. Fragmentation is a requirement for mitophagy, but it alone is not sufficient and other factors are needed [10]. Mitochondrial depolarization, as outlined above in the YFP-Parkin example, can also trigger some forms of mitophagy. Certain stresses, such as exposure to the pesticide rotenone, can cause the phospholipid cardiolipin to externalize from the inner to the outer mitochondrial membrane [15]. This externalization results in the recruitment of microtubule-associated protein light chain 3B (LC3B) to the mitochondria where it interacts with cardiolipin to initiate mitophagy [15]. Various autophagy-related proteins (Atgs) are recruited, including beclin1, to form a complex that aids in the formation of a double-membrane structure engulfing the mitochondria, known as an autophagosome. The autophagosome then fuses with a lysosome to form an autophagolysosome, where controlled degradation of the mitochondria takes place (Figure 1.2C) [10]. In this way, cells can selectively eliminate dysfunctional mitochondria. If this process is impaired, as in Parkin and PINK1 mutations of PD, the dysfunctional mitochondria are not degraded and contribute to cellular pathology.
The implication of loss-of-function mutations in Parkin and PINK1, and the resulting impairment of mitophagy is evidence of the role of mitochondrial dysfunction in PD. Further to that, it is common to use compounds that disrupt the mitochondrial electron transport chain to induce PD pathology in cellular and animal models, such as the pesticides rotenone and paraquat, and the compound MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). MPTP was first discovered to cause motor impairment comparable to that of PD in the 1980s, when three drug users were admitted to hospital for immobility and a fourth showed similar symptoms [16]. They had taken MPPP (1-methyl-4-phenyl-4-propionoxypiperidine) contaminated with MPTP, a by-product of MPPP production [16]. The authors described the symptoms as parkinsonian, and found that administration of L-dopa and carbidopa, used to treat PD, cleared symptoms in all four patients [16]. It has since been shown that MPP+ (1-methyl-4-phenylpyridinium), the active metabolite of MPTP, acts in part by inhibiting complex I of the electron transport chain in the IMM, disrupting cellular respiration and mitochondrial function [17]. Like MPP+, rotenone and paraquat also inhibit complex I [18–20], resulting in the generation of reactive oxygen species (ROS) such as superoxide anion radicals (O$_2^-$) [21]. Superoxide can be reduced to hydrogen peroxide (H$_2$O$_2$), which can be further reduced to the hydroxyl radical (·OH) in the presence of reduced metals, or react with nitric oxide (NO) to form peroxynitrite (ONOO$^-$) and other reactive nitrogen species (RNS) [21]. These reactive oxygen and nitrogen species can cause oxidative and nitrosative stress through protein nitration, lipid peroxidation and DNA damage, contributing to PD pathology [22].

With the evidence that external chemicals can lead to parkinsonian-like pathology and that loss of mitochondrial maintenance proteins are causal in many familial cases of PD, it is evident that mitochondrial dysfunction is central to PD. Further, the knowledge that PD cases
with mutations in Parkin and PINK1 lack the otherwise characteristic α-synucleinopathy [23] suggests that mitochondrial dysfunction is crucial to PD pathology, and that α-synuclein aggregation may play a role in initiating mitochondrial dysfunction in cases where it is present.

(1.4) Mitochondrial-associated membranes

Mitochondrial-associated membranes (MAMs) are regions of the endoplasmic reticulum (ER) that are in close proximity to mitochondria [24]. They are known to be involved in calcium and phospholipid exchange between the ER and mitochondria, as well as intracellular trafficking, the unfolded protein response, autophagy, mitochondrial biogenesis and the formation of inflammasomes [25]. Interestingly, all of these processes are impacted in PD, as well as other neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [25]. This is likely due to alterations in MAMs that have been reported in PD [25]. Specifically, studies have shown that MAMs can be altered by Parkin and DJ-1 [25], both proteins that cause PD when mutated. In addition to its other roles, it has been reported that MAM is a site of localization for DRP1 [24]. It has been suggested that MAMs may loop around mitochondria, facilitating the binding of DRP1 and Fis1 [24]. DRP1 can then form large multimers that assemble in a ring-like structure to facilitate fission at the MAM site [26]. Therefore, alterations in MAMs, like that present in many cases of PD, could contribute to fragmentation and later mitophagy of mitochondria.
(1.5) α-Synucleinopathy

α-Synuclein (α-syn) is a small, 14 kDa protein implicated in PD and other neurodegenerative diseases (reviewed in [27]). A neurodegenerative disorder is classified as a synucleinopathy if it is characterized by aggregation of α-synuclein [28]. Common examples of other synucleinopathies include Lewy body dementia (LBD), multiple system atrophy, and some cases of Alzheimer’s disease (AD) [9]. The role of α-syn in PD is also supported by the observations that mutations in the SNCA gene are causal in familial diseases cases. These mutations include amino acid substitutions such as A53T, A30P, and E46K, as well as gene duplication and triplication events that increase α-syn expression [9]. It has been suggested that mutated proteins aggregate more easily than their wild-type counterparts, and that a greater abundance of α-syn would also lead to more aggregation [7,8]. While these gene mutations are causal in familial PD, aggregation of wild-type α-syn is nonetheless abundant in sporadic PD cases [9], emphasizing the importance of improving our understanding of synucleinopathy to elucidating mechanisms of PD pathology and therapeutic development.

To understand the pathology of α-syn in PD and other synucleinopathies, it is first important to understand the many structural conformations in which the protein can exist (Figure 1.3). Three different regions make up the protein: the N-terminus (residues 1-60), the non-Aβ-amyloidogenic component (NAC; residues 61-95) and the C-terminus (residues 96-140; reviewed in [9]), and inter- and intra-molecular interactions involving these regions contribute to the variety of conformations α-syn can assume. In solution, recombinant α-syn is intrinsically disordered, with no ordered secondary structure when observed by circular dichroism spectroscopy (Figure 1.4A-D) [29]. It is widely believed to adopt this same conformation in the cytosol (reviewed in [9]). However, a study by Bartels and colleagues suggested that it primarily
exists as helically-folded multimers, predominantly tetramers of approximately 58 kDa, in human brain cells and red blood cells, with the monomer much less abundant [30]. These researchers report that the use of detergents and heat during purification of recombinant α-syn expressed in bacteria irreversibly degrade the tetramers into the commonly observed monomeric species [30]. More recently they have shown that the A53T and E46K mutated proteins result in decreased tetramer to monomer ratios [31]. Combined with their previous observation that tetrameric α-syn is less prone to aggregation [30], this suggests that a shift to monomeric α-syn with an increased propensity for aggregation could be involved in pathology.

Regardless of whether monomeric or tetrameric species are more abundant, it is believed that monomeric α-syn can aggregate into short β-sheet-like oligomers, which can then further aggregate into pathogenic fibrils (Figure 1.3; [27]). Further modifications of fibrils, such as hyper-phosphorylation, nitration and ubiquitylation, promote their ultimate deposition into Lewy bodies [27], which are the hallmark pathology of PD and LBD neurons. The C-terminal region is thought to contribute to the aggregation of α-syn into β-sheets, in part because it has many phosphorylation sites [9,32]. For example, α-synuclein phosphoserine-129 (PS129), a well-established marker for α-syn aggregation [33], is located within the C-terminal region.

Conversely, it has been found that α-syn can also adopt an α-helical conformation in the presence of lipid vesicles [34]. The helices are formed by 7 copies of an imperfect repeating 11-residue sequence that contains a conserved KTKEGV motif in the N-terminal and NAC regions of α-syn [9]. The resulting helix is amphipathic, such that the hydrophobic surface is embedded within the lipid membrane with the acyl chains and the hydrophilic surface can interact with the phospholipid head groups [9]. α-Syn is known to have an affinity for anionic phospholipids, because the positively charged lysines of the conserved motif can form favourable interactions
with the negatively charged head groups [9]. The C-terminal region is not involved in the formation of α-helices, likely due to its large number of acidic residues, which would not interact favourably with the anionic head group, and instead remains disordered [9]. The physiological role of α-syn is not fully understood, but is thought to involve this α-helical lipid-associated conformation. Studies have shown that α-syn localizes to neuronal synapses, and it is thought to play a role in transporting and releasing neurotransmitter vesicles [2,32,35]. Recent studies have suggested it is specifically involved in SNARE-mediated vesicle fusion, interacting with both the SNARE and the lipid vesicle (reviewed in [32]). Interestingly, the tetrameric conformation, which naturally adopts an α-helical conformation, was reported to have increased binding to negatively charged lipid membranes when compared to monomeric α-syn [30]. This suggests that regardless of whether monomeric or tetrameric α-syn is more abundant, α-helical α-syn has an important physiological function.

It is therefore apparent that α-syn can adopt a variety of conformations, and there is debate as to which of these species may be toxic and confer pathology (Figure 1.3) [27]. For example, some studies have suggested that Lewy bodies are the primary mediator of neuronal loss in PD [36], while others indicate a protective role for LBs and implicate oligomers or fibrils in neurodegenerative toxicity [37,38], arguing that LBs are a mechanism for sequestering α-syn fibrils from the rest of the cell, inhibiting their potential for toxicity. There have also been many studies that suggest that α-syn fibrils act as prions, whereby they induce the formation of fibrils from non-aggregated α-syn, propagating further synucleinopathy [39]. Recent studies have explored these phenomena by focusing on pre-formed fibrils (PFFs) that can be generated in vitro from recombinant α-syn and applied to neurons to induce aggregation of endogenous α-syn [39–41]. PFF-based models of PD recapitulate motor deficits in rodents in as little as 30 days
The protein α-synuclein can adopt many conformations. It has traditionally been thought to be natively intrinsically disordered, though recently new studies have suggested α-syn exists predominantly as a tetramer (not shown; [30]). Both monomers and tetramers can form α-helices in the presence of curved lipid membranes. Conversely, monomers can oligomerize into β-sheet-like oligomers, then become further modified by phosphorylation at serine129, nitration and ubiquitination to form fibrils. Ultimately, they are deposited as insoluble Lewy bodies.

that would require 18-months to be visible in α-syn transgenic animals, highlighting their utility over conventional model systems [41,42]. The NAC region is thought to be responsible for this prion-like characteristic, and studies have shown that fibrils made up of recombinant truncated α-syn containing only the NAC region are sufficient for seeding recruitment of endogenous α-syn into aggregates [40]. The same studies have demonstrated that PFFs can transmit between neurons, thereby seeding α-syn aggregation in previously healthy neurons [40]. This provides a potential explanation as to why grafted neurons develop PD pathology in PD patients many years after transplantation [43].
However, there is another prevalent hypothesis, known as the selective vulnerability hypothesis (reviewed in [44]). This hypothesis suggests that aggregation first occurs in neurons that are particularly vulnerable then later spreads to less-vulnerable neurons, perhaps involving transmission of metabolic factors to spread the adverse condition, rather than the protein itself. Additionally, the appearance of Lewy body pathology in grafted neurons has been associated with microglial activation [44]. Walsh and Selkoe have speculated that the two hypotheses are not mutually exclusive, and that it is possible both prion-like propagation and selective vulnerability play a role in PD pathology [44].

Irrespective of how PD pathology is transmitted between neurons, the application of PFFs composed of recombinant α-syn has been demonstrated to be a good model to study synucleinopathy in PD [39–41]. Volpicelli-Daley and colleagues were the first group to use PFFs to recruit endogenous α-syn into aggregates in primary neurons without the use of factors to aid PFF entry into the cell or mutant or over-expressed α-syn [40]. They characterized the appearance of Lewy body- and Lewy neurite-like inclusions in their experiments, using immunofluorescence, transmission electron microscopy and immune-electron microscopy, and confirming that they resembled LBs and LNs observed in the brains of human PD patients [40]. Overall, these findings combined with the observations that PFFs produce PD-like motor deficits in rodents [41], provide evidence that use of PFFs neurons is useful in modeling PD pathology.
Relationship between mitochondria and α-synuclein

In addition to secreted vesicles, α-syn can likely also interact with other intracellular membranes. Specifically, studies have shown that it can colocalize with the mitochondria [45–48]. Our lab has shown through circular dichroism (CD) spectroscopy that while both WT and A53T α-syn bind to large unilamellar vesicles (LUVs) containing the same proportion of phospholipids as the OMM in a cardiolipin-dependent manner, WT α-syn binds to a greater extent (Figure 1.4A-D; Ryan and Bamm submitted). Further, additional CD spectroscopy experiments showed that fibrils composed of either WT or A53T α-syn could be re-folded into an α-helical conformation in the presence of the OMM-like LUVs, again in a cardiolipin-dependent manner (Figure 1.4E-F). This suggests that mitochondria may be able to remove α-syn from fibrils by inducing an α-helical structure. Notably, the WT fibrils adopted an α-helical conformation more quickly and to a greater extent than the A53T fibrils (Figure 1.4G). Cardiolipin is typically found in the IMM [15], so any re-folding of α-syn would require its externalization to the OMM. As outlined above, cardiolipin externalization is known to be involved in mitophagy in response to stress including low doses of the pesticide rotenone [15], so perhaps if cardiolipin was externalized for too long, or if too much was externalized, it would trigger mitophagy. This could help to explain why A53T is disease-causing, as the CD spectroscopy results show it takes longer to refold A53T fibrils, which would provide more opportunities of attaining such a threshold.

Recent studies claim that instead of the general mitochondrial membrane, α-syn specifically localizes to MAMs [48,49]. Further, it is reported that A30P and A53T mutated α-syn do not localize to MAMs to the same extent as their WT counterparts [49]. Though the role of α-syn in MAMs is unknown, the absence of WT syn impairs MAM function and leads to
mitochondrial fragmentation [49], and therefore promoting mitophagy. This suggests that in addition to Parkin and DJ-1 impacting MAMs, α-syn could also have a role to play at MAMs.

Figure 1.4 – Interactions of cardiolipin with monomeric and fibrillar α-synuclein

A-D Binding of 4 µM WT or A53T α-syn monomers to LUVs containing 8% (A), 12% (B), 17% (C) or 30% (D) cardiolipin. The CD signal at 222 nm was taken for each trial to calculate the fraction of bound α-syn (y-axis) against the concentration of LUVs (bottom axis) and cardiolipin (top axis). Increasing cardiolipin concentration increased the fraction of bound protein, indicating cardiolipin binds α-syn directly. Cardiolipin has a higher affinity for WT over A53T α-syn (n=3). E-F Representative CD spectra for WT (E) or A53T (F) PFFs incubated in the presence of 1.2 mM LUVs containing 30% cardiolipin. Over time, PFFs shift from a fibrillar structure to an α-helical conformation. G The relative change in CD signal at 222 nm over time for the interaction of PFFs with LUVs containing 30% cardiolipin. The WT PFFs changed conformations more quickly and to a greater extent than A53T PFFs (n=3, **P < 0.01). (Ryan and Bamm submitted).
(1.7) Autophagy impairment in PD and the role of α-synuclein

In addition to impairment of mitophagy, disruption of general autophagy is also common in PD [32,50–53]. Mutations in a number of different trafficking proteins involved in autophagy can contribute to PD [51]. Further, patients with Gaucher disease, a lysosomal storage disease where the lysosomal glucocerebrosidase enzyme is non-functional, causing a toxic build-up of glucocerebroside, have a significantly increased risk for developing PD [51].

α-Syn has been shown to contribute to this pathology in a number of ways [48,50–53]. There are several reports that it disrupts trafficking between the ER and Golgi, leading to Golgi fragmentation [32,50,52]. A study that over-expressed α-syn in cells found that there was an increase in autophagosomes, but that these vesicles were not fusing with lysosomes, resulting in cytotoxicity in the cells [53]. They were able to improve viability by inhibiting Atg16L1 and beclin1, proteins involved in formation of autophagosomes, by partial knockdown [53]. This suggests that increased levels of α-syn increases biogenesis of autophagosomes, while preventing their maturation and subsequent fusion with lysosomes [51]. There are claims that α-syn disrupts the trafficking of hydrolases from the Golgi to lysosomes, resulting in lysosomal dysfunction [50]. Finally, there is controversy as to whether aggregated α-syn can be degraded by lysosomes. Some studies suggest it is degraded through the lysosome [54], others claim that it is not degraded [55], and still others report that it is specifically degraded by chaperone-mediated autophagy (CMA) [56]. It is unclear how the stage of the disease would impact each of these claims.
CHAPTER 2: RESEARCH PROPOSAL

(2.1) Rationale

Mitochondrial dysfunction, MAM alteration, α-synucleinopathy and autophagic impairment are all known to be involved in PD pathology. However, it is not yet fully understood how they are related or how the disease progresses. Our lab routinely cultures A53T and corrected (Corr) human induced pluripotent stems cells (hiPSCs). The nucleotide causing the A53T substitution in the SNCA gene was corrected to produce the Corr cell line, resulting in isogenic cell lines that differ at that nucleotide only and ensuring that any differences in the cell lines are due to the mutation and not to other genetic causes [57]. Our lab also uses a second set of isogenic cell lines, human embryonic stem cells (hESCs), where the wild-type (WT) cell line had the A53T mutation introduced. These stem cells can then be differentiated into DA human neurons. The A53T cells show PD pathology due to the presence of A53T α-syn, which has been reported to aggregate more readily than its wild-type counterpart. Using these cell lines and various molecular techniques, I aim to clarify the relationship between mitochondrial dysfunction, MAMs, α-synuclein and autophagy in PD.

(2.2) Hypothesis

I hypothesize that A53T α-synuclein initiates mitochondrial fragmentation by altering MAM contact, leading to mitophagy and synuclein degradation by lysosomes to prevent further PD pathology.
(2.3) Research Objectives

1. To characterize differences in mitochondrial diameter and MAM contact between A53T and control neurons by transmission electron microscopy (TEM)

2. To investigate changes in the autophagy pathway by looking at Golgi dilation and vacuolar area between A53T and control neurons by transmission electron microscopy

3. To determine if aggregated α-syn is localized to lysosomes for potential degradation in A53T neurons

4. To determine if inhibition of mitophagy in A53T neurons has a detrimental effect on cell survival

5. To investigate competition between α-syn and the mitophagic protein LC3B in the binding of cardiolipin
CHAPTER 3: MATERIALS & METHODS

(3.1) hESC and hiPSC cultures

Corrected (Corr) and A53T human induced pluripotent stem cells (hiPSCs) and wild-type (WT) and A53T human embryonic stem cells (hESCs) were routinely cultured and maintained using a previously described protocol [58] with slight modifications. Briefly, hiPSCs and hESCs were plated on γ-irradiated mouse embryonic fibroblasts (MEFs) in embryonic stem cell (ESC) medium with 20% knockout serum replacement (KSR) and 8 ng/mL basic fibroblast growth factor (bFGF). The media was changed daily and the cultures passaged weekly.

(3.2) Neuronal differentiation

The hESCs and hiPSCs were differentiated into A9-type dopaminergic (DA) neurons as per a protocol described previously [58,59] with slight modifications. Briefly, a single cell suspension was attained with accutase, which was then placed in dishes coated with gelatin to allow the fibroblasts to adhere, leaving the hESCs and hiPSCs in the supernatant which was collected and re-plated on Matrigel-coated dishes at a density of 4x10⁴ cells/cm². The cells then underwent floor-plate induction with medium containing KSR, 100 nM LDN193189, 10 μM SB431542, 100 ng/mL Sonic Hedgehog C25II, 2 μM purmorphamine, 100 ng/mL fibroblast growth factor 8 (FGF8), and 3 μM CHIR99021 (CHIR). After 5 days, the medium was changed to 25% N2 medium, increasing to 50%, and 75% every 2 days. The medium was then changed to Neurobasal (NB)/B27/Glutamax supplemented with CHIR on day 11 of differentiation. Two days later the CHIR was replaced with 20 ng/mL brain-derived neurotrophic factor (BDNF), 0.2 mM ascorbic acid, 20 ng/mL glial-derived neurotropic factor (GDNF), 1 ng/mL transforming
growth factor beta-3 (TGFβ3), 0.5 mM dibutyryl cyclic AMP (dbcAMP), and 10 µM DAPT. On day 20 of differentiation the cells were again dissociated into a single cell suspension with accutase and re-plated at a density of 4x10^5 cells/cm^2 in terminal differentiation medium containing NB/B27 + BDNF, ascorbic acid, GDNF, dbcAMP, TGFβ3 and DAPT, or DA neuron (DAN) medium on dishes coated with 15 µg/mL poly-ornithine, 1 µg/mL laminin, and 2 µg/mL fibronectin. Cells were maintained until day 30 or day 60 of differentiation as appropriate.

(3.3) Electron microscopy and analysis

At day 30 or day 60, the hNs were then prepared for transmission electron microscopy (TEM). The cells were treated with HyQTase to lift the cells from the well, then pelleted at 300xg for 5 minutes. The pellet was fixed in 4% paraformaldehyde (PFA) and 0.1% glutaraldehyde in DMEM at 4°C overnight. It was then washed with phosphate buffered saline (PBS), twice with MOPS (100 mM MOPS containing 1.2 mM sodium azide, pH 7.4), and once with cacodylate, followed by incubation in 2% osmium tetroxide in cacodylate for 2 hours at room temperature to stain membrane lipids. Next it was washed with cacodylate and distilled H₂O before incubation in 2% uranyl acetate for 1 hour. The pellet was then washed twice with distilled H₂O, followed by incubation in 50%, 75% and 95% ethanol containing 0.1% sodium chloride, and twice in 100% ethanol, each for 10 minutes at room temperature. It was next incubated in 10%, 50% and 75% LR White (LRW) resin in ethanol for 15-30 minutes each, followed by an overnight incubation in 100% LRW at room temperature. The following day, the pellet was incubated in 100% LRW for 15-30 minutes, and was then transferred to an EM capsule containing 100% LRW, which was sealed and placed in a 60°C oven overnight to polymerize the resin. Following polymerization, the sections were cut with a diamond knife into
slices 50-70 nm thick. The slices were then placed on copper grids, and stained with uranyl acetate and lead citrate. Images were acquired using a Tecnai F20 transmission electron microscope at 6000X – 10000X magnification.

The images were analyzed using ImageJ (National Institute of Health). Mitochondrial diameter was measured along the longest visible axis. The MAM length was calculated by measuring the length of electron-dense ER that was within 50 nm to mitochondria. These measurements were summed for each mitochondrion, and divided by the perimeter of that particular mitochondrion, resulting in a ratio of MAM length to mitochondrial perimeter that could compare mitochondria of various sizes. The diameter of each cisterna of the Golgi apparati was measured as an indication of Golgi dilation. Vacuoles were defined as globular membrane-bound regions, and include lysosomes, autophagosomes, and autophagolysosomes. For vacuole analysis, the area of cell soma and the sum of vacuolar volume were measured. Using these data, the area of vacuoles per 100 nm² of cell soma was calculated. Statistical analysis was determined by a one- or two-tailed t-test, where $P < 0.05$ was considered significant.

(3.4) Immunofluorescence and analysis

Cells were fixed on 12-mm glass coverslips via incubation in 4% PFA in PBS for 15 minutes. The cells were incubated in a primary antibody buffer (3% bovine serum albumin and 0.3% Triton-X 100 in PBS with appropriate primary antibodies) at 4°C overnight. The coverslips were washed 3 times with PBS then incubated in antibody buffer containing appropriate Alexa Fluor-conjugated secondary antibodies (1:2000 dilution) and DAPI counterstain for 1-2 hours at room temperature. The primary antibodies used were against lysosome-associated protein 1
(LAMP1 or CD107a; 1:500; Biolegend 121602), α-syn phosphoserine129 (PS129 81A; 1:1000; Biolegend 825701) and cleaved caspase-3 (Asp175; 1:500; Cell Signalling Technology 9662). Cells were imaged using an Axio Observer Z1 laser-scanning microscope (Zeiss) at 40X magnification, with z-stacks taken at 1μm intervals for slides used for colocalization analysis.

Colocalization analysis of the z-stack images was performed using Volocity (PerkinElmer). The volume of signal for each of LAMP1 and PS129, as well as colocalized signal, was measured for the cell soma of A53T and Corr hNs, then corrected for soma volume. To verify that any differences in colocalization volume could not be merely attributed to differences in total volume of lysosomes and aggregated synuclein, colocalization coefficients were calculated using the Manders equation.

\[
M_{\text{LAMP1}} = \frac{\sum_i L\text{AMP1}_{i,\text{coloc}}}{\sum_i L\text{AMP1}_i}, \quad M_{\text{PS129}} = \frac{\sum_i P\text{S129}_{i,\text{coloc}}}{\sum_i P\text{S129}_i}
\]  \quad (1,[60])

The Manders coefficient looks at the number of colocalized pixels over the total number of pixels for each of LAMP1 and PS129. It is an indication of colocalization that ranges from 0 to 1, where a value of 0 is no colocalization, and a value of 1 represents complete colocalization. When looking at volumes of signal, the equations are simplified

\[
M_{\text{LAMP1}} = \frac{\text{volume}_{\text{coloc}}}{\text{volume}_{\text{LAMP1}}}, \quad M_{\text{PS129}} = \frac{\text{volume}_{\text{coloc}}}{\text{volume}_{\text{PS129}}}
\]  \quad (2)

Therefore, the Manders coefficient for LAMP1 is an indication of how much of the total LAMP1 volume also colocalized with PS129, or the percentage of lysosomal volume that contains PS129. Likewise, coefficient for PS129 likewise how much PS129 volume colocalized with LAMP1, or the percentage of aggregated synuclein that is located at the lysosomes. Statistical analysis was performed by a two-tailed t-test, where \(P < 0.05\) was considered significant.
(3.5) Knockdown, validation and analysis of BECN1

The beclin (BECN1) knockdown (KD) was achieved through use of a short hairpin RNA (shRNA) lentiviral expression vector containing the sequence CACCATGCAGGTGAGCTTCGTGTGCCAGC in a pGFP-C-shLenti backbone (Origene TL314484). The plasmid was transfected along with the packaging plasmids pMDLg/pRRE (Addgene 12251) and pRSV-Rev (Addgene 12253) and the envelope plasmid pMD2.G (Addgene 12259) into LentiX 293T cells using the reagent polyethyleneimine to generate lentivirus. The virus was validated in LentiX 293T cells at 70% confluency. After 72 hours from initial transduction the cells were harvested and RNA was extracted and purified using an RNeasy kit (Qiagen 74104). The RNA was converted to cDNA using a Quantitect Reverse Transcriptase kit (Qiagen 205311). Next qPCR was performed using SYBR green and a StepOnePlus qPCR machine (ThermoFisher Scientific) with 40 cycles (95°C for 3 seconds and 60°C for 20 seconds). The primers used were GGACTTGGGTGACGGGCTCC (forward) and GGTGACACGGTCCAGGATCT (reverse). Neurons to be used for analysis of mitophagy inhibition were transduced at Day14 (before differentiation) and fixed for analysis at Day35.

Cell survival was assessed by immunofluorescence. The shRNA was conjugated with GFP, such that neurons that were successfully transduced with the BECN shRNA were identified by the presence of GFP. Apoptotic cells were identified by the presence of cleaved caspase-3 (casp3) in the nucleus (identified by DAPI counterstain) and considered dead or dying. Cell death of BECN1 KD hNs was calculating by determining the percentage of GFP+ cells containing cleaved caspase-3 in the nucleus for each of 79 A53T and 106 WT neurons. Statistical significance was determined by two-tailed t-test, where $P < 0.05$ was considered significant.
(3.6) Protein purification

A plasmid containing WT human α-synuclein in the pET21a backbone was purchased from Addgene (plasmid #51486), and had been deposited by the MJFF. The A53T mutant was generated using the Q5 site-directed mutagenesis kit (NEB) with the primers GCATGGTGTGACCAACAGTGCG (forward) and ACCACTCCCTCCTTGGTT (reverse). The plasmids were transformed into *Escherichia coli* BL21-CodonPlus (DE3)-RIPL competent cells (Agilent Tech). The resulting colonies were inoculated into a 10-mL LB starter culture with 50 µg/mL ampicillin, which was then shaken overnight at 37°C. The following day the starter culture was added to 1 L of LB (lysogeny broth) with 50 µg/mL ampicillin and grown with shaking at 37°C until the OD reading at 600 nm was at least 0.6. Expression was induced with 250 µg/mL ampicillin and returned to the 37°C shaking incubator for 3 hours. The cultures were then centrifuged at 6000 rpm in a JA-10 rotor for 10 minutes at 4°C and the pellets stored at -80°C until needed. α-Syn was purified as per a previously described protocol [61] with modifications. Pellets from 500 mL of culture were thawed on ice, resuspended in 50 mL water and boiled in a water bath for 20 minutes with regular shaking to degrade most proteins, with heat-stable α-syn remaining intact due to its intrinsically disordered nature. After being cooled on ice the lysate was centrifuged at 50,000xg for 30 minutes at 4°C to pellet cell debris and degraded proteins. The supernatant was adjusted to a concentration of 20 mM Tris-HCl (pH 8.0) and filtered through a 0.45-µm PES membrane before being separated via anion-exchange chromatography using a 1-mL Pall AcroSep column on a DuoLogic system (Bio-Rad). Fractions containing α-syn, as confirmed by SDS-PAGE, were separated using a Symmetry 300, C18 reversed-phase HPLC column on a Waters system with Millennium 32 software. The purified proteins were lyophilized and confirmed by mass spectrometry.
A second plasmid containing His-tagged LC3B in the pET15b backbone was also purchased from Addgene (plasmid #73949) and had been deposited by Dieter Willbold’s lab. The plasmid underwent transformation and induction of expression as above. LC3B was purified from *E. coli* as per the Willbold lab’s protocol [62]. Briefly, the protein was purified on a nickel-nitrilotriacetic acid affinity chromatography column on the same DuoLogic system.

(3.7) Large unilamellar vesicle preparation

Lipid vesicles containing 30% cardiolipin were prepared from the phospholipids phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and cardiolipin (CL), purchased from Avanti Polar Lipids in chloroform and stored at -20°C. The lipids PI, PC, PS, PE and CL were mixed in the molar ratio 6.6:38.6:5.1:19.7:30 to resemble the outer mitochondrial membrane (OMM) [63]. Large unilamellar vesicles (LUVs) with a 100 nm diameter were formed as previously described [64]. Briefly, the lipid mixture was dried under nitrogen to remove chloroform and placed under vacuum overnight. The following day the lipid film was hydrated with double-distilled water for a lipid concentration of 20 mM. The solution was placed at 50°C with frequent vortexing to form large multilamellar vesicles which were then disrupted by three freeze/thaw cycles. The lipids were then extruded through an Avanti mini-extruder, resulting in LUVs of 100 nm diameter. Following extrusion, the lipid concentration was confirmed by phosphorus assay [65]. Lipids were stored at -20°C until needed and at 4°C for up to 3 weeks, and were layered with argon gas each time the tube was opened to prevent lipid oxidation.
(3.8) Flotation assays

Flotation assay experiments were based on a protocol by Anton and colleagues [66]. Each experiment contained 10 μM of protein (LC3B, WT syn, A53T syn, or LC3B and either WT or A53T syn) and LUVs ranging from 0.1 to 10 mM in 125 μL potassium phosphate buffer (KPB). Mixtures were incubated at 37°C for 1 hour, after which they were mixed with 175 μL of 2.4 M sucrose in KPB. Using a syringe, 400 μL of 0.8 M sucrose and 300 μL of 0.5 M sucrose were carefully layered for each tube, for a total volume of 1 mL. Samples were centrifuged at 90,000 rpm for 3 hours at 4°C using a MLA-130 rotor (Beckmann), allowing for lipid-bound protein to float to the top at low sucrose densities and unbound protein to remain at the bottom where the sucrose was most concentrated. Following centrifugations, fractions were collected using a syringe. The bottom 250 μL was collected first and contained free protein, whereas the remaining 750 μL contained lipids and lipid-bound protein.

(3.9) Slot blots and Western blots

Slot blots were performed for the flotation assays due to the increased number of sample capacity compared to a Western blot. Fractions were diluted 1:15 in KPB and 200 μL applied to a 0.2-μm nitrocellulose membrane previously soaked in PBS via slot blot apparatus (Bio-Rad) and allowed to enter the apparatus by gravity for 10 minutes before vacuum was applied. Following sample application, wells were washed with 400 μL PBS, the membrane was removed and blocked in 5% milk in PBS for 1 hour, followed by incubation in primary antibody in 5% milk in PBS with 0.1% Tween (PBS-T) overnight at 4°C (mouse anti-α-synuclein; 1:1000; BD Biosciences 610787). The next day the membrane was washed with PBS-T, blocked in 5% milk,
incubated in secondary antibody (donkey anti-mouse-700; Licor; 1:10,000) for 1 hour at room
temperature and again washed in PBS-T before imaging via Licor-Fc.

Western blots were carried out using Tricine gels as both LC3B and α-synuclein are low
molecular mass proteins of a similar size. Samples were mixed with denaturing loading buffer,
then boiled for 5 minutes before loading and separation via SDS-PAGE at 100-120V. Proteins
were then transferred to a 0.2-µm nitrocellulose membrane over 50 min at 0.4 mA at 4°C.
Membranes were then blocked for 1 hour and incubated in primary antibody (rabbit anti-LC3B;
1:1000; Abcam ab48394) overnight as described above. The following day membranes were
washed, blocked, incubated in secondary antibody (donkey anti-rabbit-800; Licor; 1:10,000),
washed, and imaged via LicorFc.

It was found that the non-denaturing slot blots were inappropriate for analysis of the
apparently bound fractions, as high lipid concentrations had less protein signal (not shown),
contrary to the Western blots which were performed in denaturing conditions. This was
potentially due to interactions between the lipids and bound protein preventing antibody binding,
so only the free fractions were assessed by slot blot, and the apparently bound protein calculated.

Quantitative densitometry was performed with normalization to protein standards. The
amount of free protein was used to calculate the amount of apparently bound protein, knowing
10 µM of each protein was present, and this value was reported. The experiments were run in
triplicate, and statistical significance was determined by one-way ANOVA, where \( P < 0.05 \) was
considered significant.
CHAPTER 4: RESULTS

(4.1) Cellular model

Four isogenic lines of stem cell-derived neurons were used for the experiments. The first two lines were derived from human embryonic stems cells (hESCs) and consisted of either wild-type cells, or wild-type cells that had the disease-causing A53T mutation introduced at the α-synuclein locus, resulting in isogenic WT and A53T cell lines. The second two lines were of human induced pluripotent stem cell (hiPSC) origin. The first was derived from a PD patient that harboured the SNCA-A53T mutation. The second was generated by using zinc finger nuclease to correct this mutation, resulting in two isogenic A53T and Corr cell lines. The 4 different hPSC lines were differentiated into dopaminergic neurons. The use of two sets of hPSC lines from disparate genetic background, where the only genetic difference within each set is due to the mutation in α-synuclein [57] allows for the conclusion that any phenotypic differences that are consistent between these two sets of cells are a direct result of the A53T mutation.

(4.2) Mitochondrial fragmentation is preceded by MAM elongation in A53T hNs

To determine the effect of SNCA-A53T on mitochondrial dynamics, we first examined mitochondria on an ultrastructural level. Transmission electron microscopy demonstrated that the diameter of mitochondria in Day60 A53T hNs of both hiPSC and hESC origin are smaller than their control hNs (Figure 4.1), indicative of mitochondrial fragmentation. This fragmentation can result from a combination of increased mitochondrial fission or decreased fusion [10].
Figure 4.1 – Mitochondrial diameter in human neurons

A Representative transmission electron micrographs of WT (left) and A53T (right) hESC-derived dopaminergic human neurons at differentiation day 60; scale 0.2 µm. Zoomed views of the insets for WT (top) and A53T (bottom) demonstrate the difference in mitochondrial diameter. B Mitochondrial diameter of WT hNs is greater than in A53T. Data shown represent mean ± SEM, *P < 0.05 by two-tailed t-test, n=45-90 mitochondria. C Representative transmission electron micrographs of Corr (left) and A53T (right) hiPSC-derived dopaminergic human neurons at differentiation day 60; scale 0.2 µm. Zoomed views of the insets for Corr (top) and A53T (bottom) demonstrate the difference in mitochondrial diameter. D Mitochondrial diameter of Corr hNs is greater than A53T hNs. Data shown represent mean ± SEM, *P < 0.05 by two-tailed t-test, n=8-9 mitochondria.

Previous reports have implicated mitochondrial fission and fragmentation in mitophagy (reviewed in [10]). Specifically, researchers found that fission often occurs at MAM sites and therefore speculate that MAMs are involved in some types of fission, perhaps by wrapping around mitochondria recruiting DRP1 and Fis [24]. They further suggest that alteration of
MAMs could cause excessive fission [24]. Since α-syn has been reported to localize to MAMs [49,67], we investigated whether MAM length was altered in A53T hNs prior to the observed mitochondrial fission and fragmentation. Analysis of the TEM images showed that MAM length normalized to mitochondrial perimeter is much greater in Day30 A53T hiPSC-derived hNs than

**Figure 4.2 – MAM length in human neurons**

A Representative transmission electron micrographs of A53T hiPSC-derived dopaminergic human neurons at differentiation day 30 (left) and 60 (right); scale 0.2 µm. The insets are zoomed views for day 30 (top) and 60 (bottom) of differentiation. B The MAM length to mitochondrial perimeter ratio is significantly greater in day 30 A53T hNs than day 60. Data shown represent mean ± SEM, *P < 0.05 by two-tailed t-test, n=4-20 mitochondria. C Representative transmission electron micrographs of WT (left) and A53T (right) hESC-derived dopaminergic human neurons at differentiation day 60; scale 0.2 µm. The insets are zoomed views for WT (top) and A53T (bottom). D There is no difference in MAM length in WT and A53T hNs at Day60. Data shown represent mean ± SEM, *P < 0.05 by two-tailed t-test, n=20-40 mitochondria.
at Day60 (Figure 4.2A,B). This suggests that MAMs are elongated at earlier timepoints, and
decrease after fragmentation has occurred by Day60. Furthermore, there was no difference in
normalized MAM length between WT and A53T hESC-derived hNs at Day60 (Figure 4.2C,D),
indicating that after fragmentation A53T MAM length returns to normal levels. These results
indicate that the mitochondrial fragmentation reported above could be a result of MAM-induced
fission in A53T hNs, as the ability of MAMs to wrap around the mitochondria and initiate
recruitment of fission proteins [24] is likely facilitated by greater MAM contact. This alteration
in MAMs is probably due to either the conformation of the A53T mutant itself, or the aggregated
α-syn that accumulates in these neurons.

(4.3) Golgi dilation and vacuolar volume are increased in A53T hNs

Next, we investigated if other steps of the degradation pathway were also affected, such
as the Golgi apparatus and vacuoles. Analysis of the TEM images demonstrated that the cisternae

![Figure 4.3 – Golgi dilation in human neurons](image)

**Figure 4.3** – **Golgi dilation in human neurons**

A Representative transmission electron micrographs of day 60 WT (left) and A53T (right)
hESC-derived hNs; scale 0.2 μm. Zoomed views of the insets for WT (top) and A53T (bottom)
demonstrate the differences in Golgi apparatus. B Cisternae of the Golgi apparatus are dilated in
A53T hNs. Data shown represent mean ± SEM, *P < 0.05 by one-tailed t-test, n=9-10 cisternae.
of Golgi apparati in A53T Day60 hNs have a larger diameter than that of WT hNs (Figure 4.3), indicating Golgi dilation. This dilation could be a result of α-syn-impaired trafficking between the ER and Golgi, as previous studies have claimed [27,32,50]. Alternatively, the increased dilation and blebbing could indicate increased membrane budding and formation of lysosomal vacuoles.

**Figure 4.4 – Vacuole area in human neurons**

A Representative transmission electron micrographs of day 60 WT (left) and A53T (right) hESC-derived hNs; scale 0.5 µm. B There is more lysosomal area per 100 nm² of soma in A53T hNs than WT. Data shown represent mean ± SEM, *P < 0.05 by two-tailed t-test, n=7-15 neurons. C Representative transmission electron micrographs of day 60 Corr (left) and A53T (right) hiPSC-derived hNs; scale 0.5 µm. D There is more lysosomal area per 100 nm² of soma in A53T hNs than Corr. Data shown represent mean ± SEM, *P < 0.05 by two-tailed t-test, n=8 neurons.
To help distinguish between the possibilities, vacuolar area was compared between A53T and control hNs, where a vacuole encompasses lysosomes, autophagosomes and autophagolysosomes. It was found that both hESC-derived and hiPSC-derived A53T Day60 hNs had greater vacuolar area than their control counterparts (Figure 4.4). The values for WT hESC-derived hNs (7%) and Corr hiPSC-derived hNs (4%) are comparable to the previously reported value of 5% for lysosomal volume under physiological conditions [68]. The increased vacuolar area in A53T hNs of both cell lines is likely in direct response to increased autophagic demands, and supports the possibility that increased Golgi diameter represents an increase in lysosomal vacuoles, potentially for the degradation of mitochondria and α-synuclein.

(4.4) Aggregated α-synuclein localizes to lysosomes

Next, immunofluorescence was used to investigate if increased vacuolar area corresponded to degradation of α-synuclein aggregates, stained for the lysosomal and autophagolysosomal marker LAMP1 (lysosome-associated membrane protein 1), and PS129, a marker of aggregated synuclein that has been phosphorylated at serine 129 (Figure 4.5A). First, volume of LAMP1 signal per volume of cell soma was determined (Figure 4.5B), and it was found that A53T hiPSC-derived Day31 hNs had more lysosomal volume in their soma than Corr hNs. This correlates with the TEM data above, and suggests that the increase in vacuolar area can be attributed, at least in part, to increased lysosomal and autophagolysosomal volume, as opposed to autophagosomes as they would not contain LAMP1. Next, volume of PS129 signal was measured, and it was found that the A53T hNs also had more PS129 per soma volume than Corr hNs. This indicates that A53T hNs have more aggregated α-syn present, characteristic of synucleinopathy in PD. Lastly, comparison of volumes of signal from both LAMP1 and PS129
Figure 4.5 – Colocalization of lysosomes and PS129 α-synuclein in human neurons

A  Representative micrographs of day 30 Corr (top) and A53T (bottom) hiPSC-derived hNs stained for DAPI (blue), the lysosome marker LAMP1 (green) and PS129 (red); scale 5 µm. B  A53T hNs have more lysosomes and PS129 in the soma than Corr hNs. Similarly, A53T hNs also have more lysosomes and PS129 that are colocalized. Data shown represent mean ± SEM, **P < 0.01 by two-tailed t-test, n=20-30 neurons. C The Manders colocalization coefficients indicate that a greater percentage of lysosomes are colocalized with PS129 in A53T hNs. Similarly, a larger percentage of PS129 is localized to lysosomes in A53T. Data shown represent mean ± SEM, **P < 0.01 by two-tailed t-test, n=20-30 neurons.
found that there was greater colocalization of LAMP1 and PS129 in A53T hNs compared to Corr. To verify that this was an actual difference between aggregated α-syn localized at lysosomes, and not merely a result of increased lysosomal and aggregated synuclein volume in A53T hNs, the Manders coefficients were calculated. The Manders coefficient for LAMP1 gives an indication of the amount of LAMP1 that was colocalized with PS129 α-syn, or the percentage of lysosomes containing PS129 α-syn. Similarly, the Manders coefficient for PS129 gives an indication of the amount of PS129 α-syn that was colocalized with LAMP1, or the percentage of aggregated α-syn located at lysosomes. Both of these colocalization coefficients were greater for A53T than Corr (Figure 4.5C). This suggests that A53T hNs both have a larger percentage of lysosomes containing aggregated α-syn, and a larger percentage of their aggregated syn in lysosomes, indicating potential attempted degradation of aggregated synuclein.

(4.5) Mitophagy inhibition increases cell death in A53T hNs

MAM elongation, subsequent mitochondrial fragmentation, and activation of the degradation pathway are all precursors to mitophagy [10,24,25]. Additionally, colocalization of LAMP1 and PS129 indicates potential degradation of aggregated α-syn. To investigate if mitophagy is a protective mechanism in PD neurons to promote the degradation of toxic synuclein, mitophagy was inhibited to observe the consequences. To accomplish this, WT and A53T hESC-derived hNs were transduced with a lentiviral vector containing shRNA against beclin1 (BECN1). Beclin1 is involved in a protein complex that is crucial for autophagosome formation [53]. Therefore, knocking down BECN1 reduces the capacity of the cells for autophagy, by blocking uptake of dysfunctional organelles, including mitochondria, into autophagosomes. Knockdown was validated by qPCR in HEK293T cells, with transduced
cultures expressing less BECN1 than cells infected with a scrambled control (Figure 4.6A). Cell death of GFP+ cells was determined by the presence of caspase-3 signal in the nuclei of green cells (Figure 4.6B). A53T hNs has significantly increased cell death in GFP+ cells than WT hNs (Figure 4.6C), indicating that inhibiting mitophagy in A53T was more detrimental to cell survival. These results suggest that mitophagy could be protective in PD, perhaps as a mechanism for the clearance of aggregated α-syn.

Figure 4.6 – Inhibition of mitophagy in human neurons

A Gene expression of BECN1 is decreased in HEK293T cells transduced with the BECN KD virus compared to those infected with a scrambled control. B Representative micrographs of WT (top) and A53T (bottom) hESC-derived hNs with GFP+ cells (green) indicating shBECN delivery; stained for DAPI (blue), and cleaved caspase-3 (pink); scale 10 µm. C A53T hNs have increased cell death compared to WT hNs. Data shown represent mean ± SEM, *P < 0.05 by two-tailed t-test, n=79-106 neurons.

LC3B and α-synuclein compete for binding to cardiolipin on OMM-like vesicles

To assess the possibility that α-syn may be targeted to lysosomes through association with the mitochondria, the ability of α-syn and LC3B to bind lipids in each other’s presence was investigated. Previous studies in our lab showed that monomeric WT and A53T α-syn bind
OMM-like LUVs containing 30% cardiolipin to the same extent (Ryan and Bamm submitted). LC3B is known to also bind cardiolipin to initiate mitophagy [15]. We therefore began by performing flotation assays to measure the relative binding of both LC3B and α-syn to cardiolipin containing vesicles. For the initial analysis of LC3B binding, OMM-like LUVs containing 30% cardiolipin at concentrations ranging from 0-7.5 mM were incubated with 10 µM LC3B. Samples were subsequently centrifuged on a sucrose gradient, and four 250-µL fractions were collected, starting from the bottom fraction (denoted B1; Figure 4.8). Analysis by Western blot demonstrated that while fraction B1 contained unbound protein, as seen by the sample containing no lipid, fractions B2 through B4 contained varying amounts of bound protein (Figure 4.8). These results suggest that different conformations of bound LC3B may be present, 

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**Figure 4.7 – Separation of LC3B and LUVs in a sucrose gradient**

Varying concentrations of LUVs were incubated with 10 µM LC3B before being applied to a sucrose gradient and centrifuged. After ultracentrifugation 250-µL fractions were collected, starting from the bottom. 75 and 100 ng standards are included as a reference for signal sensitivity.
which separate into different sucrose densities. To ensure our synuclein/LC3B/cardiolipin binding experiments encompassed all potential bound forms of LC3, subsequent experiments were split into 2 fractions; the bottom “free” fraction (formerly fraction B1) and the top “apparently bound” fraction (formerly fractions B2, B3 and B4).

Using the experimental parameters established above, we next sought to determine if LC3B impacts the ability of α-syn to bind LUVs and vice versa. Equimolar amounts of WT or A53T syn and LC3B were incubated with the same OMM-like LUVs. As expected, amounts of both α-syn bound to LUVs increase with increasing LUV concentration (Figure 4.8A). The presence of LC3B impaired the binding of both WT and A53T syn to LUVs at low lipid concentrations (Figure 4.8A). However, the binding of A53T α-syn is only affected at low concentrations of LUVs, and is comparable to the no LC3B control at 1 mM LUVs (Figure 4.8A). Conversely, binding of WT syn is impaired until 7.5 mM lipids. Even at low lipid concentrations such as 0.5 mM LUVs, A53T syn is impacted to a lesser extent than WT (Figure 4.8B). These data suggest that if WT synuclein is bound to cardiolipin, it can block LC3B binding and therefore mitophagy. However, since A53T syn has a reduced ability to impair LC3B binding, it can be speculated that A53T is more susceptible to mitophagy, perhaps due to an ability for A53T α-syn and LC3B to bind cardiolipin concurrently. Similarly, the presence of either WT or A53T α-syn impaired the ability of LC3B to bind LUVs at a wide range of lipid concentrations (Figure 4.8C). Unlike with α-syn, LC3B binding is restored to control levels at 5 mM LUVs for both WT and A53T. However, although A53T syn causes impairment at the same range of concentrations as WT, it is to a lesser extent, significantly so at 0.75 mM lipid (Figure 4.8D).
Figure 4.8 – Impairment of synuclein and LC3B binding OMM-like LUVs in vitro

A Binding of α-synuclein to LUVs increases as LUV concentration increases. The presence of equimolar LC3B impairs the ability of both WT and A53T synuclein to bind, particularly at low concentrations. 

B WT α-syn is impaired to a significantly greater extent than A53T with 0.75 mM LUV concentration. 

C Binding of LC3B to LUVs reaches a maximum at low concentrations of LUV. The presence of equimolar WT or A53T α-syn impairs the ability of LC3B to bind LUVs at low concentrations. 

D WT α-syn impairs LC3B binding to a greater extent than A53T when LUV concentration is 0.5 mM. Data shown represent means of 3 replicates ± SEM, **P < 0.01 by one-way ANOVA.
(4.7) Results Summary

In summary, mitochondrial diameter is decreased in A53T hNs by Day60, indicative of fragmentation. MAM length is increased in A53T hNs at an earlier timepoint, suggesting MAM elongation precedes mitochondrial fragmentation, a precursor to mitophagy. Golgi dilation and increased vacuolar volume in Day60 A53T hNs similarly indicate these cells have an increased autophagic load. This is further supported by findings that aggregated α-syn localizes to lysosomes, suggestive of its attempted degradation. Additionally, mitophagy appears to be protective in A53T hNs since its inhibition resulted in increased cell death compared to controls. Further, A53T hNs may be more susceptible to mitophagy due to an impaired ability of A53T syn to block the protein LC3B from binding cardiolipin, an initiating step in mitophagy. These results support the hypothesis that A53T α-syn initiates mitochondrial fragmentation by altering MAM contact, leading to mitophagy and synuclein degradation by lysosomes to prevent further PD pathology.
CHAPTER 5: DISCUSSION

A53T hNs have more mitochondrial fragmentation than control hNs, possibly as a result of MAM-induced fission as evidenced by increased MAM length prior to fragmentation. They also appear to have an increased autophagic load as seen by the increase in Golgi diameter and the abundance of vacuoles present in A53T hNs. This upregulation in autophagic vacuoles could be an attempt to degrade aggregated synuclein through lysosomes, as A53T hNs have increased colocalization of PS129 α-syn at lysosomes. Further, mitophagy may be a protective mechanism to facilitate aggregated α-syn degradation, as its inhibition was lethal in A53T hNs. A53T syn is less able to impair LC3B binding compared to WT, leaving mitochondria in A53T hNs more vulnerable to mitophagy. These results suggest that mitophagy in A53T hNs may be a mechanism for the degradation of A53T synuclein.

(5.1) Role for α-synuclein at mitochondria and MAMs

A number of studies have claimed that α-syn localizes to mitochondria [45–47]. Here I present results supporting these claims as both WT and A53T α-syn can bind OMM-like LUVs containing 30% cardiolipin. However, in the past few years researchers have demonstrated that α-syn is specifically localized to mitochondria-associated membranes, though its role there is not understood [49]. My results seemingly contradict these findings, but it is possible that when MAMs are present α-syn preferentially localizes there, but in their absence, such as in the in vitro experiments, α-syn binds mitochondria. Alternatively, previous results from our lab show that cardiolipin is required for α-syn to bind OMM-like LUVs (Ryan and Bamm submitted), so under normal physiological conditions α-syn may localize to MAMs, as there is minimal
externalized cardiolipin present. In this model, we suggest cardiolipin could become externalized in response to α-syn fibrils in order to re-fold α-syn into an α-helical conformation (Ryan and Bamm submitted). This could cause α-syn to localize to the mitochondrial membrane instead, thereby altering the amounts of α-syn present at MAMs. This model would coincide with previous results, as one group has shown that the A30P and A53T mutations resulted in decreased α-syn localized to MAMs compared to WT in M17 cells [43]. This change in abundance of α-syn at MAMs results in reduced MAM function and a consequential increase in mitochondrial fragmentation [49]. The data presented here support these findings, as A53T hNs had increased MAM length at Day30 compared to Day60, and mitochondria in both hiPSC- and hESC-derived A53T hNs were fragmented at Day60, suggesting that elongated MAMs could promote fragmentation. Researchers have previously suggested that MAMs may be actively involved in fission by wrapping around mitochondria and facilitating binding of fission proteins [48], so it is reasonable that longer MAM contact sites would be better able to wrap around mitochondria, promoting fission and fragmentation. Similarly, other researchers have also reported an increase in MAM contact as a result of α-syn overexpression [70] and in patient samples [71]. However, different studies have instead found a decrease of MAM contact in DJ-1 knockout cells, another PD model system [72]. Differences in cell type, cell age, and the type of PD model (such as α-syn overexpression, α-syn mutant, or DJ-1 knockout) could all contribute to these contradictory findings. The results from this study would indicate that it is also important whether or not mitochondrial fragmentation has occurred, as MAMs returned to WT levels by later time points. Therefore, future studies should report the timepoint when making conclusions about MAM contact, and consider reporting on numerous timepoints where appropriate.
It is known that A53T synuclein is more prone to aggregation than WT, as evidenced by previous studies [7,8] and the greater volume of PS129 α-syn signal in A53T cells presented in this study. Therefore, further research should also investigate whether the MAM elongation preceding fragmentation is due to α-syn aggregation, and therefore would be present in all PD cases involving synucleinopathy, or whether it is specific to the structure of the A53T mutant and a potential lowered localization to MAMs, as previous studies suggest.

(5.2) Effects of α-synuclein on Golgi apparati

The Golgi apparati of A53T hESC-derived hNs were more dilated than in WT at Day60, coinciding with previous studies that indicate that α-synuclein interferes with the Golgi apparati in PD [32]. However, there are conflicting reports as to the mechanism as to how it occurs [32]. Cooper and colleagues were the first to identify the Golgi as a target when they were looking for genetic suppressors of α-syn toxicity in yeast [48]. They found that overexpression of Ypt1/Rab1, a protein involved in tethering vesicles between the ER and Golgi was the most successful at rescuing α-syn toxicity [48]. The authors further identified ER-Golgi deficits as the first defect to appear as a result α-syn over-expression [48]. Since then, other studies have confirmed over-expression of α-syn disrupts trafficking between the ER and Golgi (reviewed in [32]). One of these studies specifically confirmed that ER-to-Golgi transport is inhibited first, which then leads to Golgi fragmentation in rats [73]. However, another study has since reported that the Golgi is fragmented due to Rab and SNAREs before ER-to-Golgi trafficking is impaired [74]. The results presented here show that the Golgi is dilated in A53T hNs at Day60, by which time mitochondrial fragmentation has already occurred, so it is not possible to infer the order of the events. However, while visually it appears the Golgi could be fragmented, as the previous
reports suggest, it could also be blebbing to form more lysosomes to meet increased autophagic demands.

(5.3) Effects of α-synuclein on lysosomal trafficking

The TEM data of vacuolar area support the suggestion that the Golgi is producing more autophagic vesicles in A53T hNs, as vacuolar area is greatly increased compared to control hNs. This is in line with studies that suggest that autophagosomes, a type of vacuole, are greatly increased in PD [53]. However, this study also found that the autophagosomes cannot mature and fuse with lysosomes, leading to cytotoxicity [53]. This result is in contrast to the immunofluorescence experiments presented here that suggest that autophagolysosomes are increased in this model of PD, and that these results could partially explain the presence of the large number of vacuoles in A53T neurons observed by TEM.

Previous studies have investigated whether α-syn is degraded by lysosomes. Mak and colleagues were the first group to show α-syn localized to lysosomes in vivo using the lysosomal marker LAMP2A [56]. They then used proteinase K to digest proteins bound to the exterior of lysosomes to show that there was α-syn specifically inside the lysosomes [56]. They also examined the lysosomes at different time points to confirm that α-syn was being degraded after 5 minutes [56]. However, they never specifically looked for aggregated α-syn, so it is unclear what conformations of α-syn were degraded by lysosomes in their study.

A second study from Watanabe and colleagues used PFFs to induce α-syn aggregation in HEK293 cells and found that phosphorylated and ubiquitinated α-syn was localized to lysosomes and degraded with 24 hours [54]. Further, inhibition of lysosomes with bafilomycin A1 reduced
some of that clearance [54], further suggesting α-syn can be degraded by lysosomes. However, a short time later, a third group, Tanik and colleagues, published a study contradicting many of those findings [55]. They also used HEK293 cells with PFF-induced α-syn aggregation to look at degradation of p-α-syn [55]. However, their cells expressed A53T α-syn, and the PFFs were from truncated α-syn, in order to increase the number of cells with successful seeding [55]. Further, their results directly conflict with the results presented here. They stated that only 10.2 ± 6.0% of α-syn aggregates colocalized with LAMP1 in their HEK293 cells after 24 hours, with similar findings in primary mouse neurons [55], compared to the 62 ± 3% in the soma of A53T hiPSC-derived hNs reported here. They claim that α-syn aggregates are found near, but not in, autophagolysosomes, and that the lysosome inhibitor chloroquinone had no effects on phosphorylated α-syn (p-α-syn) aggregates [55], also in opposition to the Watanabe study [54].

Tanik’s group criticized the Watanabe paper, suggesting that the p-α-syn found in the lysosomes was from the exogenous PFFs they had added, because naïve HEK293 cells do not have high enough levels of endogenous α-syn to promote seeding and aggregation [55]. However, these claims counter a previous study demonstrating that exogenous PFFs made from recombinant α-syn, as in the Watanabe paper [54], are not phosphorylated [75], so concrete conclusions cannot be drawn.

Additionally, the Tanik paper continually refers to their α-syn aggregates as “LB-like” [55], but it is unclear how similar they are to LBs. The PFFs comprised of truncated α-syn were chosen because of their increased propensity to cause aggregation [55], so perhaps not only did they cause more aggregation, but also a greater degree of aggregation. This scenario could represent a much later timepoint in PD, that is not represented by either the Watanabe study that used PFFs from wild-type α-syn [54], nor by this study, that looked at relatively young hNs.
Regardless, there needs to be more research as to whether aggregated α-syn may be degraded as an initial defense against PD, but once the disease has progressed too far degradation is inhibited, or otherwise unable to occur, perhaps due to aggregate-induced impairment of autophagy.

(5.4) Role for α-synuclein in mitophagy

Mitophagy is a potential protective mechanism in A53T hNs, as its inhibition resulted in increased cell death compared to controls. This observation further contradicts findings that α-syn aggregation causes trafficking disruptions in the degradation pathway, leading to decreased autophagosome maturation and consequent fusion with lysosomes [32,50,53]. If autophagolysosome formation was blocked in the A53T hNs, then inhibition of mitophagy through BECN1 KD would presumably not have as pronounced an effect, as degradation by lysosomes would already be prevented. Again, the results presented here support the findings of the Watanabe group, who reported that cells containing p-α-syn did not have a decreased capability for mitophagy [54]. My results showing that BECN1 KD induces cell death in A53T hNs does, however, contradict another recent study that shows partial BECN1 knockdown resulted in increased cell survival in cells overexpressing α-syn [53]. However, that study reports that total BECN1 knockdown resulted in increased death, as opposed to a partial knockdown [53], and it is unclear the degree of knockdown that they found effective. Additionally, as there was a significant difference between A53T and WT hNs, cell death can also be contributed to the PD-causing mutation, rather than just an inhibition of autophagy. The knockdown of BECN1 would limit mitophagy and autophagy [53], suggesting that they are crucial in combatting pathology in the A53T PD-model, likely through degradation of aggregated α-syn and dysfunctional organelles.
Competition between LC3B and α-synuclein at mitochondria

LC3B and α-syn impair the ability of the other to bind cardiolipin-containing OMM LUVs, meaning if WT α-syn is present on cardiolipin, LC3B is not likely able to bind. However, A53T synuclein has a reduced ability to impair LC3B binding, leaving the mitochondria vulnerable to mitophagy. This means that if cardiolipin is externalized as a mechanism to re-fold fibrillar synuclein into α-helices as our lab has previously postulated (Ryan and Bamm submitted), A53T hNs would be more susceptible to LC3B-mediated mitophagy as a result of exposed cardiolipin.

Since WT syn both impairs LC3B binding to LUVs and is itself impaired from binding LUVs in the presence of LC3B, it is very likely synuclein and LC3B interact with each other. For example, WT syn and LC3B could bind each other, impairing the ability of either to bind LUVs, and leaving many cardiolipin molecules unbound to protein. In this model, A53T would have less affinity to LC3B and instead both proteins would bind LUVs, leaving less cardiolipin unbound. Alternatively, perhaps A53T and LC3B both bind cardiolipin together. In this model, one molecule of cardiolipin would be bound to either WT or LC3B, but could be bound to A53T and LC3B concurrently.

It was recently reported that the region of LC3B that binds cardiolipin is the N-terminus, leaving the C terminus still exposed [66]. This is likely due to the abundance of positive charges at the N-terminus, allowing it to interact preferentially with cardiolipin in a similar manner to α-syn. The A53T mutation may cause a conformational change in α-syn that allows for both proteins to bind cardiolipin concurrently. Alternatively, the C-terminus of LC3B has many negatively charged amino acids, so it could potentially bind α-syn at the same lysines in the N-terminus and NAC that bind cardiolipin. The possibility that LC3B and α-syn may interact is supported by data obtained during optimization of the slot blots (not shown), which found
LC3B-probed slot blots had less signal than expected in the free fraction containing LC3B and synuclein without lipids. We postulated that α-syn may be interacting with LC3B, preventing binding of the anti-LC3B antibody. Indeed, we found that the same samples gave a more appropriate signal when analyzed by Western blot, and speculate that the denaturing conditions of a Western blot disrupted interactions between LC3B and α-syn. Future research should further investigate possible interaction between LC3B and α-syn, both in terms of their ability to bind cardiolipin concurrently, and their ability to bind each other without lipids.

(5.6) Potential therapeutic targets

This study presents evidence that the SNCA-A53T mutation impacts much of the autophagic pathway. Therefore, future therapeutic approaches should target the beginning of this pathway. If later stages were targeted, resulting in the proper degradation of α-syn and mitochondria, large amounts of mitophagy would still disrupt normal mitochondrial function. Inhibition of mitophagy is also not an option, because not only does it increase cell death as reported here, but mitophagy is also a necessary mechanism for the cell to dispose of dysfunctional mitochondria that could otherwise cause harm. Therefore, steps before mitophagy occurs should be targeted. Hence, future studies should focus on elucidating the specific role of α-syn at MAMs, so therapies could try to correct any loss-of-function present in SNCA mutants, which may in turn prevent mitochondrial fission and subsequent mitophagy. Alternatively, restoring the levels of localization of α-syn at MAMs in SNCA mutants to that of WT may also rescue downstream effects. However, this may not be effective if conformational changes caused by mutations in α-syn prevent it from performing its normal function at MAMs, regardless of improved localization. Similarly, impairing the ability of LC3B to bind cardiolipin in SNCA-
A53T mutants, either by directly impeding LC3B or by improving the ability of A53T syn to impair LC3B, could render A53T mitochondria less vulnerable to mitophagy, and therefore reduce its frequency. Some studies suggest that ER-Golgi trafficking and Golgi fragmentation as the first symptoms to occur following α-syn overexpression [48, 74]. One particular study even suggests that Golgi fragmentation precedes α-syn aggregation [74]. Further research should investigate if this is also true in SNCA mutants and other mutations of PD. The most beneficial therapy would be one that targets a mechanism that is affected in as many types of PD as possible.

(5.7) Conclusions

In summary, the evidence presented here demonstrates that the SNCA-A53T mutation greatly impacts the autophagic pathway. It initially results in elongation of MAMs at Day30, leading to mitochondrial fragmentation and decreased MAM contact by Day60, potentially as a result of MAM-induced fission. By Day60 Golgi apparati are dilated and more lysosomes are present in A53T hNs, indicative of heightened activity by the autophagic pathway. This may be an attempt to degrade aggregated α-syn, as it was colocalized with lysosomes in A53T hNs. This degradation could be in concert with mitophagy, as inhibition of autophagy resulted in decreased cell survival. A53T syn has a reduced ability to impede LC3B binding and therefore initiation of mitophagy, indicating A53T hNs are more vulnerable to mitophagy. Future research should investigate the relationship between α-syn, LC3B, and OMM lipids, including binding conformations of each protein to lipids, and the interactions of the proteins with each other with and without lipids. Potential therapies should focus on targets upstream of mitophagy, to reduce the strain on the autophagic pathway.
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Isogenic.


