The Unconventional Secretion of Alpha-Synuclein by Autophagic Mechanisms and the Novel Characterization of Pathological Alpha-Synuclein Associated Extracellular Vesicles

Kevin Burbidge
Loyola University Chicago

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THE UNCONVENTIONAL SECRETION OF ALPHA-SYNUCLEIN
BY AUTOPHAGIC MECHANISMS AND THE NOVEL CHARACTERIZATION OF
PATHOLOGICAL ALPHA-SYNUCLEIN ASSOCIATED EXTRACELLULAR VESICLES

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE PROGRAM IN NEUROSCIENCE

BY
KEVIN BURBIDGE
CHICAGO ILLINOIS
AUGUST 2017
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<tbody>
<tr>
<td>α-syn</td>
<td>Alpha-Synuclein</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AMBRA1</td>
<td>Activating Molecule of Beclin 1-Related Autophagy 1</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant Response Element</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-Related Protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATP2A/SERCA</td>
<td>ER Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B Cell Lymphoma 2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone mediated autophagy</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
</tr>
<tr>
<td>CTD</td>
<td>Carboxy-Terminal Domain</td>
</tr>
<tr>
<td>DFCP1</td>
<td>FYVE-containing protein</td>
</tr>
<tr>
<td>DJ-1</td>
<td>Protein Deglicase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DLB</td>
<td>Dementia with Lewy Bodies</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DSP</td>
<td>Dual-Split Protein</td>
</tr>
<tr>
<td>EPG5</td>
<td>Autophagic Protein Homologue 5</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular Vesicle 12-kDa FK506-binding</td>
</tr>
<tr>
<td>EVs</td>
<td>Extracellular Vesicles</td>
</tr>
<tr>
<td>FIP200</td>
<td>200 kDa FAK family kinase interacting protein</td>
</tr>
<tr>
<td>GABARAP</td>
<td>Gamma-Aminobutyric Acid Receptor-Associated Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GSK-3beta</td>
<td>Glycogen Synthase Kinase-3beta</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High Mobility Group Box 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSC70</td>
<td>Heat Shock Protein 70 kDa Protein 8</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILVs</td>
<td>Intraluminal Vesicles</td>
</tr>
<tr>
<td>KEAP</td>
<td>Kelch like-ECH-associated Protein</td>
</tr>
<tr>
<td>LAMP1</td>
<td>lysosomal associated membrane protein 1</td>
</tr>
<tr>
<td>LAMP2A</td>
<td>lysosomal associated membrane protein 2A</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy Body</td>
</tr>
<tr>
<td>LBs</td>
<td>Lewy Bodies</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-Associated Protein 1 Light Chain 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LRRK2</td>
<td>Leucine-Rich Repeat Kinase 2</td>
</tr>
<tr>
<td>MSA</td>
<td>Multiple Systems Atrophy</td>
</tr>
<tr>
<td>MSA-C</td>
<td>Multiple Systems Atrophy Cerebellar Ataxia</td>
</tr>
<tr>
<td>MSA-P</td>
<td>Multiple Systems Atrophy Parkinsonism</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>MVBs</td>
<td>Multivesicular Bodies</td>
</tr>
<tr>
<td>NRF</td>
<td>Nuclear Factor Like</td>
</tr>
<tr>
<td>P25a/TPPP</td>
<td>Tubulin Polymerization-promoting protein</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's Disease</td>
</tr>
<tr>
<td>PE</td>
<td>phosphotidylethanolamine</td>
</tr>
<tr>
<td>PFF</td>
<td>Preformed Fibrillar</td>
</tr>
<tr>
<td>PFFs</td>
<td>Preformed Fibrils</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Phosphate Kinase</td>
</tr>
<tr>
<td>PI4KII</td>
<td>Phosphatidylinositol 4-Phosphate Kinase II</td>
</tr>
<tr>
<td>PINK-1</td>
<td>PTEN-induced Putative Kinase 1</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol-3-Phosphate</td>
</tr>
<tr>
<td>PI4P</td>
<td>Phosphatidylinositol-4-Phosphate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF Attachment Receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi-Network</td>
</tr>
<tr>
<td>Tx</td>
<td>Treatment</td>
</tr>
<tr>
<td>ULK</td>
<td>UNC51 like Ser/Thr Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin Proteasome System</td>
</tr>
<tr>
<td>UVRAG</td>
<td>Ultraviolet Irradiation Resistance-Associated Gene</td>
</tr>
<tr>
<td>VPS34/ PI3KC3</td>
<td>Vacuolar Protein Sorting 34</td>
</tr>
<tr>
<td>WIPI2</td>
<td>WD-repeat domain phosphoinositide-interacting protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
</tr>
</tbody>
</table>
CHAPTER ONE:

OVERVIEW AND HYPOTHESES

Synucleinopathies are a group of neurodegenerative diseases associated with the intracellular accumulation of insoluble, pathological alpha-synuclein (α-syn) [1]. Although the exact cause of why α-syn becomes misfolded and begins to aggregate is unclear, the process is associated with neurotoxicity, eventually resulting in cell death [2-4]. Research indicates that α-syn behaves in a prion-like fashion; meaning native α-syn is capable of misfolding, and can self-propagate by inducing further misfolding, as well as, be transferred from cell-to-cell, resulting in the spread of pathology [5-7]. While the exact mechanism associated with the pathological transmission of α-syn has yet to be identified, recent evidence suggests that α-syn is secreted into the extracellular environment from cells utilizing an unconventional secretory mechanism [8, 9]. Furthermore, dysregulation of this mechanism can increase the secretion of pathological α-syn, including exosome associated α-syn [8, 9]. The secretion this exosome associated α-syn is particularly important as it has been shown to be readily taken up into recipient cells, where it is capable of causing further aggregation, neurotoxicity, and cell death [7-8, 10].

The first goal of this thesis was to examine how dysregulation of autophagy influenced the unconventional secretion of our novel α-syn dual-split protein model (described in greater
This unique model measures the luciferase intensity of complemented dual-split protein constructs, allowing for the measurement of non-monomeric α-syn variants which are associated with α-syn aggregation and synucleinopathy disease pathology. Current research suggests that unconventional secretion is dependent on the upregulation of autophagy, and that inhibiting the formation of the autophagophore, the precursor to the fully developed autophagosome, prevents this process [9]. Additionally, in a study conducted by Ejlerskov P et al. in 2013, they demonstrated that α-syn secretion can be increased by preventing the fusion of the autophagosome with the lysosome [9]. They hypothesized that this inhibition of lysosome and autophagosome fusion was increasing α-syn secretion due to the cell’s attempt to clear the accumulating undegraded autophagic cargo [9]. Based on the observations reported Ejlerskov and others, we hypothesized that if the formation of the autophagophore is an essential component to α-syn secretion then inhibiting its formation will result in decreased secretion of α-syn [9]. However, if autophagy is inhibited by preventing the fusion of the autophagosome with the lysosome, then an increase in α-syn secretion will still occur due to the cell’s attempt to clear the accumulating undegraded cargo within the autophagosomes, consistent with Ejlerskov’s hypothesis. Furthermore, because inhibition of proteasome activity and mitochondrial function cause an upregulation in autophagic activity [214-216, 221, 234], we hypothesized that the inhibition of either of the proteasome or the mitochondria will increase the secretion of α-syn.

The second goal of this thesis was to develop a methodology that allowed for the analysis of extracellular vesicles, including exosomes on a single vesicle basis. The creation of methodologies to better study exosomes is of paramount importance as increasing evidence indicates that exosomes play a role in the progression of a variety of diseases [55, 56]. Included among these, are synucleinopathies, as misfolded, oligomeric species of α-syn are capable of
spreading cellular disease pathology from cell-to-cell [9]. Furthermore, because increasing evidence suggests that exosomes are not a homogenous population, the ability to isolate individual exosomes provides an opportunity to identify specific population subsets [55, 56]. In this thesis, we explain how our methodology is capable of analyzing single extracellular vesicles through the use of microscopy and imaging analysis techniques allowing for the identification of extracellular vesicle based on protein co-localization analysis. We then validated our methodology by showing what extracellular vesicles associated with exogenously added sonicated preformed fibrillar α-syn based on their co-localization with canonically accepted exosomal markers.

In this next chapter, a review of the literature is provided to first acquaint the reader with synucleinopathies and the protein α-syn. As the chapter progresses, an explanation of unconventional secretion via autophagy will be provided, including what is currently known that distinguishes secretory and degradative mechanisms. Afterwards, a review of how this unconventional secretion affects the release of α-syn associated exosomes and how autophagic mechanisms can further influence this process. Finally, we will review the proteasome and briefly look at its intersecting role in both synucleinopathies and autophagy, including the clearance of mitochondria.
CHAPTER TWO:
REVIEW OF THE LITERATURE

**Synucleinopathies**

Synucleinopathies are a group of neurodegenerative diseases that are characterized by their abnormal intracellular accumulation of the insoluble, aggregated protein deposits referred to as Lewy bodies (LBs) of which misfolded α-syn is the primary protein component [1, 7, 11, 18]. Three major types of Synucleinopathies exist: Multiple systems atrophy (MSA), Dementia with Lewy bodies (DLB), and Parkinson’s disease (PD) [11].

Motor dysfunction is central to PD symptoms which are collectively referred to as Parkinsonisms. These symptoms include bradykinesia, resting tremors, rigid mobility and instable posture [12-13]. While individuals with DLB also develop parkinsonisms, they also show classic dementia symptoms such as short-term memory loss, cognitive impairment, hallucinations, and emotional instability [14].

MSA can be classified into two categories, cerebellar ataxia (MSA-C) or parkinsonism (MSA-P). The classifications depend on α-syn distribution, phenotypic manifestation, and pattern of spread [15-16]. Individuals with MSA-C manifest with disrupted gait and classic ataxia, while individuals with MSA-P have the classical Parkinsonisms previously described [15-16]. Both categories of MSA also show autonomic dysfunction in addition to the previously stated symptoms [15-16].
The localized accumulation of α-syn within the brain regions of the central nervous systems (CNS) varies among these diseases [15-17]. However, the dopaminergic neurons within the brain regions of the substantia nigra and basal ganglia are affected in all cases of synucleinopathies [17]. Why these regions of the CNS are specifically affected among synucleinopathies is not well understood [17, 20].

**Lewy Bodies and Alpha-Synuclein.**

In both familial and idiopathic cases of synucleinopathies the formation of intracellular, proteinaceous inclusions, known as the Lewy Body (LB) occurs [1, 18-19]. The LB is the histopathological hallmark of synucleinopathies, of which the primary protein is composed of aggregated α-syn [1, 18-19]. This aggregated α-syn is misfolded and contains a variety of higher order oligomerized structures that are post-translationally modified [20-22, 242]. The most abundantly observed among these modifications are the additions of phosphorylations and ubiquitinations [20-22, 242]. Furthermore, LBs are resistant to proteinase K digestion [244].

While the formation of these LBs was once thought to cause cellular toxicity eventually resulting in cell death, this initial idea has since become challenged [242]. Current, prevailing theories suggest that sequestration of α-syn, resulting in the formations of LBs, is a protective mechanism [242]. This process occurs as a result of the cell’s inability to degrade α-syn aggregates and that by collectively isolating these aggregates within a single structure, the cell can better concentrate its degradative efforts to a single location [242]. As a result of these previously stated findings, it is believed that a pathogenic variant of α-syn exists and its accumulation within the cell is responsible for pathology thus resulting in the diseased state.

**Alpha-Synuclein**

The protein, α-syn, is 140 amino acids and is found predominantly within the central nervous system (CNS) but is also located within other tissues including the kidneys and red
blood cells [43-45]. α-syn gets its name from its localization within neurons, the synapse and the nucleus [46].

Native α-syn is generally believed to be found as a soluble monomer however some evidence has shown that it is capable of forming a tetrameric, alpha-helix [27-28]. In addition, it has been reported that a small amount of equilibrium between an oligomeric and fibrillar configurations occurs under normal conditions [27-28].

Furthermore, while generally found unfolded, α-syn is also capable of forming an amphipathic alpha-helix upon its association with a lipid membrane which is thought to be important for α-syn’s cellular function as a SNARE protein [2, 41]. The formation of this alpha-helix is achieved by the amino acid residues found within the conserved KTKEGV sequence [243]. This KTKEGV sequence is shared among the other members of the synuclein family, which totals in three with the addition of beta-synuclein and gamma-synuclein [43]. While these others synucleins share homology with the α-syn, only α-syn has been shown to be pathogenic [43].

While α-syn’s exact function in synaptic vesicle trafficking as a SNARE protein has yet to be identified, genetic studies indicate that α-syn plays a role catecholaminergic neurotransmitter vesicle release, as α-syn knock-out mice display abnormal transmission within their dopaminergic neurons [2, 41]. However, this knock-out is not embryonic lethal and phenotypically these mice are relatively normal and display minimal neurological behavioral differences when compared to wildtype (WT) mice [41]. Interestingly, mice over-expressing α-syn also display disruption in vesicular catecholaminergic neurotransmitter release, further supporting α-syn’s role in assisting in dopaminergic transmission [2, 41].
Alpha-Synuclein and Parkinson’s Disease

PD is the most common of the synucleinopathies, and is the second most common neurodegenerative disease [11]. α-syn was first linked to PD when researchers found a family with autosomal-dominant Ala53Thr mutation in the SNCA gene region, the gene associated with α-syn transcription [23]. Since then, several other genes have been identified in association with PD, including: Parkin, PTEN-induced putative kinase 1 (PINK1), protein deglicase (DJ-1), and leucine-rich repeat kinase 2 (LRRK2), and ATP13A2 [24-25].

While the Ala53Thr was the first identified, and the most frequently observed mutation, several other SNCA mutations have since been linked to familial PD including Ala30Pro and Glu46Lys [23, 25]. These mutations are thought to cause PD by increasing α-syn likelihood to misfold and subsequently, aggregate [23, 26-27]. While polymorphisms influence inherited PD, duplications and triplications of the SNCA gene locus, are also associated with causing PD [23, 27]. This process occurs simply by increasing α-syn synthesis above background basal levels. PD case’s from duplications and triplications of SNCA result in LB structure formation that are analogous to those found in SNCA mutations [1, 18-19, 23, 27]. This links WT α-syn as being capable of inducing PD pathology, an important finding, as the aggregation of WT α-syn helps to explain how idiopathic cases can occur which represent 85-90% of all PD diagnoses [23, 27].

Oligomerization and Toxicity.

As stated previously, α-syn found within LBs differs from the native unfolded monomer, as it is misfolded and oligomerized into higher order protein structures [244]. Included among these higher order structures are fibrils and oligomers which display different physiological qualities [244, 255]. The fibril species are resistant to proteinase K digestion, are detergent insoluble, and have an occluded N-terminal [244, 255]. It has been shown that sonication of
these of fibrillar species can induce $\alpha$-syn accumulations within treated cells both \textit{in vitro} and \textit{in vivo} [32-34]. In contrast to fibrils, the lower order oligomerized structures can be degraded by proteinase K and are detergent soluble but are still capable of inducing \textit{in vitro} aggregation of $\alpha$-syn and neurodegeneration within transgenic mouse models [3]. Therefore, it has led some to believe that these oligomers are the pre-existing structure to the fibrillar form [244, 255].

Furthermore, some have argued that oligomers are specifically the pathological species responsible for $\alpha$-syn’s ability to induce cellular damage and neurotoxicity [3-4, 31]. \textit{In vitro} and \textit{in vivo} studies have shown that dopamine and its metabolites are capable of inhibiting $\alpha$-syn protofilament maturation, an intermediate between the oligomeric and fibrillar species [3, 29, 31]. The implication being that the inhibition of protofilament maturation results in a specific, increased vulnerability within dopaminergic neurons due to prolonged oligomeric structure exposure [3, 29, 31]. However, in sufficient evidence exists to fully support the idea that oligomerized $\alpha$-syn is responsible for its neurotoxic properties and some level of controversy remains. While the exact $\alpha$-syn structure that induces pathogenesis remains unknown, it is generally agreed upon that the aggregation of $\alpha$-syn is responsible for its neurotoxicity [7].

\textbf{Evidence for Alpha-Synuclein Transmissibility among Cells -\textit{in vitro} and \textit{in vivo}.}

In addition to being able to induce neurotoxicity, $\alpha$-syn has also been shown to be transferrable from cell-to-cell in both \textit{in vitro}, as well as, \textit{in vivo}, as observed in PD patients who have undergone embryonic nigral cell transplants [6, 33-36]. Post-mortem observation of these patients, showed LB-like pathological inclusions within their grafted neurons 14 years later [6]. The transfer of the Parkinson's-like pathology within these stem-cells grafts in combination with $\alpha$-syn’s ability cause protein inclusion within cells has led to the formation of hypothesis stating that $\alpha$-syn is a prion-Like protein due to the similarities among the cellular spreading of prion disease [37, 38].
Prion-like proteins behave similar to prions within the same microenvironment, sharing the ability to cause cellular infectivity through similar mechanisms, they are both capable of amplification and transmissibility between cells, as well as causing dysregulation of the mechanisms that cells utilize to interfere with their degradation [33, 37, 40-41]. However, in contrast to true prions, prion-like proteins have not been demonstrated to be transmissible from person-to-person.

**Extracellular Vesicles**

Current evidence supports the idea that α-syn is secreted both within extracellular vesicles as well as in a naked, non-vesicular state. *In vitro* work shows that when sonicated preformed fibrils of α-syn are added to cells they are readily taken up into cells and can result in progressing pathology [53]. However, how the pathology transfers from cells after this addition has not been identified. It has been suggested that exosomes play a role in the transfers of α-syn pathology, as pathological species of α-syn have been isolated from the cerebral spinal fluid (CSF) of patients with PD [54].

Additionally, it has been demonstrated that the isolation and subsequent addition of exosomes from cells over-expressing α-syn to wild-type (WT) cells, increased α-syn levels within the WT as well as caused increased α-syn aggregation. Furthermore, within the same study, the isolated exosomes were shown to cause increased preferential transfer of oligomeric α-syn species *in vitro* when compared to non-vesicular oligomers, indicating that exosomes are able to transfer α-syn and its pathology to recipient cells and do so more effectively than non-vesicular α-syn [8]. Due to vesicular oligomeric α-syn’s ability to be preferentially taken up into cells it is possible that exosomes containing α-syn is a key component in the spread of synucleinopathy pathology among cells [8-9, 32-35].
Exosomes.

Exosomes are small extracellular vesicles (EVs) with a diameter of 30 to 150nm [55-58]. Exosomes are the intraluminal vesicles (ILVs) that are released into the extracellular space upon the fusion of a specific type of late endosome called the multivesicular bodies (MVBs) with the plasma membrane [55-57]. Exosomes were originally thought to simply contain the unwanted waste of cells, however, further research has shown that exosomes are a part of normal physiological intercellular communication and transfer a variety of physiological relevant cargoes including RNA, proteins, and lipids [55, 60-65]. Additionally, these EVs are highly enriched in tetraspanin proteins, a subfamily of proteins that organize membrane microdomains by forming clusters that interact with a large variety of cytosolic and transmembrane signaling proteins [55-56, 66]. Among these tetraspanins, CD9, CD63, CD81, CD82 and CD151 are broadly expressed among tissues and as a result are recognized as canonical markers of exosomes [55-56]. However, a variety of other proteins are also used as exosomal markers when such as Endosomal Sorting Complexes Required for Transport (ESCRT) proteins ALIX or TSG101. Additionally, the use of specific exosomal cargoes rather than canonical proteins can also be used to identify cell specific exosomes which may uniquely be secreted such as myelin from oligodendrocytes [67-68].

Multivesicular Bodies.

Endosomes can be classified into one of three categories: Early endosomes, late endosomes, and recycling endosomes [59]. Early endosomes containing cargoes that are fated for recycling make up the subpopulation of endosomes that develop into recycling endosomes while early endosomes that do not progress on to become secretory or degradative late endosomes [69-70]. Among these late endosomes are a specific group of MVBs which occur as a result of a large accumulation of ILVs, smaller vesicles that form within the late endosomes [59].
Before late endosomes begin to form ILVs, they go through a reorganization of their membrane which results in an enrichment of the tetraspanin proteins [66]. Afterward, the formation of ILVs is mediated by a series of proteins that form the ESCRT complexes [71]. ESCRT proteins are brought to the location where ILVs will be formed and begin to form their respective ESCRT complexes, of which four distinct ESCRT protein complexes (0, I, II, III) exist, each fulfilling a specific role in the formation of ILVs. ESCRT 0 identifies ubiquitinated cargo [72]; ESCRT I and II are recruited by varying stimuli and are thought to participate in the initiation and formation process associated with the budding of the intraluminal membrane of the endosome; and that ESCRT III completes the ILV formation process by causing scission of the budding membrane [71-75].

**Exosomes and Synucleinopathies.**

Exosomes role in increased pathology is not exclusive to PD and as evidence suggests that exosomes participate in the spreading pathology of other major synucleinopathies [7-8, 10, 54]. Exosomes containing higher levels of α-syn were found within the CSF of individuals with DLB [54]. Similar to PD, isolated exosomes from these individuals with DLB, were shown to be able to pass on pathological effects to H4, human neuroglioma cells, including increased levels of α-syn oligomerization which could be modulated in a dose dependent matter [54].

**Genetic Mutations in Parkinson’s Disease alter Exosome Biogenesis.**

LRRK2. Several of the genetic mutations associated with familial PD have been shown to play roles associated with MVB formation [76-77]. Mutations within LRRK2 account for the largest amount of both familial cases and sporadic cases of PD [78-79]. And although LRRK2 plays a role in several cellular processes, it is found to be important in the regulation of intracellular vesicle trafficking, as over-expression of LRRK2 results in decreased endocytic and exocytic vesicular release; indicating that LRRK2 plays a role in the either the release mechanism...
or increases degradation of exosomes [77-81]. Furthermore, visualization of LRRK2 indicates that it associates with MVBs and that the pathological genetic mutation R1441C of LRRK2 leads to abnormally large MVBs and results in increased exosome release [81].

**VPS35.** Other genetic mutations associated with PD have also been implicated as being part of the exosomal biogenesis pathway and MVB formation. Vacuolar protein sorting-associated protein 35 (VPS35), a protein associated with the retromer complex [82]. The retromer complex is integral to proper endosomal cargo sorting where cargoes can then be trafficked back to the cell surface or sent back to the trans-golgi-network (TGN) via retrograde transport [82-84]. In addition to cell surface and TGN sorting, the retromer complex is important for the sorting of cargoes to special endosomal membrane compartments that are not degraded via lysosomes [83]. Furthermore, it has been shown that the retromer complex plays a role in autophagy and that it directly interacts with ATG8 (yeast), human paralogues microtubule-associated protein 1 light chain 3 (LC3) and gamma-aminobutyric acid receptor-associated protein GABARAP, via VPS35 [85]. In addition, the PD familial mutation Asp620Asn of VPS35 results in inhibited formation of the WASH complex and results in decreased transport of ATG9 to autophagosomes, resulting in decreased autophagic activity [86].

**ATP 13A2.** Furthermore, the P-type ATPase ion pump, ATP 13A2, the protein transcribed and translated from PD-related gene 9, plays a role in trafficking zinc into MVB [87]. Familial mutations of ATP 13A2 result in lysosomal dysfunction and increased α-syn accumulation as a result of altered trafficking of endosomal and autophagic cargoes [88-89]. In addition, the depletion of ATP 13A2 has been shown to induce zinc homeostasis dysregulated, as well as, both increase α-syn accumulation and cause mitochondria dysfunction [90-91]. While in contrast, upon ATP 13A2 over-expression, increased survivability and decreased α-syn
accumulation [91]. Additionally, the collected culture medium from cells over-expressing ATP 13A2 resulted in increased levels of exosomal α-syn [91].

**Unconventional Secretion**

While the transfer of α-syn from cell-to-cell represents one piece in understanding the transmission of synucleinopathy pathology, another is the underlying mechanisms associated with α-syn secretion. In the previously stated paragraphs, discussing PD mutations that are associated with the endosomal pathway and exosomal α-syn release, a common theme emerges regarding the autophagic/lysosomal degradative pathway. Furthermore, the dysregulation of these processes is also associated with unconventional secretion of α-syn. While unconventional secretion is technically any process that results in the secretion of cargo that does not occur through the conventional secretory pathway, increasing evidence suggests that orchestrated unconventional secretion of specific proteins occurs by utilizing autophagic mechanisms [92].

In comparison to conventional protein secretion, very little is known about the exact mechanisms and pathways responsible for autophagic unconventional secretion. The bulk of information regarding how autophagy drives unconventional secretion was elucidated as a result of studies focusing on one of the earliest proteins identified to undergo secretion through this process, IL-1β [92, 94]. Similar to some conventional secretory proteins, IL-1β is synthesized as a pro-protein. However, in contrast to conventional secretory proteins, IL-1β has no localization signal sequence, and is translated into the cytoplasm where it is readily found [94]. Interestingly, upon activation of the specific inflammatory cascades that results in the formation of the inflammasome, IL-1β goes through proteolytic cleavage, from its inactive, pro-form to its active form, and is secreted within packaged membrane vesicles [94-97]. Until recently, the
mechanism for active IL-1β secretion was unknown; however, recent studies have indicated that active IL-1β secretion is regulated by autophagic machinery [96-97].

In addition to IL-1β, IL-18 and HMGB1 are among the other proteins that have been identified to be secreted by autophagic machinery upon inflammasome activation [98]. Similar to IL-1β, both IL-18 and HMGB1 lack a signal sequence [98]. However, HMGB1 differentiates itself from IL-18 and IL-1β, as it is thought to go through indirect proteolytic cleavage upon inflammasome activation while IL-18 and IL-1β are thought to be directly cleaved by the inflammasome [98].

Additionally both prion-like proteins α-syn and Aβ, one of the pathological proteins associated with another neurodegenerative disease, Alzheimer’s disease (AD), have also been indentified to undergo unconventional secretion as a result of autophagy [99]. However, in contrast to IL-1β, IL-18 and HMGB1, α-syn and Aβ secretion is not dependent on the activation of the inflammasome and while α-syn does not share all the previously stated traits of IL-1β, IL-18, or HMGB1, it is directly translated into the cytoplasm and does not appear to have a specific localization sequence [9, 94-99]. Therefore, it is thought that these characteristics may be shared among the proteins that undergo autophagic regulated unconventional secretion.

**Autophagy**

Evidence suggests that the degradation of α-syn occurs via both chaperone-mediate autophagy (CMA) and macroautophagy, as well as, by the ubiquitin proteasomal system (UPS) which will be talked about in later sections [104-107]. CMA is a form of autophagy where specific cargoes are directly taken to the lysosome rather than being engulfed within an autophagosome which occurs in macroautophagy. In CMA cargoes are recognized by their exposed KFERQ amino acid motif by heat shock protein 70 kDa protein 8 (HSC70/HSP8A) [100-101]. Bound cargo is then taken to lysosome where HSC70 binds lysosomal associated
membrane protein 2 A (LAMP2A) and then is unfolded [100-101]. After the specific cargo is unfolded it is then translocated into the lysosome for degradation [100]. However, it has been hypothesized that macroautophagy (referred hereafter as autophagy) is the major pathway associated with the removal of α-syn oligomers [104-107]. As elucidated in previous sections, the role of autophagy has recently expanded beyond the scope of degradation and has also been shown to be a part of the pathways associated with the unconventional secretion of specific proteins. Among these proteins includes the aggregate forming protein α-syn.

Evidence suggests that α-syn is recruited to both degradative, the process associated with autophagosome and lysosome fusion and breakdown of cargoes, and the secretory, the process associated with the autophagosome and plasma membrane fusion, autophagic pathways [107]. Our lab and others have shown that α-syn aggregates are recruited to acidified autophagic compartments associated with autophagic degradation [108-111]. As of now, only a few differences have been indentified between degradative and secretory autophagosomes. Additionally, it has been proposed that both degradative and secretory autophagosomes start from a similar origin point and become differentiated at a specific unknown branch point [112]. Therefore, having an understanding of how the precursory structure that develops through expansion into the autophagosome, the autophagophore is important for understanding both degradative and secretory autophagy. This process is also highlighted in figure 1 of the appendix.

**Autophagosome initiation.**

**ULK complex.** Classically autophagic initiation requires the ULK Complex and Beclin-1 complex. The ULK complex gets its name as result of the two proteins found within it, UNC51 like Ser/Thr kinase 1 (ULK1) and UNC51 like Ser/Thr kinase 2 (ULK2) [112-113]. Additionally, the ULK complex is made up of two other proteins ATG101 and the 200 kDa FAK family kinase
interacting protein (FIP200) [112-113]. During activation, such as starvation, ULK1 and ULK2 phosphorylate a variety of proteins including FIP200 and ATG13 resulting in the formation of the complex [112-113]. Additionally, both ULK1 and ULK2 have a carboxy-terminal domain (CTD) that allows them to associate with membranes which is thought to be important to the formation of the autophagosome [112-113]. Also, the ULK complex is predominantly negatively regulated by mTOR1 which is directly bound to ULK1 and ULK2 thus inactivating them [114-115]. In contrast, the ULK complex can is positively regulated by AMP-activated protein kinase (AMPK) [114-115].

**Beclin-1 complex and PI3K.** The Beclin-1 complex is important to autophagic initiation because of its ability to induce the formation of the autophagy specific class III PI3K [116-118]. The Beclin-1 complex is composed of Beclin-1, vacuolar protein sorting 34 (VPS34/ PI3KC3), Vacuolar sorting protein 15 (VPS15/p150), Bif-1, Rubicon, and ATG14 [116-121]. Beclin-1, VPS34, VPS15, and either ATG14 or ultraviolet irradiation resistance-associated gene (UVRAG) is necessary for its activation [117-121]. The titular Activating Molecule of Beclin-1 Related Autophagy 1 (AMBRA1) is a scaffolding protein, which interacts with Beclin-1 among other proteins and helps in assembling the proteins necessary to form the active complex [116-121]. Furthermore, AMBRA1 is positively regulated by ULK1 and is negatively by B cell lymphoma 2 (Bcl-2) and Rubicon [119-120]. Upon activation of the Beclin-1 complex VPS15 heterodimerizes with VPS34, regulating its activity [121]. This heterodimerization allows for VPS34 to catalyze the phosphorylation of phosphoinositides, an integral part of autophagophore initiation process [121].

**ATG protein complexes.** Two autophagy-related protein (ATG) systems are utilized as part of the autophagic process. These systems result in the formation of two distinct ATG complexes which are important in the elongation of the autophagophore into the
autophagosome. Additionally, these two ATG systems both rely on the E1 ubiquitin-like (UBL) protein, ATG7 [103]. However, after utilizing ATG7 they are brought to two distinct E2 UBL ATG proteins.

**ATG12-ATG5-ATG16 complex.** In the formation of the first complex, ATG7 binds to ATG12 where it brings it into close proximity with the ATG 10, the E2 UBL. ATG10 binds to ATG12 where it assistants in binding ATG12 to ATG5 resulting in beginnings of final complex, denoted as ATG12-ATG5 [103, 122-123]. The bound ATG12-ATG5 assembles with ATG16 to form the ATG12-ATG5-ATG16 complex [103, 123-126]. ATG16 acts as an E3 UBL protein where it brings the full ATG12-ATG5-ATG16 complex onto the expanding autophagophore [103, 123-126].

**ATG-LC3 complex.** During this process the second ATG complex also forms. The second of the two complexes is important in the translocation and processing of LC3 [103, 127-128]. Similar to the first ATG system, the second system also utilizes the E1 UBL protein ATG7 [103]. However, the process begins with the processing of LC3 from its precursor pro-form by ATG4, a cysteine protease [103, 122]. The cleavage of pro-LC3 into LC3, denoted as LC3-I allows for ATG7, the E1 UBL protein to recognize and bind to it. The binding of LC3-I to ATG7 allows for an E1-E2 like reaction resulting in the binding with ATG3 the E2 UBL protein [103, 122, 127-128]. The LC3-I bound to ATG3 goes through the lipization process where the now exposed glycine on the LC3-I becomes conjugated with the phospholipid, phosphotidylethanolamine (PE) [103, 129]. This process is mediated by the first ATG assembly complex, ATG12-ATG5-ATG16, which acts as the E3 UBL protein [103, 129-131]. The PE conjugation creates the lipid anchor that allows for LC3-I’s association with the autophagophore [103, 130-131]. Subsequently, this changes LC3-I’s nomenclature from LC3-I to LC3-II indicating PE is bound to LC3 [103]. This process is necessary for the autophagophore to continue elongation into an autophagosome
Additionally, this complex is also responsible for the recruitment of GABARAP to the autophagosome [83]. GABARAP plays a role in the trafficking of the autophagosome and the recruitment of specific cargoes to the autophagophore [83].

**Elongation and degradation.** During the elongation process cytoplasmic contents, such as aggregated α-syn, are engulfed by autophagophore, an isolated portion of double membrane and are fully sequestered when the autophagophore encloses to form the autophagosome [103, 128-129, 132]. The autophagosome then undergoes mediated fusion with the lysosome, resulting in the formation of the autophagolysosome. This process exposes the cytoplasmic contents enclosed within the autophagosome to the hydrolytic enzymes within the lysosome thus resulting in their degradation [9, 132].

**Autophagosome formation and Omegasomes.**

While its exact source remains unidentified, the isolated portion of double membrane that becomes the autophagophore is thought to originate primarily from the endoplasmic reticulum (ER) but may also be from another organelle such as the golgi apparatus, mitochondria, or possibly the plasma membrane, [9, 132-135]. In support of the ER being the primary source of membrane, during starvation, a specific portion of the ER, characterized as the omegasome due to its shape, has been shown to become isolated [121, 136, 137]. These isolated membranes are shown to be enriched with ATG14, and the phospholipid, phosphatidylinositol 3-phosphate (PI3P) which results in the binding of the autophagic effectors, WD-repeat domain phosphoinositide-interacting protein (WIPI2) and double FYVE-containing protein (DFCP1) [102, 121, 137]. While the exact reason for why PI3P enrichment is important for autophagic function has yet to be identified, it is essential for autophagophore’s expansion into the autophagosome [138-139].
Starvation induced versus quality control Autophagy.

Degradative autophagy has a variety of functions including: 1) The recycling of nutrients within the cell during times of starvation; 2) cellular homeostatic functions, including the regulated breakdown of damaged organelles, the removal of pathological invaders such as bacteria and viruses and the elimination of aggregated and aggregate prone proteins such as misfolded α-syn [102-103, 140-141]. However, while degradative autophagy is essential to cellular processes, the activation of autophagy can generally attributed to two different justifications: non-discriminate degradation as a result of starvation and quality control where specific cargoes are targeted for elimination [141-142]. In starvation induced autophagy, autophagophores indiscriminately engulf portions of cytoplasm with the primary goal of recycling of nutrients due to the lack availability [141-142].

In contrast, in quality control based autophagy, the autophagic cargo is selectively targeted for degradation by poly-ubiquitinated post translation modifications [140-147]. These cargoes include (but are not limited to) aggregated proteins, damaged organelles and long lived proteins [140-147]. Selectivity of the quality control based autophagy is driven by specific ubiquitin adaptor binding proteins such as p62/SQSTM1, NBR1, NDP52, Optineurin, and HDAC6 [143-149]. These adaptor proteins are able to directly link their ubiquitinated targets directly to LC3B, thus resulting in targeted degradation. It is believed that aggregated proteins, such as α-syn, are primarily targeted for autophagy via this mechanism [9, 150].

Activation mechanisms of autophagy.

The specificity of whether starvation induced or quality control autophagy occurs is thought to be differentiated through the mechanisms associated with autophagy activation.
These pathways have been elucidated via specific small molecule activators of autophagy, among which is rapamycin [151].

**Rapamycin.** Rapamycin is naturally produced substrate from the bacteria *Streptomyces hygroscopicus* and is of a macrolide composition known to have immunosuppressant effects [152]. Rapamycin is membrane-permeable and, upon treatment, rapamycin binds to 12-kDa FK506-binding protein (FKB12) forming a complex while simultaneously causing its inhibition [151, 153]. This rapamycin-FKB12 complex binds directly and inhibits mTOR1 [153-155].

**mTOR1.** mTOR1 functions to inhibit autophagy via phosphorylation of both ATG13 and ULK1 (specifically at serine 758) resulting in the inhibition of the ULK complex; one of the primary initiating complexes of autophagy [115, 155]. When mTOR1 is inhibited, ULK1/2 activity increases thus resulting in phosphorylation of the two important ULK complex subunits, ATG13 and FIP2000 [113, 156-157]. This process is thought to increase starvation induced autophagy rather than quality control based autophagy [102, 112].

**mTOR2.** While mTOR1 is thought to be important in cell cycle progression as a result of nutrient availability, it is thought that mTOR2 is influences the regulation of cell proliferation and cell survivability. Additionally, prolonged rapamycin and/or high dose treatment has shown to also result in mTOR2 inhibition [158-160]. However, due to mTOR2’s relatively recent identification, all the possible functions that mTOR2 influences have still yet to be elucidated.

**Trehalose.** Another small molecule associated with the activation of autophagy is trehalose. Although the mechanism associated with trehalose’s ability to cause an upregulation of autophagy has not been identified, it has an interesting role in neurodegenerative diseases due to its ability to increase clearance of aggregate forming proteins, including α-syn [165-166]. Trehalose is a non-reducing disaccharide that occurs when two glucose units are joined by 1,1 alpha bond [167]. Although both rapamycin and trehalose increase autophagic activity, In
contrast to rapamycin, trehalose has been shown to increase in autophagic activity through an mTOR independent pathway and appears to effect autophagosome production differently [9, 168]. Interestingly, trehalose has also been shown to activate TFEB, a transcription factor that increases lysosome biogenesis [169]. While another paper has shown that TFEB causes an increase in LC3-II positive vesicles, indicating that TFEB increases autophagy [170]. Studies conducted by DeBosch et al., within hepatocytes have proposed that trehalose’s mechanism of action for increasing autophagy occurs due trehalose’s ability to cause inhibition of glucose transports (GLUT1, GLUT2, GLUT3, GLUT4, and GLUT8) resulting in activation of AMPK which phosphorylates ULK1 [168].

In addition to trehalose’s ability to activate autophagy, trehalose has also been suggested to play a role in directly affecting protein aggregate folding. A modeling study conducted by Allison et al., 1999, proposed a mechanism on how trehalose may stabilize protein folding through the use of hydrogen bonds [171]. Additionally, trehalose has thought to alter protein folding of aggregate proteins or potentially change the thermodynamics of aggregate forming proteins resulting in change of aggregate forming proteins back to their native conformation [172].

**Secretory Autophagy.**

In contrast to degradative autophagy, the mechanisms regarding secretory autophagy are not as well studied, due to its relatively new discovery. The majority of studies that have identified the mechanisms associated with secretory autophagy were conducted within yeast; and then their protein homologues/paralogues were identified within mammalian cells.

Currently, two specific proteins have been identified to be important in the unconventional secretion of both IL-1β and α-syn: The first, GRASP55 protein found in yeast, mammalian homologue ATG5, affects autophagy in general [124, 173]. The second is RAB8a, a
GTPase, which has been identified to be necessary for secretory autophagy as a result of its function in the sorting of cargo to the plasma membrane [9]. Interestingly, a closely related GTPase, RAB8b has been suggested to be important in the degradative autophagy [174]. Potentially illustrating, a branch point in which autophagosomes may share a common origin before being distinguished for degradation or secretion. Furthermore, it has been demonstrated that yeast secretory autophagy requires the specific snare protein Sso1 which is integral for autophagosomes to associate with the plasma membrane [175]. In contrast, degradative autophagy requires the VAM7/VAM3 snare proteins for vacuoles to fuse with lysosomes [175]. These differences found within yeast autophagosomes provides further evidence suggesting that a similar precursory origin may exist before autophagosomes are distinguished as being degradative and secretory in nature.

**Compartments of unconventional protein secretion.** Research on yeast has identified compartments of unconventional protein secretion (CUPS), which resemble the mammalian omegasome, a precursory formation to the autophagophore located on the membrane of the ER [137, 176]. During starvation, GRASP re-localizes to CUPS within yeast which serves as a marker signifying the formation as secretory [176]. A true secretory marker homologue, such as GRASP, has yet to be identified within mammalian cells. Additionally, because omegasomes do not appear to form within yeast cells, and because mammalian cells have not been identified to form CUPS, whether these seemingly similar formations are actually distinct still needs to be elucidated. However, if a true GRASP homologue was identified within mammalian cells, this may begin to shed light on the possible connection between CUPS and omegasomes. Additionally, the identification of the mammalian GRASP would serve as evidence supporting the idea that degradative and secretory autophagosomes share an initial precursory origin of
ATG proteins and that the distinction between degradation and secretion occurs later in the autophagosome maturation pathway.

**Autophagosome formation is important to the secretion of alpha-synuclein.**

*Autophagosome inhibition results in decreased alpha-synuclein secretion.* Currently, the two processes thought to be important in increasing autophagic dependent secretion of α-syn are: The upregulation of autophagy, specifically increasing the formation of the autophagosomes [9, 173]; and altering the properties associated with autophagic degradation, generally due to inhibition of autophagosome and acidified lysosome fusion [9]. However, autophagosome and acidified lysosome fusion is also the result of correct microtubule trafficking.

**3-MA.** In support of the idea that α-syn secretion is dependent on autophagosome formation, the study conducted by Ejlerskov P et al., 2013, found that the treatment of 3-methyladenine (3-MA), a known inhibitor of the autophagy specific, class III PI3K (VPS34/PI3KC3), in cells over-expressing both the genetic mutant of α-syn A30P, as well as, p25α had significantly decreased levels of α-syn secretion compared to untreated controls [9]. Interestingly, 3-MA treatment is also linked to α-syn accumulation. Furthermore, siRNA mediated knock-down of ATG5, a protein important in the elongation of the autophagophore, resulted in significantly decreased α-syn secretion [9]. These results suggest that the autophagosome formation and elongation play a role in autophagy dependent secretion of α-syn [9].

*Promotion of Autophagophore formation increases alpha-synuclein secretion.* Contrastingly, promotion of autophagosome formation increased α-syn secretion. The over-expression of both α-syn A30P and Rab1A, a protein implicated in autophagophore promotion, significantly increased α-syn secretion [9, 176].
Increases in autophagy increase alpha-synuclein secretion. While in contrast, when the cells over-expressing the α-syn A30P also over-expressed p25α showed significant increases in α-syn secretion when they were treated with the autophagy initiating small molecule, trehalose [9]. In addition to trehalose treatment, rapamycin, another autophagy activating drug, in cells over-expressing the mutant form of α-syn (A30P) saw considerable fold increases in α-syn secretion and modest but significant levels, respectively [9]. This was thought to occur due to differences in the mechanisms associated with autophagic activation, as described previously [9].

Autophagosome trafficking direction. HDAC6. As stated previously, a specific subset of autophagy proteins help in the degradation of specific targets as a function of quality control autophagy. These specific adaptor proteins, such as P62/SQSTM1, and HDAC6 contain binding motifs associated with the recognition of both distinct poly-ubiquitin post-translational modifications, as well as, LC3B [144-145, 148-150]. These binding motifs allow for the specific translocation of ubiquitinated targets to center of the autophagophore [144-145, 148-150]. Among these adaptor proteins, HDAC6 also displays another function; it is also capable of linking its targets to dynein-dynactin motor proteins [148]. Within neurons P62/SQSTM1 and HDAC6 are necessary for the formation of inclusion bodies as result of their ability to direct the transportation of ubiquitinated cargo retrograde, toward the minus end of the microtubule pathway, and toward the nucleus [177]. Additionally, knocking-down HDAC6 has been shown to increase α-syn secretion [9].

P25α. Building off of HDAC6’s ability to affect retrograde transport of marked ubiquitinated cargo, several studies has been published focusing on the Tubulin Polymerization-promoting protein (P25α/TPPP), which runs conversely to HDAC6. While HDAC6 increases retrograde microtubule transport, P25α increases anterograde transport by binding to the plus
end of microtubule assemblies, decreasing their plus end growth rate and reducing depolymerization [178]. However, it was not until a study found that P25α also inhibits HDAC6’s deacetylase activity that another paper followed up on whether p25α was capable of influencing α-syn secretion [179]. The study found that the over expression, of p25α increased α-syn secretion, and that the same effect could be achieved by knocking down HDAC6 or treating with the HDAC6 inhibitor, trichostatin, and these processes were linked to increased secretion of α-syn [9]. Additionally, p25α potentially increases α-syn aggregation and is found within lewy bodies [179]. P25α is generally found with oligodendroglial cells however, dopaminergic neurons synthesize p25α in abnormal levels during PD progression [178].

**Autophagy and Exosomes**

**Amphisomes.**

As previous stated, the secretion of exosomal α-syn is thought to be one of mechanisms associated with the transfer of pathological α-syn among cells. Additionally, several of the genetic mutations associated with familial PD are associated exosome secretion but linked to dysregulation of autophagic pathways. While secretory autophagic mechanisms have been shown to influence α-syn secretion, areas of overlap between exosome biogenesis and autophagy exist. Therefore, it should come as no surprise that the exosomal secretion of α-syn is also influenced by autophagy. Specifically, one of the direct ways that exosomes and autophagy interrelate is through the production of the amphisomes.

Amphisomes are a hybridized organelle that results from the fusion of autophagosomes and endosomes which include MVBs. Amphisomes can then continue down the autophagic pathway where they can go through fusion with the lysosome resulting in degradation or bind with the plasma membrane and combined cargoes are released into the extracellular space [180-181].
Amphisome formation. The fusion of autophagosomes with endosomes to form amphisomes, as well as, the fusion of amphisomes with lysosome is a rab7 dependent process [182]. Similarly, these fusions are also dependent on VAMP8 for the priming of the SNARE protein complex that forms by the autophagosomal Syntaxin 17 and the lysosomal/endosomal SNAP29 [183, 186]. Interestingly, specific fusion of autophagosomes and late endosomes for the creation of amphisomes is also dependent on autophagosome phosphatidylinositol 4-phosphate (PI4P) that forms as a result of GABARAP’s recruitment of phosphatidylinositol 4-phosphate kinase II (PI4KII) [185]. The depletion of either PI4KII or its substrate PI4P prevents the fusion of autophagosomes and late endosomes. Conversely, while the fusion of autophagosomes with lysosomes utilizes both rab7 and the Syntaxin17-SNAP29 complex, its fusion is mediated by autophagic protein homolog (EPG5) rather than PI4P [186].

Theoretically, amphisomes seemingly provide the ideal environment for α-syn aggregation, as it has been shown that α-syn aggregation increases in mildly acid conditions due to increased fragmenting and secondary nucleation of oligomerized species that are found within amphisomes [187]. Additionally, amphisomes are particularly enriched in the glycosphingolipids GM1 and GM3 that are often found within exosomes but also shown to increase α-syn aggregation within MVBs [188]. While more information is needed before any one conclusion can be made, the formation of amphisomes may explain how autophagic drugs influence the secretion of exosomal α-syn.


Various drugs have been shown to influence autophagy and autophagic based secretion of α-syn, several of the most prominent have already been covered earlier within previous sections, however, bafilomycin-A1, the lysosomal inhibitor, provides an interesting point of intersection between autophagy, the endolysosomal pathway, and exosomes.
Bafilomycin-A1 is a naturally forming macrolide substrate that inhibits the acidification of lysosomes by blocking the vacuolar H⁺-ATPase [189]. Additionally, bafilomycin-A1 also prevents the fusion of autophagosomes and lysosomes [189-190]. A recent study in which drosophila were genetically depleted of the vacuolar H⁺-ATPase found that impotent lysosomes still fused with autophagosomes [190]. In addition, when cells were depleted of the autolysosomal SNARE protein, Syntaxin17, the fusion of autophagosomes and lysosomes were prevented despite the proper acidification of lysosomes; indicating that the vacuolar H⁺-ATPase was not responsible for this affect. The study identified that bafilomycin-A1’s also inhibits the ER Ca²⁺-ATPase, ATP2A/SERCA, which is important in maintaining proper calcium balance within the cytosol [190]. This inhibition of SERCA by bafilomycin-A1 and the subsequent calcium dysregulation is thought to prevent the fusion of autophagosomes and lysosomes due to Syntaxin17 mediated fusion dependency on calcium [190]. Supporting this idea, the treatment of thapsigargin, a specific inhibitor of SERCA, also prevents the fusion of autophagosomes and lysosomes [191].

Several studies have shown that cells over-expressing α-syn treated with bafilomycin-A1 not only caused increased levels of raw α-syn secretion but also caused a significant increase within the exosomal population [8, 49, 88]. Interestingly, the relative fold difference in the exosomal α-syn release among the bafilomycin-A1 and DMSO control condition was higher than the fold difference in general α-syn release for the same treatment paradigm [8]. One of the studies offered the explanation for the bafilomycin-A1 induced increase in exosomal α-syn secretion occurred due to elevated levels of MVB and autophagosome fusion and because of the lysosomal inhibition, the cell removes the α-syn via secretion [8]. Consistent with this idea, the increased activation of autophagy has been shown to also increase the interaction between MVBs and autophagosomes [192].
Proteasomal Inhibitors Influence Alpha-Synuclein Secretion

Introduction to the Proteasome.

In addition to the autophagy/endolysosomal pathway, the other primary degradative system within the cell is the proteasomal degradation pathway. The proteasomal degradative pathway is dynamic and changes depending on the needs of the cell and is reflected in the specificity of various types of proteosomes. These specificities are done on a genetic level by specific pathways and responses that alter the transcription of different subunits of the proteasome, thereby imparting changes on what proteasomes recognize or degrade [194-196]. Also, post-translational modification of the proteasome influences its degradative capacity and the cell can also influence proteasomal degradative systems by changing the levels of degradation of the proteasomes themselves [195]. Finally, proteasomal capacity is also influenced by other factors such as oxidative stress, aberrant or misfolded proteins, chemicals or drugs, as well as, age all of which in altering the influence of cellular levels and oxidative modifications of the proteasomes [196-197]. In particular, the 26S proteasome plays an important role in the removal of aggregate and misfolded proteins such as α-syn by utilizing the ubiquitin proteasome system (UPS) [41]. In addition to the previous stated factors, the processes that negatively influence proteasome activity are particularly important within the context synucleinopathies [197-199]. As impaired proteasomal function is associated with the accumulation of misfolded proteins such as α-syn [198-199].

Defining the 26S Proteasome.

The proteasome is made up two primary protein complexes, the 20S core particle and the 19S regulatory particle [103]. The 20S core is composed of a stack of four heptameric rings [103]. Each ring represents one of the two subunits of the 20S core particle known as the either the alpha or beta subunit [103]. The beta heptameric ring subunits (β1-7) are found in the
interior of the stacked four rings. The $\beta_1$, $\beta_2$, and $\beta_5$ of the $\beta$ subunit are catalytic, each with unique specificity for substrates due to their chymotrypsin-like, trypsin-like, and caspase-like properties [103]. While in contrast to the beta subunits, the alpha heptameric ring subunits sit on the exterior of the 20S core particle and are largely structural in nature [103].

The 19S regulatory particle recognizes ubiquitinated proteins and is important in preparing proteins for degradation by the 20S core particle. The 19S regulatory particle uses ATP hydrolysis to unfold proteins and translocates them through its narrow pore where they are degraded by the core particle [200]. The central machinery of the 19S regulatory particle is composed of a hexameric ring with six AAA ATPases abbreviated as RPT1-6 [200]. Additionally, the 19S regulatory particle is connected to the alpha-ring of the 20S core particle via the C-terminals of its six AAA ATPases and is able to open the gated entry channel where it is connected to the core particle [201].

Proteasome Response Pathway.

**NRF1.** The transcription factors nuclear factor like 1 and 2 (NRF1 & NRF2, respectively), are important in the upregulation of proteasomes [202]. NRF1 is found bound to the ER where upon activation translocates into the nucleus and binds to its response element, antioxidant response element (ARE) [202-204]. Under normal conditions, NRF1 translocates from the ER to the cytosol via the ATPase, p97, where it is then ubiquitinated and degraded [202]. Activation of NRF1 causes an increase in proteasomal transcription and occurs when the proteasomal capacity needs to be increased or when inhibition of proteasomal activity has occurred [202, 205-206].

**NRF2.** Contrastingly, NRF2 is found readily within the cytosol and becomes activated in response to oxidative stress [207]. NRF2 under normal conditions is not active, held in its inactive form by Kelch like-ECH-associated protein (KEAP1), and is readily ubiquitinated by Cullin
3 and degraded [208-210]. However, oxidative stress disrupts this interaction and leads to NRF2 accumulation. The accumulated NRF2 dimerizes, translocates within the nucleus and binds to ARE [209].

Additionally, NRF2 also associates with the mitochondrial membrane during proteasome inhibition due its association with PGAM5 [207]. Upon increased mitochondrial generated reactive oxygen species (ROS), indicating mitochondria damage/failure, activation of NRF2 occurs [210]. The resulting ROS from the mitochondria causes disassociation of NRF2 from the membrane and activation [209-201]. Activation of NRF2 via this mitochondrial pathway also results in increased transcription of heat shock proteins as well as proteasomal subunits [211]. It has been reported that increased age seems to decrease this NRF2 mitochondrial associated pathway, and as a result, less proteasomal clearance occurs along with reduced levels of heat-shock proteins transcription [207]. Due to age being the highest correlating risk factor with idiopathic PD [11], it has been implicated that this process may contribute to PD symptom exacerbation [207].

**Crosstalk between the Proteasome and Autophagy.**

In addition, to the previously talked about activation pathway of NRF1, it has been shown that mTOR1, through post-translational activation of SREBP1, is also able to positively affecting NRF1 activation [212]. As stated previously, mTOR1 is also important in the regulation of autophagy, and also important in the regulation of the proteasomal degradation, indicating that crosstalk among the two systems exists. In fact, a wide variety of evidence suggests that crosstalk between the proteasomal and autophagic pathway occurs and during increased proteasomal inhibition, quality control based autophagy will begin to compensate for the loss in activity [213-218].
Several studies have implied that both AMPK and mTOR1 have indirect mechanisms of recognizing when reduced proteasomal activity is occurring [213-214]. Additionally, it has been shown that when inhibition of the 26S proteasome occurred, an increase in AMPK and autophagic activity was observed within a variety of cell-lines [214-216]. Furthermore, within hippocampal neurons, the dysregulation of the 26S proteasome results in decreased activity of glycogen synthase kinase-3β (GSK-3β) [215]. This decreased activity in turn leads to an increase in the activation of AMPK thus resulting in upregulating autophagic activity [215]. Interestingly, this pathway within these hippocampal neurons also seems to decline with age [215].

Another example of the crosstalk between proteasome and autophagic degradative pathways is the proteasomal degradation of ULK1 which moderates the activation of autophagy as part of the ULK complex [217]. Further examples include two specific processes that are mediated by p62. p62 mediates the autophagic degradation of KEAP1, the protein response for the inactivation of NRF2 [218]. Additionally, it has been shown that degradation of LC3, which normally occurs via proteolysis, can be inhibited by the binding of p62 to LC3 resulting indirectly in contributing to increased autophagic activity [218]. Further evidence for crosstalk, has been demonstrated by proteasomal inhibition via small molecules such as MG 132 which have been demonstrated to increase autophagic activity [220-221]. Interestingly, MG 132 treatment is also linked to the secretion of α-syn [9].

**MG 132.** Carbobenzoxy-Leu-Leu-leucinal or MG 132 is an aldehyde peptide that causes inhibition of chymotrypsin-like activity and therefore, results in blockage of the proteolytic activity of the 26S proteasome [219]. While MG 132 directly inhibits the proteasome it also linked to increases in autophagic activity [221]. Lan et al., 2004, found that when PC-12 cells expressing A53T α-syn were treated with MG 132 and lysotracker, a proprietary fluorescent dye that accumulates within acidic compartments, an increase in lysotracker distinct positive puncta
occurred [221]. Additionally, the treatment of MG 132 was shown to result in an increase level LC3-II within the same PC-12 cells [221]. Furthermore, it has been reported that MG 132 treatment also results in an increase in α-syn accumulation [220-221]. These results indicate that MG 132 treatment leads to an increase of autophagosome formation, likely as a consequence of MG 132 inhibition of UPS, while also implicating the UPS in removal of α-syn based on its accumulation.

In addition to MG 132’s ability to increase α-syn accumulation, the treatment of MG 132 has been shown to increase α-syn secretion [222]. A study looking at α-syn secretion as result of chemical stressors found that MG 132 increased α-syn secretion by seven fold over control [222]. Additionally, the exosomal fraction of cultured medium from the MG 132 cells indicated that a 2-fold increase in vesicular α-syn occurred [222]. Although the mechanism for how α-syn secretion is occurring has not been identified, it is possible that the inhibition of the 26S proteasome by MG 132 is causing an increase in autophagic based secretion of α-syn. However, more information is necessary before one can make this conclusion.

While studies conducted on proteasome inhibition due MG 132 treatment link proteasome function to autophagy, as well as, α-syn accumulation and secretion. Mitochondrial degradation and turnover is another pathway directly showing a link between the proteasome and autophagy. MG 132 treatment has been shown to increase ROS levels within the cell and the depletion of GSH, indicative of mitochondrial dysfunction [219]. Furthermore, genetic mutations directly associated with mitochondrial turnover, such as PINK1 and Parkin, also tie cooperative degradation of the proteasome and autophagy to PD pathology [222-223].

**Proteasome’s role in Mitochondrial Regulation and Degradation.**

The regulation of mitochondria integrity is an example in which both the UPS and autophagic pathway play an orchestrated role in maintaining mitochondrial balance and
subsequently the management of mitochondrial related ROS. However, mitochondria dysfunction and subsequent ROS generation has been shown to play a role in a variety of neurodegenerative diseases including synucleinopathies [223]. Dysregulation of either of the autophagic or UPS degradative pathways is associated with decreased clearance of mitochondria resulting in further damaging ROS exposure and exacerbate general cellular dysfunction [223].

Mitochondria are essential organelles within mammalian cells and they participate in a variety of functions including high efficiency energy production via oxidation [224]. Under normal conditions, the mitochondria, and its subsequent systems associated with maintaining oxidative stress, are equipped to deal with this oxidation [224]. However, upon damage or dysregulation of the mitochondria, the management of oxidation becomes unregulated resulting in the mitochondria becoming a large source of ROS for the cell [225-227]. Depending on the level of dysfunction or damage, mitochondria can provoke a few different interconnected responses. Among these responses are the adaptive stress response and mitophagy [224-227]. The first-line of response upon mitochondrial dysfunction is the recruitment of the 26S proteasome, tasked with the responsibility of degrading the damaged outer membrane proteins of the mitochondria [229]. If the removal of the damaged outer membrane does not rescue the mitochondrial function, the mitochondria will be targeted for degradation via mitophagy, a specialized form of quality control based autophagy [229]. Mitophagy is occurs via one of two pathways, the receptor mediated pathway or the ubiquitin dependent pathway [230-231]. Of these two pathways, the ubiquitin dependent pathway is particularly relevant to PD pathology as it is regulated by both PINK1 and Parkin, in which mutations in both PINK1 and Parkin are associated with familial PD [231]. This ubiquitin dependent pathway occurs upon depolarization of the mitochondrial membrane resulting in PINK1 activation [231-232]. PINK1 then functions to
recruit the E3 ubiquitin ligase Parkin [231-232]. Parkin activation results in increased poly-ubiquitination of multiple outer-membrane mitochondrial proteins [231-232]. The poly-ubiquitination allows for the recruitment of the ubiquitin mediators of quality control based autophagy such as NDP52, OPTN, p62/SQSTM1, and NBR1 [232]. Additionally, it has been shown that mitophagy is also dependent on the proteasome, as Parkin been shown to also associate with the UPS via the ubiquitin receptor, Rpn-13. Furthermore, the deletion of Rpn-13 was shown to slowdown the turnover of mitochondrial clearance time [233].

**Rotenone.** In addition to genetic mutations in PINK1 and Parkin being linked to PD and mitochondrial dysfunction, the pesticide rotenone which causes mitochondrial dysregulation, has been shown to cause PD pathology. Rotenone was first linked to PD after a paper published results showing increased α-syn aggregation within the dopaminergic neurons of the substantia nigra by Betarbet et al., 2000 [234]. Rotenone, as the name implies, is a naturally forming rotenoid, that is lipid soluble and upon treatment induces mitochondrial dysfunction as a result of its ability cause uncoupling of mitochondrial complex 1 by inhibiting NADH-coenzyme Q oxidoreductase [235]. This process is thought to increase mitochondrial ROS and decrease glutathione levels; a primary protective antioxidant within the cell [236].

Rotenone treated rats expressing human α-syn were shown to have exacerbated PD-like pathology including decreased motor function and large amounts of dopaminergic death within the substantia nigra [234, 237]. Additionally, the dopaminergic neurons from these rats were shown to have increased levels of the autophagic proteins LC3-II, Beclin-1, as well as, p62 a protein almost exclusive degraded via autophagy and therefore an indicator of general autophagic quality [234]. As a result, the study indicates that increased levels of both Beclin-1 and LC3-II indicates that rotenone treatment increases autophagy, while also causing increased in dysfunctional autophagosomal degradation as indicated due to increased p62 levels [234].
In addition to mitochondrial dysfunction and autophagic upregulation and dysfunction, rotenone treatment has been also been shown to decrease both the 20S and the 26S proteasome activity while not influencing their mRNA levels [238-239]. These results suggest that rotenone treatment also indirectly inhibits general proteasomal degradation and ubiquitin related proteasomal degradation albeit through an indirect mechanism that has yet to be elucidated [240].

**Conclusion**

What mechanisms are important in regulating the secretion of pathological α-syn? And what role does exosomal α-syn play in the subsequent transmission of disease pathology among cells are two among many questions that are important in understanding synucleinopathies, such as PD. The unconventional secretion of α-syn among autophagic mechanisms is a relatively new discovery and a lot still needs to be elucidated; including whether the secretion of misfolded α-syn is generally beneficial or detrimental due to its association with increased clearance of α-syn within cells despite its role in the spreading of the disease pathology among its neighboring cells. Additionally, how exosomes play a role in the transfer of α-syn to associated brain regions and how doe autophagic mechanisms influence this process are questions that are just beginning to be investigated.

The next chapters will focus on our lab’s research on unconventional secretion of α-syn through autophagic mechanisms followed by the characterization of extracellular vesicles that contain α-syn.
CHAPTER THREE:

MATERIALS AND METHODS

Autophagic Mechanisms influence the unconventional secretion of

Non-Monomeric Alpha-Synuclein

Cell Culture.

The SH-SY5Y human neuroblastoma cell-line and the HeLa immortalized cell-line were acquired from the American Type Culture Collection (ATCC). Cells were cultured in an incubator at 37°C and 5% CO2 in Dulbecco's modified Eagle's Medium (DMEM) containing phenol red (Invitrogen), supplemented with the addition of 10% fetal bovine serum (FBS) (Hyclone), 10 ug/ml ciprofloxacin hydrochloride, 100 IU/ml penicillin, and 100 ug/ml streptomycin.

Over-expression of Alpha-Synuclein Dual Split Protein Construct.

SH-SY5Y cells and HeLa cells were dually transduced to stably over-express both complements of our α-syn dual split protein construct (DSP) using two individual lentiviral viral vector (pLVX) containing either α-syn-DSP-A or α-syn-DSP-B, each driven by a CMV promoter. Lentivirus was generated for transduction in HEK 293T cells transfected using the transfection reagent PEI with equal parts concentration of VSV-g, ΔNRF or psPax2, and pLVX-CMV-Alpha-synuclein-DSP-A or pLVX-CMV-Alpha-synuclein-DSP-B plasmid. 48 hours post-transfection lentiviral particles were collected and purified from the 293T cells cultured media using a 0.45um Millipore syringe. The purified lenti-viral particles were then used to treat SH-SY5Y cells and spinoculated at 13°C for 2 hours at 1200 x g. The treated cells were then selected for those
positive for the resulting DSP constructs by supplementing the previously mention DMEM with 5 ug/ml puromycin. Cells were then sorted for GFP intensity by flow cytometry for further selection, to ensure the cells were dually expressing both complements of the α-syn DSP.

**CRISPR Knock-outs.**

The respective knock-outs/knock-downs in SH-SY5Y and HeLa cell lines were created using the LentiCRISPRv2 (Addgene plasmid 52961). The guide sequences used with the LentiCRISPRv2 were identified with the help of the CRISPR design tool (see [http://www.crispr.mit.edu](http://www.crispr.mit.edu)). Respective oligonucleotide guide sequences for ATG7: 5=-CACCGTTTGAAGATTGGCCTAGGGGG-3=, and Beclin-1: 5=-CACCGATCTGCGAGAGACACCATCC-3=, were annealed and then cloned into the Lenti-CRISPRv2 plasmid. Lentivirus was generated for transduction in HEK 293T cells transfected using the transfection reagent PEI with equal parts concentrations of VSV-g, ΔNRF or psPax2, and the respective LentiCRISPRv2 plasmid. 48 hours post-transfection lenti-viral particles were collected and purified from the 293T cells cultured media using a 0.45um Millipore syringe. The purified lenti-viral particles were then added to our stable SH-SY5Y cells or HeLa α-syn DSP A&B cells and spinoculated at 13°C for 2 hours at 1200 x g. The transduced SH-SY5Y or Hela cells were then selected under hygromycin (HyClone) and cell lysates were acquired for genomic knockout assessment via Western blot.

**Western blotting & Western Antibodies.**

Western blot cell lysates were acquired by lysing cells in NP-40 lysis buffer composed of 100mM Tris pH 8.0, 1% NP-40, and 150 mM NaCl with the addition of the protease inhibitor cocktail (Roche) for 30 minutes on ice. Afterwards, the lysates were centrifuged for 10 minutes at 14,800g and the supernatant collected. Collected supernatants’ protein concentrations were measured by the Pierce BCA protein assay kit (Thermo Scientific) and protein was equally loaded
and ran on a 10% polyacrylamide gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

After separation, the proteins were then transferred to a nitrocellulose membrane (Bio-Rad).

The nitrocellulose membranes were treated with respective primary antibodies overnight; Rabbit ATG7 (Invitrogen #PA5-35203) 1:350; Beclin-1 (Cell Signaling #3738) 1:1000; Tubulin 1:200, diluted in powdered milk block solution 5g/50mL TBST. Primary antibodies were then washed and then subjected to their corresponding Horseradish Peroxidase (HRP) (Thermo Scientific) conjugated antibodies. HRP conjugated antibodies were then detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Chemiluminescence levels were analyzed by using the FlourchemE Imaging System (Protein Simple).

**Luciferase Plate reading and Drug Treatments.**

SH-SY5Y α-syn DSP A&B Cells or HeLa α-syn DSP A&B Cells were equally plated in 24-well at a density of 65,000-210,000 per well depending on the experiment. The cells were then treated with DMSO vehicle of .5uL/500ul, Rapamycin 100uM, Bafilomycin-A1 100 nM, MG 132 1mg/ml, Trehalose 100mM, 3-Methyladenine (5mM), or Rotenone 1um.

The cell cultured medium was then collected 24 hours later and centrifuged at 4C for 5-10 minutes and 12,000g. Afterward, the supernatant was collected and plated in triplicate at 50ul per well, in a 96-well, for a luciferase assay via Veritas microplate luminometer (Turner Biosystems). The Veritas (version 1.9.2) software was used in tandem with Veritas microplate luminometer in which the standardized Renilla Luciferase reading protocol was utilized. Depending on the experiment 50-100ul of Coelenterazine (NanoLight) solution was added via machine administration
Imaging Based Growth Rate Assessment in Tandem with Luciferase Plate Reading.

After counting by hemocytometer, 60,000-75,000 cells were added to each well of a 24-well plate containing fibronectin (sigma) treated coverslips and allowed to adhere for 4 hours in 500ul of DMEM cultured medium. After 4 hours, the cultured medium was replaced with DMEM supplemented with either DMSO .5ul/500ul or bafilomycin-A1 100mM. The cultured medium was collected 24 hours later for Luciferase Assay and was subjected to the same methodology as described previously. Simultaneously, coverslips were collected and treated with fixative solution immediately before treatment and immediately after the 24hrs cultured medium was collected. The fixative solution was composed of 3.7% formaldehyde (Polysciences) and .1 M PIPES buffer solution for 5 minutes. The coverslips were stained with Dapi in PBS for 20 minutes and were imaged by the on a DeltaVision wide field fluorescent microscope (Applied Precision, GE) outfitted with a digital camera (CoolSNAP HQ; Photometrics), while using a 1.4 numerical aperture, and 60x objective lens. After their acquisition, the images were stitched together and deconvolved using the SoftWoRx deconvolution software (Applied Precision). The final stitched and deconvolved images were then analyzed on Bitplane: Imaris software version 7.6.4

Statistical Analysis.

All statistical analyses were done and graphs were generated using GraphPad Prism version 5.00 or 6.00 (GraphPad Software, Inc.). All data shown was analyzed using two-way ANOVA with Tukey’s post-hoc test and depicts the standard error of the mean unless otherwise specified.
The Novel Characterization of Preformed Fibrillar Alpha-Synuclein’s Association with Extracellular Vesicles on a Single Vesicle Level

Cell Culture.

The SH-SY5Y human neuroblastoma cell-line was acquired from the American Type Culture Collection (ATCC). Cells were cultured in an incubator at 37°C and 5% CO2 in Dulbecco's modified Eagle's Medium (DMEM) containing phenol red (Invitrogen), supplemented with the addition of 10% fetal bovine serum (FBS) (Hyclone), 10 ug/ml ciprofloxacin hydrochloride, 100 IU/ml penicillin, and 100 ug/ml streptomycin.

Over-Expression of S15 mCherry Construct.

For extracellular vesicle visualization experiments, SH-SY5Y cells were transduced to stably over-express S15-mCherry using a lentiviral viral vector (pLVX) containing our S15-mCherry construct and driven by a CMV promoter. Lentivirus was generated for transduction in HEK 293T cells transfected using the transfection reagent PEI with equal parts concentration of VSV-g, ΔNRF or psPax2, and pLVX-CMV-S15-mCherry plasmid. 48 hours post-transfection lentiviral particles were collected and purified from the 293T cells cultured media using a 0.45um Millipore syringe. The purified lenti-viral particles were then used to treat SH-SY5Y cells and spinoculated at 13°C for 2 hours at 1200 x g. The treated SH-SY5Y cells were then selected for those positive for the resulting S15 mCherry by treating supplementing the previously mention DMEM with 5 ug/ml puromycin (Hyclone).

Alpha-Synuclein Aggregation and Labeling.

For the extracellular vesicle visualization experiments, recombinant α-syn aggregates were created via a constant shaking of purified α-syn monomers on a table-top shaker at 200
rpm (Benchmark: incu-shaker mini) for 7-10 days. The purified α-syn monomers were shaken at a concentration of 5 mg/ml and a constant temperature of 37°C in 20 mM Tris-Cl, pH 7.4. After the 7 days, A-syn aggregate were labeled with DyLight™ 488 NHS-ester fluorophores (ThermoFisher). Prior to α-syn treatment, α-syn fibrils were sonicated for 30 seconds. Sonicated fibrils of α-syn were confirmed by EM (see Effects of Serine 129 Phosphorylation on A-Synuclein Aggregation, Membrane Association, and Internalization).

**Extracellular Vesicle Collection.**

S15 mCherry SH-SY5Y Cells were plated in equal amounts at ~40% confluence within a 60mm tissue culture dish and were left untreated or treated with labeled sonicated α-syn aggregates for 24 hours in DMEM at a concentration of 100nM. Afterwards, the cell cultured medium was removed and the cells were gently washed. Fresh medium was added and collected 72 hours later; the cells were given fresh medium and cultured medium was recollected again 48 hours after the last medium change.

**Immunofluorescent staining and Preparation for Extracellular Vesicles.**

For the extracellular vesicle visualization experiments, 1mL of cultured medium was spinoculated at 13°C for 2 hours at 1200 x g onto glass coverslips and subsequently fixed with a solution of 0.1 M PIPES with 3.7% formaldehyde (Polysciences) for 5 minutes. Immediately after fixation, cells were incubated with one of four possible primary antibodies, mouse anti-CD9 (BD Pharmigen #555370), mouse anti-CD63 (BD Pharmigen #5556019), mouse anti-CD81 (BD Pharmigen #555675), or rabbit anti-Lamp1 antibodies (Abcam #24170), in a PBS block solution supplemented with 10% normal donkey serum (NDS), and 0.01% NaN3 for 1 hours. The extracellular vesicles were then subjected to their respective secondary donkey antibody conjugated to Alexa fluorophore 647; anti-mouse 647-conjugated or donkey anti-rabbit 647-
conjugated secondary antibody at a concentration of 1:400, for 20 minutes diluted in the same PBS block solution supplemented with 10% (NDS), and 0.01% NaN3.

**Wide-field Fluorescence Deconvolution Microscopy and Analysis.**

Extracellular vesicle images were taken on a DeltaVision wide field fluorescent microscope (Applied Precision, GE) outfitted with a digital camera (CoolSNAP HQ; Photometrics), while using a 1.4 numerical aperture, and 60× objective lens. 25-30 images were taken from different locations on the cover-slip to create a representative population. The resulting collected images were deconvolved after their acquisition with the SoftWoRx deconvolution software (Applied Precision). The deconvolved images were then analyzed on Bitplane: Imaris software version 7.6.4, where the spots algorithm was built around either the S15 signal or α-syn signal and the maximum fluorescence intensity found within these spots was then analyzed. All acquired images were subjected to the same spots signal algorithm via the Batch Coordinator tool (Bitplane) to each respective signal. Images with statistical outlying levels of signal were subjected to individual analysis and thrown out if deemed non-representative.

**Statistical Analysis.**

All statistical analyses were done using Microsoft Excel while the graphs were created using GraphPad Prism version 5.00 or 6.00 (GraphPad Software, Inc.).
CHAPTER FOUR:

AUTOPHAGIC MECHANISMS INFLUENCE THE UNCONVENTIONAL SECRETION OF NON-MONOMERIC ALPHA-SYNUCLEIN

Introduction: Verification of Methodology

Autophagy is one of the primary degradative pathways within the cell. However, in the past decade, autophagy’s role has expanded past the scope of purely degradation and has been linked to the unconventional secretion of several proteins [9, 92, 94, 124, 173]. Furthermore, dysregulation or modulation of autophagic activity can further influence the secretion of these proteins. Among these proteins are IL-1β and the neurodegenerative associated proteins α-syn and Aβ.

Several studies have shown that chemical dysregulation of autophagic mechanisms influenced the secretion of α-syn. In one specific example by Ejlerskov P et al. 2013, when PC12 cells over-expressing α-syn were treated with the autophagic initiators rapamycin or trehalose, an increase in α-syn secretion was observed [9]. Additionally, they found that when cells over-expressing both A30P α-syn and p25α, a protein important in the polymerization of microtubules and anterograde microtubule trafficking, were treated with the autophagic inhibitor 3-MA it resulted in a decrease in α-syn secretion [9]. These results were recapitulated when they knocking down the essential autophagic protein, ATG5 by siRNA within PC12 cells over-expressing the familial mutant variant of α-syn A30P, which also resulted in a decreased secretion of α-syn [9]. Therefore, based on these observations, increasing autophagic activity
positively increased α-syn secretion while decreasing autophagic activity by inhibiting the autophagophore formation and elongation decreases α-syn secretion.

Seemingly in contrast to the previously stated conclusion, several studies have found that the autophagic inhibitor, bafilomycin-A1 (BAF-A1), was also able to positively influence α-syn secretion. However, bafilomycin-A1’s mechanism of action varies from 3-MA’s mechanism of action or the mechanism associated with the depletion of ATG5, in that bafilomycin-A1 is a late phase inhibitor of autophagy. Rather influencing the autophagophore formation or elongation, bafilomycin-A1 inhibits the acidification and fusion of the lysosome with the autophagosome [189-190]. As a result, bafilomycin-A1 treatment increases the accumulation of autophagosomes [9]. Therefore, it has been speculated that simply increasing autophagic activity is not the only driving mechanism behind autophagic induced α-syn secretion. Rather, increased autophagic activity, as well as, the dysregulation of the degradative process also also influences α-syn’s secretion.

Therefore, we hypothesized that if autophagophore formation is an essential component to α-syn secretion then inhibition of its formation would result in decreased secretion of our α-syn construct. However, if autophagy is inhibited by preventing the fusion of the autophagosome with the lysosome, then an increase in α-syn secretion will still occur due to the cell’s attempt to clear the accumulating undegraded cargo within the autophagosomes. Additionally, if we increase autophagic activity then we should see an increase in our constructs secretion based on the results observed by others [9]. To test these hypotheses, we created a SH-SY5Y cell-line with α-syn tethered to both halves of our dual-split protein construct (DSP). By creating our SH-SY5Y α-syn DSP A&B cell-line that expresses α-syn with halves of our GFP-renilla luciferase dual-split protein construct we our able to measure α-syn that corresponds closer to
the pathogenic state as the enzymatic activity of measured luciferase must be non-monomeric. This is one of the first examples of this model being used to measure α-syn secretion.

**Experimental Design: Verification of Dual-Split Protein α-syn Construct**

**Luciferase Plate reading and Drug Treatments.**

SH-SY5Y α-syn DSP A&B Cells Cells were equally plated in 24-well at a density of 65,000-210,000 per well depending on the experiment. The cells were then treated with DMSO vehicle of .5uL/500ul, Rapamycin 100uM, Bafilomycin-A1 100 nM, Trehalose 100mM, 3-Methyladenine (5mM). All cells were cultured in a total of 500ul of DMEM for 24hours regardless of treatment condition. The cell cultured medium was then collected 24 hours later and centrifuged at 4C for 5-10 minutes and 12,000g. Afterward, the supernatant was collected and plated in triplicate at 50ul per well, in a 96-well, for a luciferase assay. Depending on the experiment 50-100ul of Coelenterazine (NanoLight) solution was added via machine administration. Each experiment was repeated three separate times unless specified otherwise.

**Results: Verification of Methodology**

Before testing whether non-monomeric α-syn secretion was dependent on autophagic mechanisms we first created are SH-SY5Y α-syn DSPA&B cell-line by dually transducting WT SH-SY5Y cells with the aforementioned constructs. Cells were grown up under puromycin selection and then a homogenous population was selected via flow cytometry based on GFP intensity (Figure 1). By selecting the DSP cells above a specific GFP intensity, we hoped to rule out the possibility of singly transduced cells or cells with imbalanced transduction levels of each of the respective α-syn DSP compliments.
After the selection process, cells were allowed to grow for a few passages to insure stability among experiments. These DSP constructs were first created and published by the Matsuda lab and utilize corresponding halves of a renilla luciferase and green fluorescent protein (GFP). Individually each half is non-functional, however upon complementation, the complementing halves come together and the renilla luciferase and GFP regain function.

**Figure 1. Cartoon representation of how the α-syn DSP A&B construct functions and the selection of the SH-SY5Y α-syn DSP A&B cell-line.** A) Cartoon indicating how the α-syn dual-split protein constructs A and B work. Individually neither construct A nor B functions. However, upon complementation, the construct regains GFP and Renilla Luciferase activity. B) Flow cytometry for the selection of GFP positive cells of high intensity. The selection was conducted to select for a homogenous cell population in which cells transduced both DSP constructs A and B in relative amounts to get the desired intensity.

By tethering each respective, corresponding half of the DSP construct to α-syn and over-expressing both complements within the same cell, we are able to measure DSP complementation by either the renilla luciferase activity or visualize the complementation via the fluorescence of the GFP (example shown in Figure 5). Increases in measurements indicates that the increased complementation of dual-split protein α-syn counterparts are occurring which one would expect to find within aggregated, pathological α-syn oligomers; in contrast to non-pathological α-syn which is thought to be found primarily in its monomeric form, as stated
early within the review of the literature. Therefore, this DSP α-syn model should more accurately represent pathogenic α-syn in contrast to simply a luciferase-α-syn construct.

To verify our DSP model was functioning the way we intended, we first treated with known chemical activators of autophagy, rapamycin and trehalose, as well as, the known chemical inhibitors of autophagy, 3-methyladenine and bafilomycin-A1, in which other studies have shown influence the secretion of α-syn [9]. Cells were exposed to each of the respective conditions for 24 hours before having their cell culture medium collected and then plated in triplicate for a luciferase assay. Based on previous findings, we would expect that autophagic activators rapamycin, trehalose, as well as, the autophagic inhibitor bafilomycin-A1 should increase complemented α-syn DSP secretion; while in contrast, 3-MA treatment should decrease complemented DSP α-syn secretion, if our model is functioning properly.

Similar to results found by previous studies, we found that when our SH-SY5Y α-syn DSPA&B cells were treated with the known activators of autophagy, rapamycin and trehalose, we saw a significant fold increase, which corresponded to roughly 3x amount of complemented DSP secretion when compared to the DMSO vehicle control (Figure 2). In addition, we found that when our cells were treated with the known late phase autophagic inhibitor bafilomycin-A1 we also saw a significant increase of roughly 3x fold in complemented DSP secretion while in contrast, when cells were treated with autophagic inhibitor, 3-methyladenine, which inhibits autophagy by directly inhibiting the autophagophore specific class III PI3K, we did not see any significant change in our complemented DSP signal (Figure 2). These results indicate that our DSP-α-syn model functions similarly to how we would have hypothesized and provides evidence verifying the authenticity of our model. Furthermore, it supports the idea that increased autophagophore formation is an important part of non-monomeric α-syn secretion.
Figure 2. Autophagic activators and inhibitors influence DSP α-syn secretion. SH-SY5Y cells expressing both α-syn DSP A&B constructs were either treated with DMSO (.5μL/500μL), 3-MA (5mM), Bafilomycin-A1 (100 nM), Trehalose (100mM), or Rapamycin (.1mM) for 24 hours. The cell culture medium was then collected and plated in a 96 well plate in triplicate for a renilla luciferase reading was taken with the automated addition of 66μL of coelenterazine substrate. Each treatment condition was replicated 3 separate times. Data was analyzed by two-way ANOVA with Tukey’s post-Hoc test. Error bars depict standard error of the mean.

Introduction: ATG7 and Beclin-1 Knock-Down

Next, we investigated how CRISPR-CAS9 knock-down of the essential autophagic proteins, ATG7 and Beclin-1, influenced the secretion of our complemented α-syn DSP construct. ATG7 is an essential autophagic protein that plays a role in both the formation and the elongation of the autophagophore [103]. ATG7 acts as an E1 ubiquitin like protein and plays a role in forming of the primary ATG complexes, as well as, part of the core ATG proteins essential to LC3 conjugation [103]. In the ATG-LC3 conjugation system, ATG7 initiates the
shuttling of LC3-I to the autophagophore, as well as, later in the elongation process shuttling GABARAP [83]. Similarly, ATG7 also shuttles ATG12 to ATG10 thereby assisting in forming the ATG12-5-16 complex, that converts LC3-I into the active LC3-II [103, 123-126]. Without LC3, an autophagophore cannot go through elongation [103, 123-126].

Based on our previous findings reported by others showing decreased α-syn secretion occurred when ATG5 was depleted by siRNA knock-down, we would expect, similarly, that decreased secretion of complemented DSP-α-syn should be observed [9].

While Beclin-1 is also essential to autophagy, it functions in distinct manner to promote the formation of the autophagophore [116-121]. Beclin-1 is enzymatically inactive but has several binding domains, including a domain that allows it to bind to the class III PI3K (VPS34) which allows it to recruit the other proteins that are essential to the formation of the Beclin-1 complex [118]. Therefore, by knocking-out Beclin-1 we would expect similar results to those observed when cells are treated with the class III PI3K inhibitor, 3-MA; decreased secretion of complemented α-syn.

**Experimental Design: ATG7 and Beclin-1 knock-down**

SH-SY5Y α-syn DSP A&B Cells or HeLa α-syn DSP A&B Cells were equally plated in 24-well at a density of 65,000-210,000 per well depending on the experiment. The cells were then treated with DMSO vehicle of .5uL/500ul or Bafilomycin-A1 100 nM, Trehalose 100mM. All cells were cultured in a total of 500ul of DMEM for 24hours regardless of treatment condition. The cell cultured medium was then collected 24 hours later and centrifuged at 4C for 5-10 minutes and 12,000g. Afterward, the supernatant was collected and plated in triplicate at 50ul per well, in a 96-well, for a luciferase assay. Depending on the experiment 50-100ul of Coelenterazine
(NanoLight) solution was added via machine administration. Each experimental condition was repeated 3-7 separate times.

**Results: ATG7 and Beclin-1 Knock-Down**

To investigate whether depletion of ATG7 influenced the secretion of our α-syn DSP construct we used CRISPR-CAS9 technology in an attempt to knock-out the ATG7 gene. We tried to knock-out ATG7 within our SH-SY5Y α-syn DSP cell-line several times with a couple different guide sequences; each time resulted in seemingly lethal knock-out and we were unable to successfully introduce the knock-out into our stable α-syn DSP cell-line. As results, we moved to knocking out ATG7 within our HeLa α-syn DSP cell-line. We were able to successfully achieve a stable heterogeneously knock-down of ATG7 within a population of HeLa α-syn DSP cell-line as indicated by our western blot (Figure 3, A).

To investigate whether α-syn secretion was influenced as a result of our ATG7 knock-down we treated control HeLa cells and the ATG7 knock-down cells with either DMSO vehicle control or bafilomycin-A1, allowing us to control for any possible changes in growth rate, and differences in cell number between replicates of the two cell-lines. We choose bafilomycin-A1 among our drugs for two reasons, it is the best documented to induce α-syn secretion and it has fewer off-target effects when compared to autophagic initiators trehalose and rapamycin. We found that when we normalized our bafilomycin-A1 results to their respective cell-line controls that a significant decrease in the fold change between the WT HeLa α-syn DSP cells was observed compared the ATG7 knock-down (Figure 2B).
Figure 3. HeLa ATG7 KO and WT DSP α-syn secretion: bafilomycin-A1 treatment. A) Depicts the Western Blot confirming the knock-down of ATG7 within our HeLa cell-line that also has stailble expression of both our α-syn DSP A&B construct. B) Shows the relative fold difference in complemented α-syn DSP A&B secretion among our WT HeLa α-syn DSP A&B cells and ATG7 KO HeLa α-syn DSP A&B. Each condition shows the relative fold change among the DMSO to bafilomycin-A1 treatment when compared to WT or ATG7 knock-down, respectively. Each treatment was replicated 8 times, where each replicate was plated in triplicate in a 96-well and the automated injection of 50-100ul coelenterazine was added with respect to the replicate. Data was analyzed by two-way ANOVA with Tukey’s post-Hoc test. Error bars depict standard error of the mean.

In contrast to our attempts to knock-out ATG7, we were able to successfully and robustly knock-down Beclin-1 via CRISPR-CAS9 within our SH-SY5Y α-syn DSP cell-line and produce a stable population as confirmed by our western blot (Figure 3 A). Similar to our HeLa knock-out cell line, we plated equal amounts of our WT SH-SY5Y α-syn DSP cell-line and our Beclin-1 SH-SY5Y α-syn DSP cell-line and either treated the cells with DMSO vehicle or bafilomycin-A1. We normalized the secretion relative light intensity values of the bafilomycin-A1 to DMSO vehicle rule out the possibility of changes in growth experience between the 24 hour incubation periods with the respective treatments between the two cell-lines. We found that there was a significant decrease in complemented α-syn DSP secretion between our Beclin-1 knock-down variant and our WT α-syn DSP cell-lines when treated with bafilomycin-A1.
Therefore, both Beclin-1 and ATG7 appear to be important for the secretion of α-syn, in concurrence with Ejlerskov P et al., 2013, ATG5 observations indicating decreased α-syn secretion.

Figure 4. SH-SY5Y Beclin-1 KO and WT DSP α-syn secretion: bafilomycin-A1 treatment. A) Depicts the Western Blot confirming the knock-down of Beclin-1 within our SH-SY5Y cell-line that also has stable expression of both our α-syn DSP A&B construct. B) Shows the relative fold difference in complemented α-syn DSP A&B secretion among our WT SH-SY5Y α-syn DSP A&B cells and Beclin-1 KO SH-SY5Y α-syn DSP A&B. Each condition shows the relative fold change among the DMSO to bafilomycin-A1 treatment when compared to WT or Beclin-1 knock-down, respectively. Each treatment was replicated 8 times, where each replicate was plated in triplicate in a 96-well and 50-100ul of coelenterazine was added with respect to the replicate. Data was analyzed by two-way ANOVA with Tukey’s post-Hoc test. Error bars depict standard error of the mean.

Next, we investigated the differences in α-syn secretion while controlling for growth rate over a 24 hour period among our respective ATG7 knock-down HeLa α-syn DSP cell-line and its WT HeLa α-syn DSP cell-line counterpart, as well as, our Beclin-1 knock-down SH-SY5Y α-syn DSP cell-line and its WT SH-SY5Y α-syn DSP cell-line counterpart. The purpose of this experiment
was to show the relative light intensity results after controlling for growth rate. This allows for the accurate comparison among the DMSO condition between respective WT and KO cell-lines. Each cell type was counted and 60,000 of both HeLa cell variants and 75,000 of both SH-SY5Y cell variants were plated onto fibronectin coated cover-slips within a 24 well plate. Cells were allowed to sit down for 4 hours and were then collected as time 0hrs or had their medium changed and treated with DMSO or bafilomycin-A1. 24 hours later the medium was collected for luciferase assay and the cells were fixed and stained for their nuclei via Dapi stain. The cells from untreated condition at both 0 hours and the 24hrs DMSO treated were then imaged via fluorescent microscopy among the respective and WT and knock-downs. Three separate stitched images were taken that resulted in the formation of a 5x5 panel of images, spanning a total of 550um in each direction. The images were deconvolved and the nuclei were counted at a constant Dapi brightness, with respective images being shown for each time point and cell variant (Figure 5). The number of nuclei for each stitched 5x5 panel was then average among all three images and each condition was replicated a second time.

We found no significant difference in growth rate among the cell-lines with respect to their knock-out and control. Additionally, the number of cells at the 24 hours mark was roughly comparable between the ATG7 knock-down HeLa α-syn DSP cell-line and its WT HeLa α-syn DSP cell-line counterpart, as well as, our Beclin-1 knock-down SH-SY5Y α-syn DSP cell-line and its WT SH-SY5Y α-syn DSP cell-line counterpart as one would expect.
Figure 5. Representative images of cell growth rate among WT and ATG7 KO HeLa α-syn DSP and WT and Beclin-1 KO SH-SY5Y α-syn DSP. The image shows representative stitched 5x5 image panel for untreated WT and ATG7 knock-down HeLa α-syn DSP cell-line and Beclin-1 knock-down SH-SY5Y α-syn DSP cell-line at both 0hrs and 24hrs. The total stitched distances is 550um in both the horizontal and vertical direction. Each cover-slip had 3 non-overlapping 5x5 image panels taken and the nuclei was counted at min intensity of 300 and a max intensity of 1500. 2 replicate cover-slips for each cell was taken for each respective time point.
Figure 6. Quantification of cell growth rates and raw luciferase intensity of the secreted complemented α-syn DSP construct. A&B) Shown in A&B are the average number of nuclei among the respective untreated cells at 0 and 24 hours for the HeLa α-syn DSP A&B WT and ATG7 KO and the SH-SY5Y α-syn DSP A&B WT and Beclin-1 KO cell-lines. The graphs show the standard deviation of from the mean values of the 2 replicates. C&D) the graphs shown in C&D show the raw RLU intensity of collected culture medium from each respective cells and treatments that correspond with the 24 hour DMSO condition shown from graph A&B which indicated no significant difference in cell growth or cell number occurred between time points. The data shows the standard deviation of the two replicates in which each replicate was plated in triplicate within a 96-well plate and subjected to an automated injection of 100ul of coelenterazine substrate as part of the renilla luciferase assay. Data was analyzed by two-way ANOVA with Tukey's post-hoc test.

Additionally, the renilla luciferase readings among our untreated and bafilomycin-A1 treated, with respect to WT and knock-down, had relatively comparable RLU values when
compared to our normalized fold change values reported previously (Figure 3&4). However, the untreated between both our ATG7 and Beclin-1 knock-down cell-lines trended toward having a decreased basal level of complemented α-syn DSP secretion although they were not significant. Additionally, the Beclin-1 knock-down bafilomycin-A1 treatment lost its significance when compared to its respective WT Beclin-1 knock-down DMSO treatment. This is likely due to the fact that only two replicates (due to time restrictions) being conducted but illustrates the differences between the WT and Beclin-1 knock-down α-syn DSP cell-lines.

**Introduction: MG 132 and Rotenone Treatment**

The ubiquitin proteasome system (UPS) is another one of the primary cellular degradative pathways. In addition to autophagy, the UPS is also important in the removal α-syn. Studies show, that upon inhibition of the proteasome, an increase in misfolded α-syn accumulates intracellularly [220-221]. This accumulation of α-syn results exacerbation of parkinsonian pathology. Furthermore, inhibition of the UPS is sensed by autophagic pathways, resulting in inhibition of mTOR1 and increased activation of AMPK, both of which upregulate autophagy [213-216]. This upregulation of autophagy is thought to occur in an attempt to compensate for proteasomal inhibition, and within the context of PD pathology, helps increase degradation of α-syn accumulations. However, others studies have shown that pharmaceutical inhibition of the proteasome by MG 132 treatment also results in an increase in α-syn secretion within PC12, rat adrenal medulla cells, over-expressing α-syn mutant A53T [221-222]. Therefore, we decided to investigate whether MG 132 would influence α-syn DSP secretion within our system.

Finally, another drug we were interested in testing within our system is rotenone, a pesticide that has been associated with PD pathology [234]. Rotenone is primarily a
mitochondrial inhibitor; however, it has also been linked to indirect proteasomal inhibition by an unknown mechanism [234, 238-239]. Interestingly, the UPS system is important in the turnover of damaged mitochondria and its inhibition results in decreased clearance of damaged mitochondria [229]. Additionally, mitochondrial dysfunction and poor turnover of mitochondria results in an increase in ROS, which is thought to be a main contributor of PD pathology [223]. Therefore, we would expect that rotenone would increase α-syn secretion due to an upregulation in autophagy from increased dysfunctional mitochondria and aggregate α-syn.

**Experimental Design: MG 132 and Rotenone Treatment.**

SH-SY5Y α-syn DSP A&B Cells Cells were equally plated in 24-well at a density of 65,000-210,000 per well depending on the experiment. The cells were then treated with DMSO vehicle of .5μL/500μl, MG 132 .1mg/ml, or Rotenone 1μm, respectively. All cells were cultured in a total of 500μl of DMEM for 24 hours regardless of treatment condition. The cell cultured medium was then collected 24 hours later and centrifuged at 4C for 5-10 minutes and 12,000g. Afterward, the supernatant was collected and plated in triplicate at 50μl per well, in a 96-well, for a luciferase assay. Depending on the experiment 50-100μl of Coelenterazine (NanoLight) solution was added via machine administration. Each experiment was repeated three separate times unless specified otherwise.

**Results: MG 132 and Rotenone Treatment.**

To test whether rotenone or MG 132 influenced α-syn secretion, we used our SH-SY5Y α-syn DSP cell-line, we plate equal concentrations of cells and treated our cells with our DMSO vehicle control, MG 132, or rotenone. We hypothesized that if we treated our cells with drugs that influenced α-syn aggregation then we would see increases in α-syn DSP complementation and that it would result in increased secretion of aggregate α-syn.
Upon collecting and testing of the cell culture medium by luciferase assay from the various conditions, we found that MG 132 resulted in significant ~5x fold increases in α-syn secretion when compared to the DMSO control (Figure 7). Additionally, rotenone treatment also resulted in a significant fold increase of complemented α-syn secretion of ~7x fold when compared to the control. Furthermore, the rotenone treated cells secreted significantly more complemented α-syn DSP when compared to MG 132. Therefore, based on our observed results non-monomeric α-syn secretion increases as a result of proteasomal inhibition by MG 132 as well as when mitochondrial dysfunction and indirect proteasomal inhibition occurs via rotenone treatment.

Figure 7. Direct and indirect proteasome inhibitors influence the secretion of the complemented α-syn DSP construct. The graph depicts how the treatment of DMSO, MG 132, or Rotenone influenced the secretion of our complemented α-syn DSP construct from our SH-SY5Y α-syn DSP A&B cell-line. The graph shows the relative fold change intensity comparing each treatment to the DMSO control. Each treatment was replicated 3 times, where each replicate was plated in triplicate in a 96-well and the automated injection of 50ul coelenterazine was added. Data was analyzed by two-way ANOVA with Tukey’s post-Hoc test. Error bars depict standard error of the mean.
Discussion

The goal of our study was to investigate how small molecule activators and inhibitors of autophagy, genetic depletion of important autophagic proteins, ATG7 and Beclin-1, proteasomal inhibition, and mitochondrial dysfunction influence the complemented secretion of our novel α-syn DSP construct. By measuring the renilla luciferase enzymatic activity that only occurs upon DSP complementation, our goal was to more accurately model pathogenic α-syn secretion, associated with the oligomeric species α-syn. In addition to looking at non-monomeric α-syn, another strength associated with our DSP model is the bioluminogenic substrate of renilla luciferase, coelenterazine, has some level of cell-membrane penetrance. This property of coelenterazine is particularly important as α-syn oligomers have been implicated to preferentially be included within the extracellular vesicles, such as exosomes [8]. In addition, autophagic dysregulation likely induces the secretion of both vesicle associated and naked, non-vesicular species of α-syn. Therefore, by measuring the the renilla luciferase activity we are not unintentionally neglecting either naked or vesicular α-syn.

We began by validating our model and hypothesized that if autophagophore formation is essential to our complemented DSP α-syn secretion, then inhibition of its formation would result in decreased secretion of our complemented α-syn construct. However, if inhibition of autophagic degradation, while preservation of autophagosome formation occurs, then we should observe an increase in secretion of our α-syn construct due to the cells attempt to clear the accumulating undegraded cargo. Additionally, if we increase autophagic activity then we should see an increase in our constructs secretion due to α-syn being a natural target for autophagic secretion.
Therefore, if cells are treated with an autophagic activator, such as rapamycin or trehalose, or an autophagic inhibitor, such as 3-MA or bafilomycin-A1, as long as an increase in the formation or accumulation of autophagosomes due to inhibition of the degradation then we would expect an increase α-syn secretion to occur. Based on the results of Figure 2, we found that our hypothesis fit with our observations, when our cells were treated with autophagic activators rapamycin or trehalose, respectively. These results are particularly interesting as trehalose and rapamycin activate autophagy through different mechanisms [9].

Rapamycin causes an upregulation of autophagy through its inhibition of mTOR1 [151, 153-155]. Rapamycin is membrane-permeable and upon entering the cell binds to and inhibits FKB12 [153-155]. This rapamycin-FKB12 complex then goes on to bind to mTOR1, resulting in mTOR1 inhibition and subsequently an upregulation in starvation induced autophagic activity [153-155]. Additionally, recent studies have found that prolonged or high-dosage rapamycin treatment results in an inhibition of mTOR2 [158-160]. While very little is known about what cascades mTOR2 is responsible for regulating, it is thought that chaperone-based autophagy is among them [162]. Conflicting evidence exists for whether rapamycin is capable of inducing α-syn secretion. In our study we treated with a large amount of rapamycin, a possible explanation for why we saw such a profound increase in α-syn secretion may be due our large rapamycin dosage. Another possible explanation is that because our system monitors α-syn secretion within the context of non-monomeric variations, we are seeing increases in aggregated α-syn release which has been shown to be partially degradative resistant [244]. It seems plausible that when a cell is unable to properly degrade α-syn via autophagic mechanisms, the resulting autophagosome undergoes secretion instead. The results that we, and others, have observed indicate that bafilomycin-A1 treatment causes an increased in autophagic secretion of α-syn by causing an increased accumulation of autophagosomes. Furthermore, if increased α-syn
accumulation had begun to occur in a cell, autophagy may already be occurring, resulting in autophagic dysregulation and increased secretion.

In contrast to rapamycin, trehalose is thought to activate autophagy through a mechanism that is independent of mTOR1 [168]. Although the exact mechanism has not been identified, it is thought trehalose increases autophagy by somehow stimulating AMPK, which results in an increase of quality control autophagy rather than starvation induced autophagy [168]. In quality control autophagy specific targets, such as α-syn aggregates, are identified for degradation via post-translational ubiquitination linkages [148-150, 168, 170]. This quality control based activation of autophagy by trehalose explains why we see an increase in α-syn being targeted for autophagy and an increase in α-syn secretion. Moreover, others have seen that trehalose causes an increase α-syn secretion which is consistent with our findings [9].

In addition to our autophagic activators, we found that 3-MA, the early autophagic inhibitor, and bafilomycin-A1 our late phase autophagic inhibitor also met our hypothesized criteria; if autophagophore formation is an essential component to α-syn secretion then inhibition of its formation would result in decreased secretion of our α-syn construct. However, if inhibition of autophagic degradation, while preservation of autophagosome formation occurs, then we should observe an increase in secretion of our α-syn construct due to the cells attempt to clear the accumulating undegraded cargo.

Several studies have found that bafilomycin-A1 was able to induced increased levels of α-syn secretion [8-9, 49, 88]. While bafilomycin-A1 is an inhibitor of degradative autophagy, it does not alter autophagosome formation but rather indirectly inhibits autophagy through lysosomal dysregulation, as stated previously [189-190]. Therefore, it is been proposed that the reason that bafilomycin-A1 causes an increase α-syn secretion is due to increased accumulation
of autophagosomes that are unable to go through degradation [8]. This accumulation of impotent autophagosomes results in disruption of the normal degradative autophagosomal pathway and causes an increase autophagosomes going through the secretory pathway instead.

In contrast, secretion of α-syn is dependent on autophagosome formation and the treatment of 3-MA has been demonstrated to decrease α-syn secretion, as previously stated in our introduction, by Ejlerskov P et al., 2013. We found that when we treated our cells with 3-MA no observable change in α-syn secretion was observed. A possible explanation for the difference between our results and the results that Ejlerskov P et al., 2013, observed could be due to their co-transduction with P25α and mutant α-syn A30P while we used our α-syn DSP model. Additionally, the temporal differences of 3-MA treatment may also provide an explanation. However, our results support the idea that autophagic activation is important for the secretion of α-syn.

To follow up on our 3-MA data which indicates the importance of autophagophore formation, we next looked at how genetic depletion of ATG7 via CRISPR-CAS9 knock-out influenced secretion of our α-syn DSP construct. We saw a significant decrease in secretion of our complemented α-syn construct within the context of bafilomycin-A1 treatment, which indicates that autophagophore elongation is important for α-syn secretion. Additionally, are results are indirectly supported by Ejlerskov P et al., 2013, data which showed that siRNA knock-down of ATG5, substantially decreased α-syn secretion.

Furthermore, to expand on this previous stated idea, Aβ secretion has also been linked to autophagic regulation [99]. In support of the similarities between Aβ and α-syn secretion: In wild-type primary neurons, Aβ secretion increased when cells were treated with rapamycin; which corresponds with our observed results [99]. Additionally, it has been shown that Aβ
secretion decreases within wild-type primary neurons treated with the Beclin-1 complex autophagic inactivator, spautin-1 and increases with the treatment of rapamycin [99]. Furthermore, when a neuron specific ATG7 knock-out was introduced into Aβ mice, it was found that a decrease in Aβ secretion was observed [99]. These, same mice also had increased Aβ accumulation within their perinuclear regions [99].

Drawing on our ATG7 knock-out results indicating that proper formation and elongation of the autophagophore influenced complemented α-syn DSP secretion, we hypothesized that knocking out Beclin-1 by CRISPR-CAS9 would also result in decreased complemented α-syn DSP secretion. Concurrent with what we observed with our ATG7 knock-out cells, we found that when we treated our Beclin-1 knock-out cells with bafilomycin-A1 we saw a significant decrease in α-syn secretion when compared to our control, providing further evidence of autophagy’s role in α-syn secretion. As far as we know, this is the first demonstration of Beclin-1’s role being fundamental to influencing α-syn secretion but fits the expected results associated with Aβ and spautin-1 treatment.

In addition to directly modifying the autophagic mechanism via drug treatment or genetic depletion, we were also interested in investigating the role that proteasomal inhibition had on α-syn secretion. It is well documented that inhibition of UPS results in an upregulation of autophagic activity as compensatory mechanism [213-216]. For example, when the S26 proteasome is inhibited within hippocampal neurons a decrease in GSK-3β is observed [215]. Decreased GSK-3β activity results in an increase in AMPK which as previously mentioned, increases quality control autophagy [215]. Furthermore, it has been shown that the UPS serves to help alleviate the burden of accumulating α-syn aggregates. Our data suggests that an increase in complemented α-syn secretion occurred upon UPS inhibition as result of MG 132.
Although more data is necessary, a logical explanation for this observed increase may be due to an upregulation in autophagic activity in an attempting to clear the α-syn through autophagy due to inhibition of the UPS.

We also observed an increase in α-syn secretion when we treated our SH-SYSY α-syn DSP cells with the mitochondrial inhibitor and indirect proteasomal inhibitor of rotenone. As previously stated, proteasome inhibition decreases α-syn degradation and causes upregulation of autophagy [213-26]. However, the UPS has also been shown to be particularly important in the turnover of damaged mitochondria [233]. Inhibition of the proteasome also results indirectly in increasing cellular ROS due to damaged mitochondria accumulation which has been associated with PD pathology [229-231, 233]. Therefore, we believe that rotenone increases α-syn secretion via a combination of effects including its ability to cause proteasomal inhibition induced increase in autophagic activity and reduce mitochondrial turnover; as well in part due rotenone’s ability to cause inhibition of mitochondrial function leading to subsequent increases in cytoplasmic ROS generation. This perfect storm of problems likely also plays a part in α-syn aggregation which explains the very high fold increase in α-syn DSP secretion. These results are in agreement with those reported by Jang et al., 2010 [49].

Future Directions

Now that we have successfully verified our α-syn DSP construct cell model, a lot of opportunities exist due to the flexibility associated with the dual protein activity of the renilla luciferase and GFP construct. One option we are interested in pursuing is to investigate the differences associated with α-syn DSP puncta within our cell lines among the different autophagic treatment conditions. Our preliminary experiments indicate changes in DSP puncta being visualized within our cells upon drug treatment conditions, which can be further
augmented by the treatment of sonicated preformed fibrillar α-syn. Additionally, we are in the process of getting a true homogenous knock-out for our respective ATG7 and Beclin-1 cell-lines and further verification of our secretion results. Additionally, ideally we would be interested in doing these knock-outs within other more relevant cell-lines, such as primary neurons. Another opportunity to investigate is to look at the GFP within the secreted EVs by our DSP cell-line and investigate the changes specifically associated autophagic secretion upon induction by our small molecule treatments utilizing the methodology that will be explained in detail in the next chapter.
CHAPTER FIVE:

THE NOVEL CHARACTERIZATION OF PREFORMED FIBRILLIAR ALPHA-SYNUCLEIN’S ASSOCIATION WITH EXTRACELLULAR CELLULAR VESICLES ON A SINGLE VESICLE LEVEL

Introduction

Exosomes are one of the subtypes of the total extracellular vesicle (EV) population and range from 30nm to 150nm in size [55-58]. Exosome biogenesis occurs as part of the endo-lysosomal pathway, where exosomes were once the intraluminal vesicles (ILVs) within a multivesicular body (MVB) [55-57, 59]. Upon MVB fusion with the plasma membrane the ILVs undergo extracellular exocytosis where they are now considered exosomes [55-57]. Initially the exosomes released as part of MVB and plasma membrane fusion were thought to be the cells unwanted waste [55]. However, it is now recognized that exosomes serve an important physiological role in intercellular communication through the transference of specific cargoes. Among these cargoes are RNA, protein, and lipids which can have dramatic effects to influence the receipt cells [60-65]. As a result of the reorganization late endosomes undergo before they can begin to form ILVs and progress to MVBs, they become enriched with tetraspaninin proteins, a subfamily of proteins that organize membrane microdomains by forming clusters that interact with a large variety of cytosolic and transmembrane signaling proteins [55-56, 66]. After the enrichment process, cargo is sorted into ILVs by the ESCRT protein complexes. As a result the tetraspanins, which are broadly expressed among tissues, as well as, specific highly conserved ESCRT proteins are recognized as canonical exosomal markers [55-56].
In addition to exosomes serving a role in intercellular communication, they are also associated with disease propagation. One of the predominant hypotheses for cell-to-cell transmission of PD pathology is through the release and uptake of α-syn associated exosomes [8]. Tissue culture studies have shown that WT α-syn can be transmitted among cells in an exosome dependent capacity. In addition, α-syn transferred via exosomes is capable of inducing aggregation within the recipient cell [8]. Recent *in vivo* evidence found that pathological α-syn species could be purified from the exosomes from CSF of patients with both DLB and PD [59], thus indicating that exosomes play a role in the spread of synucleinopathy pathology.

Furthermore, it was demonstrated by Danzer et al., 2012, that transmission of exosome associated α-syn oligomers, the α-syn variant thought to be responsible for neurotoxicity, are taken up within the cell preferential and were more likely to induce cell death than naked, non-vesicular α-syn oligomers. Indicating that transmission of α-syn via exosomes is more neurotoxic and potentially more pathogenic.

While exosomes have been shown to play a role in the transmission of cargoes, a growing body of evidence also suggests that exosomes are not a homogenous population and that specific subpopulations exist among them. Therefore, the goal of our study was to develop a new methodology that allows for the characterization of EVs on a single EV level. To do this we formulated a novel methodology that utilizes wide-field deconvolution microscopy and immunofluorescent staining in tandem with imaging analysis software to identify individual EV contents through protein co-localization analysis. We then validate our methodology by investigating whether sonicated preformed fibrils (PFFs) of α-syn specifically associate one of our tested canonical exosomal markers: CD9, CD63, CD81, and LAMP1. As far as we know, these techniques are currently not utilized within the exosome field. Furthermore, this adaptation of
techniques represents a strong alternative to those currently used that rely on first isolating specific fractions of EVs via ultracentrifugation and then looking at total isolated EV proteins due to the intrinsic nature of the readily used western blot, ELISA, and immunoblot methodology.

**Experimental Design**

We created our stable mCherry S15 SY5Y cell-line by transducing wild type SH-SY5Y cells with S15 mCherry vector that resulted in the stable expression of the S15 mCherry construct within these cells; hence forth referred to simply as S15 SH-SY5Y cells. We plated equal concentrations of our S15 cells into two 60mm dishes at ~40% confluency. Cells were either left untreated or treated with Alexa 488 fluorescently stained α-syn (PFFs) at concentration of 100nm. 24 hours after PFF treatment, both conditions cultured medium was replaced with 4mL of fresh DMEM. 72 hours later (96 hours after the initial PFF treatment) the cultured medium was collected from both dishes and fresh DMEM was re-added to each condition. 48 hours later (144 hours after the initial α-syn treatment) the cultured medium was collected for the final time. The cultured media was then centrifuged at 1200g for 5 mins, and the supernatant was collected. The supernatant was then added to a 24 well plate at 1mL per well, and spinoculated via centrifuge at 1200g, 13C for 2 hours onto cover-slips. Afterwards, the spinoculated supernatant was aspirated off and the cover-slips were fixed in our formaldehyde fix solution. Cover-slips were then treated with one of the respective primary antibodies: no treatment, CD9, CD63, CD81, or LAMP1 for 1 hour. Afterwards, cover-slips were treated with their respective Alexa 647 conjugated secondary antibody for 20 minutes. Cover-slips were then imaged by wide-field microscopy with each condition being imaged 25-30 times. Afterwards, images were deconvolved and then analyzed by Imaris software from Bitplane. A spots algorithm was then created to analyze the either the S15 or the α-syn channel, respectively. The maximum
fluorescent intensity of the respective other channels were then plotted in GraphPad Prism version 6 as an X&Y scatter plot. The above background analyses used to find above background levels for each channel were done in Microsoft Excel.

**Results**

In order to use fluorescence microscopy, we created a stable SH-SYSY cell-line that expresses our S15 mCherry construct that labels plasma membranes with a mCherry fluorophore. This process occurs due to our constructs utilization of the first 15 amino acids of the proto-oncogene tyrosine-protein kinase Src (SRC) protein sequence followed by a mCherry fluorophore. These first 15 amino acids from SRC signal for, and undergo, a myristoylation reaction which results in the addition of a myristoyl (14-carbon) lipid which acts as a plasma membrane anchor at the N-terminal glycine (Figure 8.). Additionally, due to the large amount of arginines and lysines found within this 15 amino acid sequence, it is particularly positively charged resulting in an increased attraction to the negatively charged head groups of phospholipids increasing its propensity for plasma membrane association. Therefore, our S15 mCherry construct (From here on referred to simply as S15) effectively labels our cell’s plasma membranes with the mCherry fluorophore resulting in red plasma membranes. Furthermore, and most importantly, it is also readily secreted within extracellular vesicles (Reference image of EVs Figure 10).
Figure 8. A cartoon version of the S15 construct myristoylation. The image shows how the S15 mCherry construct undergoes the addition of the myristoyl lipid anchor utilizing the first 15 amino acids from the SRC protein.

Next to validate our methodology and assess whether our S15 SH-SY5Y cells were capable of taking up PFF α-syn and secreting it within exosomes, we treated our S15 cells with sonicated Alexa 488 labeled PFF α-syn (green). 24 hours after the treatment, the cell cultured medium was replaced. 72 hours after the replacement the cultured medium collected (96 hours since initial treatment), and was once again replaced. The subsequently cultured medium was collect a second time 48 hours after the second replacement (144 hours since initial treatment). Afterwards the medium was spinoculated onto cover-slips and stained for our markers of interest. As result of the S15 label we are able to readily determine whether our PFF α-syn is taken up and secreted within EVs due to the incorporation of the S15 construct, which serves as pan-EV marker (although it does not get incorporated into all EVs, it is readily incorporated). Therefore, the resulting vesicles can be identified by microscopy based on green and red fluorophore co-localization.
After PFF α-syn treatment, we collect the conditioned cultured medium and divided it into 1mL increments among cover-slips, followed by spinoculation, in preparation for imaging, as shown in Figure 9.

**Figure 9. Schematic of EV imaging methodology.** The image is a graphical representation of the methodology used to collect and spinoculate cultured medium onto the cover-slips in order to image secreted EVs. Cells are transduced with the S15 mCherry construct and are either left untreated or treated with FITC (Green) labeled PFF α-syn. 24 hours later the medium was changed. 72 hours after the medium change the cultured medium was collected and medium was replaced. 48 hours after the second medium change the cultured medium was collected again. The cultured medium was centrifuged at 1200g, for 5 minutes, at 13°C and supernatant was collected. The supernatant was then separated into 1mL intervals and spinoculated onto cover-slips at 1200g, for 2 hours, at 13°C. Afterwards respective cover-slips were fixed and then stained for LAMP1, CD9, CD63, or CD81 or 2° only with respect to the replicate. This was replicated a total of 3 separate times with two time points results being analyzed as part of each replicate.

The cover-slips were then stained respectively for CD9, CD63, or CD81, from the tetraspanin family, to identify if our PFF α-syn was found within the exosomal subpopulation of total EVs, as these proteins represent canonically accepted exosomal markers. Furthermore, we choose to also stain for the lysosomal associated membrane protein 1 (LAMP1 or CD107a), another transmembrane protein commonly associated with exosomes but also found within lysosomes.

After staining for the exosomal marker of choice, we imaged our cover-slips by wide-field deconvolution microscopy; a representative image is shown of our extracellular vesicles on a cover-slip (Figure 10).
**Figure 10. Representative example of triple co-localization.** The panel shows a representative example of triple co-localization of all three channels. Each image of the panel indicates its associated color channel and as well as a merge image of all the channels being displayed as one image. Triple co-localization events are denoted by the arrow and mark the location the in each separate image, as well as, the merge.

The resulting images taken by our microscope were then analyzed by the Imaris imaging software. Imaris allows for analysis of EVs by creating a spots algorithm around the immunofluorescent channel of interest through the use of creating a puncta specific mask which will be referred to as a spot. We began by building our spot’s algorithm around our S15 mCherry channel shown in Figure 11, depicted as a gray orb and our S15 mcherry construct is shown in red. The spots algorithm, which is specifically designed by Bitplane for analyzing puncta, allows one to look at the fluorescence intensity of all the fluorescent channels one uses within any specific spot. Therefore by building a spots algorithm around our S15 puncta, the red channel, we were then able to analyze the fluorescence intensity of our other fluorescent channels within each spot. IE, the fluorescence intensity of the green or far-red channel found within our S15 red spots.
Afterwards, we exported our S15 spots algorithm to the Imaris batch coordinator tool, where the algorithm we created was extrapolated to all of our images and subsequently all replicates. This process allows for an unbiased, high-throughput analysis of all our images to identify all spots based on our established criteria. Additionally, the batch coordinator gives us an organized numerical value (among other things) of the max fluorescence intensity of each fluorescent channels for every spot among all images. The max fluorescence intensity of our PFF α-syn and antibody stain was then plotted against one another on an X&Y scatter plot for co-localization analysis. Within the scatterplot each point represents an S15 positive spot; where the X-value is the green channel intensity, either representing background noise or PFF α-syn fluorescence intensity depending on the treatment condition; and where the Y-value is the respective antibody stained protein intensity. Additionally, the individual axes are shown at a log2 scale to account for microscopy bit rate, resulting in a less congested and more linear representation of their relationship (Figure 12, A-E).

The above background threshold, indicating that an S15 spot was positive for a respective channel, was formulated by taking the background levels of each channels control condition, α-syn PFF vs. untreated and respective antibody staining to the secondary only control, and then calculating the average and the standard deviation of the intensity value for each channel, respectively. The respective average was then added to 2x the standard deviation of the same respective channel’s control (control channel: μ + 2σ). IE the above background, indicating positive co-localization of α-syn, was established by identified the average plus 2x the standard deviation of the intensity of the green channel within the S15 spots of the untreated cells (μ + 2σ).
Figure 11. Representative image from a cover-slip of spot algorithm built around the S15 construct. The S15 mCherry construct is shown in red while the gray indicates where a spot is made around the S15 signal. The left image shows a S15 mCherry channel alone. The right image shows a merge of the S15 mCherry channel with the S15 Spots algorithm.

As evidence of our above background methodology, the corresponding S15 spots co-localization graph for the α-syn untreated condition, in which the cover-slip were stained with the secondary only antibody is shown in Figure 12, A, indicating that minimal positive co-localization, shown as percentage, occurs within its S15 spots, as one would expect.

Representative images of S15 co-localization graphs are provided for the PFF α-syn treatment condition, in which each graph shows co-localization for each respective antibody stains. The same process was done for the α-syn untreated condition (not shown). This process was repeated 3 times, in which each replicate represents 2 collections, resulting in a total of 6 co-localization analyses for each antibody stain among the untreated and PFF α-syn treated conditions (Except for LAMP1 staining which was replicated 3 times with 2 collections per 1 replicate and 1 collection for 2 replicates, resulting in 4 measurements of which 3 independent experimental replicates occurred).
Figure 12. Representative co-localization analysis scatter plot of S15 spots on an individual cover-slip and its respective antibody stain. The figure shows the co-localization analysis scatter plot for the third replicates 96-144 hr (second time point). The percentage shown in each quadrant indicates the amount of S15 spots that co-localize with the S15 and/or the antibody stain and PFF α-syn. Each quadrant percentage is shown adjacently. Quadrants consist of: positive for S15 and PFF α-syn (bottom right quadrant of any individual graph); positive for S15 and the respective antibody stain, indicated by the title (top left quadrant of any individual graph); Triple positive for S15, PFF α-syn, and the respective antibody stain (top right quadrant of any individual graph); or finally, S15 only (bottom left quadrant of any individual graph). Each stain was performed between 4-6 times on individual cover-slips with 20-25 images taken per cover-slip.
Figure 13. Summation each respective antibody stain’s S15 spots co-localization data for all of α-syn PFF treated cells. The figure shows summation of each antibody stain’s replicates and time points with respect to the quadrants identified in Figure 12. Each stain has 3 total data replicates with 2 time points being analyzed except for LAMP1 which has 3 replicates with only the third replicate having both time points. Error bars show the standard error of the mean.

The summation of these analyses are shown in Figure 13 where each of the quadrants are broken down into one of the four possibilities and the percentage of the total S15 positive spots for each treatment and each antibody stain are shown: S15 only (negative for α-syn and the respectively indicated antibody stain); S15 & α-syn positive (Positive for S15 and α-syn but negative for the respective antibody stain); S15 & Antibody positive (positive for S15 and the respective antibody stain but negative for α-syn); or Triple positive (S15 positive, α-syn positive, and positive for respective antibody stain).
Figure 14. Summation of S15 spots positive for the respective antibody stains among untreated and α-syn PFF cells secreted EVs. The figure shows summation of each antibody stain’s replicates and time points with respect to the S15 & Ab stain and the Triple positive quadrants of the S15 spots shown in Figure 12 & 13. Error bars show the standard error of the mean.

Based on our summations we found that our S15 construct co-localizes preferentially with CD81 and CD63 in both are untreated and PFF treated conditions while CD9 and LAMP1 co-localize substantially less. Interestingly, we found that the percent co-localization between our S15 positive spots resulted in similar levels of stain co-localization regardless of the α-syn treatment condition, as shown in Figure 14. Also, we found that S15 and α-syn co-localized at a low percentage among all our S15 spots regardless of stain, likely due to a low incorporation within EVs. However, when it was incorporated into S15 positive vesicle it was also seemingly always positive for CD81, and was likely positive for either CD63 or LAMP1, based comparing the percentages between the Figure 13, A-E.
**Figure 15. S15 spots identified by the Imaris spots algorithm per image.** The figure shows the average amount of S15 positive spots identified among all cover-slips. All time points from each cover slip regardless of stain were pooled. The average amount of S15 mCherry spots created per image from the supernatant from both α-syn treated and untreated S15 SY5Y cells are compared against one another. Student’s T-test was conducted, p < .001. The error bars show the standard error of the mean.

In addition to creating co-localization analyses, we were also able to assess the number of S15 spots per cover-slip among our treatment conditions. This allowed us to get a relative sample measurement of total EV’s being secreted among our untreated and α-syn PFF treatment conditions. We pooled images among replicates and then averaged the amount of S15 spots from each image among all antibody staining paradigms, to compare how α-syn PFF’s influenced total EV secretion. We found that there was a significantly greater amount of S15 spots in our α-syn PFF condition when compared to untreated (Figure 15).

Next, due to the low percent of α-syn PFFs observed within the S15 positive spots, we chose to build a new algorithm around the α-syn channel to better characterize what extracellular markers α-syn co-localizes with and verify that α-syn was being secreted in association with S15 positive EVs. To do this, we simply built a spots algorithm around α-syn and plotted the batched coordinator results in a similar fashion as the S15 positive spots (Figure 16).
However, the X-axis for the α-syn spots was replaced with S15 channel intensity. Representative co-localization graphs for the respective antibody stains are shown in Figure 16. The summation of all replicates is shown in Figure 17. The antibody staining above background threshold was already established by our S15 spot algorithm and the same value was used. The above background for the S15 was established via visual observation of images comparing areas where no puncta of any location was observed and by using our intensity threshold used within our S15 Spots algorithm.

As indicated in Figure 17, A-E, the majority of α-syn spots that were positive for S15 were also positive CD81 the largest amount of time among our antibody stains. In contrast, CD9 was negative for S15 positive, α-syn spots a large percentage of the time; While, the S15 positive, α-syn spots, were roughly found to be positive and negative for CD63 and LAMP1 in roughly equal ratios.

Interestingly, a percentage of α-syn is not positive for S15, which may indicate that PFF α-syn is not contained with an extracellular vesicle, was not taken up into cells, or is contained within an EV without the S15 construct. Regardless of the scenario, we found that the majority of our PFFs co-localized with our S15 construct at roughly ~65% (Secondary only, α-syn spots, S15 positive, Figure 17, A) indicating that PFFs may be preferentially associated with EVs.
Figure 16. Representative co-localization analysis scatter plot of α-syn PFF spots on individual cover slip and its respective antibody stain. The figure shows the co-localization analysis scatter plot for the third replicates 96-144hr (second time point). The percentage shown in each quadrant indicates the amount of α-syn PFF spots that co-localize with the α-syn PFF spots and/or the antibody stain and S15 mCherry. Each quadrant percentage is shown adjacently. Quadrants consist of: positive for S15 and PFF α-syn (bottom right quadrant of any individual graph); positive for α-syn PFF spots and the respective antibody stain, indicated by the title (top left quadrant of any individual graph); Triple positive for S15, PFF α-syn, and the respective antibody stain (top right quadrant of any individual graph); or finally, α-syn PFF spots only (bottom left quadrant of any individual graph). Each stain was performed between 4-6 times on individual cover-slips with 20-25 images taken per cover-slip.
Figure 17. Summation of α-syn PFF spots identified for each respective antibody stain’s co-localization data. The figure shows summation of each antibody stain’s replicates and time points with respect to the quadrants identified in Figure 12. Each stain has 3 total data replicates with 2 time points being analyzed except for LAMP1 which has 3 replicates with only the third replicate having both time points. Error bars show the standard error of the mean.

Discussion

The goal of study was to create a methodology for single EV analysis. As a part of our investigation of α-syn secretion we wanted to analyze which EVs PFF α-syn associates with in an attempt to better understand the processes associated with α-syn secretion. The implication being that secreted misfolded α-syn may be contained within specific EVs and the proteins associated within these EVs may better explain the mechanisms associated with its secretion.
Due to the majority of researchers utilizing techniques, such as Western Blot, ELISA or immunoblot, to analyze the entirety of the EV population in association with the vesicles containing α-syn, we developed methodology that allows us to assess the only vesicles that contain the immunofluorescent marker of our choice. This process allows for specific analysis of only the EVs we are interested in which is particularly important due a growing body of evidence suggesting that EVs are heterogeneous with specific vesicular subsets existing within the entirety of the total EV populations. By using deconvolution microscopy combined with imaging software, we are able to analyze vesicles on an individual basis without the assumption of all vesicles being the same.

As part of our validation process we found that our S15 construct, which we used as a pan-EV marker, was positively associated with CD81 a majority of the time and that CD63 was also found associated a little over 50% of the time. In contrast, CD9 and LAMP1 were found to be positively associated with S15 a much lower percentage of the time. We found that PFF α-syn treatment did not impact the percent S15 spots positive for our respective antibody stains (CD9, CD63, CD81, or LAMP1) when compared to our control untreated (Figure 14, A&B). However, an unexpected observation was made when the average number of S15 spots was quantified. The PFF α-syn treated had significantly more S15 positive spots, when compare to the untreated control (Figure 15), despite the stain distribution remaining constant between the untreated and PFF treatment condition. Additionally, when we built an α-syn PFF spots algorithm our results indicate that α-syn associated with our S15 construct ~65% of time, these results indicate that pathological α-syn, represented by our PFFs, are more highly associated with our EVs. Furthermore, our α-syn PFF spots showed that the majority of the EV markers we stained for were positive for both α-syn and S15 in roughly similar percentages as their positive S15 co-localization percentage (S15 spots, Antibody positive staining percent Figure 13, A-F).
Except for LAMP1, which was particularly interesting, as based on our results extracellular PFF α-syn is EV positive ~65% of time (based on our secondary only, S15 positive, α-syn spots, Figure 17, A). When these PFFs are associated with an EV, they are also positive for LAMP1 ~30% of the time (α-syn spots, triple co-localization, LAMP1 staining, Figure 17, E). However, LAMP1 positive vesicles only make up ~%20 of the total secreted EV population (as indicated by our S15 spots, positive for LAMP1, Figure 13, E & Figure 14). Therefore, when PFF α-syn is found within an EV, roughly 45% of the time it will be found within a known specific subpopulation of EVs that only represent 20% of the total population. Furthermore, this EV population is of known lysosomal origin. These conclusions support the canonically accepted idea that lysosomal dysfunction is associated with PD pathology.

**Future Directions**

The authentication of this methodology provides an opportunity to investigate the EVs released from any set of cells. Rather than using the S15 marker one could use a fluorescent dye that stains plasma membranes if one wanted to look at WT cells which would allow for more flexibility. Realistically, this methodology opens up a large amount of possibilities for anyone interested in studying EVs from any type of cells and any type of disease state. Within the context of our PD research, one possible angle we would like to pursue is to analyze the EVs released from our α-syn DSP cell using the GFP signal with the addition of autophagic drugs, and the LAMP1 marker and other overlapping endo-lysosomal and autophagic proteins, such as the galectin proteins. Utilizing this methodology we could identify how autophagic processes influenced the vesicular portion of DSP secretion and whether specific autophagic processes are associated with differences in specific subtypes of exosomes.
CHAPTER SIX:

CONCLUSIONS

The secretion of misfolded α-syn provides a clear way to potentially explain how the transmission of disease pathology could be occurring within the brains of individuals with synucleinopathies. In support of this idea, the results of others, as well as, the work conducted in this thesis shows that rotenone, a pesticide that is heavily linked to increasing PD onset, increases α-syn secretion, including non-monomeric variants [222]. These results provide one example, among a body of evidence that indicates that secretion is likely linked to spreading synucleinopathy pathology. In further support, the dyregulation of autophagic mechanisms may further exacerbate pathology, as reviewed earlier, several PD associated genes play a role in regulating autophagy, vesicular trafficking, and α-syn release, including LRRK2 and ATP 13A2. While the secretion of α-syn may increase the spreading of the diseased state from cell-to-cell, it is unclear whether on an individual cell basis if the secretory process is inherently negative as it represents another method of removal of α-syn aggregates. Hypothetically, sharing the degradative burden of α-syn aggregates among cells may be protective in comparison to one cell attempting and failing to degrade all misfolded α-syn. In addition, evidence exists for both sides of the argument on whether individual cell secretion of α-syn is detrimental or favorable and it is likely that both are true depending on the circumstance.
ATP 13A2 mutations are associated with familial PD and exhibit a cellular phenotype characterized by dysregulation of endosomal and autophagic cargo sorting [88-89]. However, over-expression of WT ATP 13A2 increases neuronal survivability, and results in decreased α-syn accumulation but increased exosomal α-syn secretion [88-89]. Additionally, higher levels of ATP 13A2 mRNA are found within the surviving neurons of individuals with PD [91]. Therefore, these results indicate that increased α-syn secretion is important for cell survival.

A similar paradigm was found when we investigated the secretion of our α-syn DSP construct within chapter 4. We found that increasing autophagic activity through both mTOR independent and dependent pathways as a result of trehalose and rapamycin treatment, respectively, increased the secretion of our α-syn DSP construct. Additionally, while we did not investigate α-syn aggregate clearance, others have found that trehalose or rapamycin treatment is associated with decreased α-syn aggregate accumulation, and lower levels of cell death [9]. These results further support the idea that increased secretion of α-syn may also be tied to its removal. In addition, these results implicate autophagy as being a potential pathway for increasing both clearance, as well as, secretion of α-syn. Therefore, increased autophagic activity increases cell survivability but comes at the cost of increased α-syn secretion.

Interestingly this paradigm, that autophagy is responsible for both the removal of aggregate α-syn, as well as, its secretion and is subsequently linked to cell survivability, is also shared among another neurodegenerative disease associated protein, Aβ. When Aβ transgenic mice were given an ATG7 neuron specific knock-out, it was found that Aβ secretion was decreased but was also increased Aβ accumulation within the perinuclear regions of the mice [99]. While these mice experienced less Aβ secretion, these mice were shown to have decreased life spans and exacerbation of a neurodegenerative phenotype.
Similar to the cellular level results mentioned in the previous Aβ study, we found that when our respective ATG7 and Beclin-1 knock-out cells were treated with bafilomycin-A1, a decreased level of non-monomeric α-syn secretion occurred. Furthermore, similar results were recapitulated by Ejlerskov et al., 2013, where they showed that siRNA mediated knock-down of ATG5 substantially decreased α-syn secretion. Therefore, while decreased secretion of either Aβ or α-syn may seemingly indicate a lower potential for transferring disease pathology which occurred as result of depletion of essential autophagic proteins, within the context of Aβ it resulted in an exacerbation of the disease phenotype and decreased life span within mice.

Finally, the clearance of α-syn can also result in a differing paradigm as a result of decreased proteasome activity. Several studies have shown that when the 26S proteasome is inhibited, an upregulation of autophagic activity occurs in attempt to compensate [213-218]. This process was shown by us and others to cause an increase in α-syn secretion. However, 26S proteasome inhibition is also linked to an increase in α-syn accumulation, likely due to the loss of function in general protein clearance that no longer occurs as the UPS also is important for the degradation of α-syn aggregates [196-197]. Therefore, inhibition of the proteasome results in aggregation of α-syn, suggesting intracellular PD pathology, while potentially also exacerbating the cell-to-cell transfer of pathology due to increases in secretion.

Our demonstration of single EV analysis methodology indicated that extracellular PFF α-syn, representing a pathological α-syn variant, was found in low levels within the extracellular environment. However, when it was found in the extracellular environment it was associated with an EV the majority of the time (~65%). Additionally, these PFF α-syn positive EVs were also positive for the LAMP1 marker ~30%, a marker that was found in only ~20% of our total EV population. These data indicates that pathological α-syn EVs may originate from the lysosome,
which supports the axiomatically accepted hypothesis that lysosome dysfunction is central to PD pathology.

In further support of exosomes being tied to lysosomal dysfunction, several studies have linked oligomeric α-syn secretion to lysosome dysfunction. Bafilomycin-A1 treatment, which inhibits lysosome acidification, has been shown to increase both general α-syn, as well as, exosome specific α-syn secretion [8-9, 49, 88]. Furthermore, studies looking at bafilomycin-A1 treatment also provide a link that intersects both exosome secretion and autophagy. As bafilomycin-A1 treatment has also been shown to increase autophagosome accumulation as well as α-syn secretion through autophagic mechanisms [9, 49]. This is further supported by our findings, showed within our autophagic mechanisms influence the unconventional secretion of non-monomeric α-syn, that bafilomycin-A1 increased secretion our complemented α-syn DSP construct and that this secretion decreased significantly when we genetically depleted either autophagic proteins ATG7 or Beclin-1 via CRISPR-CAS9 knock-out. However, whether this influences the exosomal portion of α-syn secretion still needs to be investigated but may provide a link to autophagic inhibition and α-syn associated exosomes.

The secretion of misfolded α-syn represents a very clear way in which the spreading of synucleinopathy pathology can be transmitted between cells, resulting in further propagation. In this document we have demonstrated that non-monomeric α-syn secretion is linked to autophagic mechanisms and dysregulation can have profound effects on the levels at which it is secreted. Our data shows that specific activators and inhibitors could differentially change the secretion of our non-monomeric α-syn DSP construct. Additionally we provided evidence through chemical and genetic evidence that autophagic proteins are important to α-syn secretion; as genetically depleted either autophagic proteins ATG7 or Beclin-1 via CRISPR-CAS9
knock-out significantly reduced non-monomeric α-syn secretion within the context of bafilomycin-A1 treatment. This demonstration, through bafilomycin-A1 treatment, is particularly interesting and it serves as bridge: In the cell bafilomycin-A1 reduces the acidification of lysosomes, a process that results in autophagosome accumulation and degradative dysfunction but also increases both non-vesicular and exosome specific α-syn secretion. In this thesis, within THE NOVEL CHARACTERIZATION OF PREFORMED FIBRIILLAR ALPHA-SYNUCLEIN’S ASSOCIATION WITH EXTRACELLULAR CELLULAR VESICLES ON A SINGLE VESICLE LEVEL, we found that pathological α-syn species were often found within EVs that were often associated with lysosomal markers, demonstrating an intersection between exosomes, autophagy, and lysosomal dysfunction. However, familial PD pathology also provides a strong genetic tie for lysosomal dysregulation role in PD [77-84, 91]. As reviewed earlier, specific PD associated genes such as LRRK2 and ATP 13A2 play overlapping in roles in affecting autophagy, vesicular trafficking, and α-syn release. However, it is unclear whether on an individual cellular basis if this process is inherently negative as it represents another method of removal of α-syn aggregates. Additionally, the sharing of the degradative burden of protein aggregates, such as α-syn, among cells may actually be protective against disease propagation in some instances. Evidence exists for both arguments on whether α-syn secretion is detrimental or favorable and it is likely that both are true depending on the circumstance.
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VITA

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Figure 18. Autophagic pathway. Depicted in the figure is a cartoon flow-map of the autophagic pathway. Starting from left-to-right the figure shows how either mTOR1 or AMPK can respectively inhibit or activate ULK1 from the ULK complex resulting in the inhibition or initiation of autophagy. Activation of autophagy results in the activation of the Beclin and ULK Complex. Their respective activations induces the isolation of a section of double membrane phospholipid bilayer that develops into the autophagophore, becoming enriched in ATG proteins, and the formation of PI3KC3, subsequently increasing PI3P concentrations. The autophagophore then goes through elongation which is driven by the ATG Complexes resulting in the processing and translocation of LC3 to the autophagophore. The autophagophore continues through elongation until it becomes the autophagosome, an enclosed double-membrane compartment. The autophagosome can either fuse with the late endosome to form the amphisome or directly go through the degradative or secretory pathway. The degradative pathway results in lysosome fusion resulting in the breakdown of autophagosome or amphisome cargo. In contrast, the secretory pathway results in the plasma-membrane fusion and release of autophagosome or amphisome cargo into the extracellular space.