RICE UNIVERSITY

Reprogramming the proteostasis network to prevent the accumulation of α-synuclein aggregates

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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May 2014
ABSTRACT

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Protein misfolding and aggregation characterizes the development of a number of neurodegenerative diseases, such as Parkinson’s, Alzheimer’s and Huntington’s disease. The hallmark of Parkinson’s disease is the formation of proteinaceous inclusions, which consist primarily of α-synuclein (α-syn), a natively unstructured protein with propensity to misfold and aggregate. Cells have evolved sophisticated systems of protein quality control to prevent accumulation of non-native proteins and maintain protein homeostasis. However, the load of misfolded α-syn typically exceeds the capacity of the quality control system. Aberrant accumulation of misfolded α-syn leads to proteotoxic stress, eventually resulting in neurodegeneration.

The objective of this project was to investigate chemical and genetic approaches to modulate the protein quality control system and reduce the accumulation of aberrant α-syn species. Studying α-syn aggregation in cells presents a number of challenges mainly due to the limited availability of tools to quantitatively distinguish between different α-syn conformational species within the cellular environment. To address this need, an in vitro model system was
engineered based on neuroglioma cells that accumulate α-syn aggregates and a set of analytical tools were developed utilizing aggregation responsive probes to quantify α-syn aggregation in cells.

To test whether modulating the protein quality control system affects the accumulation of α-syn aggregates, a series of complementary approaches aimed at i) enhancing the innate cellular chaperone machinery, which promotes folding and prevents aggregation, and ii) upregulating the autophagy pathway, which mediates clearance of aggregated proteins were investigated. Chemical modulation of Hsp70, a ubiquitously expressed molecular chaperone, affected the accumulation of α-syn aggregates. Particularly, the Hsp70 upregulator carbenoxolone was found to reduce α-syn aggregation and prevent α-syn-induced cytotoxicity via activation of the heat shock response. On the other hand, activation of the transcription factor EB (TFEB), a master regulator of the autophagy-lysosomal pathway, was found to enhance autophagic clearance of α-syn aggregates. Cell treatment with 2-hydroxypropyl-β-cyclodextrin reduced the accumulation of aggregated α-syn specifically by upregulating TFEB-mediated autophagic clearance. These findings lay the foundation for the development of pharmacological strategies to reduce the accumulation of misfolded and aggregated α-syn for the treatment of Parkinson’s disease.
Acknowledgments

First and foremost, I would like to express my deepest gratitude and thanks to my advisor, Dr. Laura Segatori. I cannot overstate how her guidance, support, enthusiasm, and encouragement have contributed to my development as a scientist, engineer, and independent thinker.

I would also like to thank the members of the Segatori Lab – past and present – for their support, guidance, and laughter over the years.

Lastly, and most importantly, a special thank you to...

My friends, my balance

My brothers and sisters, my inspiration

My Dad, my rock

My Mom, my fire

Ellie, my joy

... and James, my best friend, my heart.
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>α-syn</td>
<td>α-synuclein</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>Hsp70</td>
<td>70 kDa heat shock protein</td>
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<td>NBD</td>
<td>nucleotide binding domain</td>
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<td>heat shock factor 1</td>
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<td>TFEB</td>
<td>transcription factor EB</td>
</tr>
<tr>
<td>CLEAR</td>
<td>Coordinated Lysosomal Expression and Regulation</td>
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<td>NAC</td>
<td>Non amyloid-beta component</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>DPPZ</td>
<td>dipyridophenazine</td>
</tr>
<tr>
<td>[Ru(phen)₂dppz]²⁺</td>
<td>ruthenium(II) dipyridophenazine complex</td>
</tr>
<tr>
<td></td>
<td>(phen=1,10-phenanthroline)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative RT-PCR</td>
</tr>
<tr>
<td>APF</td>
<td>aggregation propensity factor</td>
</tr>
<tr>
<td>CBX</td>
<td>carbenoxolone</td>
</tr>
<tr>
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<td>reactive oxygen species</td>
</tr>
<tr>
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<td>secreted embryonic alkaline phosphatase</td>
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Chapter 1

Introduction

Parkinson’s disease (PD) is the most prevalent neurodegenerative movement disorder. It is estimated that approximately 1 million people in the US and more than 4 million people worldwide are affected by PD. Currently, there are only symptomatic treatments, but no cure is available for PD. The most effective treatment is levodopa, which activates dopamine neurotransmission in the brain. It is only effective for the first 4 to 6 years of treatment, after which motor fluctuations and dyskinesias typically develop (Olanow and Tatton, 1999). The current health care cost of PD is estimated to exceed $6 billion per year. The frequency of idiopathic PD, which accounts for the majority of cases, rapidly rises with age. Only a small percentage of patients are diagnosed before the age of 50, whereas 1% of the population at 65-70 years of age, and about 5% of people older than 85 years are affected. As the average age of the US population increases, the prevalence of PD is expected to double over the next twenty years (Dorsey et al., 2007). Hence, the
economic burden of PD will inevitably increase without the development of efficient therapeutic solutions.

The primary hallmarks of PD are the loss of dopaminergic neurons in the substantia nigra pars compacta and the occurrence of proteinaceous cytoplasmic inclusions (Lewy bodies) in surviving neurons (Dauer and Przedborski, 2003). The main component of Lewy bodies is α-synuclein (α-syn) (Spillantini et al., 1997), a 140-residue presynaptic protein with unknown function that is natively unstructured and has the propensity to misfold and aggregate (Uversky, 2008). Misfolded monomers tend to oligomerize into soluble protofibrillar structures and eventually aggregate in the form of insoluble deposits (Uversky, 2003). PD patients harboring triplication of the gene encoding α-syn (SNCA), which results in an increase in the expression level of α-syn, present accumulation of α-syn aggregates and manifest early onset PD symptoms (Miller et al., 2004; Singleton et al., 2003). Interestingly, however, α-syn-containing Lewy bodies are found in both familial and sporadic forms of PD (Baba et al., 1998). However, the molecular mechanisms underlying α-syn misfolding and aggregation and its role in the progression of PD pathogenesis remains unclear.

Traces of molecular chaperones and components of the degradation system are found to aggregate with α-syn and accumulate within Lewy bodies, suggesting an attempt of the protein quality control system to prevent the abnormal accumulation of misfolded α-syn (Cook et al., 2012a). The protein quality control system consists of i) molecular chaperones that assist folding and prevent misfolding
and aggregation, and ii) the degradation machinery that facilitates the disposal of aberrant proteins (Dobson, 2003). The degradation machinery is mainly composed of the ubiquitin-proteasome system (UPS), which catalyzes the degradation of misfolded soluble proteins (Ciechanover and Brundin, 2003), and the autophagy system, which mediates clearance of misfolded and aggregated proteins by the lysosome (Wang and Klionsky, 2003). Genetic mutations in UPS components were linked to the accumulation of α-syn and neurodegeneration detected in the brains of PD patients (Liu et al., 2002; Shimura et al., 2000; Shimura et al., 2001). Surprisingly, a decrease in UPS activity was also observed in neurons of patients with sporadic PD (McNaught and Jenner, 2001), suggesting that a reduction in UPS activity is linked to the accumulation of misfolded and aggregated α-syn (Rideout et al., 2001). Additionally, reduced autophagic activity was also observed in postmortem brain tissues from PD patients (Crews et al., 2010). In summary, mounting evidence points to a link between α-syn misfolding and inefficient clearance of aberrant α-syn conformations and development of PD. This evidence led me to hypothesize a model in which inefficient function of the protein quality control system underlies the mechanism of α-syn-induced cytotoxicity.
Figure 1.1: Hierarchy of the Proteostasis Network. The proteostasis network is an integrated system that promotes and preserves protein folding and function in response to changes in the cellular environment. Adapted from Powers et al., 2009.

The protein quality control system plays a fundamental role in maintaining a physiologic balance between folding and accumulation of native, functional proteins, and protein misfolding and accumulation of off-pathway intermediates. As such, the protein quality control system is an integral part of the overarching network that controls protein homeostasis – the proteostasis network. Proteostasis refers to
"controlling the concentration, conformation, binding interactions (quaternary structure), and location of individual proteins making up the proteome by readapting the innate biology of the cell, often through transcriptional and translational changes" (Balch et al., 2008). The cellular proteostasis network is a highly adaptable multilayered system comprised of (Figure 1.1): 1) pathways that manage protein synthesis, folding, trafficking, aggregation, and degradation, 2) the signaling pathways that regulate the concentration, distribution, and activity of components in these pathways, and 3) intracellular and extracellular environmental factors that influence the activities of these pathways (Powers et al., 2009). An imbalance in proteostasis can lead to the accumulation of misfolded and aggregated proteins and formation of insoluble deposits that are the hallmark of many neurodegenerative disorders, including Parkinson's disease (McClellan et al., 2005b).

The objective of this study is to reprogram the proteostasis network to prevent the accumulation of aberrant α-syn conformations. This goal is accomplished by adapting the innate cellular chaperone and degradation capacity to modulate the accumulation of misfolded and aggregated α-syn and restore protein homeostasis. A closer examination of the protein quality control network, PD, and the role of α-syn aggregation in PD is described below in more detail.
The protein quality control system encompasses the coordinated action of molecular chaperones and degradation pathways. Chaperones assist the folding of newly synthesized proteins, protein trafficking, and the refolding of misfolded or damaged proteins. Proteins that are irreversibly misfolded are degraded through the ubiquitin proteasome system (UPS) or the autophagy-lysosomal system.
1.1. The Protein Quality Control System

The protein quality control system consists of a sophisticated network of macromolecular components and pathways that promote the folding and trafficking of newly synthesized proteins and regulate the degradation of misfolded and damaged proteins (Figure 1.2). As mentioned above, molecular chaperones facilitate the folding of proteins and prevent aggregation whereas the UPS and the autophagy system catalyze the disposal of misfolded proteins (Dobson, 2003). Impairment of either branch of the protein quality control system results in the accumulation of misfolded proteins (Wickner et al., 1999). Thus, I hypothesized that modulating the innate cellular mechanisms that normally maintain protein quality control might restore protein homeostasis and alleviate cytotoxicity under conditions of proteotoxic stress, such as abnormal accumulation of aberrant α-syn. The following sections will describe protein quality control mechanisms in more detail.

1.1.1. Molecular Chaperones

Chaperones assist native folding and trafficking of newly synthesized proteins and target misfolded proteins for degradation (Bukau et al., 2006; Deuerling and Bukau, 2004; Hartl and Hayer-Hartl, 2002; Ron and Walter, 2007). They recognize and shield exposed hydrophobic patches of partially folded and misfolded proteins thereby preventing misfolding and aggregation and promoting
unfolding of partially misfolded proteins (McClellan et al., 2005b). Not surprisingly, chaperones of the heat shock protein (HSP) family are upregulated in response to cellular stress caused by an increase in protein synthesis or accumulation of misfolded proteins—a process referred to as the heat shock response (Hartl and Hayer-Hartl, 2002). Additionally, HSPs can assist in the degradation of proteins by directing misfolded proteins to the UPS or autophagy (Arndt et al., 2007; Kettern et al., 2010), thereby providing a link between protein folding and degradation and suggesting an essential triage role of HSPs in maintaining proteostasis.

The 70 kDa heat shock protein (Hsp70) is a highly conserved, ubiquitous molecular chaperone. Basal levels of Hsp70 are observed under resting conditions, and are critically involved in multiple cellular processes, including protein folding, degradation, translocation across membranes, and protein-protein interactions (Hartl and Hayer-Hartl, 2009). Hsp70 contains an N-terminal ATPase domain (nucleotide binding domain, NBD) and a substrate binding domain (SBD) (Figure 1.3). When bound to ATP, Hsp70 exhibits an open conformation, with both high on and high off rates of unfolded substrate binding. ATP hydrolysis induces a high affinity substrate binding conformation by closing the Hsp70 lid, while ADP exchange for ATP induces substrate release (Kampinga and Craig, 2010).
Figure 1.3: Hsp70 Chaperone Cycle. Hsp70 comprises two distinct domains: a nucleotide binding domain (NBD) for ATP binding and hydrolysis and a substrate binding domain (SBD) that recognizes misfolded proteins. Misfolded proteins are targeted to Hsp70 by Hsp40 and associate with ATP-bound Hsp70 with low affinity. A high affinity Hsp70–substrate complex forms upon Hsp40–mediated hydrolysis of ATP. The substrate is released upon exchange of ADP for ATP.

The cellular activity of Hsp70 is modulated by cochaperones, including members of the Hsp40 family. There are 44 different human Hsp40s that are characterized by the presence of a J-domain, which is essential for Hsp40 interaction with Hsp70 ATPase domain (Caplan et al., 1993; Cheetham and Caplan, 1998).
Hsp40 regulates the ATPase activity of Hsp70 by binding to Hsp70 NBD and induces substrate binding and release by stimulating nucleotide hydrolysis (Fan et al., 2003; Jiang et al., 2005). Hsp40s targets substrates to Hsp70 (Cheetham and Caplan, 1998; Fan et al., 2003) or to the protein degradation machinery (Grove et al., 2011; McClellan et al., 2005a; Metzger et al., 2008; Youker et al., 2004). Hsp40 cochaperones regulate the formation of Hsp70-substrate complexes and determine whether Hsp70 facilitates substrate folding or degradation (Kampinga and Craig, 2010).

The heat shock response is regulated by heat shock factor 1 (HSF1), which mediates the upregulation of Hsp70 and other HSPs. HSF1 activation is a multistep process that involves the phosphorylation and translocation of HSF1 from the cytoplasm to the nucleus. A number of different stressors can lead to HSF1 activation including heat shock, oxidative stress, and even cell growth (Morimoto, 1998). The heat shock response is an essential component of the proteostasis network by preventing protein misfolding, inducing cytoprotection, promoting signaling pathways necessary for cell growth, and protecting cells from apoptosis (Westerheide and Morimoto, 2005).

As described in Chapter 4, chemical upregulation of Hsp70 via induction of the heat shock response was discovered to reduce α-syn aggregation and α-syn-induced cytotoxicity in a cell culture model system of PD. Furthermore, chemical modulation of Hsp70 ATPase activity was found to prevent α-syn aggregation.
1.1.2. Degradation Pathways

The proteostasis network relies on efficient mechanisms of degradation to eliminate defective and misfolded proteins. The proper function of the cellular degradation machinery prevents accumulation of unwanted proteins, which could induce deleterious consequences by forming aberrant interactions with other cellular components or by associating to form insoluble aggregates (Wong and Cuervo, 2010). The two main pathways that facilitate disposal of proteins – the UPS and autophagy – are described below in more details.

1.1.2.1. The Ubiquitin Proteasome System

The UPS is the primary route for degradation of short-lived and soluble misfolded proteins (Ciechanover and Brundin, 2003). It comprises a complex system of pathways for the recognition, targeting, and degradation of misfolded proteins (Goldberg, 2003). Proteins targeted for degradation are conjugated to a poly-ubiquitin chain (Voges et al., 1999) (Figure 1.4). Ubiquitination involves the concerted action of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ligase. An ubiquitin molecule is first activated upon binding to an E1 ubiquitin-activating enzyme and then transferred to an E2 ubiquitin-conjugating enzyme. E3 ligases recognize misfolded substrates and present them to ubiquitin-conjugated E2 enzymes. Ubiquitin molecules are transferred to the substrate by covalent linkage to lysine residues (Cook and Petrucelli, 2009). Subsequent rounds
of ubiquitination occur through the formation of an isopeptide bond between the glycine residue at position 76 of one ubiquitin and the lysine residue at position 48 of the adjacent ubiquitin (Glickman and Ciechanover, 2002). Poly-ubiquitinated proteins linked to a chain of at least four ubiquitin molecules are recognized by the proteasome (Glickman and Ciechanover, 2002).

The proteasome is composed of a 20S barrel shaped core and two 19S caps. The 19S cap, also known as PA700, is a large complex consisting of several subunits involved in many functions, including ATPase, ubiquitin binding, and deubiquitinating activities (Nandi et al., 2006). The 20S proteasome contains the proteolytic core, which encompasses three different peptidase activities that cleave the substrate into short peptides (Voges et al., 1999). The 19S cap acts as a proteasome activator by removing ubiquitin, unfolding target proteins in an ATP-dependent manner, and facilitating the opening of the 20S core (Nandi et al., 2006). (Figure 1.4) The PA28 subunits also functions as a proteasome activator by associating with the terminal rings of the 20S proteasome and activating the hydrolysis of short peptides (Gray et al., 1994; Mott et al., 1994). It is suggested that PA28, like PA700, activates the 20S proteasome by opening the channel at the terminal rings, thereby increasing access to the proteolytic core (DeMartino and Slaughter, 1999). PA28 overexpression was found to recover proteasomal function and to improve viability of cells treated with inhibitors of proteasomal degradation, such as MG-132 (Seo et al., 2007).
Figure 1.4: The Ubiquitin Proteasome System (UPS). Misfolded proteins are targeted for degradation upon poly-ubiquitination. Ubiquitin is activated by an E1 ubiquitin-activating enzyme and is transferred to an E2 ubiquitin-conjugating enzyme. An E3 ligase recognizes substrates for degradation and recruits the E2-ubiquitin complex to facilitate substrate ubiquitination. A chain of at least four ubiquitin is required for degradation by the 26S proteasome. The 19S lid removes the ubiquitin chain, unfolds the substrate and facilitates substrate insertion through the 20S proteolytic core where it is cleaved into short peptides.

1.1.2.2. Autophagy

In contrast to the UPS, autophagy is responsible for degradation of long-lived proteins by the lysosome (Wang and Klionsky, 2003). Specifically, autophagy
mediates disposal of intracellular waste material, such as aberrant proteins, protein aggregates, and damaged organelles (Mizushima and Komatsu, 2011), as well as viruses and parasites (Deretic and Levine, 2009). Cytoplasmic proteins can reach the lysosome for degradation through three different pathways (Figure 1.5): macroautophagy, chaperone-mediated autophagy, and microautophagy. Macroautophagy, the most common route of autophagic clearance, involves the delivery of cytoplasmic material to the lysosome via an intermediate organelle, called an autophagosome. Autophagic cargo is sequestered into autophagosomes. Fusion of autophagosomes with lysosomes results in delivery of the autophagic cargo to the lysosomes through the formation of autophagolysosomes (Mizushima and Komatsu, 2011). Although widely recognized as a nonselective process, recent evidence suggests that proteins can be targeted for degradation through macroautophagy. Autophagic receptors, such as p62, recognize ubiquitinated proteins and tether them to autophagosomal membrane proteins, thereby selectively placing proteins for degradation within autophagosomes (Nixon, 2013). Chaperone-mediated autophagy involves selective translocation of soluble cytoplasmic proteins directly into the lysosome through the coordinated action of Hsc70 (constitutively expressed isoform of Hsp70) and Lamp-2A, a lysosome transmembrane protein (Wong and Cuervo, 2010). Microautophagy proceeds through direct engulfment of small cytoplasmic components by the lysosome (Li et al., 2012). Proteins degraded through chaperone-mediated autophagy and microautophagy are recognized by Hsc70, which interacts with a KFERQ polypeptide motif in misfolded or damaged proteins (Massey et al., 2006; Sahu et al.,
Once the autophagic cargo enters the lysosome, degradation is catalyzed by lysosomal hydrolases, including proteases, lipases, glycosidases, and nucleotidases (Appelqvist et al., 2013).

**Figure 1.5: Autophagic Pathways.** Cytoplasmic proteins are delivered to the lysosome for degradation through three different pathways: a) macroautophagy, b) microautophagy, or c) chaperone-mediated autophagy (CMA). Adapted from Wong and Cuervo, 2010.

The autophagy pathway is regulated by the transcription factor EB (TFEB), a master regulator of the CLEAR (Coordinated Lysosomal Expression and Regulation) gene network (Sardiello et al., 2009; Settembre et al., 2011). Activation of TFEB involves TFEB dephosphorylation and translocation from the cytoplasm into the nucleus where it binds to promoters containing a CLEAR sequence (Palmieri et al., 2011; Sardiello et al., 2009). TFEB activation upregulates the expression of more
than 500 genes, which are involved in lysosomal biogenesis and proteostasis (Sardiello et al., 2009; Song et al., 2013), formation of autophagosomes, lysosome-autophagosome fusion, and autophagic clearance (Settembre et al., 2011).

In this study, the role of TFEB and autophagy in mediating the clearance of α-syn aggregates was investigated in a cell culture model of PD and chemical activation of TFEB was demonstrated to enhance α-syn clearance (Chapter 5).

### 1.2. Parkinson’s Disease

PD is a progressive neurological disorder. As mentioned above, the hallmark of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta. Symptoms of PD manifest when approximately 80% of dopamine producing neurons are damaged, resulting in loss of dopamine production. Motor symptoms of PD include uncontrollable resting tremor, rigidity, bradykinesia, and postural instability, which can be exacerbated by cognitive defects, such as speech and memory loss. Such a diverse range of symptoms and the absence of a clear correlation between phenotype and genotype have challenged the diagnosis of PD, which currently relies on medical history, neurological examination, and elimination of closely resembling diseases. The most effective strategy to alleviate PD motor symptoms is administration of levodopa, a dopamine precursor that can cross the blood brain barrier and replenish the dwindling supplies of dopamine in the affected neurons (Olanow and Tatton, 1999). Due to the progressive character of PD
neurodegeneration, most patients benefit from levodopa treatment only during the first 5 years, and become less responsive as the number of dopamine producing neurons decreases (Fahn, 2006).

The causes of PD are still far from understood. The majority of PD cases are idiopathic, but approximately 10% are of familial origin (Klein and Westenberger, 2012; Thomas and Beal, 2007). Our current understanding suggests that a variety of risk factors including genetic predisposition, exposure to environmental agents, and aging-associated metabolic and biochemical cellular dysfunction, may contribute to the development of PD. The discovery of several genes linked to the development of PD has led to a better understanding of the molecular mechanisms that underlie neurodegeneration. Six genes have been conclusively identified as PD markers (Table 1.1) (Gasser, 2009; Houlden and Singleton, 2012). These genes are involved in cellular pathways that are typically dysfunctional in PD affected-cells, which include protein folding and homeostasis (α-syn), protein degradation (Parkin and ATP13A2), mitochondrial function (DJ-1 and PINK1), and phosphorylation (LRRK2) (Franssens et al., 2010; Gasser, 2009). Genetics alone cannot explain PD pathogenesis since sporadic PD represents the large majority of cases. However, evidence that common pathological traits occur in sporadic and hereditary cases suggest a link between genes associated with familial PD and PD development. As a result, PD model systems are typically developed by generating cell lines or transgenic animals in which the function encoded by one or multiple marker genes is inactivated to recapitulate hereditary PD traits (Cookson and van der Brug, 2008; Orth and Tabrizi, 2003).
Table 1.1: Parkinson’s disease associated genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>NCBI Reference Sequence</th>
<th>Function</th>
<th>Pathogenic Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNCA</td>
<td>α-syn</td>
<td>NM_000345</td>
<td>Involved in synaptic vesicle formation?</td>
<td>Protein Aggregation</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkin</td>
<td>NM_004562</td>
<td>E3 ubiquitin protein ligase</td>
<td>Impaired protein degradation</td>
</tr>
<tr>
<td>PINK1</td>
<td>Pten-induced kinase 1</td>
<td>NM_032409</td>
<td>Mitochondrial kinase</td>
<td>Mitochondrial dysfunction</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>NM_007262</td>
<td>Involved in oxidative stress response?</td>
<td>Mitochondrial dysfunction</td>
</tr>
<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
<td>NM_198578</td>
<td>Kinase</td>
<td>Impaired phosphorylation</td>
</tr>
<tr>
<td>ATP13A2</td>
<td>ATPase type 13A2</td>
<td>NM_022089</td>
<td>Lysosome ATPase</td>
<td>Impaired protein degradation</td>
</tr>
</tbody>
</table>

1.2.1. α-synuclein aggregation in PD

Triplication of α-syn locus as well as three missense mutations leading to amino acid substitutions (A30P, E46K, and A53T) have been identified in association with familial cases of PD (Cookson, 2005). Genetic and biochemical studies showed that α-syn mutations typically result in increased accumulation of misfolded α-syn (Goedert, 2001). This evidence supports the hypothesis that α-syn misfolding plays a key role in the development of PD (Singleton et al., 2003; Spillantini et al., 1997).
α-syn is ubiquitously expressed in the central nervous system and enriched in the adult brain (Jakes et al., 1994). Although α-syn function is unclear, evidence suggests a regulatory role in dopamine neurotransmission and vesicle biogenesis (Fortin et al., 2004; Liu et al., 2004). Indeed, α-syn knockout mice present reduction in dopamine stores and increase in dopamine release compared to wild type mice, suggesting that loss of α-syn function impairs dopamine neurotransmission (Abeliovich et al., 2000). Independent evidence suggests that α-syn presents chaperone-like activity (Souza et al., 2000) and may be involved in lipid metabolism and brain inflammatory response (Golovko et al., 2009). Additionally, several α-syn binding partners have been identified, including Synphilin (Engelender et al., 1999; McLean et al., 2002) and Tublin (Lee et al., 2006). Because of its potential association with multiple cellular functions, it is hypothesized that the aberrant accumulation of misfolded α-syn may translate into a range of clinical manifestations, which are collectively referred to as synucleinopathies and include PD, dementia with Lewy bodies, and multiple system atrophy (Uversky, 2008).

α-syn is an intrinsically unstructured, natively unfolded protein. Natively unfolded proteins are extremely flexible, with little or no secondary structure under physiological conditions (Uversky, 2003). α-syn’s sequence includes a region of imperfect KTKEGV repeats, a hydrophobic core, and a highly acidic C-terminus tail (Cookson, 2005) (Figure 1.6). The imperfect KTKEGV repeats form an amphipathic α-helix that seems to facilitate membrane interactions (Cookson, 2005). α-syn’s propensity to associate with lipid membranes supports the theory that α-syn is involved in the formation of dopamine vesicles. The hydrophobic core, which is also
referred to as the non-amyloid β component (NAC), has a propensity to self-associate and drives α-syn aggregation (Giasson et al., 2001). Truncation of the C-terminal acidic tail increases rate of aggregation, suggesting that this region prevents α-syn aggregation (Murray et al., 2003).

![Diagram of structural domains of α-synuclein](image)

**Figure 1.6: Structural domains of α-synuclein.** α-syn consists of a N-terminal region rich in imperfect KTKEGV repeats, a central hydrophobic region, and a C-terminal acidic tail. Familial mutations in α-syn include A30P, E46K, and A53T.

The mechanism of α-syn aggregation is unclear. Evidence suggests that α-syn self-associates to form soluble protofibrils (oligomers), which mature into insoluble, protease-resistant, amyloid-like fibrils, and, eventually, aggregate in localized inclusions (Lewy bodies) (Uversky, 2003) (**Figure 1.7**). Removal of the hydrophobic core prevents the formation of amyloid fibrils (Giasson et al., 2001). Whether soluble oligomers or insoluble deposits exert cellular toxicity has been subject of intense debate. Although Lewy bodies are the hallmark of neurodegeneration, recent studies suggest that pre-fibrillar intermediates (protofibrils), and not mature amyloid-like fibrils, are the key toxic species. A number of studies provide evidence
for this hypothesis. Disease associated mutations promote α-syn oligomerization, but not its fibrilization (Conway et al., 2000b). The A53T mutation expands the hydrophobic domain of the protein favoring the formation of β-sheet enriched structures, which are typical of the oligomeric and amyloidogenic structures (Biere et al., 2000; Conway et al., 1998; Giasson et al., 1999). The A30P mutation disrupts the alpha-helical domain destabilizing its interaction with phospholipids and promoting the formation of non-fibrillar and non-amyloidogenic misfolded oligomeric intermediates (Conway et al., 2000b; Yonetani et al., 2009). These intermediates can exhibit annular morphology and were suggested to induce pore formation by integrating with the endoplasmic reticulum and mitochondrial membranes, causing oxidative stress (Hsu et al., 2000; Volles and Lansbury, 2002).

Recent studies suggest that the insoluble α-syn deposits serve a protective function in the cell and form to sequester toxic misfolded species in a localized space, and prevent deleterious interactions with other cellular components. Tanaka et al., reported that overexpression of α-syn variants that cause the accumulation of misfolded intermediates that do not lead to formation of insoluble deposits leads to a series of cytotoxic events that are not observed upon overexpression of α-syn variants that aggregate into insoluble deposits, suggesting a protective role of Lewy bodies (Tanaka et al., 2004).
Figure 1.7: Schematic representation of α-synuclein fibrilization. Natively unfolded α-syn monomers self-associate to form oligomers, a transient population of protofibrils of heterogeneous structure that may include spheres, chains, or rings. Protofibrils aggregate into amyloid-like fibrils, which eventually accumulate into Lewy bodies. α-syn is the most abundant component of Lewy bodies, as shown in the overlapping immunofluorescent stain in the figure (Lewy bodies, green, α-syn, red; NCBI).

1.2.2. UPS Dysfunction in PD

The presence of ubiquitinated proteins and components of the UPS within Lewy bodies suggests UPS dysfunction or inability to cope with the aberrant accumulation of misfolded proteins (Cook and Petrucelli, 2009). Wild type α-syn is a substrate of the 26S and 20S proteasome (Bennett et al., 1999) and inhibition of the 20S proteasome leads to the accumulation of α-syn (Rideout et al., 2001; Tofaris et al., 2001). However, A30P and A53T α-syn presents increased resistance to proteasomal degradation (Stefanis et al., 2001; Tanaka et al., 2001). Genetic
mutations in Parkin and UCH-L1 in familial cases of PD also lead to impaired proteasomal degradation (Kitada et al., 1998; Leroy et al., 1998). For instance, loss of Parkin’s E3 ligase activity results in the accumulation of its substrates, including CDCrel-1 (Zhang et al., 2000), Synphilin-1 (Chung et al., 2001), and the Parkin-associated endothelin receptor-like receptor (Pael-R) (Imai et al., 2001; Yang et al., 2003). Interestingly, loss of Parkin activity was observed in both genetic and sporadic forms of PD (Cookson, 2005; Wong et al., 2007). When proteasomal degradation is impaired or insufficient, soluble misfolded α-syn and aggregated α-syn can be degraded by chaperone-mediated autophagy and macroautophagy, respectively (Cuervo et al., 2004; Webb et al., 2003).

Misfolded α-syn can also impair the activity of the 26S proteasome – with monomeric and dimeric α-syn only slightly lowering proteasomal function and protofibrillar α-syn potently inhibiting degradation of polyubiquitinated proteins (Emmanouilidou et al., 2010; Lindersson et al., 2004; Snyder et al., 2003; Zhang et al., 2008). In vitro evidence suggests that α-syn can block the proteasome function by directly binding to the 19S cap (Snyder et al., 2003) or to the proteolytic core of the 20S proteasome (Lindersson et al., 2004). Proteolytic stress caused by α-syn-induced inhibition of proteasomal activity in in vitro models of PD was found to increase the cell’s susceptibility to other stresses and enhance the propensity to commit to apoptosis (Tanaka et al., 2001).
Chapter 2

Objectives

The objective of this research is to develop genetic and chemical strategies to reduce α-syn aggregation and enhance the clearance of aberrant α-syn species associated with the development of PD. Specifically, we aim to manipulate the innate cellular folding and degradation capacity to prevent accumulation of aberrant α-syn conformations and restore protein homeostasis. Studying α-syn aggregation in cells presents a number of challenges mainly due to the limited availability of tools to quantitatively distinguish between different α-syn conformational species within the cellular environment. Thus, the first milestone of this work was to i) establish a cell culture model system of PD based on neuroglioma cells that accumulate α-syn aggregates and ii) develop analytical tools to detect and quantify α-syn aggregation in cells. The development of an in vitro model system of α-syn aggregation enabled us to investigate genetic and chemical approaches to reprogram the proteostasis network. I hypothesized that α-syn misfolding and aggregation could be reduced by:
a) enhancing the expression and activity of molecular chaperones, and b) upregulating the autophagy-lysosomal system. This hypothesis was investigated by accomplishing the following specific aims:

**Specific Aim 1:** Developing an *in vitro* model system to study α-syn aggregation. The objective of this aim was to develop experimental tools to monitor α-syn accumulation in cell culture. This goal was achieved by generating a cell model system based on the overexpression of α-syn fused to the green fluorescent protein (GFP) to visualize and quantify α-syn in cells. To investigate α-syn aggregation, we utilized aggregation-responsive dyes that emit a fluorescent signal when bound to aggregated protein, namely the commercially available ProteoStat® dye and a dipyridophenazine ruthenium(II) complex. The *in vitro* model system generated in this study will be generally useful to investigate the molecular mechanisms underlying α-syn misfolding and deposition. Furthermore, it will provide a platform to screen modulators of α-syn aggregation.

**Specific Aim 2:** Modulating the chaperone machinery to prevent α-syn aggregation. I hypothesized that enhancing the expression and activity of Hsp70 reduces α-syn aggregation. This hypothesis was tested by investigating the effect of carbenoxolone, a small molecule that upregulates the expression of Hsp70, on my *in vitro* model system of PD. To elucidate the mechanism of Hsp70 mediated reduction of α-syn aggregation, chemical modulation of Hsp70 ATPase activity was also
investigated. Results from this study provide a proof-of-principle demonstration that chemical modulation of the Hsp70 machine is a viable therapeutic strategy for the treatment of diseases characterized by α-syn misfolding and deposition.

**Specific Aim 3: Upregulating the autophagy-lysosomal system to promote cellular clearance of aggregated α-syn.** I hypothesized that enhancing autophagic clearance mechanisms prevents the accumulation of aggregated α-syn. This hypothesis was tested by investigating TFEB, a master regulator of autophagy and lysosomal biogenesis, in my *in vitro* model system of PD. Activation of TFEB was induced genetically by overexpressing wild type TFEB and a mutated variant that is known to accumulate preferentially in the nucleus and induce activation of TFEB-target genes. Chemical activation of TFEB was also investigated by exposing the *in vitro* model system of α-syn aggregation to 2-hydroxypropyl-β-cyclodextrin, a known activator of TFEB. Results from this study provide proof-of-principle evidence that TFEB mediated enhancement of autophagic clearance prevents the accumulation of aggregated α-syn and demonstrate the role of TFEB as a therapeutic target for PD and potentially other diseases characterized by the accumulation of proteinaceous aggregates.
Chapter 3

Development of an *in vitro* model system of α-synuclein aggregation

The objective of this work is to generate a cell-based platform to investigate the molecular mechanisms involved in α-syn aggregation. Studying α-syn aggregation in cells presents a number of challenges mainly due to the limited availability of tools to quantitatively measure α-syn aggregation within the cellular environment. To address this need and monitor cellular aggregation of α-syn, I developed a cell culture model system based on the overexpression of α-syn in neuroglioma cells and a set of analytical tools based on the use of aggregation responsive fluorescent probes.
3.1.1. Cell culture model systems of PD

*In vitro* model systems recapitulate the pathological features of a disease and allow investigating the molecular mechanisms underlying the cellular pathogenetic events. Cell culture models provide a controlled environment to study specific pathogenic mechanisms and the genes and proteins involved (Alberio et al., 2012). Cell cultures can be easily manipulated to selectively induce or repress the expression of a gene of interest and analyze the structural, biochemical, and functional consequences. Unlike *in vivo* model systems, *in vitro* systems do not encompass the effect of the cellular pathogenetic cascade on organismal physiology. Nevertheless, cell culture model systems of human diseases provide useful tools to conduct rapid and high-throughput analyses aimed at characterizing and modifying a specific phenotypic event, such as α-syn aggregation and formation of proteinaceous inclusions (Cookson and van der Brug, 2008; Orth and Tabrizi, 2003).

When constructing a cell culture model system, it is important to carefully select the host cell type and control the level of protein expression. Ideally, post-mitotic human dopaminergic neuronal cell lines should be used as host cells in order to address the selective loss of dopaminergic neurons in the *substantia nigra* of PD patients (Orth and Tabrizi, 2003). Furthermore, cytotoxicity caused by experimental procedures commonly used for transient transfections can be avoided by creating stably transfected cells. Excessive constitutive expression of a transgene often leads to the synthesis of large quantities of transgenic protein, which may be deleterious to the cells. Thus, cell culture model systems should be engineered to
achieve the desired and precisely controlled expression level of transgenic protein. Finally, monoclonal stable populations are typically needed to avoid experimental artifacts associated with a heterogeneous population.

The presence of Lewy Bodies is an important pathological feature of PD and was previously recapitulated in cell cultures. In a recent study, Ko et al. detected the presence of insoluble inclusions by using an inducible expression system to overexpress α-syn in differentiated neuronal cells (Ko et al., 2008). After 28 days of expression, inclusions were detected by both immunofluorescence and using thioflavin S, a benzothiazole molecule that binds to the beta sheet rich structures characteristic of amyloidgenic proteins, and emits a fluorescent signal (Biancalana and Koide, 2010). The formation of α-syn inclusion bodies has also been induced by treating cells with stress-inducing agents, such as the mitochondrial inhibitor rotenone (Lee et al., 2002), or by inducing overexpression of proteins encoded by other genes associated with familial PD, such as Parkin and Synphilin-1 (Engelender et al., 1999). A more recent study reported the formation of inclusions in SH-SY5Y cells expressing wild type α-syn that were treated with the proteasome inhibitor MG-132 (Dyllick-Brenzinger et al., 2010). To test the overarching hypothesis that reprogramming the proteostasis network can prevent α-syn aggregation and promote α-syn clearance, this study aimed to generate a cell culture model system based on the overexpression of wild type α-syn that accumulates α-syn aggregates.
3.1.2. Detection of α-syn aggregates in cells

Detection and quantification of protein aggregation in cells and, particularly, distinguishing between soluble and insoluble conformations of proteins in a biologically complex cellular environment, is experimentally challenging. Many techniques are available to analyze protein aggregation, but few directly monitor protein aggregation in cells. Separation techniques, including size exclusion chromatography and ultracentrifugation, can be used to quantify the amount of soluble and insoluble α-syn, but require cell lysis and protein purification for analysis (den Engelsman et al., 2011). For example, cell lysates can be separated into detergent soluble and insoluble fractions and analyzed by polyacrylamide gel electrophoresis followed by western blotting to evaluate the relative abundance of α-syn in each fraction (Dyllick-Brenzinger et al., 2010; Ko et al., 2008; Lee et al., 2002). However, these methods are time consuming and not amenable to high throughput applications.

Microscopy based techniques that rely on the use of protein specific antibodies or fluorescent probes have been widely used to image protein aggregation in cells (den Engelsman et al., 2011). Fusion of α-syn to a reporter protein such as the green fluorescent protein (GFP) facilitates detection of α-syn in cells (McLean et al., 2001; Schwach et al., 2010). Changes in GFP fluorescence does not necessarily correlate with the extent of α-syn misfolding and aggregation. Thus, protein aggregation can be verified within the cell when reporter proteins are used in combination with molecular probes of protein aggregation, such as thioflavins
Thioflavin T (ThT), a benzothiazole molecule that displays minimal fluorescence in aqueous media and enhanced fluorescence when bound to amyloid aggregates, has been extensively used to probe and quantify fibril formation (Antony et al., 2003; Biancalana and Koide, 2010). Particularly, ThT has been used to characterize the structure of α-syn fibrils and to investigate the aggregation kinetics of different purified α-syn mutants (Conway et al., 1998, 2000a), which is crucial to elucidate the molecular mechanisms of PD pathogenesis.

The main drawback associated with the use of ThT to detect protein aggregates in cells is its green fluorescence emission, which overlaps with the intrinsic fluorescence properties of other cellular components, such as flavins or reduced NAD(P)H (Viegas et al., 2007). Aldehyde-containing reagents typically used to fix cell and tissue samples generate an autofluorescent background signal with spectral properties similar to ThT (MS Viegas, 2009). Furthermore, polyphenols, such as curcumin and quercetin, which inhibit aggregation in vitro, present strong absorptive and fluorescent properties that overlap with ThT photoluminescence and may compete with ThT for binding to fibrillar binding sites (Hudson et al., 2009). In summary, there is an urgent need for fibrillization responsive probes with large stokes shifts and red-shifted fluorescence emissions, which display low background signal and could be used as alternatives to ThT when other commonly used green fluorescent reporters are present. The development of tools to monitor cellular aggregation of amyloidogenic proteins, in turn, will provide new avenues to
study the cellular pathogenesis of numerous human diseases that result from deposition of proteinaceous aggregates (Dobson, 2003).

Ruthenium(II) dipyridophenazine (DPPZ) complexes have been extensively studied as luminescent DNA probes on the basis of their strong binding to double stranded DNA (Erkkila et al., 1999; Murphy and Barton, 1993). Ruthenium(II) complexes do not exhibit luminescence in aqueous solutions; however, integration of the DPPZ ligand between the base pairs of DNA results in a strong luminescent signal (Friedman et al., 1990; Jenkins et al., 1992). Given the remarkable similarity between the structure of double stranded DNA and amyloid-like fibrils, it was proposed that ruthenium (II) DPPZ complexes could likewise intercalate between stacked cross beta sheets and emit luminescence. Although amyloidogenic proteins do not share sequence homology, the secondary structure of amyloid fibrils are remarkably similar (Sunde et al., 1997). Ruthenium (II) DPPZ complexes were found to bind to a number of fibrillization-prone proteins, including bovine serum albumin (Svensson et al., 2011; Tan et al., 2008), amyloid beta (Cook et al., 2011), and α-syn (Cook et al., 2012b). Specifically, fibrilization of purified α-syn was detected in real time using the ruthenium(II) DPPZ complexes, [Ru(bpy)₂dppz]²⁺ (bpy = 2,2'-bipyridine) and [Ru(phen)₂dppz]²⁺ (phen = 1,10-phenanthroline) (Cook et al., 2012b). The luminescent properties of ruthenium (II) DPPZ complexes are favorable over traditional aggregation probes, such as ThT because they have red-shifted fluorescence emissions that do not overlap with commonly used green fluorescent reporters, large Stokes shifts (180 nm), and long
photoluminescence lifetimes, making these complexes an ideal probe in highly fluorescent environments in cells.

In this study, a cell culture model system was generated to study α-syn aggregation and [Ru(phen)$_2$dppz]$^{2+}$ was validated as a molecular probe to detect α-syn aggregation in cells. Monocolonal populations of human neuroglioma cells stably overexpressing α-syn fused to GFP were isolated and α-syn-GFP was found to accumulate into aggregates. Treatment with small molecule inhibitors of the proteasome was also shown to enhance α-syn aggregation, confirming that α-syn aggregation is influenced by changes in the protein quality control system as observed in PD cells. Furthermore, the use of the [Ru(phen)$_2$dppz]$^{2+}$ complex to detect α-syn aggregation was investigated in this cell model system in collaboration with the Marti research group (Rice University). An increase in [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence was observed under conditions that induce protein aggregation, such as inhibition of proteasomal degradation. We also demonstrated colocalization of α-syn-GFP and [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence in cells, indicating a correlation between the intensity of [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence and the formation of α-syn aggregates.

In summary, a cell-based model system was established that can be utilized to monitor the accumulation of α-syn aggregates and to study how modulating protein quality control mechanisms alters α-syn accumulation and aggregation. [Ru(phen)$_2$dppz]$^{2+}$ was also demonstrated as a molecular probe for the detection of α-syn aggregation in cells, thereby providing a novel and much needed tool to
quantify α-syn aggregation and to study the cellular pathogenesis of α-syn and potentially other amyloidogenic proteins involved in protein deposition diseases.

3.2. Results

3.2.1. Generation of an in vitro model system of Parkinson’s disease based on the overexpression of α-synuclein

To study α-syn aggregation in cell cultures, stable cell lines that express α-syn fused to GFP were generated. The cDNA encoding wild type α-syn was generated by assembly PCR and cloned into pcDNA™6.2/C-EmGFP-DEST using the Gateway® cloning technology (Invitrogen). The resulting plasmid, pcDNA6.2/α-syn-EmGFP, contains a C-terminal fusion of α-syn to EmGFP, a variant of the Enhanced Green Fluorescent Protein (EGFP) that has been optimized for mammalian expression. The use of α-syn-GFP fusion as a reliable reporter for disease-associated phenotypes has been previously established (McLean et al., 2001; Pandey et al., 2006; Schwach et al., 2010). H4 cells were transfected with pcDNA6.2/α-syn-EmGFP and stable monoclonal cell lines were isolated by subculturing the cells to 0.5 cell/well in 96-well plates in medium supplemented with Blasticidin (Figure 3.1). GFP-positive clones were expanded for further characterization of α-syn-GFP expression in monoclonal populations.
Figure 3.1: Schematic representation of the procedure used to generate a monoclonal stable cell line expressing α-synuclein fused to GFP.
To characterize \( \alpha \)-syn-GFP expression in the stable monoclonal populations, GFP fluorescence of H4 cells expressing \( \alpha \)-syn-GFP was first analyzed by flow cytometry. GFP fluorescence was quantified by calculating the percentage of cells that exhibit GFP fluorescence within each monoclonal population, the mean GFP fluorescence intensity of each clone, and the relative standard deviation of GFP fluorescence (Figure 3.2 and Table 3.1). The average GFP fluorescence intensity of polyclonal H4/\( \alpha \)-syn-GFP cells was found to be 12,738 ± 125.8% and displayed a bimodal frequency distribution, due to the presence of a population of untransfected cells (left) and a population of transfected, GFP-positive cells (right) (Figure 3.2a; gray plot). A series of monoclonal populations displaying a range of GFP fluorescence intensities were isolated (Figure 3.2a). Clone 2 exhibited the most desirable properties – high GFP fluorescence with a small relative standard deviation. The average GFP fluorescence intensity of clone 2 was found to be 27,263 ± 60.4% with 99.2% of the total cell population displaying positive GFP fluorescence greater than untransfected H4 cells (Figure 3.2b and Table 3.1). In comparison, clone 4 was found to exhibit the lowest GFP fluorescence intensity (6,106 ± 152.3%) among all the clones tested, with 90.1% of cells displaying positive GFP fluorescence. Fluorescence microscopy was used to confirm the measurements obtained by flow cytometry. As expected, GFP fluorescence was not detected in microscopy images of parental H4 cells that do not contain the \( \alpha \)-syn-GFP transgene. Microscopy images of clone 2 demonstrated higher GFP fluorescence signal compared to clone 4 (Figure 3.2c), which is consistent with the measurements.
obtained by flow cytometry. In summary, clone 2 presents the highest percentage of cells with positive GFP fluorescence as well as the highest GFP mean fluorescence intensity with the lowest relative standard deviation, suggesting that this cell population is genetically homogeneous. Clone 2 was selected as a cell model system to study α-syn aggregation and is hereafter referred to as “H4/α-syn-GFP cells”.

**Table 3.1: Flow cytometry analysis of stable monoclonal populations of H4 cells expressing α-syn-GFP.**

<table>
<thead>
<tr>
<th></th>
<th>Cells with GFP Fluorescence (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean GFP Fluorescence Intensity (a.u.)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Coefficient of Variation&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal</td>
<td>74.3</td>
<td>12738</td>
<td>125.8</td>
</tr>
<tr>
<td>Clone 1</td>
<td>99.2</td>
<td>21103</td>
<td>58.8</td>
</tr>
<tr>
<td>Clone 2</td>
<td>99.2</td>
<td>27263</td>
<td>60.4</td>
</tr>
<tr>
<td>Clone 3</td>
<td>92.0</td>
<td>7816</td>
<td>129.6</td>
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<tr>
<td>Clone 4</td>
<td>90.1</td>
<td>6106</td>
<td>152.3</td>
</tr>
<tr>
<td>Clone 5</td>
<td>96.8</td>
<td>19065</td>
<td>74.4</td>
</tr>
<tr>
<td>Clone 6</td>
<td>96.9</td>
<td>20273</td>
<td>68.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of cells with positive GFP fluorescent signal. Gating for GFP-positive cells was based on H4 cells not expressing α-syn-GFP used as a control to account for background fluorescence.  
<sup>b</sup> Arbitrary units (a. u.)  
<sup>c</sup> % Coefficient of Variation (%CV) = SD/mean fluorescence intensity *100
Figure 3.2 Characterization of GFP fluorescence in monoclonal populations of H4 cells expressing α-syn-GFP.  

a) Representative fluorescence histograms of polyclonal and monoclonal populations of H4 cells expressing α-syn-GFP. 

b) Mean fluorescence intensity of polyclonal and monoclonal populations of H4 cells expressing α-syn-GFP. The mean GFP fluorescence intensity of each clone was measured by flow cytometry. 

C) Fluorescence microscopy analyses of H4 cells, clone 2 and clone 4. α-syn-GFP fluorescence was imaged at 40X and 100X. DAPI (blue, row 3) was used to visualize the nucleus.
To verify α-syn-GFP expression in H4/α-syn-GFP cells, cellular accumulation of α-syn and GFP were evaluated by Western blot. Accumulation of α-syn and GFP proteins was not observed in non-transfected H4 cells, as expected (Figure 3.3a). Moreover, α-syn and GFP bands detected in H4/α-syn-GFP cells presented similar size and were both located at 41.5 kDa, which corresponds to the predicted size of the α-syn-GFP fusion protein, suggesting that full length α-syn and GFP are expressed at the same level and as a fusion protein. The increase in expression of α-syn and GFP in H4/α-syn-GFP cells compared to H4 cells was also confirmed at the transcriptional level by monitoring α-syn and GFP mRNA expression levels using quantitative RT-PCR (qRT-PCR) (data not shown).

To confirm the expression of α-syn-GFP as a fusion protein, colocalization between α-syn and GFP was tested in H4/α-syn-GFP cells. Cells were incubated with a α-syn-specific antibody and analyzed by confocal microscopy. Fluorescent images (Figure 3.3b, column 1 and 2) were merged and quantified using the ImageJ script, Colocalization Colormap (see Section 3.4.5) to evaluate the extent of colocalization of α-syn and GFP. The results are presented as a colocalization colormap where “hot” colors represent a positive correlation and “cold” colors represent a negative correlation (Figure 3.3b, column 4) (Jaskolski et al., 2005). Colocalization colormaps were then filtered using a color threshold script in ImageJ to display only pixels with positive correlation (Figure 3.3b, column 5). As expected, α-syn and GFP fluorescence were not detected in non-transfected H4 cells. In H4/α-syn-GFP cells, GFP and α-syn colocalization was observed as indicated by the hot colors in the
filtered colocalization colormaps (high colocalization, column 5). These findings confirm that α-syn is stably expressed in H4/α-syn-GFP cells and that α-syn-GFP is expressed as a fusion protein.

Figure 3.3 α-syn-GFP expression in H4/α-syn-GFP cells. a) Western blot analyses of α-syn and GFP in clone 2 cells. GAPDH was used as a loading control. b) Immunofluorescence microscopy analyses of α-syn in clone 2 cells. Images of GFP (green, column 1) and α-syn (blue, column 2) were merged (column 3) and colocalization was evaluated using the NIH ImageJ Colocalization Colormap plugin (column 4). High colocalization represented by hot colors was depicted by filtering colormap images based on hue as described in Section 3.4.5 (pixels 1-60) (column 5). Scale bar represents 20 µm.
3.2.2. α-synuclein accumulates into aggregates in H4/α-syn-GFP cells

To determine if the overexpression of α-syn-GFP in H4/α-syn-GFP cells results in α-syn aggregation, the relative amount of α-syn-GFP that accumulates into insoluble aggregates was evaluated. GFP fluorescence and binding of the ProteoStat® dye, a 488-nm excitable red fluorescent molecule that specifically interacts with denatured proteins within protein aggregates (Shen et al., 2011), was monitored by confocal microscopy. The ProteoStat® dye has red shifted emissions making it suitable for studies in combination with commonly used green fluorescent probes (Shen et al., 2011). Fluorescent images (Figure 3.4a, column 1 and 2) were merged and quantified using the ImageJ script Colocalization Colormap (see Section 3.4.5) to evaluate the extent of colocalization of α-syn-GFP and the ProteoStat® dye, which provides a readout for α-syn-GFP aggregation. GFP fluorescence formed punctate structures in untreated H4/α-syn-GFP cells (column 1), which is consistent with previous reports demonstrating that aggregated α-syn-GFP fusion proteins form small, spherical structures in cells (McLean et al., 2001). ProteoStat® dye fluorescence was also detected in H4/α-syn-GFP cells (column 2), which indicates the presence of aggregated proteins within the cell (Shen et al., 2011). Furthermore, colocalization of GFP and ProteoStat® dye fluorescence signals was observed as shown by the hot colors in the filtered colocalization colormaps (high colocalization, column 5). As expected, GFP fluorescence and ProteoStat® dye binding was not detected in non-transfected H4 cells. These results suggest that α-syn-GFP forms aggregates in the cell culture model system of PD created in this study.
To evaluate whether α-syn-GFP aggregation in H4/α-syn-GFP cells is influenced by changes in the protein quality control system, ProteoStat® dye binding was monitored upon inhibition of proteasomal degradation. α-syn degradation is mediated by the UPS (Bennett et al., 1999) and inhibition of the proteasome leads to the accumulation and aggregation of α-syn (Rideout et al., 2001; Tofaris et al., 2001). Proteasome inhibitors, such as MG-132 and lactacystin (Table A.1), act by blocking the hydrolytic activities in the proteasome (Kisselev and Goldberg, 2001) and have been found to induce protein aggregation in cells (Shen et al., 2011). H4/α-syn-GFP cells were treated with MG-132 (0.5 µM) and lactacystin (2 µM) and imaged as described above. High levels of α-syn aggregation was detected in H4/α-syn-GFP cells treated with proteasome inhibitors as demonstrated by the punctate GFP fluorescence signal (Figure 3.4a, column 1), ProteoStat® binding (column 2), and colocalization of GFP and ProteoStat®. The extent of α-syn aggregation was found to be higher in cells treated with MG-132 or lactacystin than in untreated H4/α-syn-GFP cells, which is consistent with previous reports demonstrating that inhibition of proteasomal degradation promotes α-syn aggregation (Bennett et al., 1999; McLean et al., 2001). These findings suggest that α-syn accumulation in H4/α-syn-GFP cells is sensitive to perturbations in protein homeostasis and modulation of the protein quality control system.
Figure 3.4 α-syn-GFP accumulates into aggregates in H4/α-syn-GFP cells and α-syn aggregation is enhanced by proteasome inhibitors. a) Fluorescence microscopy analyses of H4/α-syn-GFP cells untreated and treated with MG-132 (0.5 µM) or lactacystin (2 µM) for 16 h. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ Colocalization Colormap plugin (column 4). High colocalization represented by hot colors was depicted by filtering colormap images based on hue as described in Section 3.4.5 (pixels 1-60) (column 5). Scale bar represents 20 µm. b) Total protein aggregation in H4/α-syn-GFP cells untreated and treated with MG-132 (0.5 µM) or lactacystin (2 µM) for 16 h. Total protein aggregation was quantified by measuring binding of the ProteoStat® dye by flow cytometry. The APF was calculated as described in Section 3.4.7 (n ≥ 3; p < 0.05).
To confirm that inhibition of the proteasome promotes protein aggregation in H4/α-syn-GFP cells, the extent of total protein aggregation was quantified by calculating the aggregation propensity factor (APF; see Section 3.4.7) of H4/α-syn-GFP cells treated with proteasome inhibitors relative to untreated H4 cells. This experiment measures how small molecule treatments might generally impact cellular protein homeostasis, since a significant percentage of all proteins are aggregation-prone (David et al., 2010; Dobson, 2003). Cells were treated with MG-132 (0.5 µM) and lactacystin (2 µM) and the fluorescence of ProteoStat® dye was measured by flow cytometry. Untreated H4/α-syn-GFP cells displayed an APF of 22.7% compared to untreated H4 cells and treatment of H4/α-syn-GFP cells with MG-132 and lactacystin increased the APF to 33.5% and 39.3%, respectively (Figure 3.4b). These findings confirm the results obtained with fluorescence microscopy and suggest that α-syn aggregation in H4/α-syn-GFP cells is responsive to small molecule modulators of the protein quality control system. Taken together, these results demonstrate that H4/α-syn-GFP cells generated in this study represent a reliable model system to study α-syn aggregation.

3.2.3. Detection of α-syn aggregates using a Ruthenium(II) dipyridophenazine complex

Ruthenium(II) complexes, and particularly, [Ru(phen)_2dppz]^{2+} (Figure 3.5a) bind to purified fibrillar α-syn aggregates and can be used to monitor real time
formation of α-syn fibrils (Cook et al., 2012b). H4/α-syn-GFP cells developed in Section 3.2.1 were used to investigate the use of [Ru(phen)$_2$dppz]$^{2+}$ as a probe to monitor α-syn aggregation in cells. Binding of [Ru(phen)$_2$dppz]$^{2+}$ to α-syn aggregates was first evaluated by quantifying [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence in H4 and H4/α-syn-GFP cells. Cells were incubated with a range of [Ru(phen)$_2$dppz]$^{2+}$ concentrations and [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence intensity was quantified by flow cytometry. As expected, untreated H4 cells (that do not overexpress α-syn) did not display significant [Ru(phen)$_2$dppz]$^{2+}$-photoluminescence. However, a significant increase in [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence signal was observed in H4/α-syn-GFP cells (Figure 3.5b). MG-132 was used to induce aggregation of misfolding-prone proteins as described above. MG-132 treatment resulted in dramatic increase in [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence in H4 cells, suggesting that [Ru(phen)$_2$dppz]$^{2+}$ binds to protein aggregates. The increase in [Ru(phen)$_2$dppz]$^{2+}$ photoluminescence was even more dramatic when MG-132 treatment was applied to H4/α-syn-GFP cells, which is expected to cause accumulation of α-syn aggregates.

The optimal [Ru(phen)$_2$dppz]$^{2+}$ concentration that leads to minimal non-specific binding and background photoluminescence was determined by analyzing microscopy images of H4 and H4/α-syn-GFP cells treated with [Ru(phen)$_2$dppz]$^{2+}$ under the same conditions. Representative images (0.5µM [Ru(phen)$_2$dppz]$^{2+}$) are reported in Figure 3.5c and confirm the results obtained by flow cytometry. Similar to what was observed using the ProteoStat® dye, the aggregates detected in MG-132
Figure 3.5: [Ru(phen)$_2$dpmpz]$^{2+}$ photoluminescence intensity in H4 and H4/α-syn-GFP cells. a) Structure of [Ru(phen)$_2$dpmpz]$^{2+}$. b) Flow cytometry analysis of [Ru(phen)$_2$dpmpz]$^{2+}$ photoluminescence intensity in H4 and H4/α-syn-GFP cells untreated and treated with MG-132 (2 µM) for 16 h. [Ru(phen)$_2$dpmpz]$^{2+}$ fluorescence was measured using a 488-nm laser and a 585/42 band pass filter. The relative fluorescence was calculated by subtracting the background fluorescence of [Ru(phen)$_2$dpmpz]$^{2+}$ in untreated H4 cells. Data are reported as mean ± SD (n ≥ 3; *p < 0.05, **p < 0.005). c) Fluorescence microscopy images of H4 and H4/α-syn-GFP cells untreated and treated with MG-132 (2 µM) for 16 h. Aggregation was detected using 0.5 µM [Ru(phen)$_2$dpmpz]$^{2+}$. 
treated cells using [Ru(phen)$_2$ddpz]$^{2+}$ are granular and dispersed throughout the cell as previously reported for aggregated GFP fusion proteins in cell cultures (McLean et al., 2001).

To demonstrate that [Ru(phen)$_2$ddpz]$^{2+}$ photoluminescence observed under conditions that promote aggregation of cellular proteins is due to [Ru(phen)$_2$ddpz]$^{2+}$ binding to α-syn aggregates, colocalization of GFP fluorescence and [Ru(phen)$_2$ddpz]$^{2+}$ photoluminescence was evaluated in H4 and H4/α-syn-GFP cells. Fluorescence images of H4 and H4/α-syn-GFP cells incubated with the optimal [Ru(phen)$_2$ddpz]$^{2+}$ concentration (0.5µM) (Figure 3.6a, column 1 and 2) were merged and quantified using the ImageJ script Colocalization Colormap (as described previously in the Sections 3.2.1 and 3.4.5). High [Ru(phen)$_2$ddpz]$^{2+}$ photoluminescence signal, which co-localizes with α-syn-GFP as indicated by the hot colors in the filtered colocalization colormaps, was detected in H4/α-syn-GFP cells treated with MG-132 (high colocalization, column 5). Control studies demonstrated that GFP fluorescence is not observed in the channel used to detect [Ru(phen)$_2$ddpz]$^{2+}$ photoluminescence (Figure 3.7), suggesting that the emission spectra of GFP and [Ru(phen)$_2$ddpz]$^{2+}$ do not overlap and that [Ru(phen)$_2$ddpz]$^{2+}$ can be utilized in the presence of other commonly used green fluorescent reporters.
Figure 3.6: Detection of α-syn aggregation in H4 and H4/α-syn-GFP cells. Fluorescence microscopy images of H4 and H4/α-syn-GFP cells untreated and treated with MG-132 (2 µM) for 16 h. Aggregation was detected using [Ru(phen)_2dppz]^{2+} a) or ProteoStat® dye b). Images of α-syn-GFP fluorescence (green, column 1) and aggregates (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Colocalization of GFP and [Ru(phen)_2dppz]^{2+} or ProteoStat® dye were evaluated using the Colocalization Colormap plugin (column 4). High colocalization represented by hot colors was depicted by filtering colormap images based on hue as described in Section 3.4.5 (pixels 1-60) (column 5).
Figure 3.7: \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) spectra do not overlap with GFP. Fluorescence microscopy images of H4 and H4/α-syn-GFP cells untreated and treated with MG-132 (2 µM) for 16 h. \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) was not added to cells in order to determine if GFP fluorescence is observed in the channel used to detect \([\text{Ru(phen)}_2\text{dppz}]^{2+}\), demonstrating lack of spectra overlap.

To further evaluate the use of \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) to detect protein aggregation in cell cultures, \([\text{Ru(phen)}_2\text{dppz}]^2\) was compared to the commercially available ProteoStat® dye. The results obtained using \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) to detect α-syn aggregation described above was found to be similar to what was observed using ProteoStat® dye (Figure 3.6b). However, the overall fluorescence intensity of ProteoStat® dye signal and colocalization of ProteoStat® dye signal with GFP was higher compared to \([\text{Ru(phen)}_2\text{dppz}]^{2+}\). These differences are likely due to different
affinities of these molecules for α-syn aggregates or to different photoluminescence properties of these two molecules.

To quantify [Ru(phen)$_2$dppz]$^{2+}$ binding to aggregated proteins, the APF of H4 and H4/α-syn-GFP cells treated with MG-132 relative to untreated H4 cells was calculated (as described in Section 3.4.7). Cells were treated with MG-132 and binding of [Ru(phen)$_2$dppz]$^{2+}$ (0.5µM) or ProteoStat® dye (1:14,000 dilution) was measured by flow cytometry. In samples treated with [Ru(phen)$_2$dppz]$^{2+}$, the APF of H4 cells treated with MG-132 was 54.8% compared to untreated H4 cells (Figure 3.8). H4/α-syn-GFP cells displayed an APF of 12.3%, which was further enhanced to 58.2% upon MG-132 treatment. In cells treated with ProteoStat® dye, reported here for comparison, MG-132 treatment resulted in a dramatic increase in APF in H4 cells (70.5%) and in H4/α-syn-GFP cells (from 30.9% in untreated cells to 58.7% in MG-132 treated cells), which is similar to what observed using [Ru(phen)$_2$dppz]$^{2+}$. These findings confirm the results obtained with fluorescence microscopy and demonstrate the use of [Ru(phen)$_2$dppz]$^{2+}$ as a molecular probe to monitor protein aggregation in cell culture.
Figure 3.8: Aggregation propensity factor (APF) of H4 and H4/α-syn-GFP cells. H4 and H4/α-syn-GFP cells untreated and treated with MG-132 (2 µM) for 16 h were stained with [Ru(phen)2dppz]2+ (0.5 µM) or ProteoStat® dye and fluorescence was monitored by flow cytometry. The APF was calculated as described in Section 3.4.7. Data are reported as mean ± SD (n ≥ 3; *p < 0.05, **p < 0.01, ***p < 0.005).

3.3. Discussion

Given the central role of α-syn aggregation in the development of PD, a cell culture model system based on the overexpression of α-syn in H4 human neuroglioma cells was generated. α-syn was fused to GFP to enable visualization of α-syn cellular localization. To ensure homogeneous expression, a monoclonal population of H4 cells stably expressing α-syn-GFP was isolated and correlation between GFP fluorescence and α-syn expression level was verified. Furthermore, overexpression of α-syn-GFP in H4/α-syn-GFP cells resulted in the accumulation of α-syn containing aggregates as indicated by colocalization of GFP with aggregate-
responsive Proteostat dye. Finally, proteostasis regulators were shown to influence α-syn aggregation, as demonstrated by the increase in α-syn aggregation observed in H4/α-syn-GFP cells treated with the proteasome inhibitors MG-132 and lactacystin.

To address the current need for molecular probes that bind to specific protein conformations and enable detection of amyloidogenic proteins in cells, a phenanthroline derivative of ruthenium (II) DPPZ complex, ([Ru(phen)₂dppz]²⁺) was investigated. Ruthenium (II) DPPZ complexes exhibit a number of chemical properties that make these compounds an attractive alternative to more widely used organic dyes, including long lifetimes, photostability, large Stokes shifts, and red fluorescence emission. Moreover, their large Stokes shift will allow their simultaneous use with other fluorescent probes emitting in the blue and green spectral regions. In this study, [Ru(phen)₂dppz]²⁺ was used to detect the presence of α-syn aggregates in the cell culture model of PD developed. [Ru(phen)₂dppz]²⁺ photoluminescence signal was shown to correlate with the amount of cellular aggregates and to respond to modulation of the protein quality control system achieved via inhibition of proteasomal degradation (Figure 3.5, Figure 3.6, and Figure 3.8). Furthermore, I hypothesized that the changes in photoluminescence due to protein aggregation in H4 cells treated with MG-132 are a consequence of partially denatured structure of protein aggregates, which expose hydrophobic cavities that [Ru(phen)₂dppz]²⁺ can bind. Binding of [Ru(phen)₂dppz]²⁺ to these hydrophobic sites would favor the excited state population of "bright state" over the
energetically favorable “dark state” of these ruthenium complexes in aqueous solution (Brennaman et al., 2002). [Ru(phen)₂dppz]²⁺ photoluminescence was also quantified by flow cytometry, paving the way for applications of this novel, highly sensitive molecular probe in high-throughput screens for the discovery of therapeutic targets for PD.

In summary, a cell-based model was established to study α-syn aggregation, which will be a valuable tool to screen for genetic targets of the proteostasis network and small molecule proteostasis regulators that prevent α-syn aggregation and promote α-syn clearance. In collaboration with Marti’s research group, we also provided proof-of-principle demonstration of the use of ruthenium (II) dipyridophenazine complexes to monitor aggregation of amyloidogenic proteins in cells. Results from this study open the way to more detailed investigations of the unique photoluminescence properties of this diverse class of metal compounds enabling their use to study protein misfolding diseases and develop therapeutic strategies to prevent the aberrant accumulation of proteinaceous aggregates.

3.4. Materials and Methods

3.4.1. Primers and Plasmids

The cDNA encoding human wild type α-syn (P37840) was generated by assembly PCR using primers reported in Table 3.2. The PCR product was first
cloned into pENTR™11 and then transferred into pcDNA™6.2/C-EmGFP-DEST using Gateway® recombination cloning technology (Invitrogen) according to the manufacture’s protocol. This construct is referred to as pcDNA6.2/α-syn-EmGFP.

Table 3.2: Primers for α-syn assembly

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3.4.2. Cell Lines and Stable Transfections

Human H4 neuroglioma cells (HTB-148, ATCC) were cultured in high glucose DMEM (Thermo Scientific, HyClone) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin Glutamine (PSQ), and maintained at 37 °C and 5% CO₂. Cells were plated 24 h before transfection in medium without PSQ and transfected with pcDNA6.2/α-syn-EmGFP using Lipofectamine2000 according to the manufacturer’s instructions (Invitrogen). After 16 h, the transfection medium was replaced with fresh, complete medium and cells were incubated for an additional 24 h. Stably transfected cells were selected by subculturing cells to a concentration of 2.5 x 10⁴ cells/mL in complete medium with 5 µg/mL Blasticidin S HCl (Invivogen). Blasticidin-resistant cells were plated in 96-well plates at a concentration of 0.5 cells/well to isolate monoclonal populations. GFP fluorescence was detected by microscopy (BC-364 Inverted Epifluorescent Microscope, Jenco) and by flow cytometry (FACSCanto™II, BD Biosciences).

3.4.3. Western blots

H4/α-syn-GFP cells were plated in 10-cm culture dishes at a concentration of 1.0 x 10⁵ cells/ml for 24 h. The total protein content was extracted by incubating the cells in Complete Lysis-M buffer (Roche) according to manufacturer’s protocol. Protein concentrations were determined by Bradford assay; samples were diluted to the same concentration and separated by gel filtration using a 12% SDS-PAGE gel.
Western blot analyses were performed using mouse anti-α-syn (Sigma, 1:12,500), chicken anti-GFP (Anaspec, 1:6000), and rabbit anti-GAPDH (Santa Cruz, 1:10,000) antibodies and appropriate secondary antibodies (HRP conjugated anti-mouse, anti-chicken and anti-rabbit (Santa Cruz, 1:10,000). Blots were visualized using Luminata™ Forte Western HRP Substrate (Millipore).

3.4.4. Immunofluorescence Studies

Cells were cultured on acid-washed glass coverslips. Cells were fixed using 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with 8% bovine serum albumin. To detect colocalization of α-syn and GFP, cells were incubated with mouse anti-α-syn antibody (Sigma, 1:1000) for 1 h, washed with 0.1% Tween/PBS, and incubated for 1 h with DyLight 549 goat anti-mouse antibody (Rockland Immunonochemical, 1:500). Images were collected at 100X using a confocal microscope (FluoView FV1000, Olympus) and analyzed using NIH ImageJ software.

3.4.5. Colocalization Analyses

Colocalization in H4/α-syn-GFP cells was evaluated using the Colocalization Colormap script, an ImageJ plugin that calculates the correlation of intensity between complementary fluorescent signals. The results are presented as a colormap where hot colors represent positive correlation and cold colors represent
negative correlation (Jaskolski et al., 2005). Colormaps were analyzed using the ImageJ plugin *Threshold Colour*, to filter RGB images based on hue, saturation, and brightness (http://www.dentistry.bham.ac.uk/landinig/software/software.html). To indicate high colocalization, the hue was filtered to display pixel intensities from 0 to 35 and designated as red pixels. To indicate low colocalization, the hue was filtered to display pixel intensities from 35 to 60 and designated as yellow pixels. Pixels in the hue range from 60 to 255 were considered negative correlation and not evaluated in this study.

### 3.4.6. Quantitative RT-PCR

RT-PCR analyses was conducted as previously described (Wang et al., 2011a). Total RNA was extracted using RNAGEM™ Tissue reagent (ZyGEM) according to the manufacturer’s protocol. cDNA was synthesized from total RNA using qScript™ cDNA SuperMix (Quanta Biosciences) and quantified using a NanoDrop (Thermo Scientific). Quantitative PCR reactions were performed using PerfeCTa™ SYBR Green FastMix (Quanta Biosciences) in a CFX96 Real-Time PCR Detection System (Bio-Rad) with corresponding primers (Table 3.3). Samples were heated for 2 min at 95°C and amplified using 45 cycles of 1 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Analyses were conducted using CFX Manager software (Bio-Rad) and the threshold cycle (C_T) was extracted from the PCR amplification plot. The ΔC_T value was used to describe the difference between the C_T of a target gene and the C_T
of the housekeeping genes *GAPDH* and *ACTB*. The relative mRNA expression level of treated cells was normalized to that of untreated cells: relative mRNA expression level = $2^{\Delta Ct (treated cells) - \Delta Ct (untreated cells)}$.

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### 3.4.7. Aggregation Studies

For microscopy analysis of α-syn aggregation, H4 and H4/α-syn-GFP cells were cultured on acid-washed glass coverslips. Cells were fixed for 30 min using 4% paraformaldehyde, permeabilized for 30 min on ice with 0.5% Triton X-100, and incubated for 30 min with [Ru(phen)$_2$dppz]$^{2+}$ (0.5µM) or with the ProteoStat® Aggregation detection dye (Enzo Life Sciences, 1:3,500) according to the manufacturer’s protocol. Protein aggregation was analyzed by fluorescence microscopy (Olympus Fluoview 1000) using a 458-nm laser and 560-660 nm band-
pass filter to detect \([\text{Ru(phen)}_2\text{dppz}^2\text{]^2+\text{}}\) photoluminescence and the Texas Red filter to detect the ProteoStat® dye.

Protein aggregation was evaluated by measuring fluorescence intensity by flow cytometry (FACSCanto™ II, BD Biosciences) using a 488-nm argon laser and 585/42 band pass filter. H4 and H4/α-syn-GFP cells were fixed, permeabilized, and incubated with \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) (0.5µM) or the ProteoStat® dye (1:14,000) as described above. The aggregation propensity factor (APF) was calculated using the following formula: \(\text{APF} = 100 \times (\text{MFI}_{\text{treated}} - \text{MFI}_{\text{control}}) / \text{MFI}_{\text{treated}}\), where MFI is the mean fluorescence intensity of the aggregation dye and untreated H4 cells were used as the control.

3.4.8. Statistical Analysis

All data are presented as the mean ± SD and statistical significance was calculated using a two-tailed t-test.
Chapter 4

Chemical modulation of Hsp70 expression and activity reduces \(\alpha\)-synuclein aggregation

The objective of this work is to investigate how modulation of the cellular chaperone capacity affects the accumulation and aggregation of aberrant \(\alpha\)-syn species. Specifically, the effect of chemical modulators of Hsp70 expression and activity on \(\alpha\)-syn aggregation was examined in the \textit{in vitro} model system described in Chapter 3.

4.1.1. Heat shock proteins modulate \(\alpha\)-synuclein aggregation

HSPs are highly conserved molecular chaperones that protect cells from proteotoxic stress by preventing protein misfolding and aggregation and by
promoting the degradation of misfolding-prone proteins (Hartl and Hayer-Hartl, 2002) (refer to Chapter 1 for a detailed description). HSPs, ubiquitinated proteins, and components of the UPS accumulate within Lewy bodies, implicating both protein misfolding and UPS dysfunction in the development of PD (McNaught et al., 2001). In vitro studies revealed colocalization of HSPs with insoluble α-syn aggregates (McLean et al., 2002; Muchowski, 2002). Similar evidence was obtained from post mortem analyses of brains from individuals afflicted with dementia with Lewy bodies (McLean et al., 2002), suggesting an attempt of the HSP system to cope with the aberrant load of misfolded α-syn.

The role of Hsp70 in α-syn accumulation and aggregation has been extensively investigated. Hsp70 directly interacts with α-syn, prevents α-syn aggregation, and protects cells from α-syn-induced toxicity (Huang et al., 2006). Overexpression of Hsp70 was reported to suppress the loss of dopaminergic neurons caused by α-syn accumulation in a Drosophila model (Auluck et al., 2002). Consistent with these data, Hsp70 overexpression was shown to prevent α-syn aggregation, promote α-syn clearance, and alleviate α-syn-induced cytotoxicity in mouse models and in cell culture (Klucken et al., 2004). Specifically, Hsp70 inhibits α-syn self-association by interacting with prefibrillar α-syn, thereby converting insoluble α-syn to a soluble species (Dedmon et al., 2005; Huang et al., 2006). HSPs, such Hsp70, are upregulated upon activation of HSF1, which typically occurs as part of the cellular stress response to restore protein homeostasis (Morimoto, 1998). Not
surprisingly, HSF1 overexpression reduced α-syn accumulation and aggregation by enhancing Hsp70 expression (Gestwicki and Garza, 2012; Liangliang et al., 2010).

As discussed in Chapter 1, Hsp40 mediates the formation of Hsp70 complexes with client proteins by regulating Hsp70 ATPase cycle (Kampinga and Craig, 2010; Langer et al., 1992). The Hsp40 homologs Hdj-1 and Hdj-2, were found to localize with Hsp70 in α-syn inclusions and Hdj-1 overexpression was shown to suppress α-syn aggregation in neuroglioma cells (McLean et al., 2002). Furthermore, Hdj-1 overexpression was demonstrated to increase ubiquitination of α-syn and reduce stress induced α-syn aggregation in neuroblastoma cells (Fan et al., 2006).

4.1.2. Chemical modulators of Hsp70

Chemical modulation of Hsp70 has been mainly achieved via upregulation of Hsp70 expression or regulation of Hsp70 ATPase activity. The quinone methide triterpene celestrol induces HSF1 activation, and thus, Hsp70 upregulation, with kinetics similar to heat shock (Westerheide et al., 2004) and was demonstrated to reduce polyglutamine aggregation and toxicity in Huntington’s disease models (Zhang and Sarge, 2007). Geranylgeranylacetone (GGA), a known antiulcer drug, was also reported to upregulate Hsp70 through protein kinase C-mediated phosphorylation of HSF1 (Fujiki et al., 2006; Nagai et al., 2005; Uchida et al., 2006; Yamanaka et al., 2003) and repressed polyglutamine aggregation in a mouse model of spinal and bulbar muscular atrophy (Katsuno et al., 2005). HSF1A, a benzyl
pyrazole-based molecule, directly activates HSF1 by enhancing its trimerization, translocation into the nucleus, and phosphorylation, which results in Hsp70-induced reduction of polyglutamine aggregation and cytotoxicity in cell culture and in a *Drosophila* model of Huntington’s disease (Neef et al., 2010).

Modulators of Hsp70 activity typically regulate the assembly of the Hsp70-Hsp40 complex, which affects the rate of ATP hydrolysis and substrate binding and release (Jinwal et al., 2009; Wisen et al., 2010). Chemical modulation of Hsp70 ATPase activity, for instance, alters Huntingtin aggregation *in vitro*: the dihydropyrimidine derivative SW02 enhanced Hsp70 ATPase activity, resulting in a reduction in Huntingtin aggregation. However, CE12, a structurally related compound, inhibited Hsp70 ATPase activity, thereby promoting the accumulation of Huntingtin aggregates (Chafekar et al., 2012).

Carbenoxolone (CBX), a semisynthetic derivative of glycyrrhizic acid, widely used for the treatment of peptic ulcers (Pinder et al., 1976), presents neuroprotective properties in animal models of ischemia (de Pina-Benabou et al., 2005). Recent evidence showed that CBX activates HSF1 and upregulates Hsp70 levels in cells. However, it is unclear whether CBX treatment also leads to upregulation of other HSPs (Kawashima et al., 2009; Nagayama et al., 2001). In addition, the molecular mechanism involved in the activation of HSF1 induced by CBX treatment is elusive. Evidence suggests that CBX induces membrane potential collapse and reactive oxygen species (ROS) generation by interacting with the respiratory chain in the mitochondria, thereby causing oxidative stress (Salvi et al.,
It was therefore hypothesized that the heat shock response is activated as part of the cellular response to CBX-induced oxidative stress.

**Figure 4.1: Chemical modulation of Hsp70 mediates α-syn aggregation.** CBX upregulates the expression of Hsp70. 115-7c and MAL3-101 modulate Hsp70 activity by inducing activation or inhibition of Hsp70 ATPase activity, respectively.

This study investigated whether treatment with CBX prevents α-syn aggregation in human neuroglioma cells. Using the cell culture model system described in Chapter 3, treatment with CBX was demonstrated to lower α-syn aggregation. Mechanistic studies revealed that CBX treatment activates HSF1 and
thereby upregulates Hsp70. The mechanism of CBX-mediated upregulation of Hsp70 involves mild induction of oxidative stress. However, CBX treatment did not result in cytotoxicity or apoptosis induction under conditions that prevented α-syn aggregation. Additionally, direct modulation of Hsp70 activity, using MAL3-101 (Fewell et al., 2004) and 115-7c (Wisen et al., 2010; Wisen and Gestwicki, 2008), compounds that interact with the nucleotide binding domain of Hsp70 and alter ATPase activity, dramatically affects α-syn aggregation. These findings confirm the role of the Hsp70 machine as a potential therapeutic target for the treatment of PD and perhaps other neurodegenerative diseases associated with protein aggregation.

4.2. Results

4.2.1. CBX treatment prevents α-syn aggregation

In order to evaluate whether treatment with CBX influences the formation of α-syn aggregates, H4/α-syn-GFP cells were treated with CBX and the relative amount of α-syn-GFP that accumulates in insoluble aggregates was evaluated. α-syn aggregation was first investigated in H4/α-syn-GFP cells incubated with a range of CBX concentrations for 16 h by monitoring GFP fluorescence and binding of the ProteoStat® dye, as described in Chapter 3 (Shen et al., 2011). Cells treated with MG-132 (0.5 µM) were used as positive control. Fluorescent images (Figure 4.2, column 1 and 2) were merged and quantified using the ImageJ script Colocalization.
Figure 4.2: CBX decreases α-syn-containing aggregates in H4/α-syn-GFP cells. H4/α-syn-GFP cells untreated or treated with MG-132 (0.5 µM) or CBX (1, 10, 50, or 100 µM) for 16 h were analyzed by fluorescence microscopy. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Colocalization of α-syn-GFP and ProteoStat® dye were evaluated using the Colocalization Colormap plugin (column 4): “hot” colors represent a positive correlation and “cold” colors represent a negative correlation. High colocalization represented by hot colors was depicted by filtering colormap images based on hue as described in Section 3.4.5 (pixels 1-60) (column 5). Scale bar represents 20 µm.
Colormap (see Section 3.4.5) to evaluate the extent of colocalization of \( \alpha \)-syn-GFP and the ProteoStat\textsuperscript{®} dye, which provides a read-out for \( \alpha \)-syn-GFP aggregation. Detection of \( \alpha \)-syn aggregation in H4/\( \alpha \)-syn-GFP cells in the presence or absence of MG-132 was demonstrated by the punctate GFP fluorescence (column 1) and by the hot colors in the filtered colocalization colormaps (high colocalization, column 5). However, \( \alpha \)-syn-GFP aggregation decreased in H4/\( \alpha \)-syn-GFP cells treated with CBX in a concentration dependent manner. Specifically, \( \alpha \)-syn-GFP aggregation was undetectable in H4/\( \alpha \)-syn-GFP cells treated with a concentration of CBX greater than 50 \( \mu \)M, as shown by the lack of colocalization between \( \alpha \)-syn-GFP and the aggregation-specific dye, ProteoStat\textsuperscript{®} (Figure 4.2, rows 5–6).

\( \alpha \)-syn-GFP aggregation was also evaluated by quantifying the extent of colocalization between the dye and \( \alpha \)-syn-GFP in single cells (Table 4.1). MG-132 treatment resulted in an increase in the percentage of cells containing high colocalization (high: 72.2%; low: 83.3%) compared to untreated H4/\( \alpha \)-syn-GFP cells (high: 59.7%; low: 92.8%), as expected (Shen et al., 2011). Treatment with CBX caused a dramatic decrease in the percentage of cells displaying high (5.6%) and low (43.5%) colocalization compared to untreated H4/\( \alpha \)-syn-GFP cells.
Table 4.1: Quantitative analysis of α-syn aggregation in H4/α-syn-GFP cells treated with CBX (ProteoStat® – α-syn-GFP Colocalization)

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>High Colocalization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Low Colocalization&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>59.7 ± 14.1</td>
<td>92.8 ± 11.0</td>
</tr>
<tr>
<td>MG-132</td>
<td>72.2 ± 29.4</td>
<td>83.3 ± 33.3</td>
</tr>
<tr>
<td>CBX*</td>
<td>5.6 ± 9.6</td>
<td>43.5 ± 12.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>The degree of colocalization was determined by filtering colormap images based on hue: high colocalization was defined as pixels 1 to 35 and low colocalization was defined as pixels 35 to 60 (*p < 0.05). The percentage of cells that contained high and low colocalization was calculated by analyzing multiple images obtained from independent experiments and averaged over three experiments. The data are reported as the mean ± SD.

Next, the effect that CBX treatment has on protein aggregation in H4/α-syn-GFP cells was compared to H4 cells that lacked the α-syn-GFP overexpression vector. This experiment was conducted to measure how this treatment might generally impact cellular protein homeostasis, since a significant percentage of all proteins are aggregation-prone (Dobson, 2003). H4 and H4/α-syn-GFP cells were treated and imaged as described above and the average pixel intensity of the ProteoStat® dye was calculated to quantify total protein aggregation. Treatment of H4/α-syn-GFP cells with CBX (50 µM) caused a 2.3-fold decrease in cellular aggregation compared to untreated cells (Table 4.2), consistent with the data in Table 4.1. The average intensity of the aggregation-specific dye detected in H4 cells in the presence or absence of MG-132 (0.5 µM) was similar to that observed in
H4/α-syn-GFP cells under the same conditions. Interestingly, while CBX treatment again decreased aggregation in H4/α-syn-GFP cells (2.3-fold), it caused a mild increase in general protein aggregation in H4 cells (1.6-fold) compared to untreated cells. These results suggest that administration of CBX to otherwise healthy cells can promote some degree of cellular protein aggregation, perhaps due to the action of CBX inhibition on connexins, and subsequent, cell cycle control (Vinken et al., 2011). In contrast, CBX treatment of cells accumulating aberrant misfolded and aggregation-prone proteins (H4/α-syn-GFP cells) exhibits a protective effect.

Table 4.2: Quantitative analysis of total cellular aggregation in H4 and H4/α-syn-GFP cells treated with CBX

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>H4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>H4/α-syn-GFP&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>12.5 ± 2.9</td>
<td>16.3 ± 3.5</td>
</tr>
<tr>
<td>MG-132</td>
<td>15.0 ± 4.4</td>
<td>14.1 ± 2.7</td>
</tr>
<tr>
<td>CBX*</td>
<td>20.3 ± 2.7</td>
<td>7.1 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average pixel intensity of ProteoStat® dye in images of H4 and H4/α-syn-GFP cells untreated and treated with MG-132 (0.5 µM) or CBX (50 µM) using ImageJ as described in Section 4.4.2. Data are reported as mean ± SD (n≥3; *p < 0.005).
Figure 4.3: CBX reduces total protein aggregation and decreases accumulation of insoluble α-syn in H4/α-syn-GFP cells. a) Total protein aggregation in H4 and H4/α-syn-GFP cells untreated or treated with MG-132 (0.5 µM) or CBX (50 µM) for 16 h. Total protein aggregation was quantified by measuring binding of the ProteoStat® dye by flow cytometry. The APF was calculated as described in Section 3.4.7. Data are reported as the mean ± SD (n≥3; *p < 0.05, **p < 0.005). b) Western blot analyses of Triton X-100 soluble and insoluble α-syn and GFP in H4/α-syn-GFP cells treated with MG-132 (0.5 µM) or CBX (50 µM) for 16 h. GAPDH was used as a loading control.

To quantify the effects of small molecule treatment on the extent of total protein aggregation, the aggregation propensity factor (APF) of H4 and H4/α-syn-GFP cells treated with MG-132 (0.5 µM) and CBX (50 µM) was calculated relative to untreated H4 cells (see Section 3.4.7). The fluorescence of ProteoStat® dye relative to untreated H4 cells was measured by flow cytometry (Figure 4.3a). The APF of H4 cells was enhanced to 28.1% upon incubation with MG-132 and to 30.6% upon
incubation with CBX. Untreated H4/α-syn-GFP cells displayed an APF of 67.0% and MG-132 treatment increased the APF to 73.2%. However, CBX treatment caused a dramatic decrease in aggregate dye binding, thus lowering the APF of H4/α-syn-GFP cells to 37.2%. These findings confirm the results obtained with fluorescence microscopy.

α-syn-GFP aggregation in H4/α-syn-GFP cells treated with MG-132 (0.5 µM) or CBX (50 µM) was also confirmed by evaluating the relative accumulation of α-syn in Triton X-100 soluble and insoluble protein fractions by Western blot. Accumulation of soluble α-syn was largely unchanged upon MG-132 treatment but was considerably enhanced by CBX treatment. Consistent with this result, CBX also decreased the amount of insoluble α-syn compared to the untreated control (Figure 4.3b). These results are in agreement with what was observed from the florescence microscopy and flow cytometry studies above, and confirm that CBX treatment reduces α-syn aggregation. CBX treatment did not alter α-syn transcription, as evaluated by qRT-PCR (Figure 4.4) suggesting that CBX mediated reduction in α-syn aggregation is not due to an effect on α-syn expression. Interestingly, α-syn accumulation in the insoluble fraction increased upon treatment with MG-132, in agreement with the data in Figure 4.3a. The apparent net increase in the amount of α-syn-GFP in MG-132-treated cells (i.e., soluble and insoluble fractions) is consistent with a fraction of this protein being targeted for proteasome-mediated degradation (McNaught and Jenner, 2001; McNaught et al., 2001).
Figure 4.4: CBX does not alter the expression of α-syn or GFP in H4 and H4/α-syn-GFP cells. Relative mRNA expression of a) α-syn and b) GFP in H4 and H4/α-syn-GFP cells treated with CBX (50 µM) for 16 h. mRNA expression levels were evaluated by qRT-PCR, corrected for the expression of the housekeeping gene, GAPDH, and normalized to those of untreated cells.

4.2.2. CBX upregulates Hsp70 in H4/α-syn-GFP cells

CBX was previously reported to induce the expression of HSPs, particularly Hsp70, by activating HSF1 (Kawashima et al., 2009). Thus, I asked whether CBX treatment prevents α-syn aggregation by upregulating Hsp70. This study demonstrates that Hsp70 mediates a reduction of α-syn aggregation in H4/α-syn-GFP cells treated with CBX using five independent experiments designed to evaluate Hsp70 expression (Figure 4.5 and Figure 4.6), the effect of Hsp70 activity on α-syn aggregation and solubility (Figure 4.7, and Figure 4.8) and activation of HSF1 (Figure 4.9, Figure 4.10, and Figure 4.11).
The relative mRNA expression levels of Hsp70 (NM_005345) in H4 and H4/α-syn-GFP cells treated with CBX (50 µM) were evaluated by qRT-PCR as previously described (Wang et al., 2011a) and compared to the expression levels of Hsp27 (NM_001540), Hdj1 (Hsp40 (NM_006145)), and Hsp90 (NM_005348). As anticipated, Hsp70 expression was upregulated upon CBX treatment in H4 (2.1-fold) and H4/α-syn-GFP (3.0-fold) cells. CBX treatment, however, did not considerably alter the mRNA expression levels of Hsp27, Hdj1, or Hsp90 in H4 cells and resulted in a modest increase in the mRNA expression levels of Hsp27 (1.7-fold) and Hsp90 (1.9-fold) but not Hsp40 in H4/α-syn-GFP cells (Figure 4.5a). Interestingly, Hsp27, which was previously reported to modulate α-syn induced toxicity in cell culture (Zourlidou et al., 2004), was not found to be considerably upregulated by CBX, suggesting that CBX mediated reduction of α-syn aggregation does not depend on Hsp27. These results are also consistent with previous studies suggesting that CBX-induced expression of HSPs varies depending on cell type and on the concentration of CBX used (Kawashima et al., 2009; Nagayama et al., 2001). The increase in Hsp70 expression upon CBX treatment observed at the transcriptional level was confirmed at the protein level by evaluating the accumulation of Hsp70 by Western blot (Figure 4.5b). Treatment of H4/α-syn-GFP cells with CBX results in a 52% increase in Hsp70 protein (Figure 4.5c), which is comparable to what was observed in cells subjected to heat shock. However, treatment of H4/α-syn-GFP cells with CBX was found not to affect the accumulation of Hdj1, an Hsp40 co-chaperone, which regulates the formation of complexes between Hsp70 and client proteins, and is
Figure 4.5: CBX upregulates Hsp70 in H4/α-syn-GFP cells. 

**a)** Relative mRNA expression of Hsp70, Hsp27, Hdj1, and Hsp90 in H4 and H4/α-syn-GFP cells was measured after treatment with CBX (50 μM) for 16 h. mRNA expression levels were evaluated by qRT-PCR, corrected for the expression of the housekeeping gene, GAPDH, and normalized to those of untreated cells. Data are reported as the mean ± SD (n≥3; *p < 0.05).

**b)** Western blot analyses of Hsp70 in H4/α-syn-GFP cells subjected to heat shock or treated with CBX (50 μM) for 16 h. Actin expression was used as a loading control.

**c)** Quantification of Hsp70 bands detected by western blot in CBX-treated samples. Band analyses and quantifications were conducted using NIH ImageJ software. Data are reported as the mean ± SD (n≥3; *p < 0.05).
thus expected to affect Hsp70-mediated folding (Figure 4.5b-c) (Hartl and Hayer-Hartl, 2002). These data suggest that, among HSPs, Hsp70 might be the limiting factor in modulation of α-syn aggregation and is the primary and perhaps only mediator of the CBX effect on α-syn-GFP aggregation. However, these results do not exclude the possibility that CBX treatment might influence the expression of nucleotide exchange factors, which could alter modulation of α-syn aggregation by the Hsp70 machine.

The effect of CBX on Hsp70 expression was also confirmed by monitoring the activation of the Hsp70 promoter in H4/α-syn-GFP cells treated with CBX. H4/α-syn-GFP cells were transfected with pDRIVE5SEAP-hHSP70 (Invivogen), a vector engineered for the expression of secreted embryonic alkaline phosphatase (SEAP) under the control of human Hsp70 promoter, and were then treated with CBX (100 µM) for 8 h. CBX treatment resulted in a 3.2-fold and a 2.0-fold increase in SEAP expression compared to untreated H4/α-syn-GFP cells and H4 cells, respectively (Figure 4.6a–b). Activation of the Hsp70 promoter induced by heat shock (40-fold increase) is reported here for comparison. These results suggest that moderate upregulation of Hsp70 is sufficient to reduce α-syn-GFP aggregation.
Figure 4.6: CBX induces Hsp70 expression in H4/α-syn-GFP cells. SEAP reporter assay using an expression vector that contains the promoter region of human Hsp70 gene fused to the gene encoding secreted embryonic alkaline phosphatase in a) H4/α-syn-GFP and b) H4 cells untreated or subjected to heat shock or treated with CBX (50 µM). SEAP activity was evaluated by measuring the absorbance of QUANTI-Blue reagent and normalized to untreated H4/α-syn-GFP cells. Data are reported as mean ± SD (n≥3; *p < 0.05).
Figure 4.7: Inhibition of Hsp70 causes α-syn aggregation in H4/α-syn-GFP cells treated with CBX. a) Fluorescence microscopy of H4/α-syn-GFP cells untreated or treated with CBX (50 µM) and/or MAL3-101 (10 µM) for 16 h. Images were analyzed as described in Section 3.4.5. Scale bar represents 20 µm. b) Total protein aggregation in H4/α-syn-GFP cells untreated or treated with CBX (50 µM) and/or MAL3-101 (10 µM) for 16 h. Total protein aggregation was quantified by measuring binding of the ProteoStat® aggregation dye by flow cytometry. The APF was calculated as described in Section 3.4.7. Data are reported as mean ± SD (n≥3; p<0.05, *p<0.01).
To evaluate whether the decrease in α-syn aggregation observed in H4/α-syn-GFP cells treated with CBX depends on Hsp70 activity, ProteoStat® dye binding was monitored upon inhibition of Hsp70 activity using MAL3-101, a compound that blocks the interaction between Hsp40 co-chaperones and Hsp70 and inhibits the stimulation of ATP hydrolysis \textit{in vitro} (Fewell et al., 2004). MAL3-101 was previously shown to inhibit Hsp70 activity in cells and in cell culture systems (Braunstein et al., 2011; Huryn et al., 2011; Patham et al., 2009; Rodina et al., 2007). α-syn-GFP aggregation was examined by evaluating GFP and ProteoStat® dye fluorescence in H4/α-syn-GFP cells treated with CBX (50 µM) and/or MAL3-101 (10 µM). MAL3-101 was found to inhibit the ability of CBX to prevent α-syn-GFP aggregation (Figure 4.7a, compare CBX to CBX+MAL3-101 images). Also, as might be anticipated, MAL3-101 on its own failed to solubilize α-syn-GFP, consistent with the requirement for Hsp70 to mediate this activity.

To quantify the effect of MAL3-101 treatment on CBX-mediated reduction in α-syn aggregation, ProteoStat® dye binding was monitored by flow cytometry in H4/α-syn-GFP cells treated with CBX and MAL3-101, and calculated the APF of cells treated with CBX and MAL3-101 relative to untreated cells. CBX treatment resulted in dramatic decrease in ProteoStat® dye binding (APF = –17%; Figure 4.7b). As expected, MAL3-101 treatment was found to increase ProteoStat® dye binding (APF = 6.3%) compared to untreated cells. The addition of MAL3-101 to CBX-treated cells resulted in an increase in ProteoStat® dye binding (APF = 9.7%). These data are in agreement with the results obtained from the fluorescence microscopy analysis.
(Figure 4.7a) and suggest that MAL3-101 prevents CBX-induced reduction in α-syn aggregation in H4/α-syn-GFP cells. Together, these results confirm that CBX-induced Hsp70 upregulation plays a key role in preventing α-syn-GFP aggregation and that inhibition of Hsp70 by MAL3-101 promotes the formation of α-syn-GFP into insoluble aggregates.

To further explore the effect of chemical modulation of Hsp70 activity on α-syn aggregation, ProteoStat® dye binding was examined upon enhancement of Hsp70 activity. Hsp70 was activated by treating H4/α-syn-GFP cells with 115-7c (UPCMLD00WMAL1-271; PubChem CID 5461551), a small molecule that binds to a region of Hsp70 adjacent to the surface involved in J-domain stimulation and that works in cooperation with Hsp40 co-chaperones to enhance nucleotide hydrolysis in vitro (Wisen et al., 2010; Wisen and Gestwicki, 2008). Treatment with 115-7c favors the accumulation of Hsp70 in the ADP-bound state, which is hypothesized to enhance its affinity for misfolded substrates and prevent aggregation (Chafekar et al., 2012). The specificity of 115-7c binding to Hsp70 was previously demonstrated, validating the use of this compound to investigate Hsp70 activity (Wisen et al., 2010). α-syn-GFP aggregation was tested by monitoring GFP and ProteoStat® dye fluorescence in H4/α-syn-GFP cells treated with a range of 115-7c concentrations for 16 h. Treatment with 115-7c lowered α-syn aggregation at all concentrations tested as shown by the loss of colocalization between α-syn-GFP and the ProteoStat® dye compared to untreated cells (Figure 4.8a). The effect of Hsp70 activation in H4/α-syn-GFP cells treated with CBX was also investigated. To quantify
Figure 4.8: Activation of Hsp70 reduces α-syn aggregation in H4/α-syn-GFP cells. Fluorescence microscopy of H4/α-syn-GFP cells untreated or treated with 115-7c (0.1, 1, and 10 µM) for 16 h. Images were analyzed as described in Section 3.4.5. Scale bar represents 20 µm. b) Total protein aggregation in H4/α-syn-GFP cells untreated or treated with CBX (50 µM) and/or 115-7c (1 µM) for 16 h. Total protein aggregation was quantified by measuring binding of the ProteoStat® aggregation dye by flow cytometry. The APF was calculated as described in Section 3.4.7. Data are reported as mean ± SD (n≥3). c) H4 cells were transfected to express the αsyn-split GFP system and treated with CBX (50µM) and/or 115-7c (1 µM) for 16 h. Fluorescence complementation was evaluated by measuring GFP fluorescence by flow cytometry. Relative fluorescence was calculated by normalizing the fluorescence of treated cells to that of untreated cells. Data are reported as mean ± SD (n≥3).
the effects of 115-7c treatment on CBX-induced decrease of total protein aggregation, ProteoStat® dye binding was monitored by flow cytometry in H4/α-syn-GFP cells treated with CBX and 115-7c, and calculated the APF relative to untreated cells. 115-7c treatment decreased ProteoStat® dye binding (APF = –13.2%) compared to untreated cells, and co-treatment with 115-7c and CBX further reduced ProteoStat® dye binding (APF = –44.4%) (Figure 4.8b). These results confirm the role of Hsp70 in preventing α-syn aggregation and suggest that chemical upregulation of Hsp70 expression and activity both affect α-syn aggregation.

To further confirm that chemical modulation of Hsp70 expression and activity prevents accumulation of α-syn aggregates, a α-syn-split GFP complementation assay was used to test α-syn solubility. In this assay, GFP is cleaved into two unequal size fragments: a large “detector” fragment (GFP<sub>1-10</sub>) and a short β-sheet “sensor” fragment (GFP<sub>11</sub>), which, when fused to a protein of interest, functions as a sensor of protein solubility (Cabantous et al., 2005). In this assay, the sensor fragment was fused to α-syn. Fluorescence complementation occurs only when α-syn is maintained in soluble form and the sensor fragment is accessible to the detector fragment. As a result, GFP fluorescence is a measurement of α-syn solubility and is inversely proportional to α-syn aggregation (Kothawala et al., 2012). A transfection control consisting of a plasmid encoding a red fluorescent protein (mCherry) was included to account for differences in expression levels of α-synGFP<sub>11</sub> or GFP<sub>1-10</sub>, as previously described (Kothawala et al., 2012). The effect of Hsp70 on α-syn-GFP solubility was evaluated by monitoring GFP fluorescence in H4
cells expressing α-syn-GFP\textsubscript{11} and GFP\textsubscript{1-10} and treated with CBX and 115-7c. GFP fluorescence increased in cells treated with CBX (16.4%) and 115-7c (17.0%) and co-treated with CBX and 115-7c (21.7%) (Figure 4.8c). These results confirm that Hsp70 prevents α-syn aggregation by enhancing its solubility.

4.2.3. CBX reduces α-syn aggregation by activating HSF1

Hsp70 and other heat shock proteins are upregulated upon HSF1 activation. In mammalian cells, HSF1 activation is a multistep process that involves the phosphorylation and translocation of HSF1 from the cytoplasm to the nucleus and the formation of large, irregularly shaped HSF1 granules (Morimoto, 1998). HSF1 granules were previously observed in the nuclei of CBX-treated cells, suggesting that CBX causes HSP upregulation by activating HSF1 (Kawashima et al., 2009). Therefore the intracellular localization of HSF1 was evaluated in H4/α-syn-GFP cells treated with CBX. Cells were incubated with the Hoechst 33342 fluorescent stain that allows visualization of the nuclei (Figure 4.9, row 1) and with a fluorescently labeled anti-HSF1 antibody (row 2), and images were analyzed as described above. Under control conditions, HSF1 resided throughout the nucleus in untreated H4/α-syn-GFP cells. Upon heat shock (42 °C for 2 h), HSF1-positive granules formed in the nuclei (arrows in Figure 4.9) are characteristic of HSF1 activation (Jolly et al., 1999). Treatment of H4/α-syn-GFP cells with CBX for 2 and 4 h resulted in the
formation of HSF1 granules similar to those observed upon heat shock, consistent with the notion that the effect of CBX is mediated through HSF1 activation.

Figure 4.9: HSF1 is activated in H4/α-syn-GFP cells treated with CBX. H4/α-syn-GFP cells untreated or subjected to heat shock (42°C for 2 h followed by 4 h incubation at 37°C) or treated with CBX (50 µM for 2 or 4 h) were analyzed by immunofluorescence microscopy to monitor HSF1 localization. The nucleus was detected with Hoechst 33342 fluorescent stain (blue, column 1) and HSF1 was detected using a rabbit anti-HSF1 antibody (red, column 2). Arrows indicate HSF1-positive stress granules. Images were analyzed and merged (column 3) using NIH ImageJ software. Scale bar represents 10 µm.

To demonstrate that Hsp70 induction by CBX and the consequent reduction in α-syn aggregation is HSF1 dependent, H4/α-syn-GFP cells were treated with
KNK-437, a known inhibitor of the heat shock response (Yokota et al., 2000). α-syn-GFP aggregation was examined by evaluating GFP and ProteoStat® dye fluorescence in H4/α-syn-GFP cells treated with CBX (50 µM) and KNK-437 (10 µM). KNK-437 treatment was verified to promote protein aggregation in H4 cells (Figure 4.10a) and resulted in a decrease in α-syn solubility as verified using the α-syn-split GFP complementation (Figure 4.10b). As expected, KNK-437 failed to solubilize α-syn-GFP (Figure 4.11a). Co-treatment with KNK-437 and CBX resulted in an increase in α-syn-GFP aggregation compared to treatment with CBX, suggesting that KNK-437 treatment counteracts the effect of CBX (Figure 4.11a, compare CBX to CBX+KNK-437 images). To quantify the effects of KNK-437 treatment on CBX mediated reduction of α-syn aggregation, ProteoStat® dye binding was monitored by flow cytometry in H4/α-syn-GFP cells treated with CBX and 115-7c, and calculated the APF relative to untreated cells. KNK-437 treatment increased ProteoStat® dye binding (APF = 12.8%) compared to untreated cells. While CBX treatment, as reported above, lowers the ProteoStat® dye binding (APF = −17%), the addition of KNK-437 to cells treated with CBX resulted in an increase in ProteoStat® dye binding (APF = 11.9%) (Figure 4.11b). Together, these results confirm that CBX-induced Hsp70 upregulation is HSF1 dependent and that inhibition of the heat shock response by KNK-437 promotes α-syn aggregation.
Figure 4.10: Inhibition of HSF1 enhances α-syn aggregation and decreases α-syn solubility in H4/α-syn-GFP cells.  
a) α-syn aggregation in H4/α-syn-GFP cells treated with the HSF1 inhibitor KNK-437. H4/α-syn-GFP cells untreated or treated with KNK-437 (1 or 10 µM) for 16 h were analyzed by fluorescence microscopy. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) as described in Section 3.4.5. Scale bar represents 20 µm. 
b) α-syn solubility in H4 cells treated with the HSF1 inhibitor KNK-437. H4 cells were transfected to express the αsyn-split GFP system and treated with KNK-437 (1 or 10 µM) for 16 h. Fluorescence complementation was evaluated by measuring GFP fluorescence by flow cytometry. Relative fluorescence was calculated by normalizing the fluorescence of treated cells to that of untreated cells. Data are reported as mean ± SD (n≥3; *p<0.01).
Figure 4.11: CBX-mediated reduction in α-syn aggregation is dependent on HSF1 activation. a) Fluorescence microscopy of H4/α-syn-GFP cells untreated or treated with CBX (50 µM) and/or KNK-437 (10 µM) for 16 h. Images were analyzed as described in Section 3.4.5. Scale bar represents 20 µm. b) Total protein aggregation in H4/α-syn-GFP cells untreated or treated with CBX (50 µM) and/or KNK-437 (10 µM) for 16 h. Total protein aggregation was quantified by measuring binding of the ProteoStat® dye by flow cytometry. The APF was calculated as described in Section 3.4.7. Data are reported as mean ± SD (n≥3; p<0.05).
4.2.4. CBX induces moderate oxidative stress

CBX induces the collapse of mitochondria membrane potential and production of ROS in neurons (Salvi et al., 2005; Zundorf et al., 2007). Oxidative stress has been repeatedly observed to induce Hsp70 upregulation via HSF1 activation, which, in turn, lowers proteotoxicity caused by ROS damage and protein misfolding (Ahn and Thiele, 2003; Morimoto, 1998; Polla et al., 1996). Hence, it was speculated that the cytoprotective effect of CBX is mediated through induction of oxidative stress. I asked whether CBX treatment induces oxidative stress in H4/α-syn-GFP cells. Oxidative stress was evaluated using dihydrorhodamine 6G, a cell permeable molecule that is oxidized to fluorescent rhodamine 6G (R6G) upon induction of oxidative stress (Qin et al., 2008). H4 and H4/α-syn-GFP cells were treated with MG-132 (0.5 µM) or CBX (50 µM) for 16 h, and R6G fluorescence was measured by flow cytometry. H$_2$O$_2$ treatment, under conditions previously reported to cause oxidative stress (100 µM for 1 h) (Hoyt et al., 1997), was used as a positive control in this experiment. H$_2$O$_2$ and MG-132 resulted in 39.4% and 34.6% increases in R6G fluorescence, respectively, in H4/α-syn-GFP cells (Figure 4.12a). Treatment with CBX resulted in an 11.7% increase in R6G fluorescence. These results suggest that CBX induces mild oxidative stress, which is likely to trigger HSF1 activation and Hsp70 upregulation. An increase in R6G fluorescence was also observed in H4 cells treated with H$_2$O$_2$ (37.4%) and MG-132 (35.7%) under the same conditions (Figure 4.12b). However, incubation of H4 cells with CBX did not result in a significant increase in R6G fluorescence, suggesting that the CBX-triggered increase in general
protein aggregation in H4 cells (Figure 4.3a) arises from another phenomenon. Based on these data, it also cannot be excluded that additional pathways, distinct from induction of oxidative stress, may contribute to CBX mediated activation of HSF1 and Hsp70 upregulation. These results suggest that the effect of CBX might be cell type specific, as previously suggested (Kawashima et al., 2009), and that CBX-mediated induction of oxidative stress is likely to have a protective effect under conditions causing proteotoxic stress such as misfolding and aggregation.

Figure 4.12: CBX induces the generation of intracellular ROS in H4 and H4/α-syn-GFP cells. a-b) ROS generation in (a) H4/α-syn-GFP and (b) H4 cells treated with H2O2 (100 µM) for 1 h and MG-132 (0.5 µM) or CBX (50 µM) for 16 h was quantified by measuring R6G fluorescence by flow cytometry. The change in fluorescence was calculated by subtracting the mean fluorescence intensity of the untreated sample from the mean fluorescence intensity of treated samples, which was normalized to the treated sample to obtain a percent value of R6G fluorescence. Data are reported as mean ± SD (n≥3; *p < 0.05, **p < 0.005).
To determine whether CBX treatment under conditions that induce oxidative stress and prevent α-syn-GFP aggregation causes cytotoxicity and cell death, apoptosis induction was evaluated in H4 and H4/α-syn-GFP cells treated with CBX (50 µM). This was accomplished by monitoring membrane rearrangement (Annexin V binding), which is characteristic of early apoptosis, and membrane fragmentation (propidium iodide (PI) binding), which is characteristic of late apoptosis, as previously described (Wang et al., 2011a; Wang et al., 2011b). Taxol stabilizes microtubules leading to G2/M cell cycle arrest and apoptosis (Bacus et al., 2001) and was thus used as a positive control in this experiment. H4/α-syn-GFP cells treated with taxol (50 nM) presented a 68.7% increase in Annexin V binding compared to untreated cells (Figure 4.13a). Treatment of H4/α-syn-GFP cells with CBX did not cause cytotoxicity, but resulted in a decrease (20.0%) in Annexin V binding. Similar results were obtained after incubating H4 cells with taxol and CBX under the same conditions, which resulted in a 75.3% increase and a 7.2% decrease in Annexin V binding, respectively (Figure 4.13b). Moreover, the small molecules tested caused no considerable changes in the relative PI-positive population in H4 and H4/α-syn-GFP cells (Figure 4.13c–d). These results indicate that CBX treatment does not cause cytotoxicity in H4/α-syn-GFP cells under conditions shown to cause mild oxidative stress and prevent α-syn-GFP aggregation.
Figure 4.13: CBX does not induce apoptosis in H4 and H4/α-syn-GFP cells. a-b) Relative Annexin V-binding affinity in (a) H4/α-syn-GFP and (b) H4 cells treated with taxol (50 nM) and CBX (50 µM) for 16 h. Data are reported as mean ± SD (n≥3; ***p < 0.01). c-d) Relative PI population in (c) H4/α-syn-GFP and (d) H4 cells treated with taxol (50 nM) and CBX (50 µM) for 16 h. Data are reported as mean ± SD (n≥3; *p < 0.05, **p < 0.01).

4.3. Discussion

CBX [3-(3-carboxy-1-oxopropoxy)-11-oxo-olean-12-en-29-oic acid] is a semisynthetic derivative of glycyrrhizinic acid, the active component in licorice root, and is used for the clinical treatment of gastritis and ulcers (Doll et al., 1965). CBX
promotes the healing of gastric ulcers by stimulating mucous secretion and cell proliferation in the stomach (Baker, 1994). CBX increases the concentration of prostaglandins by inhibiting 15-hydroxyprostaglandin dehydrogenase and A13-prostaglandin reductase, thus activating the anti-inflammatory response (Peskar et al., 1976). It was also reported that CBX increases whole body sensitivity to insulin and reduces total cholesterol and glucose production rate in humans (Andrews et al., 2003) by inhibiting 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), which converts cortisol to cortisone and is normally elevated in patients with Metabolic syndrome (Andrews et al., 2003; Sano et al., 2012). Furthermore, CBX binds to connexins, blocking gap junction intercellular communication, which has anticonvulsant and neuroprotective effects in animal models (de Pina-Benabou et al., 2005; Gareri et al., 2004). A CBX cytoprotective function was recently investigated and linked to its ability to enhance the cellular chaperone capacity. Specifically, CBX upregulates Hsp70 in HeLa and human neuroblastoma (A-172) cells through HSF1 activation (Kawashima et al., 2009; Nagayama et al., 2001). However, the molecular mechanism of CBX mediated activation of HSF1 was unclear. CBX induces the collapse of mitochondria membrane potential and production of ROS (Salvi et al., 2005; Zundorf et al., 2007). Hsp70 upregulation via HSF1 activation limits proteotoxicity caused by ROS damage and protein misfolding (Ahn and Thiele, 2003; Polla et al., 1996). Hence, I hypothesized that the cytoprotective effect of CBX is mediated through induction of oxidative stress.
This study investigated whether CBX might alleviate α-syn cytotoxicity by modulating Hsp70. CBX treatment was demonstrated to activate HSF1, thereby upregulating Hsp70, and reduce α-syn-GFP aggregation in H4/α-syn-GFP cells. This study also demonstrated that enhancement of Hsp70 activity increased α-syn solubility, and that this increase in α-syn solubility was further enhanced in cells treated to simultaneously upregulate Hsp70 expression and activate its function. Under conditions shown to reduce α-syn-GFP aggregation, CBX was found to induce mild oxidative stress. The induction of oxidative stress caused by CBX could potentially mediate activation of the heat shock response, and, particularly, upregulation of Hsp70. Furthermore, CBX reduces toxicity associated with α-syn overexpression, suggesting that i) CBX-mediated induction of oxidative stress does not cause induction of apoptosis, and ii) CBX-mediated reduction of α-syn aggregation alleviates α-syn toxicity.

In untransfected H4 cells, used here as non-diseased control cells, CBX treatment caused a low increase in total protein aggregation. In addition, Hsp70 was mildly upregulated and no oxidative stress or cytotoxicity was observed. These results suggest that the effect of CBX might be cell type specific, as previously suggested (Kawashima et al., 2009), and that CBX-mediated induction of oxidative stress has a protective effect under conditions causing proteotoxic stress such as misfolding and aggregation.

In summary, CBX-induced HSF1 activation and Hsp70 upregulation reduces α-syn-GFP aggregation and α-syn-associated toxicity in a synucleinopathy model.
Additionally, this study demonstrates, for the first time, that chemical modulation of Hsp70 activity, investigated using the Hsp70-specific modulators MAL3-101 and 115-7c, impacts α-syn aggregation, thereby validating the use of chemical modulators of Hsp70 expression and activity to reduce the accumulation of α-syn aggregates. Results from this study thus provide a proof-of-principle demonstration that chemical modulation of the Hsp70 machine is a viable therapeutic strategy for the treatment of diseases characterized by α-syn misfolding and deposition. Considering the limited penetration of CBX into the blood brain barrier (Leshchenko et al., 2006), these findings motivate the search for safer and more effective CBX analogs for the treatment of PD and potentially other protein misfolding diseases caused by deposition of proteinaceous aggregates.

4.4. Materials and Methods

4.4.1. Reagents and Cell Cultures

Carbenoxolone (CBX) was purchased from Sigma-Aldrich, KNK-437 was from EMD Millipore, and taxol was from Fisher Scientific. MAL3-101 and 115-7c were generous gifts from Dr. Jeffrey L. Brodsky and Dr. Peter Wipf (University of Pittsburgh, Pittsburgh, PA)

H4 cells stably transfected for the expression of α-syn-EmGFP (H4/α-syn-GFP) were generated as previously described in Chapter 3. Human H4 neuroglioma
(HTB-148, ATCC) and H4/α-syn-GFP cells were cultured in high glucose DMEM (Fisher Scientific) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin glutamine, and maintained at 37 °C and 5% CO₂.

4.4.2. Aggregation Studies

Aggregation studies were conducted as described in Sections 3.4.5 and 3.4.7.

4.4.3. Cell Fractionation

Detergent solubility and cell fractionation was conducted as previously described (Klucken et al., 2004). H4/α-syn-GFP cells were plated in 10-cm culture dishes at concentration of 1.0 x 10⁵ cells/mL and treated with small molecules for 24 h. The soluble protein fraction was extracted by incubating the cells in Complete Lysis-M Buffer (Roche) supplemented with 1% Triton X-100 on ice with gentle agitation for 30 min followed by centrifugation at 15,000 x g for 60 min at 4 °C. The pellet was resuspended in Complete Lysis-M Buffer supplemented with 2% SDS and 8M urea and sonicated to collect the insoluble protein fraction.
4.4.4. Western blots

Western blot analyses of Triton X-100 soluble and insoluble fractions were performed as described in Section 3.4.3.

Western blot analyses were performed using mouse anti-α-syn (Sigma, 1:12,500), chicken anti-GFP (Anaspec, 1:6000), and rabbit anti-GAPDH (Santa Cruz, 1:10,000) antibodies and appropriate secondary antibodies (HRP conjugated anti-mouse, anti-chicken and anti-rabbit (Santa Cruz, 1:10,000)). Blots were visualized using Luminata™ Forte Western HRP Substrate (Millipore).

H4/α-syn-GFP cells were plated in 10-cm culture dishes at a concentration of 1.0 x 10^5 cells/mL and subjected to heat shock (42°C for 2 h followed by 4 h incubation at 37°C) or treated with small molecules for 24 h. Protein was extracted by incubating the cells in TNT buffer (50mM Tris pH 7.2, 150 mM NaCl, 1% Triton X-100) on ice with gentle agitation for 30 min followed by centrifugation at 15,000 x g for 15 min at 4 °C. Total protein of each lysate was determined using BCA Protein Assay Reagent (Thermo Scientific, Pierce). Equal protein amounts, totaling 10 µg for each treatment, were separated using a 10% polyacrylamide gel. Western blot analyses were performed using mouse anti-Hsp70 (StressMarq, 1:1000), mouse anti-Hdj1 (StressMarq, 1:2000), and rabbit anti-beta actin (Cell Signaling, 1:2500) and the appropriate HRP conjugated secondary antibody (anti-mouse and anti-rabbit, Cell Signalling, 1:5000). Blots were visualized using SuperSignal WestPico
Chemiluminescent substrate (Thermo Scientific, Pierce) and quantified using ImageJ software (NIH).

4.4.5. Quantitative RT-PCR

RT-PCR was conducted as described in Section 3.4.6 using the primers reported in Table 4.3.

Table 4.3: Heat shock protein primer sequences used in qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Code</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
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<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>GTCGGAGTCAACGGATT</td>
<td>AAGCTTCCGTTCCTCAG</td>
</tr>
<tr>
<td>Hsp27</td>
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<td>AAGTTTCCTCCTCCCTGTCC</td>
<td>CGGGCTAAGGCTTTACTTGG</td>
</tr>
<tr>
<td>Hdj1</td>
<td>NM_006145</td>
<td>CGCCGAGGAGAAGTTC</td>
<td>CATCAATGTCCATGCTTT</td>
</tr>
<tr>
<td>Hsp70</td>
<td>NM_005345</td>
<td>GGAGCCGGAGAAGTACA</td>
<td>GCTGATGATGGGGTCTACA</td>
</tr>
<tr>
<td>Hsp90</td>
<td>NM_005348</td>
<td>GATAAACCCTGACCATCC</td>
<td>AAGACAGGAGCGCCATTTCA</td>
</tr>
</tbody>
</table>
4.4.1. SEAP Reporter Assay

Activation of the promoter region of human Hsp70 was monitored by measuring the accumulation of secreted embryonic alkaline phosphatase (SEAP) in the medium of H4 and H4/α-syn-GFP cells transfected with pDRIVE5SEAP-hHSP70 (Invivogen) and treated with CBX. H4 and H4/α-syn-GFP cells were plated in 24-well plates at 1 x 10^5 cells/mL and grown for 16 h. Cells were transfected with 0.3 µg of pDRIVE5SEAP-hHSP70 using Lipofectamine 2000 (Invitrogen). After 16 h, the transfection medium was replaced with fresh medium and cells were treated with CBX (100 µM), or heat shock (42°C for 2 h followed by 4 h incubation at 37°C). The medium was collected and centrifuged for 5 min at 10,000 rpm. SEAP activity was analyzed using QUANTI-Blue according to manufacturer’s instructions (Invivogen) and light emission was measured at OD_{620} using a microplate reader (BioTek Synergy HT).

4.4.2. Immunofluorescence Studies

Immunofluorescence studies were conducted as described in Section 3.4.4 using rabbit HSF1 antibody (Enzo Life Sciences, 1:1000) and DyLight 549 goat anti rabbit antibody (Rockland Immunocchemical, 1:500).
4.4.3. Split GFP Assay

The split GFP assay was conducted as previously described (Kothawala et al., 2012). H4 cells were plated in 6-well plates and incubated for 24 h at 37°C. The media was removed and replaced with fresh media containing 0.33 µg of pCMV-mGFP/αsyn-GFP11, and 0.67 µg of pcDNA4/TO/GFP1-10 per well and transfected using Lipofectamine 2000 (Invitrogen). Transfection reactions were incubated for 16 h and fluorescence was measured using a flow cytometer (FACSCanto™ II, BD Biosciences).

4.4.4. Toxicity Assay

Induction of apoptosis was measured as previously described (Wang et al., 2011b). H4 and H4/α-syn-GFP cells were treated with taxol (50 nM) and CBX (50 µM) for 16 h at 37 °C. Cells were collected and resuspended in 100 µL of 1X binding buffer (BD Biosciences). Cell toxicity was tested by incubating samples with 5 µL of Annexin V-Cy5 (BD Biosciences) and 5 µL of PI for 20 min in the dark at room temperature. Samples were diluted with 400 µL 1X binding buffer and analyzed by flow cytometry (FACSCanto™ II, BD Biosciences) with a 533-nm Helium Neon laser for Cy5 fluorescence.
4.4.5. Measurement of intracellular ROS generation

Dihydrorohodamine 6G was used to measure oxidative stress as previously described (Qin et al., 2008). Cells were cultured in medium containing MG-132 (0.5 µM) or CBX (50 µM) for 16 h at 37 °C. Samples treated with 100 µM hydrogen peroxide (H$_2$O$_2$) for 1 h at 37 °C were used as positive control. Cells were washed with PBS and incubated with 5 µM dihydorohodamine 6G (Anaspec) in serum-free DMEM for 30 min at 37 °C. Cells were collected in PBS, centrifuged at 300 x g for 5 min, and washed with PBS. R6G fluorescence was analyzed by flow cytometry using a 488-nm argon laser and 585/42 band pass filter.
Chapter 5

Genetic and chemical modulation of the autophagy-lysosomal system enhances clearance of \(\alpha\)-synuclein aggregates

The objective of this work is to investigate how modulation of autphagic clearance affects accumulation and aggregation of aberrant \(\alpha\)-syn species. Specifically, genetic and chemical approaches were used to activate a master regulator of the autophagy-lysosomal system (the transcription factor EB) and monitored \(\alpha\)-syn aggregation in the \textit{in vitro} model system described in Chapter 3.

The two main cellular quality control system that mediate protein degradation are the ubiquitin-proteasome system (UPS) and autophagy (Wong and Cuervo, 2010) (refer to Chapter 1 for a detailed description). The UPS provides the primary route for degradation of short-lived and soluble misfolded proteins
A reduction in proteasome activity appears to be linked to the accumulation of misfolded and aggregated α-syn (Rideout et al., 2001) and genetic mutations in UPS components have been associated with reduced UPS activity and neurodegeneration in familial forms of PD (McNaught et al., 2001). Autophagy provides an alternative pathway for protein degradation. Primarily responsible for mediating the degradation of long-lived proteins by the lysosome (Wang and Klionsky, 2003), autophagy plays a key role in promoting clearance of misfolded and aggregated α-syn (Cuervo et al., 2004; Webb et al., 2003). The UPS and autophagy mediate coordinated and complementary roles, which become particularly critical under conditions of proteotoxic stress (Nedelsky et al., 2008). Not surprisingly, recent evidence suggests that adaptive or pharmacologically induced activation of autophagy may restore protein homeostasis when the UPS capacity is insufficient or compromised (Iwata et al., 2005; Pan et al., 2008b; Pandey et al., 2007; Rideout et al., 2004).

Alterations in the autophagy pathway have been specifically observed in association with development of PD. Autophagy activity generally declines with age and autophagy markers are found to be decreased in postmortem brain tissues from PD patients (Crews et al., 2010; Cuervo et al., 2005), suggesting a link between reduced autophagic clearance and accumulation of aggregated α-syn. α-syn transgenic mice are characterized by lowered autophagic activity and progressive neurodegeneration (Crews et al., 2010). These phenotypes can be rescued by upregulating essential components of the autophagy system, such as Beclin-1, Atg7,
and Rab1a (Crews et al., 2010; Spencer et al., 2009; Winslow et al., 2010). Pathogenic variants of α-syn were also reported to block protein translocation into the lysosome and reduce α-syn degradation by CMA (Cuervo et al., 2004). Genetic mutations in the lysosomal protein ATP13A2 is linked to the development of familial PD (Di Fonzo et al., 2007; Park et al., 2011; Ramirez et al., 2006). Loss of ATP13A2 function resulted in lysosomal dysfunction, accumulation of α-syn, and cytotoxicity in primary neurons (Usenovic et al., 2012). Interestingly, evidence also suggests an increased susceptibility to α-syn aggregation in diseases characterized by lysosomal dysfunction, such as Gaucher and Niemann-Pick disease, underscoring the role of the lysosomes in mediating autophagic clearance of α-syn (Saito et al., 2004; Tayebi et al., 2001; Varkonyi et al., 2003). Taken together, these studies point to the important role of autophagy in mediating clearance of α-syn and led us to hypothesize that \textit{enhancement of autophagic clearance could ameliorate the phenotypes associated with accumulation of α-syn aggregates}, thereby providing a therapeutic strategy for the treatment of PD.

Novel insights into the mechanisms of autophagy regulation emerged with the recent discovery that the transcription factor EB (TFEB) controls the coordinated activation of the CLEAR (Coordinated Lysosomal Expression and Regulation) network (Sardiello et al., 2009; Settembre et al., 2011). TFEB regulates lysosome biogenesis and proteostasis (Sardiello et al., 2009; Song et al., 2013), as well as autophagosome formation and autophagosome-lysosome fusion, thereby promoting cellular clearance (Settembre et al., 2011). The regulatory mechanism of
TFEB induced activation of the CLEAR network involves a lysosome to nucleus signaling mechanism where transduction of the lysosomal status is mediated by TFEB activation and subsequent nuclear localization (Martina et al., 2012; Rocznia-Ferguson et al., 2012; Settembre et al., 2012). Not surprisingly, enhancing TFEB expression and activation restores lysosomal proteostasis in diseases characterized by lysosomal dysfunction, such as Gaucher disease and Tay–Sachs disease (Song et al., 2013).

Recent evidence suggests a role of TFEB in mediating clearance of aggregated proteins that are normally degraded through autophagy. Specifically, overexpression of TFEB was found to decrease the accumulation of polyglutamine-containing huntingtin aggregates in a rat striatal cell model of Huntington’s disease (Settembre et al., 2011) and reduce Huntingtin aggregate formation in Neuro2a cells subjected to oxidative stress (Tsunemi et al., 2012). Furthermore, overexpression of TFEB was shown to reduce neurodegeneration in in vitro and in vivo models of PD by restoring lysosome levels and increasing autophagic clearance (Decressac et al., 2013; Dehay et al., 2010). Decressac et al., reported that the presence of oligomeric α-syn species was reduced in α-syn transgenic mice in which TFEB is overexpressed, suggesting that TFEB plays a role in reducing the accumulation of aggregated α-syn. However, the molecular mechanisms underlying TFEB-mediated clearance of aggregated α-syn remains uncharacterized.

Based on evidence that α-syn misfolding and aggregation is often linked to inefficient function of cellular quality control mechanisms that regulate degradation
of aberrant proteins and that TFEB is a master regulator of lysosomal biogenesis and autophagy, I hypothesized that TFEB activation might prevent accumulation of α-syn aggregates by enhancing its clearance through the autophagy pathway. This hypothesis was tested by using the cell culture model system described in Chapter 3 (Kilpatrick et al., 2013). Overexpression of TFEB reduced the accumulation of aggregated α-syn. Specifically, the reduced accumulation of α-syn aggregates correlated with TFEB activation and with upregulation of the CLEAR network and the autophagy system. Chemical activation of TFEB using 2-hydroxypropyl-β-cyclodextrin (HPβCD) was also shown to mediate autophagic clearance of aggregated α-syn. These results support the role of TFEB as a therapeutic target for the treatment of PD and potentially other neurodegenerative diseases characterized by protein aggregation.

5.1. Results

5.1.1. TFEB activation promotes clearance of α-syn aggregates

The role of TFEB in modulating α-syn aggregation was investigated using H4/α-syn-GFP cells developed as described in Chapter 3. TFEB fused to a FLAG tag (TFEB-3xFLAG) was overexpressed in H4/α-syn-GFP cells by retroviral transduction and evaluated the relative amount of α-syn-GFP that accumulates in insoluble aggregates. α-syn aggregation was evaluated by monitoring GFP
Figure 5.1: TFEB overexpression results in reduced accumulation of α-syn aggregates. a) Fluorescence microscopy analyses of H4/α-syn-GFP cells transduced to express TFEB-3xFLAG or S142A TFEB-3xFLAG. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Scale bar represents 20 µm. b) Total protein aggregation in H4/α-syn-GFP cells transduced to express TFEB-3xFLAG or S142A TFEB-3xFLAG. Total protein aggregation was quantified by measuring binding of the ProteoStat® dye by flow cytometry. The APF was calculated as described in Section 3.4.7 and normalized to TFEB mRNA expression. Data are reported as mean ± SD (n ≥ 3; p < 0.01). c) Fluorescence microscopy analyses of H4/α-syn-GFP cells treated with control siRNA or TFEB siRNA. Images were analyzed as described in (a). Scale bar represents 20 µm. d) Total protein aggregation in H4/α-syn-GFP treated with control siRNA or TFEB siRNA. Total protein aggregation was quantified as described in (b). Data are reported as mean ± SD (n ≥ 3; p < 0.01).
fluorescence and binding of the ProteoStat® dye, as described in Chapter 3 (Shen et al., 2011). Fluorescence microscopy images of α-syn aggregation in control (non-transduced) H4/α-syn-GFP cells show punctate GFP fluorescence (Figure 5.1a, column 1, green) that colocalizes with the ProteoStat® dye signal (column 2, red), as shown in merged images (column 3, yellow). GFP fluorescence appeared diffuse throughout H4/α-syn-GFP cells that were transduced to induce TFEB overexpression and was found not to colocalize with the ProteoStat® dye signal, suggesting that increasing TFEB expression reduces the accumulation of α-syn aggregates.

TFEB localizes predominantly in the cytoplasm of resting cells and translocates into the nucleus upon activation (Sardiello et al., 2009). To investigate whether the reduction of α-syn aggregates depends on activation of TFEB, α-syn aggregation was evaluated in cells expressing a TFEB variant (TFEB-S142A) that localizes preferentially in the nucleus (Settembre et al., 2011). Overexpression of S142A TFEB via retroviral transduction of H4/α-syn-GFP cells with TFEB-S142A-3xFLAG was found to result in diffuse GFP signal and, importantly, to reduce ProteoStat® dye binding to a larger extent than overexpression of wild type TFEB (WT TFEB). Complete lack of colocalization of GFP and ProteoStat® signal was also observed, suggesting that activation of TFEB prevents accumulation of aggregated α-syn (Figure 5.1a, row 3). The decrease in protein aggregation observed upon overexpression of WT TFEB is likely due to TFEB activation induced by
accumulation of α-syn aggregates, similar to what was previously reported in
disease cells presenting deposition of storage material (Sardiello et al., 2009).

To confirm that TFEB expression and activation levels mediate reduction in
protein aggregation, the extent of total protein aggregation was quantified by
evaluating the aggregation propensity factor (APF; see Section 3.4.7) of H4/α-syn-
GFP cells overexpressing WT TFEB or S142A TFEB relative to non-transduced
control cells (Figure 5.1b). APF values were corrected by TFEB mRNA expression
levels (evaluated by qRT-PCR) to account for differences in transduction efficiencies.
The total protein aggregation was decreased in H4/α-syn-GFP cells expressing WT
TFEB compared to control cells (APF = −5.0%), which further decreased upon
overexpression of S142A TFEB (APF = −10.9%), suggesting that TFEB prevents
accumulation of aggregated proteins.

To confirm the role of TFEB in regulating the accumulation of α-syn
aggregates, the extent of α-syn aggregation was monitored upon silencing TFEB
expression. Small interfering RNA (siRNA) was used to silence TFEB expression in
H4/α-syn-GFP cells and α-syn-GFP aggregation was analyzed by confocal
microscopy. Treatment with TFEB siRNA resulted in 60% reduction in TFEB
expression compared to cells transfected with control siRNA, as determined by qRT-
PCR. Microscopy images of H4/α-syn-GFP cells treated with TFEB siRNA presented
diffuse GFP fluorescence and strong colocalization of GFP and ProteoStat® dye
signals (Figure 5.1c). Interestingly, the APF of cells treated with TFEB siRNA was
found to increase to 81.9% compared to cells treated with control siRNA suggesting
that TFEB plays a key role in regulating accumulation of α-syn aggregation (Figure 5.1d).

**Figure 5.2: TFEB localizes in the nuclei of H4/α-syn-GFP cells.** a) Immunofluorescence microscopy analyses of TFEB subcellular localization in H4/α-syn-GFP cells transduced to express TFEB-3xFLAG or S142A TFEB-3xFLAG. TFEB nuclear localization was monitored using a FLAG-specific antibody and DAPI nuclear stain. Colocalization of DAPI (blue, column 1) and TFEB-3xFLAG (red, column 2) is shown in purple (column 3). Scale bar represents 10 µm. b) Percentage of cells transduced as described in (a) presenting TFEB nuclear localization. Representative fields containing 50-100 cells were analyzed (p < 0.05).

TFEB nuclear localization was evaluated to investigate the correlation between TFEB activation and accumulation of aggregated α-syn. H4/α-syn-GFP cells transduced to overexpress WT TFEB-3xFLAG or TFEB-S142A-3xFLAG were
analyzed using a FLAG-specific antibody and DAPI nuclear stain (Figure 5.2a). Immunofluorescence analyses revealed complete lack of TFEB signal in control cells lacking transgene expression, as expected, and nuclear localization of TFEB in cells overexpressing WT TFEB or S142A TFEB, as indicated by colocalization of anti-FLAG and DAPI signals. TFEB nuclear localization was quantified by calculating the fraction of transduced cells (FLAG-positive) that present nuclear localization of TFEB (Figure 5.2b). TFEB was found to localize in the nucleus of 50.7% of cells expressing WT TFEB and in 78.1% of cells expressing S142A TFEB. TFEB activation was also confirmed by testing the expression of genes that are known targets of TFEB and are upregulated upon TFEB activation. The mRNA levels of representative genes, namely *GBA* (Glucocerebrosidase), *HEXA* (Hexosaminidase A), and *LAMP1* (Lysosome-associated membrane glycoprotein 1), were monitored by qRT-PCR and were found to be significantly upregulated upon overexpression of WT and S142A TFEB (Figure 5.3).

These results, taken together, indicate that the reduction in α-syn aggregation observed upon overexpression of TFEB correlates with the extent of TFEB expression and activation.
Figure 5.3: Overexpression of TFEB upregulates the CLEAR network in H4/α-syn-GFP cells. Relative mRNA expression levels of representative CLEAR network genes in H4/α-syn-GFP cells transduced to express WT TFEB-3xFLAG. GBA, HEXA, and LAMP1 mRNA expression levels were obtained by qRT-PCR, corrected for the expression of the housekeeping genes, GAPDH and ACTB, and normalized to those of untreated cells (dashed line). Data are reported as the mean ± SD (n≥3; p < 0.05, *p < 0.01)).

5.1.2. TFEB-mediated clearance of α-syn involves activation of autophagy

To determine whether the reduced accumulation of α-syn aggregates observed upon TFEB overexpression results from TFEB-mediated activation of autophagy and autophagic clearance of α-syn aggregates, a series of autophagic markers were tested. LC3 (microtubule-associated light chain protein 3) was first monitored in H4/α-syn-GFP cells overexpressing TFEB. LC3 is recruited to autophagosomal membranes (Kabeya et al., 2000) and is widely used as a marker of autophagy induction (Mizushima et al., 2010). H4/α-syn-GFP cells overexpressing
TFEB were first analyzed by confocal microscopy using an LC3-specific antibody to visualize LC3 structures (Figure 5.4a, column 1). As expected, LC3 signal was diffused in control cells, indicating basal autophagic activity. The formation of punctate LC3 structures in cells overexpressing TFEB indicates the accumulation of autophagosomes. These results are consistent with previous reports demonstrating that TFEB activation results in enhanced formation of autophagosomes (Settembre et al., 2011).

The formation of autophagolysosomes was evaluated to verify whether TFEB activation, under conditions that result in reduced accumulation of aggregated α-syn, enhances autophagic clearance (Mizushima and Komatsu, 2011). Autophagolysosomes were detected by evaluating the extent of colocalization between LC3, an autophagosomal membrane protein, and LAMP2, a lysosomal membrane protein (Mizushima et al., 2010). H4/α-syn-GFP cells overexpressing TFEB were incubated with antibodies specific for LC3 and LAMP2 and analyzed by confocal microscopy (Figure 5.4a). Overexpression of TFEB resulted in enhanced colocalization of LC3 (column 1, red) and LAMP2 (column 2, blue), as shown in the merged images (column 3, purple), compared to control cells. The extent of colocalization was quantified by calculating the percentage of pixels exhibiting positive correlation between LC3 and LAMP2 (Figure 5.4b; see Section 5.3.3). LC3-LAMP2 colocalization was found to increase from 3.5% in control cells to 12.8% in cells overexpressing TFEB.
Figure 5.4 TFEB mediates reduction of α-syn aggregates by inducing autophagic clearance.  

a) Immunofluorescence microscopy analyses of LC3 and LAMP2 in H4/α-syn-GFP cells transduced to express TFEB-3xFLAG. Colocalization of LC3 (red, column 1) and LAMP2 (blue, column 2) is shown in purple (column 3). Scale bar represents 20 µm.  

b) Quantification of LC3- LAMP2 colocalization was calculated as described in Section 5.3.4 using randomly selected images containing 30-50 cells obtained from three independent experiments (p < 0.001).  

c) Relative mRNA expression levels of representative genes involved in the autophagy pathway in H4/α-syn-GFP cells transduced to overexpress TFEB. MAPLC3, SQSTM1, BECN1, and UVRAG mRNA expression levels were obtained by qRT-PCR, corrected for the expression of the housekeeping genes, GAPDH and ACTB, and normalized to those of untreated cells (dashed line). Data are reported as mean ± SD (n ≥ 3; p < 0.05, *p < 0.01).  

d) Total protein aggregation in H4/α-syn-GFP cells transduced to express TFEB-3xFLAG and treated with bafilomycin (100 nM). Total protein aggregation was quantified by measuring binding of the ProteoStat® dye by flow cytometry. The APF was calculated as described in Section 3.4.7. Data are reported as mean ± SD (n ≥ 3; p < 0.05).
To confirm that TFEB-mediated reduction in α-syn aggregates parallels activation of autophagy, the expression of genes involved in different steps of the autophagy pathway was tested. H4/α-syn-GFP cells were transduced to express TFEB and the mRNA levels were measured by qRT-PCR (Figure 5.4c). TFEB overexpression resulted in significant upregulation of MAPLC3 (LC3; 2.2-fold), which is involved in the formation of autophagic vesicles as described above, SQSTM1 (p62; 1.7-fold), which is involved in cargo recognition, and BECN1 (Beclin-1; 2.1-fold) and UVRAG (UV Radiation Resistance-Associated Gene; 1.8-fold), which are required for the formation of autophagosomes. Interestingly, MAPLC3, SQSTM1, and UVRAG are known to be direct targets of TFEB (Settembre et al., 2011).

To directly assess whether the decrease in α-syn aggregates depends on TFEB mediated activation of autophagy, the extent of protein aggregation was evaluated in H4/α-syn-GFP cells overexpressing TFEB and treated to block the autophagic flux. Specifically, H4/α-syn-GFP cells transduced with TFEB-3xFLAG were treated with bafilomycin, an inhibitor of vacuolar H+ ATPase (V-ATPase) activity that prevents fusion of autophagosomes with lysosomes (Yamamoto et al., 1998). As expected, bafilomycin treatment increased ProteoStat® dye binding (APF = 39.1%) compared to control cells (Figure 5.4d). The addition of bafilomycin to cells overexpressing TFEB also resulted in an increase in ProteoStat® dye (APF = 5.8%) compared to cells overexpressing TFEB and not treated with bafilomycin (APF = –20.7%). These results indicate that inhibition of downstream steps of the
autophagy pathway (i.e., autophagolysosome formation) prevents TFEB-mediated reduction in protein aggregation.

In summary, these results demonstrate that TFEB activation reduces accumulation of α-syn aggregates in neuroglioma cells overexpressing α-syn-GFP and that autophagy plays a key role in TFEB-mediated clearance of aggregated α-syn.

5.1.3. Pharmacologic activation of TFEB by HPβCD results in clearance of aggregated α-syn

2-hydroxypropyl-β-cyclodextrin (HPβCD), an FDA-approved pharmaceutical excipient commonly used to enhance drug solubility (Rajewski and Stella, 1996), was recently reported to function as an activator of TFEB. Particularly, HPβCD treatment induces TFEB nuclear translocation, upregulation of the CLEAR network, and an increase in autophagic clearance (Song et al., 2014). Additionally, previous studies showed that methyl-β-cyclodextrin (MβCD) reduces α-syn aggregation in rat neuroblastoma cells and in transgenic mice overexpressing α-syn (Bar-On et al., 2006); however, the molecular mechanisms underlying MβCD-induced reduction in α-syn aggregation are unclear. Based on evidence that TFEB activation results in reduced accumulation of α-syn aggregates (Figure 5.1 and Figure 5.2), I hypothesized that cell treatment with HPβCD, by inducing activation of TFEB, could enhance clearance of α-syn aggregates, thereby providing a pharmacologic strategy
to reduce deposition of α-syn aggregates. The formation of α-syn aggregates in H4/α-syn-GFP cells treated with HPβCD was first evaluated by monitoring GFP and ProteoStat® dye fluorescence. Preliminary studies were conducted using a range of HPβCD concentrations to determine the optimal HPβCD dosage that reduces α-syn aggregates without altering cell viability (not shown). Treatment with HPβCD (1 mM) resulted in the appearance of diffuse GFP fluorescence, reduction in ProteoStat® dye binding, and lack of colocalization between GFP and ProteoStat® dye signals (Figure 5.5a, row 2) to an extent similar to what observed upon genetic activation of TFEB (Figure 5.1), suggesting that HPβCD treatment prevents accumulation of α-syn aggregates. HPβCD treatment under these conditions was confirmed not to induce activation of early or late apoptosis, as evaluated by monitoring Annexin V and PI binding (Figure 5.6).

To directly assess whether HPβCD-induced reduction in α-syn aggregates is mediated by TFEB, the effect of HPβCD on α-syn aggregation was evaluated upon silencing of TFEB expression (Figure 5.5a, row 3). Silencing TFEB in HPβCD-treated cells resulted in the reappearance of punctate GFP signal, binding of ProteoStat® dye, and colocalization of ProteoStat® and GFP signals, indicating that TFEB mediates HPβCD-induced reduction of α-syn aggregates. Treatment with control siRNA did not alter HPβCD-induced reduction of α-syn aggregates (Figure 5.7).
Figure 5.5: HPβCD treatment results in clearance of α-syn aggregates. a) Fluorescence microscopy analyses of H4/α-syn-GFP cells treated with HPβCD (1 mM) for 24 h with or without TFEB siRNA. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Scale bar represents 20 µm. b) Total protein aggregation in H4/α-syn-GFP cells treated with control siRNA or TFEB siRNA for 48 h and with or without HPβCD (1 mM) for 24 h. Total protein aggregation was quantified by measuring binding of the ProteoStat® dye by flow cytometry. The APF was calculated as described in Section 3.4.7. The data are reported as mean ± SD (n ≥ 3; p<0.05).

Flow cytometry analyses confirmed that HPβCD treatment lowers the extent of total protein aggregation (APF = -15.7%) (Figure 5.5b). Silencing TFEB in cells treated with HPβCD, on the other hand, resulted in a dramatic increase in ProteoStat® dye binding (APF = 39.6%). These findings confirm that HPβCD treatment reduces the accumulation of α-syn aggregates and that this effect is mediated by TFEB.
Figure 5.6: HPβCD does not induce cytotoxicity in H4/α-syn-GFP cells. a-b) Relative Annexin V-binding affinity (a) and PI population (b) in H4/α-syn-GFP cells treated with taxol (25 nM) and HPβCD (1 mM) for 16 h. Data are reported as the mean ± SD (n≥3; p < 0.05).

Figure 5.7: Treatment with control siRNA does not alter HPβCD-induced reduction in α-syn aggregates Fluorescence microscopy analyses of H4/α-syn-GFP cells treated with control siRNA and HPβCD (1 mM) for 24 h. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Scale bar represents 20 µm.
Figure 5.8: HPβCD treatment induces activation of TFEB in H4/α-syn-GFP cells. 

**a-b)** Immunofluorescence microscopy analysis of TFEB subcellular localization in H4/α-syn-GFP cells treated with HPβCD (1 mM). TFEB nuclear localization was monitored using a TFEB-specific antibody and DAPI nuclear stain. Colocalization of DAPI (blue, row 1) and TFEB (red, row 2) is shown in purple (row 3). Scale bar represents 10 µm. 

**c)** Percentage of HPβCD-treated cells presenting TFEB nuclear localization. Representative fields containing 50-100 cells were analyzed (p < 0.05).

**d)** Relative mRNA expression levels of representative CLEAR network genes in H4/α-syn-GFP cells treated with HPβCD (1 mM) for 24 h. GBA, HEXA, and LAMP1 mRNA expression levels were obtained by qRT-PCR and calculated as described in **Figure 5.4c**. Data are reported as mean ± SD (n ≥ 3; p < 0.01).
To verify whether HPβCD induced reduction in α-syn aggregates involves TFEB activation, TFEB nuclear localization and expression of representative genes that are known targets of TFEB were evaluated. Intracellular localization of TFEB was monitored by confocal microscopy using a TFEB-specific antibody. Microscopy images were taken at various time points after the addition of HPβCD in the culturing medium (Figure 5.8a). TFEB was found to progressively translocate into the nucleus of HPβCD-treated cells. TFEB nuclear translocation, quantified by calculating the fraction of cells that present nuclear localization of TFEB, was found to increase from 25.5% to 71.6% after 24 h of HPβCD treatment (Figure 5.8b and c). Significant upregulation of the TFEB target genes tested (Figure 5.8d), namely GBA (2.1-fold), HEXA (2.3-fold), and LAMP1 (2.1-fold), was also detected confirming that TFEB nuclear translocation induced by HPβCD results in activation of the CLEAR network.

5.1.4. HPβCD-induced activation of TFEB enhances autophagy in H4/α-syn-GFP cells

To determine whether autophagy is involved in HPβCD-mediated reduction of α-syn aggregates, a series of markers of the autophagic pathway was monitored in H4/α-syn-GFP cells treated with HPβCD. To confirm enhancement of the autophagy system in H4/α-syn-GFP cells treated with HPβCD, upregulation of representative genes involved in the autophagy pathway were verified (Figure
5.9a), namely MAPLC3 (1.9-fold), SQSTM1 (2.2-fold), BECN1 (2.2-fold), and UVRAG (1.9-fold).

Activation of autophagy in H4/α-syn-GFP cells treated with HPβCD was confirmed by immunoblotting of LC3 isoforms (Figure 5.9b). HPβCD treatment results in increase in LC3-II, suggesting enhanced formation of autophagic vesicles (Mizushima et al., 2010). The further increase in LC3-II levels observed in cells treated with HPβCD in the presence of the autophagy inhibitor bafilomycin, compared to cells treated only with HPβCD, indicates an increase in autophagic flux. These results confirm that HPβCD treatment induces activation of autophagy in H4/α-syn-GFP cells.

To monitor the formation of autophagosomes and autophagolysosomes, the formation of LC3 puncta and colocalization of LC3 and LAMP2 were evaluated, respectively (Figure 5.9c). Punctate LC3 structures were observed in cells treated with HPβCD (column 1, red), indicating enhanced autophagosome formation, as well as increase in colocalization of LC3 and LAMP2 (column 2, blue) as shown in merged images (column 3, purple), indicating enhanced autophagolysosome formation. LC3-LAMP2 colocalization was found to increase from 3.5% to 13.7% upon treatment with HPβCD (Figure 5.9d).

These results, taken together, demonstrate that treatment of H4/α-syn-GFP cells with HPβCD, under conditions that result in TFEB activation and reduced accumulation of α-syn aggregates, activates autophagy.
Figure 5.9: HPβCD treatment results in autophagic clearance of α-syn aggregates in H4/α-syn-GFP cells. a) Relative mRNA expression levels of representative genes of the autophagy pathway in H4/α-syn-GFP cells treated with HPβCD (1 mM) for 24 h. MAPLC3, SQSTM1, BECN1, and UVRAG mRNA expression levels were obtained by qRT-PCR and calculated as described in Figure 5.4c (p < 0.05). b) Western blot analyses of LC3 isoforms and GAPDH (used as loading control) in H4/α-syn-GFP cells treated with HPβCD (1 mM) for 24 h and quantification of LC3-II bands. Band intensities were quantified with NIH ImageJ software and corrected by GAPDH band intensities (p < 0.05). c) Immunofluorescence microscopy analysis of LC3 and LAMP2 in H4/α-syn-GFP cells treated with HPβCD (1 mM) for 24 h. Colocalization of LC3 (red, column 1) and LAMP2 (blue, column 2) is shown in purple (column 3). Scale bars represent 20 µm. d) Quantification of LC3- LAMP2 colocalization was calculated as described in Section 5.3.4 using randomly selected images containing 30-50 cells obtained from three independent experiments (p < 0.001).
Figure 5.10: Bafilomycin prevents HPβCD-induced autophagic clearance of α-syn. **a)** Fluorescence microscopy analyses of H4/α-syn-GFP cells untreated or treated with HPβCD (1 mM) and/or bafilomycin (100 nM) for 24 h. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Scale bar represents 20 µm. **b)** Total protein aggregation in H4/α-syn-GFP cells untreated or treated with HPβCD (1 mM) and/or bafilomycin (100 nM) for 24 h. Total protein aggregation was quantified by measuring binding of the ProteoStat® aggregation dye by flow cytometry. The APF was calculated as described in Section 3.4.7. Data are reported as mean ± SD (n ≥ 3; p < 0.05).

To directly assess whether the decrease in α-syn aggregates observed in H4/α-syn-GFP cells treated with HPβCD depends on autophagic activity, α-syn aggregation was monitored upon inhibition of autophagy using bafilomycin. α-syn-GFP aggregation was examined by evaluating GFP and ProteoStat® dye fluorescence in H4/α-syn-GFP cells treated with HPβCD and bafilomycin (100 nM). Bafilomycin treatment was found to inhibit the ability of HPβCD to reduce α-syn-GFP...
aggregation (Figure 5.10a, compare HPβCD to HPβCD+bafilomycin images). Flow cytometry analyses conducted to quantify ProteoStat® dye binding confirmed that bafilomycin treatment results in an increase in protein aggregation in HPβCD-treated cells (Figure 5.10b; APF = 36.3%). These results indicate that HPβCD-mediated reduction in α-syn aggregates depends on autophagic clearance.

5.1.5. HPβCD-mediated clearance of aggregated α-syn does not depend on the ability of HPβCD to extract cholesterol

A number of studies demonstrate that cyclodextrins can alter the cellular concentration of cholesterol by extracting cholesterol from the plasma membranes (Christian et al., 1997; Kilsdonk et al., 1995) or by reducing lysosomal cholesterol content (Rosenbaum et al., 2010). Cholesterol depletion from plasma membranes has been demonstrated to affect many cellular processes (Maxfield and Tabas, 2005), particularly, autophagy (Cheng et al., 2006). Therefore, I asked whether the reduction in α-syn aggregates observed in cells treated with HPβCD is due to the ability of HPβCD to alter cellular levels of cholesterol. To address this question, TFEB activation and the accumulation of α-syn aggregates in cells treated with HPβCD-cholesterol inclusion complexes were tested. HPβCD-cholesterol complexes were prepared by saturating HPβCD with cholesterol as previously described (Christian et al., 1997). H4/α-syn-GFP cells were treated with HPβCD (1 mM) or HPβCD-cholesterol complexes (1 mM) and TFEB subcellular localization was
examined by confocal microscopy (Figure 5.11a). Microscopy analyses revealed that HPβCD-cholesterol complexes induce nuclear translocation of TFEB and that the extent of nuclear translocation is comparable to that observed in cells treated with HPβCD that is not saturated with cholesterol. These results suggest that HPβCD-induced activation of TFEB is independent of HPβCD ability to deplete the intracellular levels of cholesterol.

To investigate whether the ability of HPβCD to deplete the intracellular levels of cholesterol affects clearance of α-syn, the accumulation of α-syn aggregates in H4/α-syn-GFP cells treated with HPβCD or HPβCD-cholesterol complexes was evaluated as described above (Figure 5.11b). Cell treatment with HPβCD-cholesterol complexes resulted in reduction of α-syn aggregates as indicated by reduced binding of ProteoStat® dye and lack of colocalization between GFP and ProteoStat® dye signals. Moreover, cell treatment with HPβCD-cholesterol complexes resulted in reduction in α-syn aggregates to an extent comparable to that observed upon treatment with HPβCD. These results suggest that HPβCD-induced activation of TFEB and HPβCD-mediated clearance of α-syn aggregates does not depend on the ability of HPβCD to alter the intracellular concentration of cholesterol.
Figure 5.11: HPβCD-mediated clearance of α-syn aggregates does not depend on the ability of HPβCD to alter cholesterol levels. a) Immunofluorescence microscopy analyses of TFEB subcellular localization in H4/α-syn-GFP cells untreated or treated with HPβCD (1 mM) or HPβCD–cholesterol complexes (1 mM) for 24 h. TFEB nuclear localization was monitored using a FLAG-specific antibody and DAPI nuclear stain. Colocalization of DAPI (blue, column 1) and TFEB (red, column 2) is shown in purple (column 3). Scale bar represents 10 µm. b) Fluorescence microscopy analyses of H4/α-syn-GFP cells untreated or treated with HPβCD (1 mM) or HPβCD–cholesterol complex (1 mM) for 24 h. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Scale bar represents 20 µm.

5.2. Discussion

The accumulation and aggregation of misfolded α-syn is critically linked to the development of PD (McNaught and Olanow, 2003). Upregulation of autophagy was found to prevent the accumulation of α-syn in vitro and in transgenic mice (Crews et al., 2010; Spencer et al., 2009; Winslow et al., 2010). Enhancing
autophagic clearance has been thus proposed as a therapeutic strategy to promote the disposal of aberrant forms of α-syn associated with the development of PD (Rubinsztein et al., 2012).

The recent discovery of TFEB provided a novel target for upregulating clearance of autophagic cargo by the lysosome (Settembre and Ballabio, 2011; Settembre et al., 2011). Indeed, TFEB was found to provide neuroprotection in vivo by restoring lysosomal function and enhancing autophagy (Decressac et al., 2013; Dehay et al., 2010). This study demonstrates that TFEB promotes autophagic clearance of aggregated α-syn (Figure 5.12). Specifically, overexpression of TFEB reduced the accumulation of aggregated α-syn in neuroglioma cells. Additionally, increased expression of TFEB resulted in upregulation of the CLEAR network and autophagy genes, and increased formation of autophagosomes and autophagolysosomes, indicating that reduction in α-syn aggregates correlates with increase in autophagic flux. Chemical inhibition of downstream steps of the autophagy pathway was found to prevent TFEB mediated clearance of aggregated α-syn, suggesting that the observed reduction in α-syn depends on autophagic degradation.

The studies presented herein focus primarily on clearance through macroautophagy; however, the possibility that TFEB also affects chaperone-mediated autophagy and that degradation of misfolded α-syn directly by the lysosome could also contribute to the observed reduction in α-syn aggregates cannot be excluded (Cuervo et al., 2004). Nevertheless, results from this study
identify TFEB as a therapeutic target to reduce the accumulation of aberrant α-syn aggregates and motivate the discovery of chemical activators of TFEB for therapeutic intervention.

Figure 5.12: Schematic representation of the mechanism underlying TFEB-mediated clearance of aggregated α-syn. Upon translocation from the cytoplasm to the nucleus, TFEB regulates the expression of genes involved in lysosome and autophagosome biogenesis, fusion of lysosome and autophagosomes, and clearance of aggregated α-syn. Pharmacological activation of TFEB-can be achieved by exposing cells to HPβCD treatment.

Pharmacological activation of autophagy is a promising therapeutic strategy for the treatment of PD (Decressac et al., 2013; Pan et al., 2008a). Rapamycin, for example, induces autophagy by inhibiting the phosphorylation of the mammalian target of rapamycin (mTOR), a negative regulator of autophagy (Sarbassov et al.,
and was shown to confer neuroprotection in α-syn transgenic mice (Decressac et al., 2013) and promote autophagic clearance of α-syn in PC-12 cells (Webb et al., 2003). Lithium chloride induces autophagy through an mTOR-independent pathway and was also shown to enhance α-syn clearance in vitro (Sarkar et al., 2005). Currently available compounds that activate autophagic clearance, however, lack specificity for the autophagy pathway. A more promising strategy to enhance autophagic clearance may be through activation of TFEB.

In an attempt to identify compounds that enhance autophagic clearance of α-syn by activating TFEB, the response of H4/α-syn-GFP cells to treatment with HPβCD was investigated. The autophagy-activating properties of β-cyclodextrins (βCDs), a family of cyclic oligosaccharides known to deplete cholesterol from biological membranes (Atger et al., 1997; Christian et al., 1997; Kilsdonk et al., 1995) have been previously reported in different model systems (Cheng et al., 2006). Davidson et al. reported evidence of βCD-mediated clearance of cholesterol and lipoproteins in lysosomal storage disorders (Davidson et al., 2009). Our lab recently demonstrated that activation of autophagy and enhanced clearance of autophagic substrates in HPβCD-treated cells is mediated by TFEB (Song et al., 2014). This study demonstrated that HPβCD treatment mediates reduction in α-syn aggregation through activation of TFEB and upregulation of autophagy (Figure 5.12). These results are in agreement with those obtained in cells treated to achieve genetic activation of TFEB. These findings are also in agreement with previously reported evidence demonstrating that βCD treatment results in reduction of α-syn
aggregation (Bar-On et al., 2006; Fortin et al., 2004), suggesting that βCD induced neuroprotection observed in α-syn transgenic mice might be the result of α-syn autophagic clearance mediated by TFEB.

Because βCDs are also known to deplete intracellular cholesterol (Christian et al., 1997; Kilsdonk et al., 1995)—a process that may be crucially involved in the mechanism of βCD-induced autophagy activation—I also asked whether activation of TFEB and subsequent reduction of α-syn aggregation in cells treated with HPβCD was dependent on the ability of HPβCD to alter the cellular concentration of cholesterol. Treatment of H4/α-syn-GFP cells with cholesterol saturated-HPβCD resulted in TFEB activation and reduction in α-syn aggregation. These results suggest that the autophagic response to HPβCD treatment that leads to α-syn clearance does not depend on the ability of HPβCD to extract cholesterol from cellular membranes. Bar-On et al. found that MβCD-mediated reduction in α-syn aggregation is inhibited in cells treated with cholesterol-saturated MβCD, which led to the hypothesis that changes in the concentration of cholesterol affects α-syn accumulation and aggregation (Bar-On et al., 2006). The findings presented here are in disagreement with this study and suggest that the reduction in α-syn aggregation observed in cells treated with HPβCD under the conditions used in the present study is independent of the ability of HPβCD to extract cellular cholesterol, but rather involves TFEB-mediated activation of autophagic clearance.

The observation that cell exposure to HPβCD results in enhanced autophagic clearance independent of HPβCD ability to alter the cellular pool of cholesterol
suggests that induction of autophagy is likely linked to the adaptive cellular response, which is activated upon internalization of HPβCD. βCDs enter the cell through endocytosis (Rosenbaum et al., 2010) and endocytic delivery of HPβCD to the lysosome was shown to alter lysosomal proteostasis (Chen et al., 2010; Giocondi et al., 2004; Graham et al., 2003). These observations suggest a model in which TFEB activation upon internalization of HPβCD occurs to restore lysosomal proteostasis (Song et al., 2013) and is likely to proceed via mTOR, a key regulator in the autophagy pathway that monitors the status of the lysosome and controls TFEB activation (Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Activation of TFEB and upregulation of the autophagy-lysosomal system in response to HPβCD internalization, in turn, mediates clearance of α-syn aggregates.

In summary, genetic and chemical activation of TFEB reduces the accumulation of aggregated α-syn and promotes α-syn clearance by enhancing the autophagy pathway. This study also provides proof-of-principle evidence that chemical activation of TFEB is a viable therapeutic strategy to enhance the degradation of α-syn aggregates and motivates the discovery of alternative compounds that can effectively cross the blood-brain barrier (Camargo et al., 2001; Monnaert et al., 2004; Pontikis et al., 2013) for the treatment of PD and potentially other neurodegenerative diseases characterized by protein deposition. Finally, results from this study describing the impact of HPβCD on the lysosome-autophagy system are likely to inform a variety of drug delivery applications in which βCD is
routinely used as excipient to increase the solubility and bioavailability of drugs (Davis and Brewster, 2004).

5.3. Materials and Methods

5.3.1. Reagents and Cell Cultures

2-hydroxypropyl-β-cyclodextrin (HPβCD) and cholesterol were purchased from Sigma-Aldrich, and bafilomycin was from Cayman Chemical, TFEB siRNA (Cat. No. SI00094969) and control siRNA (Cat. No. 1027280) were purchased from Qiagen. pMSCV-PIG, gag-pol, and VSVG plasmids were from Addgene and TFEB-3XFLAG plasmid was a generous gift from Dr. Marco Sardiello (Baylor College of Medicine, Houston, TX).

H4 cells stably transfected for the expression of α-syn-EmGFP (H4/α-syn-GFP) were generated as previously described in Chapter 3. Human H4 neuroglioma (HTB-148, ATCC), H4/α-syn-GFP, and HEK-293T cells (CRL-11268, ATCC) were cultured in high glucose DMEM (Fisher Scientific) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin glutamine, and maintained at 37 °C and 5% CO₂.
5.3.2. TFEB Retrovirus Plasmid Construction and Transduction

The plasmid, pMSCV-PI650/TFEB, was constructed as follows: first, the GFP cassette in the pMSCV-PIG plasmid was replaced with eqFP650 using Ncol and SalI restriction enzyme sites creating pMSCV-PI650. TFEB-3XFLAG was inserted into the MCS of pMSCV-PI650 using BglII and Xhol generating pMSCV-PI650/TFEB. pMSCV-PI650/TFEBS142A was obtained by site directed mutagenesis of pMSCV-PI650/TFEB using a reverse primer containing the S142A point mutation, 5’-TGGCCATGGGAGCATTGGGAGCAC-3’.

Retrovirus particles were generated as follows: HEK-293T cells were cultured in 10 cm dishes and transfected with 10 µg of pMSCV-PI650/TFEB or pMSCV-PI650/TFEB-S142A and 5 µg each of plasmids expressing the helper genes, gag-pol and VSVG, using Lipofectamine 2000 according to manufacturer’s instructions (Invitrogen). After 8 h, the transfection medium was replaced with fresh medium and incubated for 48 h. Retrovirus particles were collected by removing the medium using a sterilized syringe and filtered with 0.45 um syringe filter. Polybrene (8 µg/ml) was added to the retrovirus before transducing cells.

Retroviral gene transduction experiments were conducted as follows: H4/α-syn-GFP cells were plated in 6-well plates at a concentration of 5 x 10⁴ cells/ml and cultured overnight. The medium was removed and replaced with medium containing retrovirus particles and the plates were centrifuged at 2500 rpm for 90 min at 30 °C. Cells were incubated at 37 °C for 24 – 48 h before analysis.
5.3.3. Aggregation Studies

Aggregation studies were conducted as described in Sections 3.4.5 and 3.4.7. APF was corrected for TFEB expression level (evaluated by qRT-PCR) to eliminate differences in ProteoStat® binding due to the variability of transduction efficiency.

5.3.4. Immunofluorescence Studies

Immunofluorescence studies were conducted as described in Section 3.4.4. To detect TFEB nuclear localization, cells were incubated with rabbit anti-TFEB antibody (Genetex, 1:100) overnight at 4°C, washed with 0.1% Tween/PBS, and incubated for 1 h with DyLight 549 goat anti-rabbit antibody (Rockland Immunochemical, 1:500). To detect LC3-LAMP2 colocalization, cells were incubated with rabbit anti-LC3 antibody (MBL International, 1:1000) and mouse anti-LAMP2 antibody (BioLegend, 1:1000) for 1 h, washed, and incubated with DyLight 549 goat anti-rabbit antibody and DyLight 649 goat anti-mouse antibody (Rockland Immunochemical, 1:500) for 1 h. Images were collected at 60X using a confocal microscope (FluoView FV1000, Olympus) and analyzed using NIH ImageJ software.

Colocalization of LC3 and LAMP2 was quantified using Matlab. The background signal was subtracted from LC3 and LAMP2 images by removing red (LC3) and blue (LAMP2) pixels that displayed a brightness signal below a predefined threshold (30). To ensure that both fluorescent signals are within the
same order of magnitude, LC3 and LAMP2 pixels that present brightness signal within a predefined range (0.5 and 2) were designated as positive correlation and selected to calculate the percentage of colocalization. The percentage of colocalization was calculated by normalizing the number of pixels presenting LC3 and LAMP2 positive correlation by the total number of pixels in each cell over the entire image. Average values were calculated over multiple images and replicate samples.

5.3.5. siRNA Transfection

siRNA transfection was performed using HiPerFect transfection reagent (Qiagen) as previously described (Song et al., 2013). Each well of a 6-well plate was seeded with 150 ng of siRNA in 25 µl of RNase-free water. 12 µl of HiPerFect transfection reagent were diluted with 63 µl of serum-free culture medium, added to each well and incubated for 10 min at room temperature. A 2 ml-solution of medium containing $8 \times 10^4$ cells was added to each well and the plates were incubated at 37 °C for 48 h. The medium was replaced with fresh medium or fresh medium containing 1 mM HPβCD and microscopy analyses were preformed after 24 h of treatment.
5.3.6. Quantitative RT-PCR

RT-PCR analyses were conducted as described in Section 3.4.6 using the primers reported in Table 5.1.

Table 5.1: TFEB-target Primer Sequences Used in qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Code</th>
<th>Forward Primer (5' → 3')</th>
<th>Reverse Primer (5' → 3')</th>
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<tr>
<td>ACTB</td>
<td>NM_001101</td>
<td>GATCATTTGCTCTTCTGGAGC</td>
<td>ACTCTCTTTGCTGTATACAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>GTCGGAGTCAACGGATT</td>
<td>AAGCTTCCCGTTCTCACAG</td>
</tr>
<tr>
<td>TFEB</td>
<td>NM_007162</td>
<td>CCAGAAGCAGAGCTACAGAT</td>
<td>TGTGATTGCTCTTCTCTGCCG</td>
</tr>
<tr>
<td>GBA</td>
<td>NM_000157</td>
<td>CCAAGCCTTTGAGTTAGGTAAG</td>
<td>CCCGTGTGATTAGCTGCGAT</td>
</tr>
<tr>
<td>HEXA</td>
<td>NM_000520</td>
<td>CAACCAACACATTTCTCTCCA</td>
<td>CGCTATCGTGACCTGCTTTT</td>
</tr>
<tr>
<td>LAMP1</td>
<td>NM_005561</td>
<td>ACGTTACAGGTCCAGCTCAT</td>
<td>TCTTGGAGCCTCCATTTG</td>
</tr>
<tr>
<td>BECN1</td>
<td>NM_003766</td>
<td>GGCTGAGAAGACTGCTGGTG</td>
<td>CTGCTGTCGAGCCATTAGA</td>
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<tr>
<td>MAPLC3</td>
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<td>GTGTCCGTTCAACACAGGAAG</td>
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<tr>
<td>SQSTM1</td>
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<tr>
<td>UVRAG</td>
<td>NM_003369</td>
<td>ATGCCAGAGCGCTCTGATACA</td>
<td>TGACCCCAAGTATTTCCGAGCCA</td>
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</table>
5.3.7. LC3 Western Blots

H4/α-syn-GFP cells were plated in 10-cm culture dishes at a concentration of 1.0 \( \times 10^5 \) cells/ml and treated with HPβCD (1 mM) for 48 h. The total protein content was extracted by incubating the cells in Complete Lysis-M buffer (Roche) according to manufacturer's protocol. Protein concentrations were determined by Bradford assay; samples were diluted to the same concentration and separated by gel filtration using a 15% SDS-PAGE gel. Western blot analyses were performed as described in Section 3.4.3 using rabbit anti-LC3 (Sigma, 1:5000) and rabbit anti-GAPDH (Santa Cruz, 1:10,000) antibodies and appropriate secondary antibodies (HRP conjugated anti-rabbit (Santa Cruz, 1:10,000)).

5.3.8. Preparation of HPβCD–Cholesterol Complexes

HPβCD–cholesterol complexes were prepared as previously described (Christian et al., 1997). 10 µl of cholesterol from a stock solution of 50 µg/mL prepared in chloroform:methanol 1:1 (v:v), was added to a glass tube and the solvent was evaporated under a gentle stream of nitrogen. 10 ml of 10 mM HPβCD dissolved in DMEM medium without serum was added to the dried cholesterol and the solution was sonicated in a bath sonicator for 3 min followed by overnight incubation in a rotating water bath at 37 °C. The solution was diluted to 1mM in DMEM and filtered through a 0.45 µm syringe filter to remove excess cholesterol crystals immediately prior to adding the HPβCD–cholesterol complexes to the cells.
5.3.9. Toxicity Assay

H4/α-syn-GFP cells were treated with taxol (50 nM) and HPβCD (1 mM) for 16 h at 37 °C and induction of apoptosis was measured as described in Section 4.4.4.
Chapter 6

Summary

In this work, a number of chemical and genetic approaches were investigated to further our understanding of the quality control mechanisms that affect α-syn aggregation. A stable neuroglioma cell line that accumulates α-syn aggregates (H4/α-syn-GFP cells) was generated as a platform to conduct in vitro studies. To respond to the increasing need for aggregation-responsive probes that can be used to monitor protein aggregation in cells, the use of a ruthenium (II) derivative [Ru(phen)²dppz]²⁺, for the detection of α-syn aggregates was established in H4/α-syn-GFP cells. Finally, modulators of the chaperone and degradation machineries were investigated in H4/α-syn-GFP cells with the ultimate goal to prevent accumulation of α-syn aggregates.

To test how modulation of the cellular chaperone capacity affects α-syn aggregation, chemical approaches were used to upregulate the expression and
activity of the cytoplasmic chaperone Hsp70 (Chapter 4). The glycyrrhizinic acid derivative CBX, was found to reduce α-syn aggregation in H4/α-syn-GFP cells. CBX prevented the accumulation of α-syn aggregates by upregulating Hsp70 expression. By using modulators of Hps70 activity, this study also provided evidence that CBX-mediated reduction in α-syn aggregation depends on Hsp70 activity.

To test how modulation of cellular clearance mechanisms affects α-syn aggregation, genetic and chemical strategies were used to activate TFEB, a master regulator for the autophagy-lysosomal system, which is the main catabolic pathway that mediates degradation of aggregated proteins (Chapter 5). TFEB overexpression was demonstrated to prevent the accumulation of aggregated α-syn in H4/α-syn-GFP cells and that reduction in α-syn aggregation depends on TFEB-induced upregulation of autophagic clearance. Pharmacological activation of TFEB was investigated by treating H4/α-syn-GFP cells with HPβCD, which was found to induce activation of autophagy and clearance of α-syn aggregates.

In summary, this study provides proof-of-principle evidence that pharmacological modulation of quality control mechanisms that control α-syn misfolding and aggregation prevent accumulation of aberrant α-syn species. These findings lay the foundation for the development of therapeutic strategies based on enhancing the innate cellular chaperone capacity and autophagic clearance mechanisms for the treatment of diseases associated with the accumulation of aggregated α-syn.
6.1.1. Future Directions

Protein misfolding and aggregation underlies the pathogenesis of a number of neurodegenerative diseases, which not only present similar cellular pathogenic events, but are also all characterized by the accumulation of β-sheet rich fibrillar aggregates with similar morphologic structures termed amyloids (Chiti and Dobson, 2006; Soto, 2003). Thus, aggregation-sensitive probes are typically used to detect aggregated structures formed with monomeric precursors that do not share any sequence homology among their primary sequence, including α-syn, amyloid-β, and Huntingtin (Chiti and Dobson, 2006; Munishkina and Fink, 2007). I anticipate that [Ru(phen)₂dppz]²⁺ complexes will be generally useful to detect amyloidogenic structures in cells and suggest that [Ru(phen)₂dppz]²⁺ be tested in other cellular models of protein deposition diseases. Although the [Ru(phen)₂dppz]²⁺ complex exhibits fluorescent properties favorable over ThT, such as red-shifted fluorescence emissions that do not overlap with commonly used green fluorescent reporters, large Stokes shifts (180 nm), and long photoluminescence lifetimes, it is unable to permeate the cell membrane. The design of fluorescent aggregation probes that passively diffuse across the cell membrane would allow for rapid, real time detection of aggregate formation without the need for fixative agents and permeablisization. This could be potentially accomplished by conjugating the ruthenium (II) DPPZ complex to a cell-penetrating peptide, which facilitates diffusion through the membrane (Bullok et al., 2006). Furthermore, modifying the luminescent properties of the ruthenium (II) DPPZ complex to enhance the signal of
the aggregate-bound complex would result in a probe with enhanced sensitivity and limit of detection.

In this work, modulation of the Hsp70 machine was demonstrated to be a promising strategy to prevent α-syn aggregation. A number of HSPs are sequestered in Lewy bodies and, particularly, Hsp90 (Uryu et al., 2006). Evidence suggests that Hsp90 binds to purified α-syn and accelerates α-syn fibrilization in the presence of ATP (Falsone et al., 2009). In the absence of ATP, Hsp90 predominately interacts with oligomeric α-syn species and prevents aggregation (Daturpalli et al., 2013). These observations suggest that Hsp90 may play a critical role in preventing cytotoxicity induced by accumulation of oligomeric α-syn. However, little is known regarding the effect of Hsp90 on α-syn aggregation in cells. I suggest that the role of Hsp90 in α-syn aggregation be investigated in vitro to understand the therapeutic potential of Hsp90 modulation for the treatment of PD.

Inefficient autophagic activity underlies the pathogenesis of a diverse range of neurodegenerative diseases characterized by accumulation of proteinaceous substrates. My findings demonstrating that TFEB activation mediates autophagic clearance of α-syn aggregates motivate future analysis of the role of TFEB in other protein deposition diseases, such as Alzheimer’s disease and amyotrophic lateral sclerosis. While the studies reported herein focus on investigating the role of macroautophagy in TFEB-mediated clearance of α-syn aggregation, it is possible that chaperone-mediated autophagy could also be involved in the degradation of α-syn. To establish whether chaperone-mediated autophagy affects the accumulation
of aberrant α-syn species upon activation of TFEB, modulation of chaperone-mediated autophagy should be investigated in H4/α-syn-GFP cells exposed to HPβCD or treated to activate TFEB genetically.
References


mutations in the P-Type ATPase ATP13A2 (PARK9) causing Kufor-Rakeb Syndrome, a form of early-onset parkinsonism. *Hum. Mutat. 32*, 956-964.


transmembrane conductance regulator degradation in yeast. *Mol. Biol. Cell*
15, 4787-4797.


Appendix

Table A.1: Small molecules used in this study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Function</th>
<th>Structure</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>MG-132</td>
<td>Proteasome inhibitor</td>
<td><img src="image" alt="Structure" /></td>
<td>(Kisselev and Goldberg, 2001)</td>
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<tr>
<td>Lactacystin</td>
<td>Proteasome inhibitor</td>
<td><img src="image" alt="Structure" /></td>
<td>(Kisselev and Goldberg, 2001)</td>
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<td>Carbenoxolone (CBX)</td>
<td>Hsp70 inducer</td>
<td><img src="image" alt="Structure" /></td>
<td>(Kawashima et al., 2009; Nagayama et al., 2001)</td>
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<td>Compound</td>
<td>Function</td>
<td>Structure</td>
<td>Reference</td>
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<tr>
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<tr>
<td>MAL3-101</td>
<td>Hsp70 ATPase inhibitor</td>
<td><img src="image" alt="MAL3-101 Structure" /></td>
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<td>115-7c</td>
<td>Hsp70 ATPase activator</td>
<td><img src="image" alt="115-7c Structure" /></td>
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<td>KNK-437</td>
<td>HSF1 inhibitor</td>
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<td>Structure</td>
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</tr>
<tr>
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<tr>
<td><strong>Taxol</strong></td>
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<td><img src="image" alt="Taxol structure" /></td>
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<td><strong>Hydroxypropyl-β-cyclodextrin</strong></td>
<td>TFEB activator</td>
<td><img src="image" alt="HPβCD structure" /></td>
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<td>(HPβCD)</td>
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<tr>
<td><strong>Bafilomycin</strong></td>
<td>Lysosome /autophagy inhibitor</td>
<td><img src="image" alt="Bafilomycin structure" /></td>
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