Investigating the effects of α-synuclein variants on mitochondrial function in Parkinson's disease

by
Rachel Allison Yates

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Rachel Allison Yates

APPROVED:

_____
Dr. David E. Nelson
Department of Biology

_____
Dr. Lynn Boyd
Department of Biology Chair

_____
Dr. Roger Heinrich
Honors Distinguished Visiting Scholar

_____
Dr. John Vile
University Honors College Dean

Abstract

Parkinson's disease is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain and affects millions of individuals worldwide each year. It is known that mitochondrial dysfunction and Lewy body formation are both associated with the disease, but the extent to which these two symptoms are related has yet to be determined. In this study, the effects of α-synuclein, the major protein component of Lewy bodies, on mitochondrial dysfunction was tested by cloning five different α-synuclein variants (each with differing aggregation and trafficking potentials) and expressing them in live human neuronal cell lines to determine if there was a significant decrease in mitochondrial health and function. The results suggested that all α-synuclein variants, including wildtype α-synuclein, decreased mitochondrial polarization, a measure of health and function. These data suggest that a more detailed investigation of α-synuclein expression and mitochondrial function is warranted.

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States, affecting approximately one million individuals, with fifty-to-sixty thousand new cases diagnosed each year. The vast majority of these cases (95%) are described as "sporadic" seemingly occurring at random with no clear cause, genetic or otherwise. Symptoms include tremors at rest, bradykinesia, stiffness of muscles, and poor balance. These clinical symptoms are caused by the loss of dopaminergic neurons (DA-neurons) in the substantia nigra pars compacta (SNpc), a region of the brain involved in the control of movement. The loss of DA-neurons is also accompanied by the formation of intra-neuronal aggregates known as Lewy Bodies (Spillantini *et al*. 1997), a histopathological 'hallmark' of the disease. Alongside these aggregates, an average of a 35% reduction in mitochondrial Complex 1 efficiency has been observed in patients with PD (Schapira 2008). Despite these clear symptoms, the underlying causes of the disease are still not understood.

1.1 *Alpha-Synuclein*

Lewy bodies are found in almost every case of sporadic PD. They are mainly composed of the pre-synaptic protein α-synuclein (α-syn), a small 140 amino acid protein with an N-terminal amphipathic α-helical domain between amino acids (aa) 1-60, a central hydrophobic region (61-95 aa) known as the non-amyloid-β component (NAC), and an acidic, unstructured C-terminus (96-140 aa). It is expressed in the central nervous system in almost all vertebrates, found predominately in presynaptic termini, and has been shown to play a role in neurotransmitter release by regulating synaptic vesicle recycling (Iwai *et al.* 1995 and Marques *et al.* 2012). Wild-type α-syn is frequently post-transcriptionally

modified in many different ways, producing N-terminal and C-terminal truncation variants in both healthy and PD brains in proportion to total α-syn (Muntane *et al*. 2012 and Liu *et al.* 2005). The function of these truncation variants is still greatly understudied and their role in both normal and PD brains has yet to be elucidated. However, truncation of α-syn is likely to alter its solubility, potentially inducing the aggregation of wild-type α-syn proteins.

1.2 *Aggregation of α-synuclein and Lewy Body Formation*

Lewy bodies and protein aggregation in PD are synonymous with α-syn. However, it is still unclear what impact the formation of Lewy bodies has on cellular function. Recently, it has been shown that a wide variety of cellular proteins are sequestered to α-syn-containing Lewy bodies, including inhibitor kappaBα (IκBα) and ring-finger protein 11 (RNF11), negative regulators of the nuclear factor kappaB pathway (NF-κB), a vital regulator of inflammatory response. Although it is unclear what effect the loss of the N-terminus of α-syn will have on its ability to form aggregates, it has been shown that C-terminal truncations of α-syn, which remove the negatively charged end of the protein (specifically aa 1-120 and 1-110), are more prone to aggregation and promote the aggregation of wild-type α-syn in the same cell (Liu *et al.* 2005), forming Lewy body-like structures. In addition to the natural forming α-syn variants, one previous study on α-syn aggreagtion cloned a fusion gene that expressed a chimeric version of α-syn containing a C-terminal CL1 aggregation tag, which was shown to form Lewy-like structures in cells (Wan & Chung 2012).

1.3 *Alpha-synuclein and Mitochondrial Health and Function*

While Lewy bodies still remain the hallmark of PD, mitochondrial dysfunction has become increasingly associated with the disease. Known mitochondrial Complex 1 inhibitors, such as the pesticide rotenone and a meperidine analog, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), have been used for many years to induce artificial PD in animal models (Burbach *et al*. 2003). Mutations in genes such as PINK-1 and parkin, which code for proteins involved in the autophagy of dysfunctional and damaged mitochondria ('mitophagy'), are associated with genetic cases of PD (Smidt *et al*. 1997). In histological studies, oxidative damage to lipids, proteins, and DNA has been observed in brain tissue of PD patients (Dias *et al.* 2013). Although mitochondrial dysfunction is clearly a major component of PD, the mechanisms that lead to it are not fully understood.

Recently, it has been discovered that wild-type α-syn binds to mitochondrial membranes and may interfere with mitochondrial health and integrity (Devi *et al.* 2008). *In vitro* studies have shown that this interaction is mediated by the first 25 amino acids of the protein, which have been shown to bind cariolipin, a phospholipid found only at mitochondrial membranes (Nakamura *et al.* 2008). This association often leads to an accumulation of α-syn in the mitochondria, reducing Complex I activity as well as increasing the production of reactive oxygen species (Devi *et al.* 2008), which can be detrimental to the cell. It has been suggested that α-syn also affects mitophagy, a mitochondrial quality control process that is mediated by parkin proteins, which destroys damaged mitochondria. However, these data are controversial, with some groups claiming that α-syn promotes mitophagy (Choubey *et al*. 2011 and Huang *et al.* 2012), while others

suggests that α-syn may inhibit the process (Winslow *et al.* 2010). To date, it is unclear whether truncated α-syn variants affect mitochondrial health or quality control.

1.4 *Hypothesis*

I hypothesize that while C-terminal variants of α-syn may affect mitochondrial health, as they retain the N-terminal domain, which mediates import into the mitochondria, N-terminal variants will not. In addition, α-syn variants that are more likely to aggregate (i.e. α-syn with CL1 aggregation tag) may have less direct effect on mitochondrial health as they are potentially immobilized in Lewy body-like structures in the cytoplasm. To test this hypothesis, I will determine whether the expression of α-syn variants in human cells affects mitochondrial membrane polarization, which is a measure of mitochondrial health and function.

2. Materials and Methods

2.1 *Annealing Double-stranded CL1 Oligonucleotide.*

Complementary single-stranded CL1 oligonucleotides (sense and antisense) were mixed at a 1:1 ratio in 1xMedium salt restriction buffer (MRB - (10x) – 100 mM Tris-Cl (pH 7.5), 500 mM NaCl, 100 mM $MgCl_2$, 10 mM DTT) to a final concentration of 25 µM. The oligonucleotide mixture was incubated for 5 minutes at 95ºC and cooled slowly to room temperature over 3 hours to allow complementary strands to anneal without forming inappropriate secondary structures. A working stock solution of 0.1 µM was made by diluting the 25 µM mixture with 10x MRB and PCR grade water. The CL1 oligonucleotide amino acid chain was ACKNWFSSLSHFVIHL (Wan & Chung 2012).

2.2 *Molecular Cloning.*

α-syn cDNA was amplified using PCR (95°C for 3 minutes; 30 sets of 95°C 30 seconds, 55°C for 45 seconds, then 68°C for 40 seconds; 68°C for 3 minutes). PCR products and pcDNA3 mammalian cell expression vector were cut using *Kpn1*, *Xho1*, and *Apa1* restriction enzymes (cut sites were incorporated into the α-syn forward and reverse primers used in PCR). Cut DNA was electrophoresed on a 0.8% gel containing Ethidium Bromide (10 mg/mL) and purified using QIAquick Gel Extraction Kit (Qiagen, NLD). Purified, cut α-syn cDNA segments were ligated into the cut pcDNA3 expression vector using T4 DNA Ligase (NEB, Ipswich, MA). EZ-load DNA ladder (Bio-Rad 170-8355) was used when running all DNA electrophoresis gels.

2.3 *Transformation and Mini Culture.*

All transformations used Subcloning efficiency DH5α competent cells (Invitrogen 18265-017). DH5α were stored at -80ºC and thawed on ice immediately before use. After

thawing, 0.5 µL of ~100 ng/µL plasmid DNA (e.g. pcDNA3 containing α-syn variants) was added to 25 µL of DH5α in 1.5 mL Eppendorf tubes and incubated on ice for 15 minutes. The cells were then heat-shocked at exactly 42ºC for 30 seconds and immediately incubated on ice for 2 minutes to recover. 75 µL of LB broth was added to the cells and spread onto LB agarose plates containing ampicillin (AMP; 50 µg/mL). The plates were incubated overnight at 37ºC in a bacteria incubator. After incubation, colonies were picked and placed into 3 mL of LB broth with AMP. Cultures were shaken at 300 rpm for 16 hours at 37ºC. Then plasmid DNA was extracted with a mini-prep kit (Omega Bio-Tek, USA) in accordance with manufacturer's guidelines. For large-scale plasmid DNA production, 3 mL starter cultures of bacteria transformed with the desired plasmid were subcultured into 50 mL LB broth and incubated for 16 hours at 37ºC with the plasmid DNA extracted using midi-prep kits (Omega Bio-Tek, USA).

2.4 *Tissue Culture.*

HeLa and SK-N-AS cells (ATCC, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat-inactivated 10% FBS, 0.1% of penicillin/streptomycin, and 0.1% of nonessential amino acids. All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

2.5 *Transfection.*

Cell culture plates were transfected when 75-90% confluent. Using a tissue culture hood, 3 µL of transfection reagent (polyethyleneimine (PEI), a 25 kDa linear from Polysciences, cat# 23966-2) was added to 100µL of serum-free media in a sterile 1.5 mL Eppendorf tube for every 1 µg of plasmid DNA being used. Mixture was vortexed for 10

seconds and plasmid DNA was added. The mixture was then added to the cells immediately after a 15 minute incubation at room temperature. *Transfections for Western Blots*: SK-N-AS (for WT and α-syn-CL1) and HeLa (for α-syn 1-110, 1-120, and 25-140) cells were used. 1μg of plasmid DNA was used for all α-syn variants and controls. *Transfections for Imaging*: HeLa cells were used for all imaging experiments. α-syn variants (including empty pcDNA3 expression vector as a control) and EYFP were co-transfected at a 3:1 ratio, totaling 1μg of DNA.

2.6 *Western Blot.*

24 hours post transfection (refer to section 2.5) cells were washed with PBS and then lysed using Laemlli lysis buffer (1.25 mL 0.5 Tris-HCl, at a pH of 6.8, 1 mL of glycerol, 2 mL of 10% SDS, 250 μL 0.1% Bromophenol blue, and 5.5 mL DI H2O). Protein lysates were heated to 90°C for 5 minutes to denature proteins, and then separated on a 12% SDS-PAGE minigel and transferred to a nitrocellulose membrane. Blots were blocked with 5% milk in PBS/T for an hour at room temperature and incubated overnight in primary antibodies (α-syn (1:1000) or actin (1:5000); Cell Signaling) at 4°C. After incubation, blots were washed 3 times (5 minutes each time) in PBS/T and incubated in HRP-conjugated secondary antibodies (anti-mouse for α-syn and anti-rabbit for actin, both at 1:2000). Blots were incubated with buffered luminol in the presence of hydrogen peroxide and bioluminescence was visualized using a ChemiDocTM MP Imaging System and Imaging Lab software (Bio-Rad).

2.7 *TMRM Staining and Imaging.*

Cells plated in 35 mm glass-bottom dishes and stained with 2uL (10 µM) tetramethylrhodamine methyl ester (TMRM) and incubated with humidity at 37°C and 5% CO_2 for 15 minutes. Cells were then washed with PBS and imaged using a Nikon Ti-Eclipse wide-field microscope, equipped with a CoolSNAP Myo camera (Photometrics, USA), Intensilight Epi-fluorescence illuminator, computer-controlled stage, and a full environmental enclosure with CO_2, humidity, and temperature control. The microscope was controlled using Nikon Elements Software (Nikon, USA). Still images of at least 40 cells were taken for each plate. EYFP was excited through a 465-495 nm excitation filter and emitted light was detected through a 515-555 nm barrier filter, reflected from a 505 nm dichroic mirror. mCherry was excited through a 535-550 nm excitation filter and emitted light was detected through a 610-675 nm barrier filter, reflected from a 565 nm dichroic mirror. Post-acquisition, 14-bit nd2 images were analyzed using FIJI (Schindelin *et al.* 2012).

3. Results

3.1 Generating α-syn-CL1 Gene Fusion and Chimeric Protein

The first goal of this project was to clone variants of the human α-syn cDNA into the pcDNA3 mammalian cell expression vector by using standard molecular cloning techniques. I began by cloning a chimeric fusion of wild-type (WT) α-syn with C-terminal CL1 aggregation tag (α-syn-CL1) (Fig.1). Serial Cloner was used to generate plasmid maps of the expected product, pcDNA3-α-syn-CL1 (Fig. 2A and 2B). Primers for both WT α-syn cDNA and complimentary DNA oligonucleotides encoding the small CL1 tag were designed with compatible restriction enzyme cut sites to ensure that both α-syn cDNA and the CL1 oligonucleotides were inserted into the pCDNA3 expression vector in the correct order and proper orientation (Table 1). The cloning process itself was performed in the following manner: The CL1 sense and antisense oligonucleotides were annealed *in vitro* to form double-stranded DNA encoding the CL1 oligonucleotide (dsCL1), and agarose gel electrophoresis was performed to verify the dsCL1 product formed correctly by comparing its mobility to an equal quantity of single-stranded CL1 (ssCL1) oligonucleotides (Fig 2C). Even though both ssCL1 and dsCL1 oligonucleotides were roughly the same length, the ssCL1 DNA ran faster (and therefore slightly farther down the gel) than the dsCL1 DNA. Furthermore, the dsCL1 band was brighter despite containing the same quantity of DNA. This was because double-stranded DNA bound the fluorescent ethidium bromide dye better than single-stranded DNA. Taken together, these data confirmed that the annealing process had worked. The dsCL1 oligonucleotides were then cloned into the pcDNA3 mammalian expression vector and a diagnostic PCR was performed on two isolated clones to ensure the CL1 sequence was present (Fig. 2D). One of the two clones (lane 2) had a stronger

signal than the other (lane 3), but both colonies contained a fragment at the expected length of 100bp (accounting for the 48bp from the CL1 oligonucleotide and miscellaneous base pairs from the primers). WT α-syn cDNA was amplified by PCR excluding the 3' stop codon, ligated into cut pcDNA3 containing CL1 (pcDNA3-CL1) and transformed into competent cells. Empty, cut pcDNA3 vector was used as a ligation control. There were significantly more colonies on the transformed plate containing pcDNA3-α-syn-CL1 than on the control plate (Fig. 2E), suggesting that the ligation was successful. To verify that the successful ligations actually contained our product and were not just re-closed plasmid, a PCR digest was performed for six discrete clones from the experimental plate. An approximately 600bp DNA fragment was expected if the ligation was successful (48bp from the CL1 oligonucleotide, 417bp from α-syn cDNA without the stop codon, and about 150 bases in between the product and the primers). All six clones contained fragments at the expected lengths (Fig. 2F), and one was selected for all further work.

3.2 *Generating α-syn Truncation Variants*

Two naturally occurring C-terminal (α-syn 1-120 and 1-110) and one N-terminal (α-syn-25-140) α-syn truncation variants were cloned using similar techniques as that used to generating α-syn-CL1. When cloning α-syn-CL1, techniques were utilized to tag on additional base pairs to the C-terminus of WT α-syn. In the case of these three truncation variants, base pairs have been removed from WT α-syn (Fig. 1). For α-syn 1-120 and 1-110, specially designed reverse primers were used in PCR amplification of WT α-syn that attached at either the 120^{th} or 110^{th} codon. Similarly, a special forward primer was used to

generate α-syn 25-140, attaching at the 25th codon (Table 1). The rest of the cloning process was the same as α-syn-CL1, described in Figure 2.

Primer Names	Sequence (5' - 3')
WT α-syn Forward	GCTTGGTACCATGGATGTATTCATGAAAGG
WT α-syn Reverse (stop)	CTTACTCGAGGGCTTCAGGTTCGTAGTCTTG
WT α-syn Reverse (no stop)	CTTACTCGAGTTAGGCTTCAGGTTCGTAGTCTTG
1-110 α-syn Reverse (stop)	CGTTACTCGAGTTATCCTTCCTGTGGGGCTCCTTC
1-120 α-syn Reverse (stop)	CGTTACTCGAGTTAGTCAGGATCCACAGGCATATC
25-140 α-syn Forward	GCTTGGTACCATGGTGGCGGAAGCGGCCGGC
CL1 Forward	CTTACTCGAGGCATGCAAGAACTGGTTCTCTAGCCTGT CCCACTTCGTCATCCATCTGTAGGGGCCCGCTTAG
CL1 Reverse (stop)	CTTAGGGCCCCTACAGATGGATGACGAAGTGGGACAG GCTAGAGAACCAGTTCTTGCATGCCTCGAGGAACTA

Table 1: List of Cloning Primers. List includes new primers designed specifically for this project. Commercially available and standard sequencing primers (i.e. T7 and SP6) are not listed.

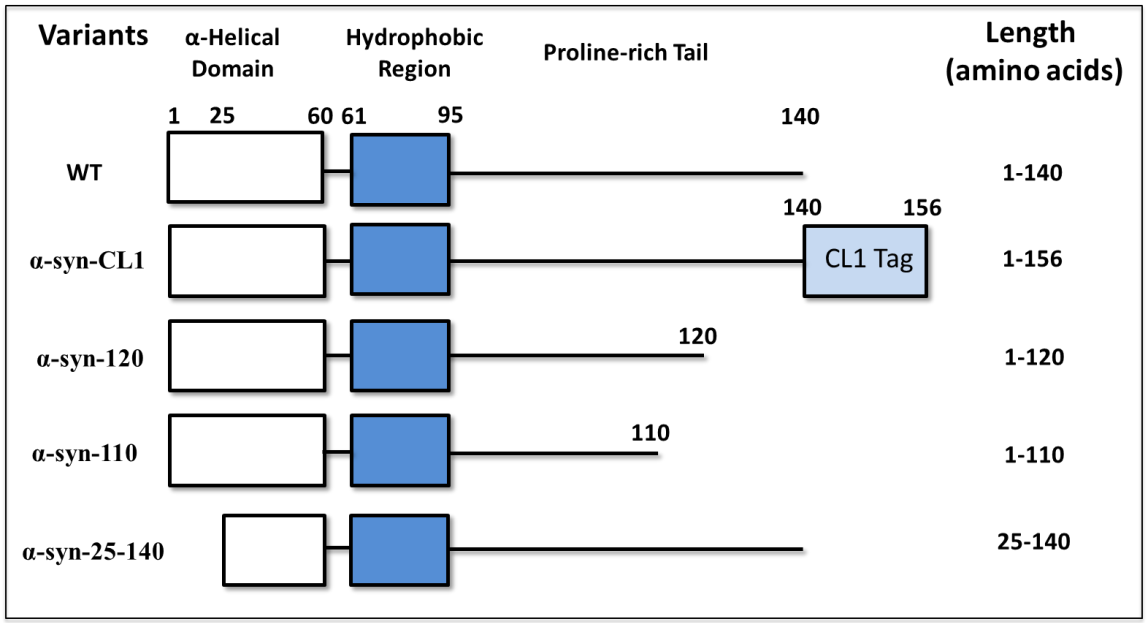

Figure 1: Representation of Naturally Occurring and Chimeric Variants of Human α-syn Proteins. From top to bottom: Wildtype (WT) α-syn, α-syn-CL1 with a C-terminal aggregation tag, α-syn-110 and 120 with truncated C-termini, and α-syn-25-140 with an N-terminal truncation.

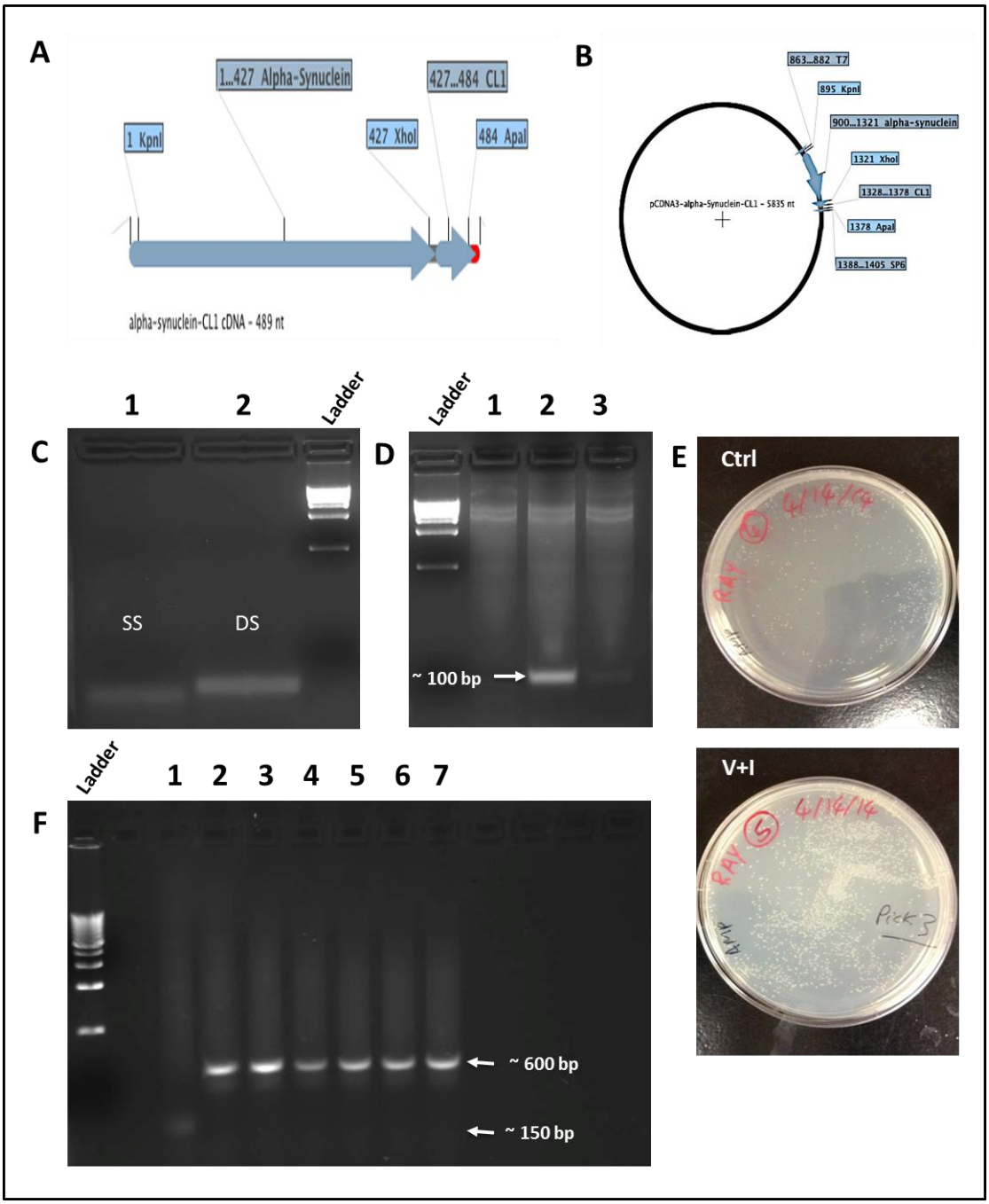

Figure 2: Molecular Cloning of α-syn-CL1 in pcDNA3. (**A**) Map of the α-syn-CL1 fusion (**B**) Representation of α-syn-CL1 cloned into pcDNA3 expression vector. (**C**) Annealing complementary synthetic single-stranded CL1 oligonucleotides into double-stranded DNA (dsDNA). Lane *1* = pre-annealing and *2* = post-annealing. (**D**) Diagnostic PCR of dsCL1 inserted into pcDNA3: the CL1 forward primer and SP6 vector primer (reverse) were used. Lanes: *1*, Ctrl (empty pcDNA3 used as PCR template), *2*, and *3*, potential CL1-containing clones used as PCR template. A 100bp band was expected for CL1-containing clones. (**E**) α-syn cDNA was cloned into pCDNA3-CL1 cut with restriction enzymes Kpn1 and Xho1. Ligated plasmid was transformed into DH5α *E. coli* and plated. The top picture depicts the result of a control ligation (empty vector). The bottom represents the ligation of the α-syn cDNA insert (I) into the pcDNA3-CL1 vector (V). (**F**) Six individual colonies were picked from the transformation plate (lanes 2-7) and were tested by diagnostic PCR. The T7 and SP6 pcDNA3 vector primers were used to amplify inserted α-syn. Empty pcDNA3 vector was used as a control template (lane 1). Subsequent lanes show amplification products from individual clones. A 600bp band was expected if α-syn-CL1 was present. A 150bp band was expected in the control. Images (**A+B**) were produced in Serial Cloner. On the DNA gel ladder (**C+D+F**), the bottom fragment is 1kb.

3.3 Validation of α-syn Variants

The expression of all five α-syn variants in mammalian cells was validated by Western Blot (Fig. 3). WT α-syn has a molecular weight of 14kDa, but appears at ~17kDa when electrophoresed on denaturing SDS-PAGE gels. Knowing this, WT α-syn was expressed in separate control samples and run alongside all α-syn variants to compare band sizes. The inclusion of the C-terminal CL1 tag increases the molecular weight of α-syn CL1, and as expected, it migrated slower on the SDS-PAGE gel, appearing at a slightly higher molecular weight on the blot (Fig. 3A). α-syn-110 and 120 are both smaller than WT α-syn and therefore exhibited a greater mobility on SDS-PAGE gels than WT α-syn (i.e. appeared lower), with α-syn-110's slightly lower weight reflected accurately (Fig. 3B). Likewise, α-syn-25-140 is also smaller in weight than WT, which was apparent on the western blot (Fig. 3C). Equal quantities of actin were found to be present in each sample, demonstrating that loading was consistent between samples and that each of the proteins are expressed at comparable levels from the engineered plasmids in human cells.

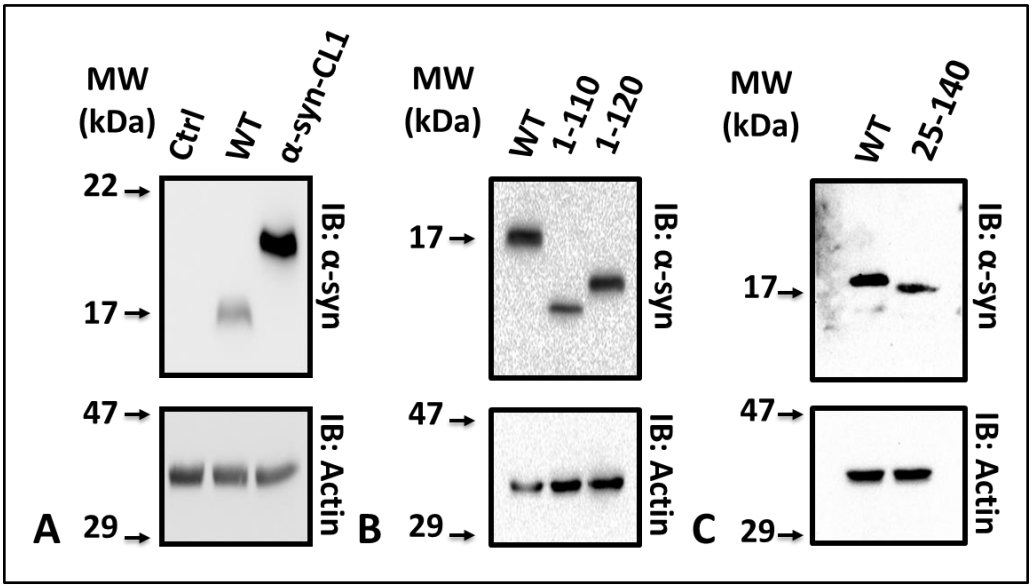

Figure 3: α-Syn Variants Tested by Western Blot. Protein lysates from either **(A)** SK-N-AS or **(B+C)** HeLa cells, expressing the indicated α-syn variants or control vector were analyzed by western blot using anti-α-syn and anti-actin antibodies. Actin was used as a loading control for all blots.

3.4 *Effect of α-syn Variants on Mitochondrial Function*

To measure changes in mitochondrial function in cells expressing α-syn variants, a lipophilic fluorescent dye, TMRM, was used to detect mitochondrial membrane polarization. TMRM's lipophilic structure allows it easily to penetrate the mitochondria's lipid bilayer membrane. TMRM will distribute throughout polarized mitochondria, giving off a strong fluorescent signal. It is less likely to associate with depolarized mitochondria, resulting in high TMRM signal in cells with healthy, polarized mitochondria, and weak or no signal from cells with depolarized, unhealthy mitochondria.

HeLa cells were co-transfected with the plasmids to express α-syn variants (or an empty expression vector, pcDNA3, as a control) and EYFP as a fluorescent marker of successfully transfected cells. Twenty-four hours later, the cells were stained with TMRM and imaged using fluorescent microscopy. TMRM fluorescence in at least 40 cells expressing each variant was imaged (Fig. 4A). By quantitatively comparing images, it was clear that the control cells exhibited higher TMRM fluorescence than cells expressing any of the α-syn variants, suggesting that the presence of α-syn was effecting mitochondrial health. The TMRM fluorescence was quantified for individual cells using FIJI software and all data was normalized to the average TMRM fluorescence in control cells (Fig. 4B). WT, 1-110, and 25-140 α-syn exhibited ~60% of the control's TMRM fluorescence, while in α-syn-CL1 expressing cells it was closer to 70%. It is noteworthy that α-syn 1-120 saw the greatest decrease in mitochondrial function, showing only 40% TMRM staining compared to the control. There was no obvious correlation between the amount of EYFP to TMRM signal. Standard deviation bars were not represented on the graph due to the high amount of variability, making them too large to represent accurately (TMRM: Ctrl=1

+/- 0.87, WT=0.66 +/- 0.61, 1-110=0.65 +/- 0.59, 1-120 =0.41 +/- 0.28, 25-1400.63 =+/- 0.58, CL1=0.72 +/- 0.64; EYFP: Ctrl=1 +/- 1.01, WT=1.37 +/- 1.66, 1-110=1.09 +/- 1.11, 1-120=1.16 +/- 0.93, 25-140 1.10 =+/- 1.08, CL =1.09 +/- 1.21).

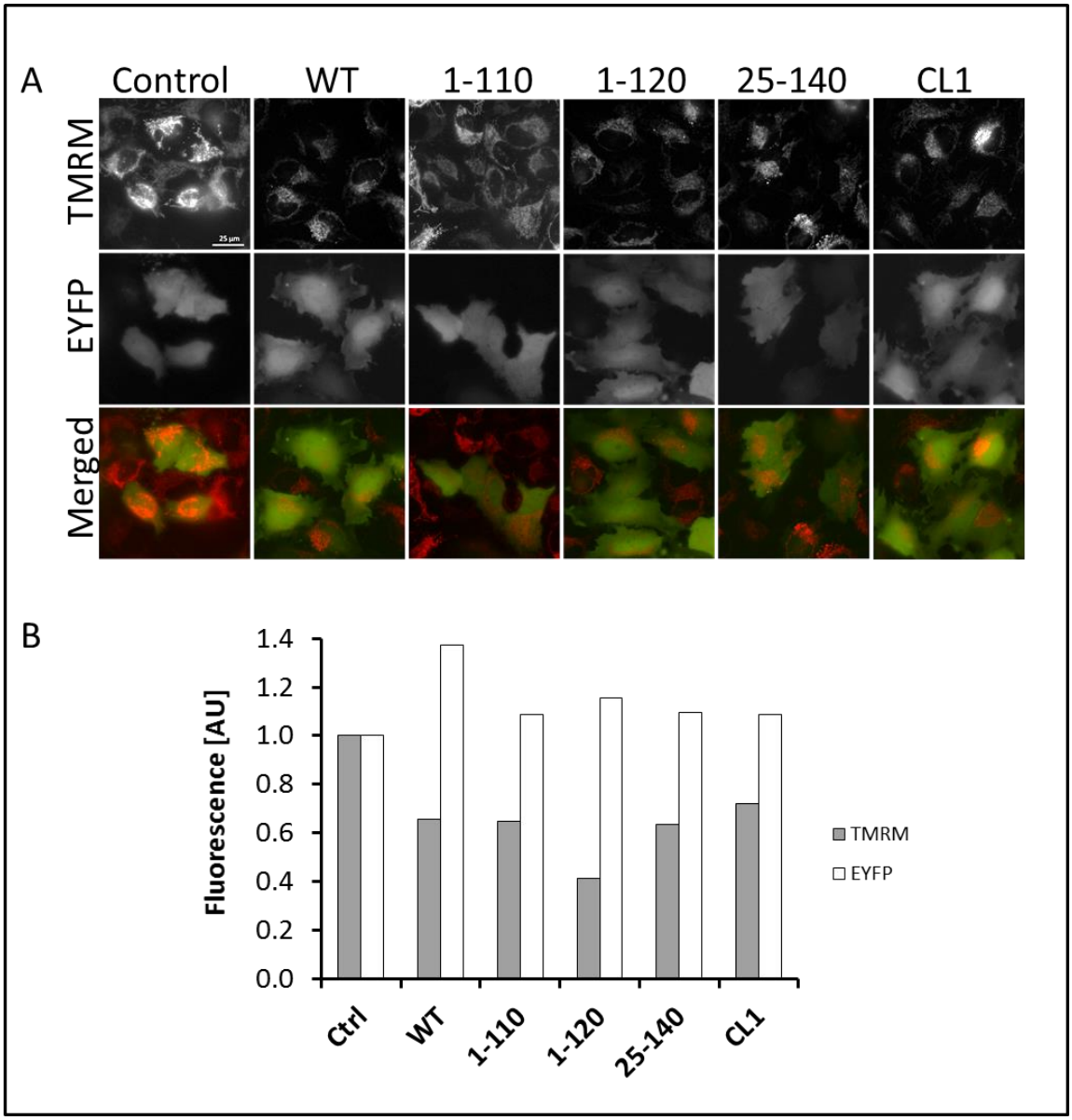

Figure 4: Effect of α-syn Variants on Mitochondrial Membrane Potential. (**A**) 24 hours post-transfection with plasmids to express α-syn variants and EYFP, HeLa cells were stained with TMRM and imaged by fluorescent widefield microscopy. EYFP and TMRM fluorescence are represented in green and red, respectively. Scale bar represents 25 µm. (**B**) Representation of the average TMRM and EYFP signals in cells imaged containing α-syn variants. All data sets were normalized to the control (Ctrl). Data from ≥ 40 cells was collected for each condition.

4. Discussion

Every year, millions of Americans are effected by PD, a neurodegenerative disease whose etiology is still not fully understood. One of the leading theories as a causative factor of DA neuronal death in PD is mitochondrial dysfunction. Many studies have shown decreases in mitochondrial health and function, but the molecular changes that cause this have yet to be elucidated. In cases of sporadic PD, it is possible that α-syn proteins, which are known to be expressed in the DA neurons of patients with PD, have a negative effect on mitochondrial function and health, leading to neuronal death. The unstructured nature of α-syn allows it to aggregate readily, most famously forming insoluble, intra-neuronal aggregates known as Lewy bodies, which are characteristic of the disease. While it is still unclear if Lewy bodies are harmful or helpful to the cell, their presence indicates that α-syn may play a significant role in PD. This study used molecular cloning techniques to generate plasmid constructs to express naturally occurring (α-syn WT, 1-110, 1-120, and 25-140) and chimeric (α-syn-CL1) α-syn variants that differ in size, how they are trafficked within the cell, and their propensity to aggregate. These constructs were used in combination with fluorescent microscopy to determine if overexpression of these variants effects mitochondrial health and function.

Many steps were taken to ensure all of the α-syn variants were properly cloned and the final products were exactly what we expected. First, the original human α-syn cDNA that we obtained from Dr. G. L. Millhauser (UCSF, USA) was sequenced to verify that there were no initial mutations. The sequencing results revealed that the cDNA sequence was codon optimized for expression in bacteria. However, this did not change the identity of the polypeptide sequence that was coded for. The Western blot analysis also confirmed

that the codon optimization did not interfere with the ability of the cDNA to be expressed in human cells (Fig. 3), with the exogenous proteins expressed at a higher level than endogenous α-syn. Additionally, throughout the cloning process, PCR and restriction digests were performed to verify that the α-syn variant cDNAs were inserted in the correct position and orientation into the pcDNA3 expression vector.

Having verified the identity of our α-syn variant expression constructs, their ability to be expressed in human neuronal cells lines was confirmed using SDS-PAGE and Western Blotting techniques. SDS-PAGE separates denatured proteins from samples of lysed cells by molecular weight. Specific proteins (e.g. α-syn) can then be identified by immunoblotting (Western Blotting). This technique utilizes primary antibodies that are raised against a unique epitope within a target protein of interest. Antibodies bound to target protein on the western blot are subsequently detected using HRP-conjugated secondary antibodies, catalyzing a light-generating chemiluminescent reaction, which is detected using either X-ray film of highly sensitive cameras. Using this methodology, we found that all of the α-syn variants were expressed in human neuronal cell lines (Fig. 3).

Once all of the α-syn variants had been generated and verified, they were transfected into HeLa cells, and their effect on mitochondrial function was measured using TMRM staining. All cells were co-transected with EYFP and one of the α-syn variants (or empty pcDNA3) at a 1:3 ratio. This was done to increase the probability that a cell expressing EYFP also contained α-syn. Only those cells exhibiting EYFP fluorescence were selected for imaging and analysis. It is important to keep in mind that while this technique is acceptable, individual cells will receive different numbers of copies of the α-syn and EYFP constructs. Therefore, it is possible that a proportion of the analyzed cells

did not contain α-syn. Another possible confounding factor is the inherent variability among cells, which will affect any assay involving quantitative measurements made from small numbers of single cells (in this case, 40-50 cells/condition). These may explain the extremely high *SD* values that were calculated for the imaging data. However, these issues could be countered by increasing the sample size, performing additional independent biological repeats, and appropriate statistical analysis. Despite these potential caveats, I will discuss the results of the single cell experiments as they are now, but it is important to recognize that these data are only preliminary.

I hypothesized that α-syn 1-110 and 1-120 would have an effect on mitochondrial health while α-syn 25-140 would not because α-syn requires the N-terminus to associate with mitochondrial membranes. After analyzing the images, the data suggests that all three of these variants decreased mitochondrial function, with 1-120 having the greatest effect (Fig. 4B). It is possible that while α-syn 25-140 did not associate with the mitochondria, it could have still formed toxic soluble fibrils (clusters of α-syn monomers that are significantly smaller than insoluble Lewy bodies) which are free to diffuse within the cell, damaging membranous structures including the mitochondria (Giasson & Waxman 2009). The central hydrophobic region of α-syn has been shown to be responsible for fibrillization (Giasson *et al.* 2001). All three of these mutants still retain this region, so it is reasonable to assume that all of them would be capable of forming soluble fibrils, but only α-syn 1-110 and especially 1-120 would likely be specifically trafficked to the mitochondria. Therefore, the heightened ability of α-syn 1-120 to decrease mitochondrial function may be due to the combination of initially trafficking to the mitochondria and subsequent formation of soluble fibrils within the mitochondria.

For α-syn-CL1, I hypothesized that it would be less likely to affect mitochondrial health and function because the CL1 aggregation tag is said to promote Lewy body formation in the cytoplasm. If α-syn-CL1 is sequestered into insoluble Lewy-like structures in the cytoplasm, it may not be readily trafficked to or be capable of affecting the mitochondria. The data suggests that it did decrease mitochondrial function, although it had the smallest effect of all of the tested variants (Fig. 4B). These data suggest that not all α-syn-CL1 aggregated into Lewy-like structures. It would be beneficial to perform a soluble/insoluble protein assay after transfection of this variant into human cells. That would tell us what proportion of the total α-syn-CL1 is fibrillized versus aggregated into Lewy bodies. It is important to note that while working with this variant for other research projects, despite published evidence to the contrary, it has never shown the ability to form Lewy bodies in our experiments when this was specifically tested. Working with a 2014 NSF FirstSTEP student team hosted by the Nelson lab (MTSU), we co-transfected HeLa cells with α-syn-CL1 and α-syn-EGFP and used live cell microscopy to visualize any Lewy body formation, but never obtained any data that suggested this variant promoted aggregation. So it is likely that the effect on mitochondrial function seen in this experiment was due to fibrillization.

In conclusion, the data collected suggests that all five of the α-syn variants (including WT) decreased mitochondrial function. This suggests that it is not necessary for α-syn to retain the N-terminal mitochondrial targeting sequence that is responsible for binding phospholipids on the mitochondrial membrane in order impair mitochondrial health and function. However, in order to make definitive statements about the effect of

the α-syn variants, more biological repeats should be performed along with additional experiments that probe other markers of mitochondrial health and function.

To expand upon this research, it may be helpful to produce new genetic tools that allow us more accurately to determine or specify the quantity of exogenous α-syn expressed in cells. These tools could include IRES expression vectors to express α-syn and a fluorescent protein from a bicistronic transcript at a fixed ratio. Additionally, in order to better understand how α-syn oligomerization and aggregation influence the ability of these proteins to affect mitochondrial function, it may also be necessary to perform soluble/insoluble protein assays.

In addition to exploring the effects of α-syn on mitochondrial health and function, the tools generated for this project will serve as a resource for other future studies in the Nelson lab. For example, neuroinflammation, which is also associated with PD and a possible causative factor of the disease (More *et al*. 2013), may be caused by inappropriate nuclear factor kappaB (NF-κB; a 'master-regulator' of the inflammatory response) activity in the brain (Sen & Baltimore 1986) due to the sequestration of key regulatory proteins to Lewy bodies. The α-syn variants created in this project could be used to further understand α-syn's role in the NF-kB pathway.

In summation, both mitochondrial dysfunction and α-syn aggregation are associated with PD, but the relationship between these two characteristics has yet to be elucidated. This project investigated the effects of five different α-syn variants on mitochondrial health and function. The data collected, while only suggestive, indicates that the N-terminal mitochondrial membrane targeting sequence on α-syn in not necessary for α-syn to decrease membrane polarization, a measure of health and function. The tools

created in this project are useful sources for further investigation into the effects of α-syn aggregation in PD.

References

Burbach, J.P. *et al*. Transcription factors in the development of midbrain dopamine neurons. *Ann. N. Y. Acad. Sci.* **991**, 61–68 (2003).

Choubey, V. *et al*. Mutant A53T α-Synuclein Induces Neuronal Death by Increasing Mitochondrial Autophagy. *Journal of Biological Chemistry* **286**, 10814-10824, (2011).

Devi, L. *et al*. Mitochondrial Import and Accumulation of α-Synclein Impair Complex I in Human Dopaminergic Neuronal Cultures and Parkinson Disease Brain. *Journal of Biological Chemistry* **283**, 9089-9100 (2008).

Dias, V. *et al*. The role of oxidative stress in Parkinson's disease. *J. Parkinsons Dis.* **3**, 461-491 (2013).

Giasson, B. I., *et al.* A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. *J. Biol. Chem.* **276**, 2380–2386 (2001).

Giasson, B. I. & Waxman, E. A. Molecular mechanisms of α-synuclein neurodegeneration. *Biochimica et Biophysica Acta (BBA)* **1792**, 616-624 (2009).

Huang, Y. *et al*. Macroautophagy in sporadic and the gentic form of Parkinson's disease with the A53T α-synuclein mutation. *Translational Neurodegeneration* **1**, 2, (2012).

Iwai, A. *et al*. The precursor protein of non-Aβ component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* **14**, 467-475, doi:10.1016/0896-6273(95)90302-X (1995).

Liu, C-W. *et al*. A precipitating role for truncated alpha-synuclein and the proteasome in alpha-synuclein aggregation: Implications for pathogenesis of Parkinson disease. *The Journal of Biological Chemistry* **280**, 22670- 22678 (2005).

Marques, O. & Outeiro, T-F. Alpha-synuclein: from secretion to dysfunction and death. *Cell Death and Disease* **3**, e350; doi:10.1038/cddis.2012.94 (2012).

More, S. V. *et al*. Cellular and molecular mediators of neuroinflammation in the pathogenesis of Parkinson's disease. *Mediators of inflammation* **2013**, 952375, doi:10.1155/2013/952375 (2013).

Muntane, G. *et al*. α-Synuclein Phosphorylation and Truncation are Normal Events in the Adult Human Brain. *Neuroscience* **200**, 106-119 (2012).

Nakamura, K. *et al*. Optical reporters for the conformation of α-synuclein reveal a specific interaction with mitochondria. *J. Neurosci* **28**, 12305–12317 (2008).

Schapira, A. H. V. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Neurology* **7**, 97-109, doi:10.1016/S1474-4422(07)70327-7 (2008).

Schindelin, J. *et al*. Fiji: an open-source platform for biological-image analysis. *Nature Methods.* **9**, 676-682 (2012).

Sen, R. & Baltimore, D. Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. *Cell* **47**, 921-928 (1986).

Smidt, M.P. *et al*. A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proc. Natl Acad. Sci. USA* **94**, 13305–13310 (1997).

Spillantini, M. G. *et al*. Alpha-synuclein in Lewy bodies. *Nature* **388**, 839-840, doi:10.1038/42166 (1997).

Wan, O. W. & Chung, K. K. The role of alpha-synuclein oligomerization and aggregation in cellular and animal models of Parkinson's disease. *PloS one* **7**, e38545, doi:10.1371/journal.pone.0038545 (2012).

Winslow, A. R. *et al*. α-synuclein impairs macroautophagy: implications for Parkinson's disease. *Journal of Cell Biology* **190**, 1023-1037 (2010).