

IDENTIFYING REGULATORY TARGETS IN PARKINSON'S DISEASE PATHOLOGY
AND SCREENING POTENTIAL THERAPEUTICS

by

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DEDICATION

I would like to dedicate my thesis to my parents Luz and Jesus Viana for helping me achieve what I thought was impossible and for always believing in me even when I did not believe in myself. Thank you for everything.

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I would like to acknowledge the NIH-INBRE fellowship for supporting me throughout my time at Delaware State University. I would like to thank my mentor Dr. Hwan Kim for his patience and understanding through my Masters. I would like to thank Michael Moore for his incredible amount of help with the confocal images. I would also like to thank my committee members Dr. Murali Temburni, Dr. Hakeem Lawal, and Dr. Hacene Boukari. Thank you to my lab members Dinesh Verma, Austin Jackson, Alex Burris, Dionne Williams, and Lindsey Ruggiero for making lab a fun environment and a teaching experience.

IDENTIFYING REGULATORY TARGETS IN PARKINSON'S DISEASE PATHOLOGY AND SCREENING POTENTIAL THERAPEUTICS

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ABSTRACT

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc). There are currently only treatments for relieving the symptoms of Parkinson's disease with no cure. All of the treatments currently available vary in efficacy and usually cause irreversible side-effects including dyskinesia after long term use (PD Med Collaborative Group, 2014). The main cause of the loss of the dopaminergic neurons is the aggregation of proteins called Lewy bodies. The formation of the Lewy bodies is mainly mediated by alpha-synuclein. Most Parkinson's disease cases are idiopathic. This has made scientists look at other reasons for the formation of Lewy bodies other than genetics such as post-translational modifications (PTM) on proteins. One such post-translational modification that has been looked at as having an important role in PD pathology is the small ubiquitin-like modifier (SUMO). SUMO is a post-translational modification that may be involved in the aggregation and toxicity of alpha-synuclein (Krumova *et al.*, 2011; Zhu *et al.*, 2018) In this study we assess the role of SUMOylation on alpha-synuclein *in vivo* and *in vitro*. Our main hypothesis is that the over-expression of SUMO conjugase, Ubc9,

protects dopaminergic neurons in the striatum. We used the Ubc9 overexpressing C57Bl/6 mouse model from the John Hallenbeck lab at NINDS (Lee *et al.*, 2012) and injecting them with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) chronically. We then quantified the levels of alpha-synuclein in the striatum through confocal microscopy. We also generated stable N27 cell lines over-expressing wild-type alpha-synuclein-HA and the K96R:K102R mutant alpha-synuclein-HA to assess different protein-protein interactions potentially. After confirming the expression of wild-type and the SUMOless alpha-synuclein mutant, the proteins were immunoprecipitated, run on a gel, and sent out for mass spectrometry. The immunoprecipitation samples were unable to be read, but the inputs were able to be read. The most interesting observation when comparing the two inputs is the difference in ubiquitin. There is more ubiquitin in the wild-type than the mutant input, which supports our hypothesis that SUMO competes with Ubiquitin for binding to lysines in target proteins. Since the current treatment options for PD only involve treating the symptoms and do not focus on the neuroprotection or neurorecovery, we looked at a pharmacological approach to develop novel compounds that could have potential therapeutic effects. In collaboration with AurimMed Pharma Inc., we screened 31 novel small compounds supplied by AurimMed Pharmaceuticals and tested for neuroprotective effects against 1-methyl-4-phenylpyridinium (MPP+) and measured in MTT and LDH assay (courtesy of Dinesh Verma). The screening of the novel compounds resulted in the identification of a few promising compounds that showed neurorecovery effects at all the concentrations used in both MTT and LDH assays. Neurorecovery is the recovery of cells from MPP+ damage.

These studies may help elucidate possible biological neuroprotective targets as well as novel compounds that do not simply target the symptoms of PD, but prevent the loss of dopaminergic neurons.

TABLE OF CONTENTS

| | Page |
|--|-------------|
| List of Tables..... | viii |
| List of Figures..... | ix |
| CHAPTER I: GENERAL INTRODUCTION..... | 1 |
| CHAPTER II: LITERATURE REVIEW..... | 3 |
| CHAPTER III: RESEARCH METHODS..... | 6 |
| MPTP Lesioned Mice..... | 6 |
| Isolating and sectioning of mouse brains..... | 6 |
| Immunofluorescence staining..... | 7 |
| Tissue lysate preparation..... | 8 |
| Generation of α -synuclein wild-type and mutant cell lines..... | 8 |
| Immunoprecipitation..... | 9 |
| Immunoblotting..... | 9 |
| MTT assay..... | 10 |
| LDH assay..... | 11 |
| CHAPTER IV: RESEARCH FINDINGS..... | 12 |
| CHAPTER V: DISCUSSION..... | 22 |
| REFERENCES..... | 26 |

LIST OF TABLES

| | | |
|-----------------|--|----|
| Table 1. | Mass spectrometry analysis of gel inputs show a decrease in ubiquitin and polyubiquitin species in the mutant K96R:K102R alpha-synuclein mutant..... | 15 |
|-----------------|--|----|

LIST OF FIGURES

| | | |
|------------------|--|----|
| Figure 1. | Confirmation of the expression of wild-type and K96R:K102R mutant alpha-synuclein. | 12 |
| Figure 2. | Hemagglutinin immunoprecipitation shows a difference in protein amount around the 70 kDa molecular weight | 14 |
| Figure 3. | Representative confocal images showing higher level of alpha-synuclein in Ubc9 transgenic mice striatum that that in WT, after chronic MPTP damage | 17 |
| Figure 4. | Alpha-synuclein Western blots show a higher level of alpha-synuclein in Ubc9 transgenic mice striatum tissue lysate than that in WT, after chronic MPTP damage | 18 |
| Figure 5. | MTT assay of novel compounds shows that the AurimMed compound-0130, -0131, -0132, are the most effective at all log scales | 19 |
| Figure 6. | LDH assay screening shows the AurimMed compound-0130, -0131, and -0132 as possible neurorecovery compounds at most log scale doses | 20 |

CHAPTER I

GENERAL INTRODUCTION

Parkinson's disease is a neurodegenerative disease categorized by the loss of dopaminergic neurons in the Substantia Nigra pars compacta. It is the second most common neurodegenerative disorder after Alzheimer's disease (Dorsey *et al.*, 2007). Currently, there is no cure for PD or treatment to prevent the pathology of Parkinson's disease. The most common therapy is levodopa (L-DOPA), which is a pre-cursor for dopamine production and can cross the blood brain barrier, while dopamine can not. The problem with L-DOPA is that dyskinesia can start to develop for most patients after long term use (Gray *et al.*, 2014). Other treatment strategies, just like L-DOPA, only work to treat the symptoms of PD and does not offer any neuroprotection against oxidative stress.

The alpha-synuclein mediated aggregation of proteins to form Lewy bodies in the Substantia Nigra pars compacta is a classic indication of Parkinson's disease. A form of post-translational modification, small ubiquitin-like modifier (SUMO) can be conjugated to target proteins by SUMO conjugase, Ubc9 and change their solubility (Mukherjee *et al.*, 2009). The preliminary results from our lab showed that the SUMOylation of dopamine transporter (DAT) increases the function of the protein (Cartier *et al.*, manuscript under final review). SUMOylation on alpha-synuclein has not been well understood due to the conflicting findings in the scientific community. SUMOylation on alpha-synuclein has been shown to inhibit aggregation and exacerbate aggregation.

The main hypothesis of my thesis is to test whether SUMOylation on alpha-synuclein can be a potential neuroprotective target in mouse models of PD and if altering the SUMO sites of

alpha-synuclein may cause different protein-protein interactions *in vitro* when compared to wild-type. Additionally, we would like to screen different novel compounds from AurimMed Pharmaceutical Inc. to develop potential compounds that can be neuroprotective or have neurorecovery effects (courtesy of Dr. Dinesh Verma).

CHAPTER II

LITERATURE REVIEW

Parkinson's disease (PD) is a neurodegenerative disease, first described by James Parkinson in 1817 (Parkinson, 1817). It is a progressive motor disease with symptoms like bradykinesia, resting tremor, stiff muscles, and unstable posture (Eckermann, 2013). The non-motor symptoms of Parkinson's disease include depression, anxiety, decreased smelling ability, and digestive issues (Braak & Del Tredici, 2017; Todorova *et al.*, 2014). Pathologically, it is characterized by the loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc). The loss of these dopaminergic neurons is due to the formation of Lewy bodies that derived from the protein aggregation including alpha-synuclein as a main mediator (Goedert *et al.*, 2017).

The main cause of the Lewy bodies formation in PD pathology is still not well understood. Most of the PD cases are idiopathic; however, there are still around 10-20% of the cases that are due to genetic mutations (Eckermann, 2013). Some of the genetic mutations for PD are identified from the parkin, Leucine rich repeat kinase 2 (LRRK2), and alpha-synuclein (SNCA) genes. The causes for idiopathic PD are not well understood, compared to the familial PD. It has been believed that environmental factors such as neurotoxins and pesticides play a role in the cause of the Parkinson's disease. These toxins created oxidative stress which can cause mitochondrial dysfunction (Langston, 1985; Zorzon *et al.*, 2002). Currently there is no cure for Parkinson's disease and numerous treatments including L-DOPA alleviate the PD symptoms, however, novel approaches such as deep brain stimulation and stem cell research can be promising in near future.

Alpha-synuclein is a 140 amino acid protein that is natively unfolded and found predominantly in the presynaptic domains of neurons in brain. It has been found in the nuclear envelope and synaptic regions, giving its name of “synuclein” (Maroteaux *et al.*, 1988). The exact role of alpha-synuclein in the body has not been well understood; however, there is evidence that alpha-synuclein is involved in synaptic transmission (Burre *et al.*, 2010). The first mutation to the SNCA gene was the A53T mutation and it was found to be autosomal dominant (Polymeropoulos *et al.*, 1997). After this discovery, the A30P mutation and the E46K mutations were discovered (Boyd *et al.*, 2012).

Alpha-synuclein has three main domains: the amphipathic region, non-amyloid- β component (NAC), and an acidic tail (Bridi & Hirth, 2018). Many of the mutations that have been identified on alpha-synuclein are in the amphipathic region such as A30P, E46K, and A53T. The non- β -amyloid domain of alpha-synuclein has been shown to be implicated in the aggregation of the protein (Giasson, 2001). The acidic tail is where some post-translational modifications such as SUMOylation occur (Krumova *et al.*, 2011). SUMOylation occurs at two lysine domains on alpha-synuclein; lysine 96 and 102 (Krumova *et al.*, 2011).

In order to prevent its aggregation, we propose to assess a novel therapeutic target on alpha-synuclein, Small Ubiquitin-like Modifier (SUMO). SUMO is involved in many different cellular processes such as protein stability, and DNA repair (Morris *et al.*, 2009). SUMO can covalently bind to the lysine residues of a variety of target proteins. Specifically, SUMO binds to lysine residues in the consensus sequence ψ KxE/D where ψ is a hydrophobic amino acid, K is lysine, x is any amino acid, and E/D are glutamic acid and aspartic acid, respectively (Dorval & Fraser, 2006). The SUMO pathway, much like ubiquitin, requires an SUMO activating enzyme, E1

ligase(SAE1/2) that activates SUMO, the E2 conjugating enzyme Ubc9, and an E3 ligase (Morris *et al.*, 2009). It has been debatable whether SUMO can be neuroprotective or promote alpha-synuclein aggregation (Kim *et al.*, 2011). It has been shown that SUMOylation of alpha-synuclein can help delay the aggregation of alpha-synuclein (Krumova *et al.*, 2011). Conversely, it has also been shown that SUMOylation can promote alpha-synuclein aggregation (Kim *et al.*, 2011; Oh *et al.*, 2011).

1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP) has been shown to produce Parkinson-like symptoms in humans and rats (Langston, 1985). MPTP can cross the blood brain barrier and is then converted to MPP⁺ that is taken up by dopaminergic cells where it causes damage to the cells. This damage to the substantia nigra causes the Parkinsonian symptoms in mice.

CHAPTER III

RESEARCH METHODS

1. MPTP Lesioned mice

All animal protocols were conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals; all procedures were approved by the Delaware State University Animal Care and Use Committee. All efforts were made to minimize animal numbers and distress. The dosage of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was 25 mg/kg administered to wildtype and Ubc9 overexpressing transgenic C57BL/6 mice through intraperitoneal injections for 7 consecutive days (Lazzara *et al.*, 2015). Saline was administered to the negative control group in the same manner. All the MPTP-treated mice are monitored daily by experimenters and/or vivarium staffs. When more than 10% of body weight loss was detected, those animals are excluded from the experiments. Three days after the last injection, mouse cages were properly disposed to prevent the contamination of MPTP.

2. Isolating and sectioning of mouse brains

Mice were sacrificed by perfusion with 150 mM of NaCl dissolved in 70% Ethanol. While the mice were anesthetized with a constant flow of isoflurane, a small incision to the right atrium was made and then the animals were perfused by a peristaltic pump. The whole brain was then removed from the animals after perfusion. The half of the brain was isolated for immunohistochemistry and the other half for biochemistry assays. The half for

immunohistochemistry were post-fixed in 4% paraformaldehyde (PFA) overnight and transferred to 30% sucrose solution for increasing the brain density (Lazzara *et al.*, 2015). After freezing the brains in OCT solution, the embedded brains were sliced using a cryostat at 14 μm sections and placed on polarized slides. The other half of the brain were isolated with cerebellum, brainstem, striatum, olfactory bulb, and the rest of the brain and stored at -20 °C to be used later for biochemistry assays.

3. Immunofluorescence staining

Tissue sections were washed in 0.1M phosphate buffer (PB, pH 7.2) three times for 15 minutes each. The samples were post-fixed in 4% PFA for 20 minutes. Next, endogenous peroxidases were inactivated with a deactivating solution containing 20% methanol, 0.2% Triton X-100, and 0.05% H_2O_2 for 10 minutes. The slides were washed in 0.2% PBT 2 times for 15 minutes per incubation. Following the wash step, the slides were blocked in 3% goat serum for 1 hour. Sections were then incubated in rabbit anti-alpha-synuclein primary antibody (1:200, Cell Signaling) at 4°C overnight. After overnight incubation, sections were washed in 0.2% PBT twice for a total of 1 hour. Alexa 555 α -goat anti-rabbit IgG secondary antibody (1:250, A21428, Thermo) was added onto the sections and incubated for 2 hours at room temperature. Following incubations, sections were washed in 0.2% PBT twice for 15 minutes each. Coverslips were mounted using Prolong diamond antifade mounting medium (Thermo). Brain Sections were imaged using LSM 510 confocal microscope (Zeiss) at the DSU imaging core, with support from Michael Moore.

4. Tissue lysate preparation

Tissue lysates were prepared from isolated C57BL/6 mouse brain tissues. The tissue lysis buffer was prepared containing 1% Triton, 0.1% NP-40, and protease inhibitors (100x from Thermo). Tissue was homogenized in a glass pestle and tissue lysate buffer was added. The tissue was then sonicated on ice at max power for 10 seconds. Samples were spun down at 4°C, 14,000 rpm for 10 minutes. The supernatant was then collected and protein concentration determined by the BCA assay (Thermo).

5. Generation of α -synuclein wild-type and mutant cell lines

An α -synuclein wild type and a K96R:K102R mutant α -synuclein construct, were conjugated with a hemagglutinin (HA) tag and were transfected into N27 parental cells. The constructs were cloned from a pcDNA 3.1 mammalian expression vector (Invitrogen) and point-mutations were generated by GenScript. Once N27 parental cells reached ~70% confluency in a 6 cm dish, they were transfected with the constructs using lipofectamine 2000 (Invitrogen) for 6 hours. The measured concentration of 6.25mg DNA from each construct was used to transfect the cells, as the manufacturer recommended. After allowing the cells to recover for 24 hours, 500 ug/mL of Geneticin (G418, Gibco) was added to each dish with fresh media for selection. After selection, the expression of the constructs was confirmed through Western blot.

6. Immunoprecipitation

Cells were lysed with cold RIPA buffer plus 20 mM of *N*-ethylmaleimide (NEM) and 1X protease inhibitor cocktail. Cells were solubilized by gentle rocking at 4°C for 1 hour. The cell lysate was spun down at 14,000 rpms at 4°C for 15 minutes. The supernatant was added to a new tube. Hemagglutinin (HA) antibodies (Thermo, 26183) were added to cell lysate in RIPA buffer plus *n*-ethylmaleimide (NEM) (Thermo) and protease inhibitor cocktail (Thermo). NEM was added to the RIPA buffer to prevent de-SUMOylation of proteins. Antibodies were incubated with rotation at 4°C overnight. On the next day, 60 uL of a 50% protein-A/G mix of Sepharose beads (Santa Cruz Biotech) were added to the samples and incubated on rotation at 4°C for 2 hours. The beads were spun down using a mini centrifuge for 3 minutes at 4°C. The supernatant was removed and the beads were washed in 1 mL of cold RIPA buffer (plus NEM and protease inhibitors). The beads were resuspended by manual agitation and then spun down again. The beads were washed four times. After the last spin and removal of the supernatant, the beads were resuspended in 60 µL of 1X sample buffer with 10% β-mercaptoethanol (Sigma). The beads were sonicated at max level for 5 seconds and then placed in a 37°C water bath for 30 minutes. The sample was then separated on a SDS-PAGE gel. This protocol was slightly modified from a recently submitted manuscript by Cartier et al. (Juan Viana is in the authorship).

7. Immunoblotting

Samples were separated using MOPS SDS-PAGE gel (GenScript), and transferred to a PVDF membrane (EMD Millipore). The transfer was done with 1X bicine buffer (3 g Tris-

base, 4.08 g bicine) at 60 volts at 4°C for 50 minutes. The PVDF membrane was blocked with 5% dry milk dissolved in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) at room temperature with gentle agitation for 1 hour. The membrane was incubated with a primary antibody such as alpha-synuclein at 1:1000 dilution in the blocking solution at 4°C with gentle rocking overnight. On the following day, the PVDF membrane was washed with TBS-T buffer with vigorous rocking three times for 10 minutes each. Horseradish peroxidase-conjugated secondary antibodies were added to the membrane in TBS-T (1:5000) and incubated with gentle rocking at room temperature. The blot was washed 3 times with TBS-T at room temperature with vigorous rocking for 10 minutes each. The blot was developed using Luminata solution and visualized using Thermo My ECL imager.

8. MTT assay

In a 96-well plate, 5,000 cells/100 µl were plated in triplicate wells. After 1 hour, cells were treated with the toxin MPP⁺ at 640µM final concentration and incubated at 37 °C, 5% CO₂ for overnight. The media was removed and different novel AurimMed Pharma compounds were given to the cells in media in log scale doses (1nM, 10nM, 100nM, 1µM and 10µM). After 24 hours of a novel compound treatment, 20 µl of MTT was added to each well and incubated at 37°C for three hours. Discard the media containing MTT reagent, then 200 µl of dimethyl sulfoxide (DMSO) was added to dissolve the insoluble purple formazan product in the solvent. The absorbance was measured at wavelengths 570/630 nm using a plate reader (Molecular Devices).

9. LDH assay

In a 96 well plate, cells were seeded at 5,000 cells per well. The cells were exposed to 640 μ M of MPP+ overnight. Media was removed and novel compounds were used to treat the cells at different doses (1nM, 10nM, 100nM, 1 μ M and 10 μ M) in log scale for 24 hours. After the 24 hours, take 50 μ L of the media from each well and put into a new plate. Add 50 μ L of the reaction mixture to it and incubated at room temperature for 30 minutes in the dark. After incubation, add 50 μ L of the stop solution to each well. The absorbance was then measured at 490/680 nm by a plate reader (Molecular Devices).

CHAPTER IV

RESEARCH FINDINGS

Confirmation of the generation of α -synuclein cell line

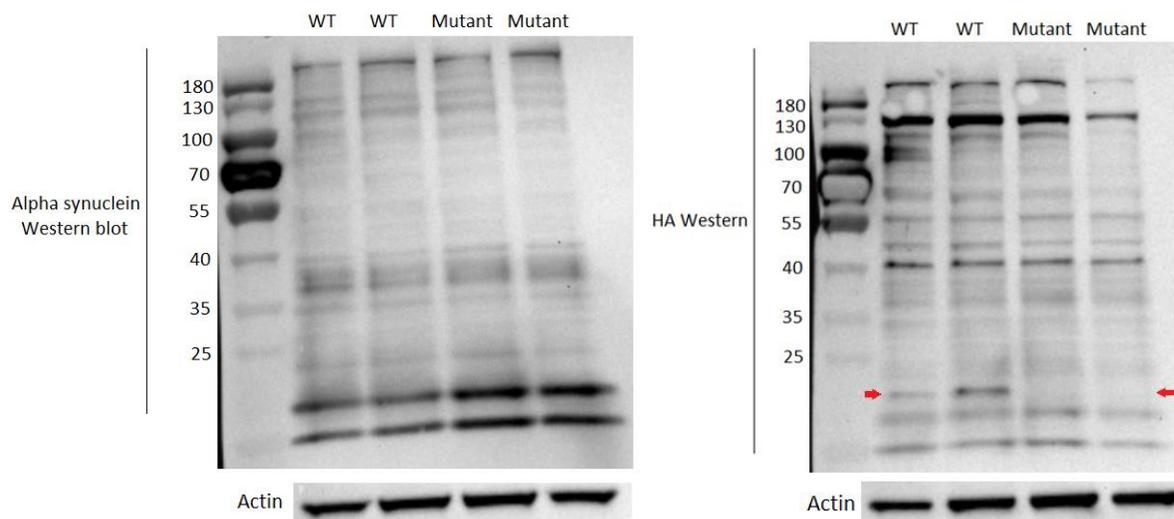


Figure 1. Confirmation of the expression of wild-type and K96R:K102R mutant alpha-synuclein. N27 cells were lysed after transfection and antibiotic selection to confirm the expression of the constructs. Cell lysates were run on a 4-20% gel then transferred and probed for either alpha-synuclein, HA, or actin.

In order to test the hypothesis of differential protein-protein interactions between SUMOylated and non-SUMOylated alpha-synuclein, N27 cells were transfected. The cells were transfected with either a plasmid containing wild-type alpha-synuclein with an hemagglutinin (HA) tag, or the K96R:K102R mutant alpha-synuclein. The cells were lysed and ran on a gel and transferred to a PVDF membrane for Western blot. Figure 1 shows the HA and alpha-synuclein Western blots used to confirm the expression of the constructs in N27 cells with actin as a loading control. The alpha-synuclein Western blot shows a band around 15 kDa consistent with the molecular weight of alpha-synuclein (Weinreb *et al.*, 1996). The HA Western blot shows the expression of the exogenous constructs. There is also a 15 kDa molecular weight band of a protein expressing

the HA tag. The two Western blots together show that the constructs with an HA-tag were successfully transfected into the N27 parental cells.

HA immunoprecipitation shows potential protein-protein interactions between wild-type and mutant alpha-synuclein

Immunoprecipitations were done on the two different cell lines using an HA antibody. The samples were run alongside the inputs on the gel that was stained with Imperial blue stain to show protein bands. Figure 2 shows a difference in the band intensity between the lanes of wild-type and the mutant form of alpha-synuclein around 70 kDa. There is a difference in the bands around the 70 kDa molecular weight. This could be due to the decrease in a higher molecular weight species of alpha-synuclein such as a multimer in the mutant form of alpha-synuclein or derived from differential protein-protein interaction based on the presence of SUMO in alpha-synuclein (Henley *et al.*, 2014). We sent out the gel for mass-spectrometry analysis to our collaborator Dr. Seo lab at the Korea Basic Science Research Institute (KBRI) in South Korea.

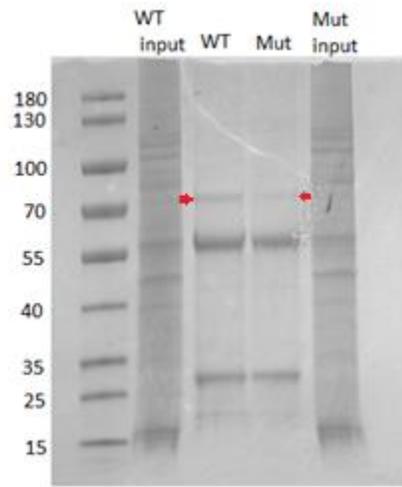


Figure 2. Hemagglutinin immunoprecipitation shows a difference in protein amount around the 70 kDa molecular weight. Immunoprecipitations were performed on cell lines with HA antibody and precipitates were run on a 4-20% gel and stained with Imperial blue (Thermo) overnight.

Mass spectrometry analysis of the protein gel

Table 1 shows the mass spectrometry analysis of the protein gel between the input for the wild-type alpha-synuclein and the mutant form. The immunoprecipitation lanes were unable to be read due to the keratin contamination masking the band signal. The table does show that there is more ubiquitin, polyubiquitin-B, and polyubiquitin-C for the wild-type alpha-synuclein input lane compared to the mutant input lane, which supports our hypothesis that SUMO competes with ubiquitin for binding to lysine residues (Rott *et al.*, 2017).

| Accession | Protein | Confidence % | Mut:WT Ratio |
|------------|---|--------------|--------------|
| Q6I8Q6 | Histone H2A | >95 | .84 |
| A0A091CM51 | Ubiquitin-40S ribosomal protein S27a | 90 | .32 |
| A0A0G2K3K2 | Actin, cytoplasmic 1 | >95 | 1.30 |
| A0A091CS77 | Gamma-enolase | >95 | 1.36 |
| A0A091DFZ4 | 60 kDa heat shock protein, mitochondrial | 90 | 2 |
| G5C5A6 | Ubiquitin | 90 | 0.31 |
| A0A091DL08 | Polyubiquitin-B | 90 | 0.39 |
| G5B8W4 | 40S ribosomal protein S27a | 90 | 0.32 |
| G5B170 | Heat shock cognate 71 kDa protein | >95 | 0.66 |
| Q5FWT0 | Ubc protein (Fragment) | 90 | 0.39 |
| G5B116 | Putative heat shock protein HSP 90-beta-3 | 90 | 2 |
| Q6P7R7 | Ubiquitin A-52 residue ribosomal protein fusion product | 90 | 0.31 |
| Q63654 | Polyubiquitin (Fragment) | 90 | 0.30 |
| F1LML2 | Polyubiquitin-C | 90 | 0.37 |
| A0A091CSH2 | Heat shock protein HSP 90-alpha | >95 | 2.51 |

Table 1. Mass spectrometry analysis of gel inputs show a decrease in ubiquitin and polyubiquitin species in the mutant K96R:K102R alpha-synuclein mutant.

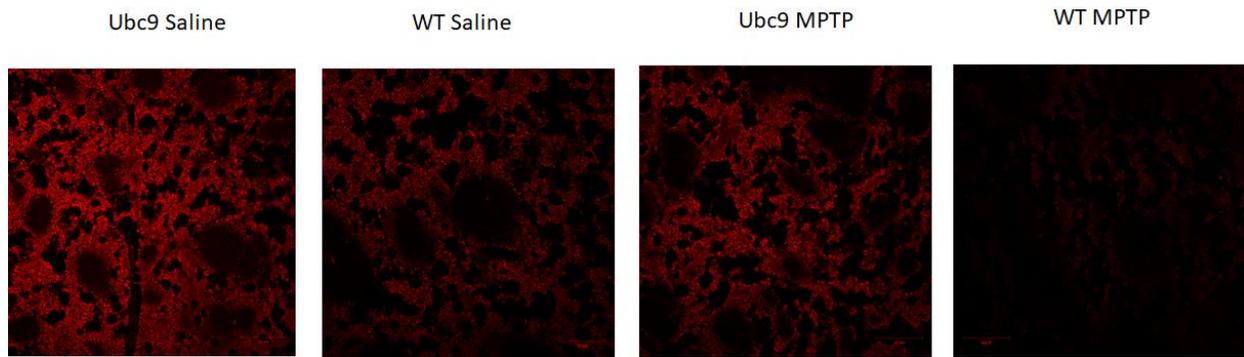
The whole gel was sent to our collaborator Dr. Seo at the KBRI to run the immunoprecipitation samples. The results provided limited information from the IPed samples due to the keratin contamination, but the input lanes show a few potential targets in different ubiquitin species between the WT and non-SUMO alpha-synuclein cell line.

Confocal images show an increased amount of alpha-synuclein in the striatum from Ubc9 transgenic mice, compared to the WT mice

The 14 µm thin slices were used from either Ubc9 or WT mice and both groups were treated with either saline or MPTP. The striatum including sections were probed for alpha-synuclein to assess their level. The representative images of striatum showed higher level of alpha-synuclein protein in the Ubc9 transgenic mice, compared to wild-type mice. The tissue and secondary antibody only-treated control sections show little to no background indicating the specificity of

the primary antibody (data not shown). When the levels of fluorescence was quantified, it showed a clear trend of higher levels of alpha-synuclein from Ubc9-Tg with MPTP, compared to the MPTP-lesioned wild-type group. There was a significant difference between the Ubc9-Tg saline group and both wild-type saline and MPTP treated groups. Although there was no significant differences between the MPTP-treated transgenic group and any other groups, we need to repeat these experiments because there were large variation, which derived from the technical issues due to the lack of expertise in the technique.

A.



B.

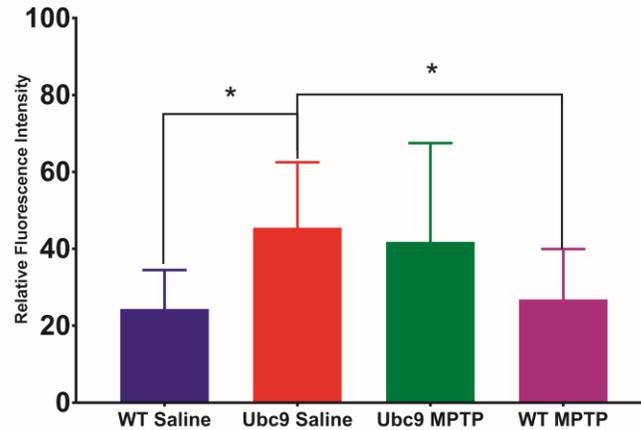


Figure 3. Representative confocal images showing higher level of alpha-synuclein in Ubc9 transgenic mice striatum than that in WT, after chronic MPTP damage. A. The 14 μ m thick slices were imaged using a Zeiss confocal microscope. These example figures show that the transgenic Ubc9 mice are less damaged over all in the striatum than that in the wild-type mice from MPTP toxicities. **B.** The relative fluorescence intensity of alpha-synuclein in the 40X striatum images shows the Ubc9 saline samples have significantly more alpha-synuclein than the wild-type saline or wild-type MPTP samples. Data analyzed by One-Way ANOVA. *: $p < 0.05$.

Figure 4 shows an alpha-synuclein Western blot with a trend of higher level of alpha-synuclein from the striatum of Ubc9 over-expressing mice than that of WT, after chronic MPTP toxicities. When the band intensity of alpha-synuclein were normalized with the loading control, the graph clearly shows higher protein levels of alpha-synuclein in the Ubc9 mice than that in WT, suggesting that the pan-Ubc9 overexpression protects the striatum from the neurotoxin MPTP-induced toxicities (Fig. 4). Due to the large error bars from the lack of technical expertise, there was no significance between the groups. Therefore, we need to replicate these experiments with the collected samples or may need to collect additional in vivo samples later.

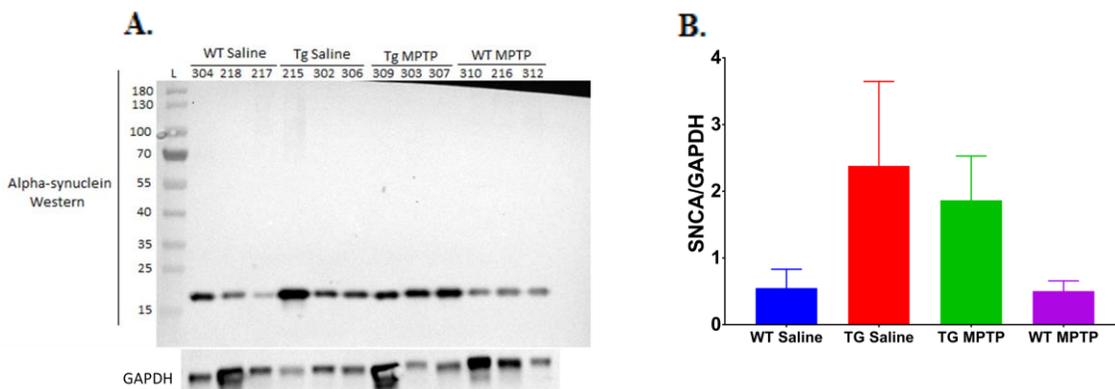


Figure 4. Alpha-synuclein Western blots show a higher level of alpha-synuclein in Ubc9 transgenic mice striatum tissue lysate than that in WT, after chronic MPTP damage. A. Extracted 20 μ g of striatum tissue lysate was loaded onto a gel, run, and transferred for Western blot. Western blot shows different levels of alpha-synuclein monomers in the striatum tissue. **B.** Normalized graph representing the levels of alpha-synuclein as seen on the Western blot. Analyzed by One-Way ANOVA. *: $p < 0.05$.

Screening of novel AurimMed compounds for neurorecovery effects from MPP+ induced damage

After identifying Ubc9 and SUMO as regulatory targets in PD pathology, a pharmacological approach was taken and novel compounds were screened to identify a potential therapeutic. Previously it has been shown by Carol Lazzara that compound AMP-X-0079 from AurimMed Pharmaceutical Company, had neuroprotective and neurorecovery effects against oxidative stress such as MPP+ or hydrogen peroxide induced toxicity in N27 cells. Janae Caviness, a previous labmate showed that the same compound had the same effects in MPTP lesioned mice. Given the success of AMP-X-0079, other compounds were also screened to see if there are other novel compounds that can be as effective as or more than AMP-X-0079. Over 31 novel compounds were screened for neurorecovery effects against MPP+ and

hydrogen peroxide-induced toxicities in cell viability (MTT) or cytotoxicity (LDH) assays (Courtesy of Dr. Dinesh Verma).

Figure 5 shows the MTT assay results from a few promising compounds screened with 2 non-efficacious compounds (Fig. 5). N27 cells were plated into a 96 well plate with 5,000 cells per well. After a 24 hour incubation period with 0.640 mM

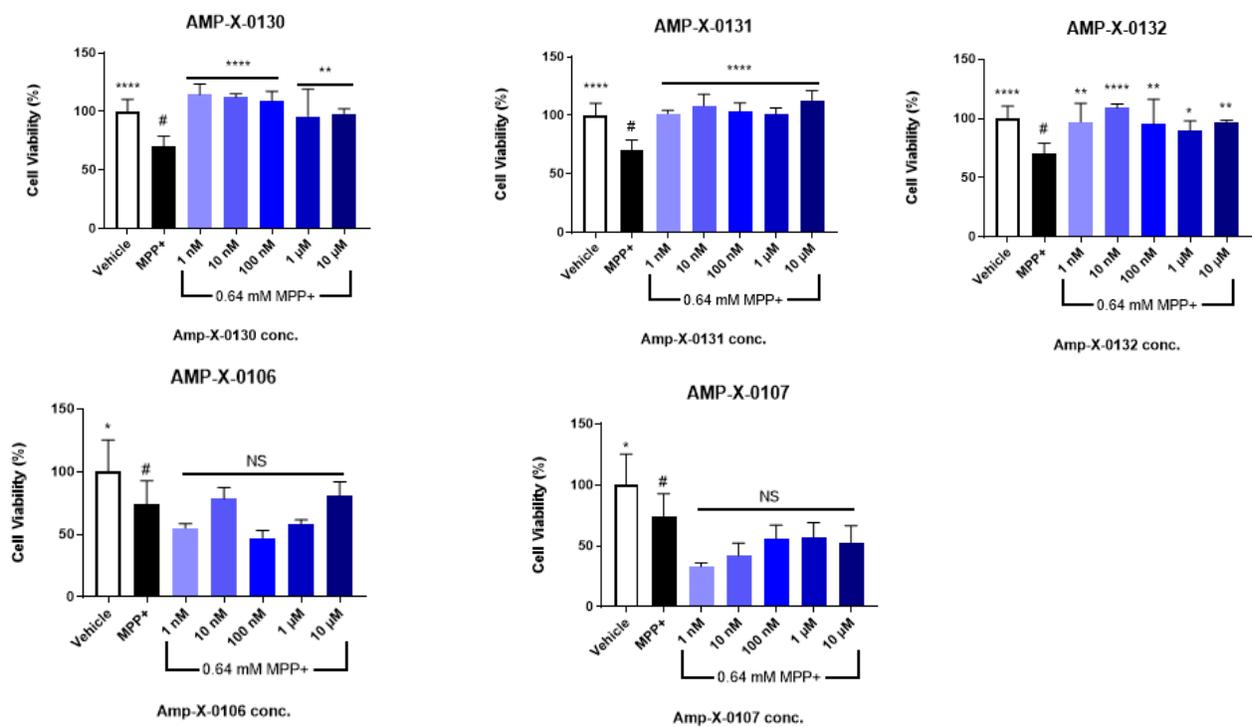


Figure 5. MTT assay of novel compounds shows that compound 0130, 0131, 0132, are the most effective at all log scales

of MPP+, each compound was added to the wells at log scale concentrations starting from 1 nM and the highest at 10 μM and incubated for 16 hours for assessing the recovery effects from the toxicity. The plate was then read to check for the recovery effects of each compound. A few compounds such as AMP-X-0130, 0131, and 0132 showed significant

recovery effects from MPP+ induced toxicities across all compound concentrations tested. Interestingly, the AMP-X-0130 had a more significant neurorecovery effect at 1 nM to 100 nM, but not as significant of an effect at the higher concentrations. The compounds AMP-X-0106 and AMP-X-0107 had no significant neurorecovery effect against MPP+ toxicity. Data was analyzed by One-Way ANOVA, Dunnett's Test (n= 3 or 4). *: p < 0.05; **: p < 0.01; ***: p < 0.001 and ****: p < 0.0001 compared to MPP+ only control (#).

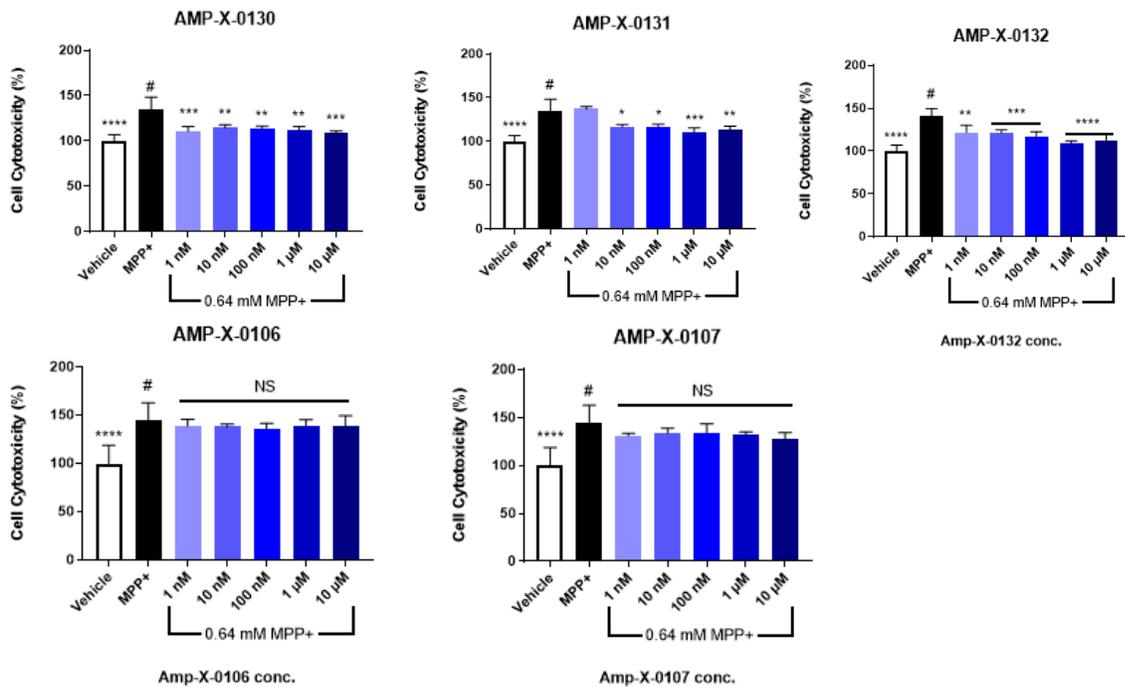


Figure 6. LDH assay screening shows compound 0130, 0131, and 0132 as possible neurorecovery compounds at most log scale doses. N27 cells were exposed to 0.64 mM MPP+ for 16 hours and then treated with the novel compounds for 24 hours. One-Way ANOVA, Dunnett's Test (n= 3 or 4). *: p < 0.05; **: p < 0.01; ***: p < 0.001 and ****: p < 0.0001 compared to MPP+ only control (#).

Figure 6 shows the LDH assay results of the same compounds used in the MTT assay. The LDH assay was used to measure the cytotoxicity in the mode of the compounds' recovery effects. The compounds were treated in the N27 cells for 16 hours after 24-hour incubation of MPP+. The results show that the compounds of AMP-X-0130, -0132 showed significant neurorecovery effects across all concentrations used in the experiments. The compound AMP-X-0131 was not significant at the lowest concentration of 1 nM. The compounds AMP-X-0106 and AMP-X-0107 showed no neurorecovery effects at any concentration as like as in the MTT assay.

Taking both MTT and LDH assays together, the compounds AMP-X-0130, -0131, and -0132 showed the most promising neurorecovery results, which can be higher than the efficacy of the compound AMP-X-79 in the previous studies done by Carol Lazzara and Janae Caviness.

CHAPTER V

DISCUSSION

There is currently no cure for Parkinson's disease, only treatments to help with the symptoms. Some of the treatments available for PD are L-DOPA, L-DOPA/carbidopa intestinal gel infusion, and deep brain stimulation. L-DOPA is usually the first course of action for PD patients; however, it is only effective for approximately 10 years before it is no longer effective (Connolly *et al.*, 2014). Deep brain stimulation is the last therapy given to PD patients and it is invasive and also only effective for 10 years. None of these therapies enables to cure or prevents PD pathology. My research focuses on identifying a potential regulatory target as well as developing novel compounds that have neuroprotective and/or neurorecovery effects on dopaminergic cells. My thesis focuses on identifying a potential regulatory target in PD pathology and screening/finding potential therapeutic novel compound. My thesis shows that there is a trend for neuroprotection against MPTP oxidative stress in Ubc9 over-expressing mice as well as novel compounds that show neurorecovery effects *in vitro*.

The mass spectrometry data were inconclusive when trying to show the differences in protein-protein interactions between SUMOylable alpha-synuclein and SUMOless (non-SUMO) alpha-synuclein, due to the IP-related technical issues. The immunoprecipitation samples were contaminated with keratin which masked the signal from being read or the concentrations of IP'ed samples were too low to be meaningfully detected in mass spectrometry. The input lanes for the wild-type and SUMOless alpha-synuclein enabled to compare the differential protein-protein interactions and it showed some interesting

differences in protein-protein interactions based on the presence of SUMO in alpha-synuclein. Specifically, there was more ubiquitin and polyubiquitin in the wild-type lane than the mutant lane. This is not surprising since ubiquitin also binds to lysine residues just like SUMO (Krumova *et al.*, 2011; Swatek & Komander, 2016). Since the known SUMO sites were mutated to arginines from lysines, neither ubiquitin nor SUMO could bind to those residues, which could explain the reduced ubiquitin levels in the mutant alpha-synuclein compared to the WT.

The Western blot showed that there is more alpha-synuclein in the Ubc9 over-expressing mice after vehicle or MPTP injections compared to the wild-type mice. This could be due to the overexpression of Ubc9 directly, or indirectly through SUMO conjugation to other protein targets for showing neurorecovery effects against MPTP toxicity. When the alpha-synuclein Western blot is normalized against GAPDH, there is no significant differences between the groups, although we detected a clear trend of protective effects by Ubc9 overexpression from MPTP toxicities. This could be due to the large error bars that came from either technical issues or sample degradation. Regardless, the graph and Western blot show an intriguing trend of the Ubc9 over-expressing mice having higher level of alpha-synuclein than the wild-type. The findings from the biochemistry experiments can be backed up by the confocal images. The confocal images also show that the Ubc9 overexpressing mice have more alpha-synuclein in the striatum than the wild-type mice with MPTP toxicity. When the fluorescence levels were quantified the Ubc9-Tg MPTP group did not have significantly more alpha-synuclein in the striatum when compared to all the other groups. This could be due to the lack of technical expertise. The graph does show that Ubc9-Tg

saline group was significantly higher than both wild-type saline and MPTP groups.

Surprisingly, we did not see a significant difference between the wild-type saline and MPTP groups. This could also be due to technical issues such as uneven fluorescence between different sections within each group. Even though we did not get the expected significant results, the confocal data does show the same trend as the Western blot. Both the biochemistry and the microscopy results show a trend that supports the hypothesis that overexpressing Ubc9, and thus having more SUMOylation, can be neuroprotective against MPTP toxicity.

Currently there is no medication that can be neuroprotective or have neurorecovery effect for PD patients. Since we looked at potential *in vivo* neuroprotective targets for PD, we decided to take a pharmacological approach to identifying a potential *in vitro* therapeutic. In our study, we observed the effects of different compounds provided by AurimMed with MPP⁺ toxicity in cell viability and cytotoxicity assays. In the MTT assay, the compounds of AMP-X-0130, -0131, and -0132 showed significant cell viability at all compound concentrations when 0.640 mM MPP⁺ was applied. According to the LDH assay, the compounds of AMP-X-0130, -0131, and -0132 showed the least cytotoxicity or the most neurorecovery effects. Compounds AMP-X-0106 and AMP-X-0107 showed no neurorecovery effects in either assay. When taking both assays together, the compounds AMP-X-0130, -0131, and -0132 are the most promising compounds that showed its potency at almost all concentrations in both assays. This could be due to the effectiveness of the compounds; however, other compounds are still effective in providing recovery effects from oxidative stress. Our data presentation was not perfectly consistent, which may be due to

technical errors since there was trouble with the effectiveness of the MPP+ toxicity that may be resulted from the changes in the N27 cell line after repeated seedings over time. Given the data, the compound AMP-X-0130, -0131, and -0132 are the most promising candidates that can be compared with the compound AMP-X-79 for developing a potential future therapeutic for Parkinson's disease.

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