Validation of Sirtuin 3 as a Neuroprotective and Neurorestorative Target in the Mutant Human A53T α-synuclein Rat Model of Parkinson’s Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Cell and Systems Biology
University of Toronto

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Abstract

Mitochondrial dysfunction plays a major role in the progressive and pathological development of Parkinson’s disease (PD). Mitochondrial protein deacetylase sirtuin 3 (SIRT3) assists in controlling bioenergetic and antioxidation processes by regulating mitochondrial enzyme acetylation levels. Based on this, overexpression of SIRT3 in the substantia nigra pars compacta (SNC) was assessed for its disease-modifying and cytoprotective effects in a well-accepted, adeno-associated virus (AAV) model of PD. SIRT3-myc AAV was infused into the SNC before or after A53T AAV induction of parkinsonism and analyzed at three and six-week time points. Results suggested that at six weeks, SIRT3 overexpression significantly reduced parkinsonian deficits, localized to the mitochondria, and displayed deacetylase function. Overall, the beneficial effects of SIRT3 demonstrate its disease-modifying implications for PD.
Acknowledgements

Notably, I would like to express appreciation for my supervisor Dr. Joanne Nash for providing me with a great educational and research opportunity where I could contribute to the field of neuroscience and Parkinson’s research. Also, I am immensely thankful for colleague Dennison Trinh for his positivity, dedication, strong work ethic, constant support, and time sacrificed to train and assist me throughout my degree. Special thanks to former postdoctoral fellow Dr. Jacqueline Gleave for performing the study optimization as well as many surgeries and analyses, and for her contribution to the publication of this work. Harsimar Brar, a volunteer in our lab, demonstrated to me a strong desire to learn and assist and I thank her for her cooperation and diligence, especially in the final stages of my experiments. I am truly grateful for the contributors in this research.

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This thesis is dedicated to my parents, who I admire so deeply. Thank you for giving me strength and fortitude, and for always believing in me and my potential.
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<th>Full Form</th>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>α-synuclein</td>
<td>Alpha synuclein</td>
</tr>
<tr>
<td>A53T</td>
<td>Human mutant alpha synuclein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>CCAC</td>
<td>Canadian Council on Animal Care</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CypD</td>
<td>Cyclophilin D</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>DJ-1</td>
<td>Protein deglycase 1</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenyl acetic acid</td>
</tr>
<tr>
<td>DOPAL</td>
<td>3,4-dihydroxyphenylacetaldehyde</td>
</tr>
<tr>
<td>E1</td>
<td>Ubiquitin-activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Substrate-specific ubiquitin ligase (E3)</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERCs</td>
<td>Extrachromosomal ribosomal DNA circles</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
</tr>
<tr>
<td>GC</td>
<td>Genome copies</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus pars externa</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus pallidus pars interna</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic Acid</td>
</tr>
<tr>
<td>IDH2</td>
<td>Isocitrate dehydrogenase 2</td>
</tr>
<tr>
<td>IH</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeats</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>MAM</td>
<td>Mitochondrial-associated membranes of the endoplasmic reticulum</td>
</tr>
<tr>
<td>MAOI</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>MLS</td>
<td>Mitochondrial localization sequence</td>
</tr>
<tr>
<td>MPP</td>
<td>Mitochondrial processing peptidase</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-methyl-4-phenyl pyridinium</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium spiny neurons</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAM</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>NDUFA9</td>
<td>NADH dehydrogenase 1 α subcomplex 9</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PFF</td>
<td>Preformed fibrils</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rAAV</td>
<td>Recombinant adeno-associated virus</td>
</tr>
<tr>
<td>RBD</td>
<td>Rapid-eye movement sleep behaviour disorder</td>
</tr>
<tr>
<td>Sir2</td>
<td>Silent information regulator 2</td>
</tr>
<tr>
<td>SIRT3</td>
<td>Sirtuin 3</td>
</tr>
<tr>
<td>SIRT3$^{H248Y}$</td>
<td>Deacetylase-deficient SIRT3</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SNC</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>SOD2</td>
<td>Superoxide dismutase 2</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer mitochondria</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>UCH-L1</td>
<td>Ubiquitin carboxy-terminal hydrolase L1</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Parkinson’s Disease

Parkinson’s disease (PD) is the second most common neurodegenerative disorder and was first described by James Parkinson as the “shaking palsy” (1, 2). PD involves degeneration of dopaminergic neurons in the substantia nigra (SN), a structure of the basal ganglia. These neurons project into the mesencephalic brain structure known as the striatum. Due to the high presence of neuromelanin-pigmented dopamine neurons in the SNc, the neurodegeneration involved with PD results in SNc depigmentation as well as lowered dopamine levels (3, 4). It is believed that PD neuropathology begins in the olfactory bulb and dorsal motor nucleus and progresses to the raphe nuclei and locus coeruleus, followed by the amygdala, hypothalamus, basal forebrain, and SNc (5, 6). Finally, pathology spreads into the cerebral cortex in sensory-association, premotor, and primary sensory and motor areas (5, 6).

Clinical symptoms of PD are not usually apparent until approximately 50% of dopaminergic cell loss has occurred in the SNc (7). Prevailing symptoms include resting tremors, bradykinesia, muscle rigidity, akinesia, loss of automatic movements, and postural instability (1, 2). In addition, the basal ganglia form networks with the ventral tegmental area, prefrontal cortex, nucleus accumbens, and the hippocampus, which play roles in learning, motivation, reward, and temporal processing (5). The basal ganglia can also form networks with the brain stem, which processes sleep-wake cycles (5). In later stages of PD, these networks may be damaged, resulting in a number of other cognitive and neurobehavioural abnormalities, including dementia, depression, hallucinations, sleep disorders, olfactory dysfunction, musculoskeletal pain, cardiovascular issues, and compulsive behaviours. For example, a longitudinal study on rapid-eye movement sleep behaviour disorder (RBD) showed that out of forty-four patients
diagnosed with RBD, twenty developed a neurological disorder with nine of those patients acquiring PD (8). This suggests that sleep disorders may also predate PD. Another study used a series of neuropsychological tests to examine executive and visuospatial function, memory, and attention in PD patients and healthy controls. Results showed that 60% of PD patients in early to middle stages of the disease displayed some type of cognitive dysfunction, with executive function and memory being most affected (9). Typically, PD patients experience a progressive decline in their motor ability, cognition, and quality of life as the disease advances.

Parkinson’s disease affects 0.3% of people worldwide and approximately 3% of individuals over the age of 65 (10). The cause of PD can vary greatly by patient. However in 90-95% of cases, PD occurs for random or unknown reasons with no apparent family history and is classified as ‘sporadic’ or ‘idiopathic’. Familial PD on the other hand occurs in 5-10% of cases, where there are nine major gene mutations known to play a role in PD pathogenesis (see section 1.2). Whether genetic or sporadic, it is likely that a multitude of factors act in combination with each other to result in a PD phenotype. These factors include age, gender, growth factors, genetics, and environmental factors. While these factors may not necessarily cause PD, they contribute to increasing the risk of acquiring the disease.

Due to the frequency of the disease and the anticipated future spike in aging disorders as the baby-boomer population enters old age, development of better symptomatic treatments or disease-modifying agents to slow or halt the disease is of high importance. In Canada, 61% of PD patients reported personal expenses for medications, mobility aids, speech and physical therapy, and other health care services relating to their condition that are not covered by government or insurance programs (11). In addition, 84% of PD patients rely on family and friends, volunteers, paid caregivers, and financial resources (11). The expected rise in PD will thus result in a higher cost of care (for example, in/outpatient care, nursing) and prescription
drug expenditure per patient. In consequence, this will lead to decreases in productivity from both patients and their caregivers. Based on this, if the impact of the disease on patients can be reduced or eliminated, a reduction in the economic impact of the disease would be observed as well. Currently, the mechanisms by which dopamine neurons degenerate in PD are not fully understood. For this reason, there is no method by which the course of the disease can be modified. However, various aspects of genetics, the environment, and cellular function that play a role in PD pathology have been identified. These aspects can be used as the basis for the discovery of new treatments and disease-modifying agents.

1.1.1 Neural Circuitry

The basal ganglia are composed of neural circuits that are important for planning motor movements and action selection (12). The basal ganglia contain bilateral nuclei known as the SN, which are then further subdivided into two parts: the more medial pars compacta (SNC) and laterally the pars reticulata (SNr). Another basal ganglia structure known as the striatum contains GABAergic medium spiny neurons (MSNs) and cholinergic neurons. The striatum provides inhibitory neuronal outputs to the thalamus via the globus pallidus and the SN. In the indirect pathway, the striatum receives inhibitory dopaminergic inputs from the SNC and excitatory glutamatergic inputs from the cortical motor areas. The striatum then releases inhibitory GABA onto the globus pallidus pars externa (GPe), another structure of the basal ganglia. This prevents the GPe from releasing GABA onto the subthalamic nucleus (STN), which disinhibits the STN and allows glutamatergic excitation of the globus pallidus pars interna (GPI) (13). The end result is inhibition of thalamocortical pathways from transmitting motor signals back to the motor cortex (13). The direct pathway, on the other hand, involves inhibitory GABAergic outputs from the striatum acting on the GPI, which disinhibits thalamocortical pathways thus allowing
movement (13). One study used optogenetics to activate MSNs of the direct pathway to elicit motor activity and the indirect pathway to suppress motor activity. Both pathways displayed increased MSN firing before action selection as well as both excitatory and inhibitory effects in the SNr possibly due to lateral inhibition or other circuits that may be involved (13). A study by Kravitz et al. (2010) showed that bilateral activation of MSNs of the indirect pathway leads to freezing, bradykinesia, and difficulty initiating movements (12). In contrast, these deficits are rescued by activation of the direct pathway. These studies provide more information about basal ganglia function that may be applicable to diseases such as PD.

Normally, control of initiation and execution of voluntary movement is achieved through balance of the direct and indirect pathways. In PD, dopaminergic neurodegeneration reduces striatal inputs from the SN, leading to excess inhibition of the thalamus downstream (14). This leads to behaviours such as rigidity, freezing, and bradykinesia as described by Kravitz et al. (2010). However, fMRI and SPECT imaging show that dopamine depletion in pallidal structures of the basal ganglia and not the striatum drives tremor generation in the cerebellothalamic circuit, which controls the tremor amplitude (15). More recently, it was shown in PD patients with tremor episodes that dopaminergic medication reduced pallidal tremor onset and directly acted on the cerebello-thalamo-cortical circuit to reduce tremor amplitude (14). It is the disordered neuronal output and synchronicity changes that occur with PD that lead to motor deficits such as tremors and dyskinesias.

1.2 Genetics

There is evidence suggesting that SNc dopamine neurons can be more susceptible to PD-related neurodegeneration based on an individual’s genetics. Gene expression profiles in rat dopaminergic neuron populations have been analyzed, showing that SN neurons express high
levels of energy metabolism transcripts (16). This is suggestive of the high susceptibility of SN
dopamine neurons to mitochondrial dysfunction (discussed further in 1.4.2). While most PD
cases are sporadic, both familial and sporadic forms of PD present comparable clinical
manifestations and involve genetic components. Therefore, although fewer PD patients suffer
from familial PD, research on mutations of PD-related genes helps create a better understanding
of the detrimental neuronal mechanisms that are characteristic of both familial and sporadic PD.
Knowledge of genetic information is vital as it may help diagnose PD earlier, predict who may
be at risk so that early intervention is possible, and allow medications, therapies, and treatments
that target genes or cellular pathways to be developed. Such treatment strategies could
potentially slow, halt, or even prevent PD progression in patients, regardless of the cause.

Years ago, there was no research proving that PD might have a genetic basis. It was not
until 1997 that the SNCA gene encoding α-synuclein was discovered, and since then many other
PD-related genes have been identified (17). The SNCA PD susceptibility gene was found on
chromosome 4 in a genome analysis study of a large Italian kindred. Analysis of the α-synuclein
gene showed evidence of recombination events and a nucleotide point mutation G209A
responsible for the A53T amino acid change (17). Population studies found that this mutation
was present in four independent PD families but not in any control chromosomes (17). Today,
there are 18 known genes denoted PARK1 through PARK18 which are proposed to have an
association with PD. However PARK1-9, PARK16 as well as PD-associated gene mutations in
GBA (not currently assigned a PARK label), have been confirmed to show an increase risk of
developing PD. Some of these will be discussed further in sections 1.2.1-1.2.6.
1.2.1 PARK1 and PARK4 - SNCA

α-synuclein is a soluble, monomeric protein consisting of 140 amino acids encoded by the SNCA gene on chromosome 4q21. α-synuclein is ubiquitously expressed throughout the central and peripheral nervous systems. In cells, α-synuclein is proposed to exist in presynaptic terminals, the nuclear envelope, and the cytoplasm. α-synuclein does not take on a folded secondary structure unless bound to a lipid membrane, where its N-terminus then adopts an α-helical formation. The primary structure of α-synuclein is outlined in Figure 1. The physiological function of α-synuclein is uncertain, however it is suggested that α-synuclein may be involved in neuroplasticity, histone interaction, vesicular transport, and regulation of neurotransmission. The function of α-synuclein is discussed further in section 1.4.5.2.

Polymorphism and mutation studies of the SNCA gene provide evidence for a causal link between α-synuclein and PD. Specifically, the A53T missense mutation is the most frequently occurring missense substitution of the SNCA gene, involving an amino acid substitution of alanine to threonine at position 53 (17). This was the first mutation identified for inherited forms of PD. The A53T amino acid substitution disrupts the protein’s natural α-helical structure and extends the β-sheet character (17). This causes a tendency for α-synuclein to aggregate and abnormally associate with other proteins, leading to Lewy body and Lewy neurite formation, disruption of cellular functions that threaten cell viability, and eventually neurodegeneration. There is also evidence that mutant α-synuclein can localize to the mitochondria where it interrupts complex I and IV activity and mitochondrial membrane potential, inevitably leading to reactive oxygen species (ROS) production, mtDNA damage, and triggering apoptotic events (18). Other identified PD-associated SNCA missense mutations include E46K, H50Q, G51D, and A30P. Interestingly, because these mutations occur within the first 53 amino acids of the α-
synuclein protein, they all exist within the N-terminal region and thus can directly impact its structure and function.

In addition to missense mutations of SNCA, gene dose due to copy number variations may also play a role in acquiring PD. There is evidence of duplication or triplication of the SNCA gene, causing high expression of α-synuclein. This SNCA gene multiplication can thus trigger the PD with a phenotype severity, onset, and disease progression correlating to gene dose, varying gene expressivity, and penetrance (19).
Figure 1: Primary structure of the human α-synuclein protein

R = xKTK(E/Q)GVxxxx
**Figure 1: Primary structure of the human α-synuclein protein**

The diagram represents the full-length, primary structure of one of the isoforms of the human α-synuclein protein (19 kDa). This particular 140-amino acid length isoform of α-synuclein is the most abundant in the brain and susceptible to aggregation compared to other shorter isoforms that exist. More specifically, it is found in the nuclear envelope, cytosol, and presynaptic terminal of neurons. It can also be found in the heart, skeletal muscle, pancreas, and placenta. The N-terminus of α-synuclein is positively charged and consists of seven imperfect repeats that are six amino acids in length (xKTK(E/Q)GVxxxx) represented by R1 to R7. Natively, α-synuclein is an unfolded monomer, however when the N-terminus interacts with lipid membranes, it takes on an α-helical structure. The non-amyloid component (NAC) core is a hydrophobic region and is associated with oligomerization. Finally, the C-terminus is a negatively charged region that remains unfolded when α-synuclein interacts with lipid membranes. When α-synuclein forms oligomers, its α-helical structure is disrupted and β-sheets become apparent. This can influence aggregation and interactions with other proteins as seen in PD.
1.2.2 PARK2 - Parkin

Parkin is a 465-amino acid, 52kDa protein encoded by PARK2 on chromosome 6q25-27. Parkin is found in dopaminergic and non-dopaminergic neurons of the SN, striatum, hippocampal formation, pallidal formation, and the cerebellum where it localizes to the cytosol and endoplasmic reticulum (ER) (20). Parkin functions as an E3 ubiquitin ligase to mark proteins such as α-synuclein at lysine residues for degradation by the ubiquitin proteasome system (UPS). Parkin also interacts with a number of other proteins such as complex I of the mitochondrial electron transport chain (ETC) and PD-associated protein, PINK1. There is evidence that parkin mutations are associated with a reduction in complex I activity, suggesting its role in the mitochondria (21). Parkin also interacts with PINK1 to help regulate mitochondrial integrity and function, promote mitophagy, and maintain cellular dynamics. This interaction will be discussed further in section 1.2.3.

Parkin mutations are the most common cause of recessive forms of PD and lead to early or juvenile-onset PD (22). Like SNCA, PARK2 gene mutations can involve missense mutations or multiplications spanning across all twelve of its exons (23). Furthermore, the PARK2 gene contains long introns and is located within a common fragile site. This makes the gene susceptible to breaks, small and large deletions, exon rearrangements, and DNA replication and transcription errors. At the subcellular level, parkin mutations destabilize its ubiquitin-like domain and disrupt its ligase activity, thus impairing the cell’s ability to recognize, ubiquitinate, and degrade neurotoxic protein aggregates (24). This compromises cellular processes that may lead to PD. Parkin mutations can also disrupt the PINK1/parkin pathway by causing PINK1 to fail to recruit parkin at the mitochondria and by causing parkin to exhibit a loss of mitophagic activity.
1.2.3 PARK 6 - PTEN-Induced Putative Kinase 1 (PINK1)

The PARK6 gene on chromosome 1p35-36 encodes PINK1, a 581-amino acid mitochondrial membrane protein found in the cortex, striatum, thalamus, cerebellum, SN, and hippocampus (25, 26). PINK1 has a C-terminal serine-threonine kinase domain with a mitochondrial localization sequence (MLS) that allows its mitochondrial import or contact with the cytosolic side of the outer mitochondrial membrane (25, 27). PINK1 is shown to accumulate on damaged mitochondria where it then recruits and phosphorylates parkin to promote mitophagy and ubiquitination of mitochondrial proteins for removal (28). Normally, when PINK1 is imported into the mitochondria, it is cleaved by proteases such as PARL from a stable 63kDa form to an unstable 52kDa form that is often targeted for proteasomal degradation. It is suggested that cleavage of PINK1 is a method by which healthy mitochondria suppress the PINK1/parkin pathway, preventing mitophagy of healthy mitochondria (28). Thus in damaged mitochondria, PINK1 is not cleaved, which is essential to allow for recruitment of parkin for mitophagy (27). There is also evidence that PINK1 phosphorylates serine-250 of the NDUFA10 subunit of complex I required for ubiquinone reduction, thus implying its role in the ETC and ATP synthesis (29).

Autosomal recessive mutations of PARK6 are responsible for early-onset of PD with Lewy bodies (25). When mutations exist in the serine-threonine kinase domain, there is a loss of kinase activity that disrupts the PINK1/parkin mitophagic pathway (30). Studies in Drosophila collectively show that PINK1 and parkin mutants lead to energy depletion, dopamine neuron and muscle degeneration, and shortened lifespan (31, 32). It was also shown that expression of parkin in PINK1 KO Drosophila, but not vice versa, may compensate this phenotype, suggesting that PINK1 functions upstream of parkin (31).
1.2.4 PARK7 – Protein Deglycase (DJ-1)

PARK7 of chromosome 1p36 encodes DJ-1, a 189-amino acid ubiquitously expressed protein abundant in the SN, striatum, hippocampus, neocortex, and cerebellum (33). Under physiological conditions, DJ-1 homodimerizes and localizes to the cytoplasm with little nuclear and mitochondrial expression (34). However, the oxidative environment modulates cycling between dimeric and monomeric forms that determines its mitochondrial or nuclear localization (33, 34). Specifically, oxidative stress increases DJ-1 expression and mitochondrial translocation (34). The function of DJ-1 is not well defined. However at the mitochondria, in vitro knockout studies show that DJ-1 is required to directly associate with the NDUFA4 subunit of complex I to maintain its activity (35). It has also been shown that DJ-1 decreases ROS-dependent translocation of parkin to PINK1 at damaged mitochondria due to its ability to control ROS and mitochondrial health so that PINK1/parkin-mediated mitophagy is not required (36). This may be due to the ability of DJ-1 to scavenge ROS via redox reactions to protect the cell (37). DJ-1 is also implicated in the regulation of tyrosine hydroxylase (TH) expression by sequestration of a corepressor from the TH promotor, thus influencing dopamine synthesis (38).

Point mutations of DJ-1 include missense, truncating, splicing, and large exon deletions that cause early-onset, slow-progressing, autosomal recessive PD (39). These mutations may prevent DJ-1 expression and the ability of DJ-1 to homodimerize, leading to loss-of-function and destabilization (37). For example, the L166P point mutation prevents typical DJ-1 homodimerization and α-helical character, which is correlated with a reduction in stability and its catalytic, protease, chaperone, and ROS-scavenging function (40, 41). The impaired structure also leads to DJ-1 polyubiquitination, which increases susceptibility to UPS degradation (40, 41).
1.2.5 PARK8 – Leucine-Rich Repeat Kinase 2 (LRRK2)

LRRK2, also known as dardarin, is a large, 2527-amino acid multi-domain protein encoded by the PARK8 gene of chromosome 12q12 (42, 43). LRRK2 is found in the cytosol and associates with the outer mitochondrial membrane and synaptic vesicles (44). Near its C-terminus, LRRK2 possesses a kinase domain that allows it to regulate various cellular processes such as protein assembly, mitochondrial function, and apoptotic mechanisms (45). LRRK2 also possesses a GTPase domain that facilitates protein interaction for signaling, maintenance of the cytoskeleton, and assistance with vesicle trafficking. For example, LRRK2 co-localizes with α- and β-tubulin in primary hippocampal neurons, suggesting its interaction with microtubules and possible involvement in microtubule assembly and dendrite length (46). There is also evidence of LRRK2 involvement in vesicle trafficking and cell signaling due to its co-localization with trafficking proteins and presence in synaptic and transport vesicle membranes (46).

A multitude of missense and nonsense mutation variants have been identified for LRRK2 in all of its domains (43, 44). The most common LRRK2 mutation is the gain-of-function glycine to serine missense substitution at position 2019 (G2019S) in the kinase domain. G2019S causes an increase in kinase activity by keeping the active site of LRRK2 open while raising the $K_m$ of ATP (47). Overexpression of mutated LRRK2 used to mimic the gain-of-function mechanism is shown to create UPS dysfunction at an unknown point in the pathway, which contributes protein accumulation such as α-synuclein, ubiquitin, and tau (48).

1.3 Environmental Factors

There are a variety of environmental elements that may influence both genetic and sporadic forms of PD. Individuals who live in rural areas or who work in welding, industrial, or agricultural industries are at an increased risk for PD. In rural areas or the agricultural industry,
this is due to chances of increased exposure to well water contaminated with various pesticides, insecticides, herbicides, and fungicides. Individuals living in regions of the world where such chemicals are or were once used may have a higher dietary intake of contaminated foods and water (49). Men exposed to pesticides through plantation work were shown to have higher PD risk with longer exposure (50). With industrial or welding industries, there is a possibility of manganese or industrial emissions exposure that puts individuals at risk for PD. It was shown that manganese exposure via intranasal administration impairs spatial memory and alters levels of neurotransmitter and their metabolites in the prefrontal cortex, hippocampus, and striatum (51). Intracranial injection of manganese into the rat striatum indirectly leads to neurotoxicity via impairment of ATP metabolism (52). Neurological and neuropsychological testing in welders show that manganese exposure may lead to reductions in motor skills, processing information, working memory, and mood, which may be indicative of early signs of a form of parkinsonism called manganism (53).

In addition to harmful environmental exposures, non-environmental neurotoxicants may also be relevant to human PD risk. For example, in the 1970’s, an attempt to synthesize street opioid known as desmethylprodine or MPPP resulted in neurotoxic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) impurities (54). Intraperitoneal injection into squirrel monkeys led to exhibition of PD symptoms such as akinesia, tremor, and rigidity (55). In addition, when injected as an illicit street drug in humans, SN dopaminergic neuron loss was confirmed after autopsy (56).

Overall, individuals exposed to these environmental contaminations are at a higher risk of PD, especially if genetics are not in their favour. Exposure to these toxins leads to significant changes in neuronal surroundings, such as alterations in the amount of microglia activation and production of inflammatory factors (56). While this may not cause PD, it can contribute to
disease progression and activation of cell death cascades. It should be noted that some issues with these environmental toxin reports include the uncertainty of specific pesticides used, the degree and length of exposure, small sample sizes, recall bias in human case studies, and the lack of studies done, making it difficult to determine the risk.

1.4 Cell Death Mechanisms

1.4.1 Mitochondrial Dysfunction and Cell Respiration Deficits

Mitochondria are cellular organelles that function to generate ATP and maintain cellular homeostasis. Mitochondria also play a major role in ROS production, regulation of Ca\(^{2+}\) ions, and the storage and release of apoptotic factors surrounding cell death. Each mitochondrion consists of an inner and outer membrane separated by an intermembrane space. The inner membrane is arranged into a highly folded structure known as cristae, which encloses the innermost region known as the matrix. The matrix is where the Kreb’s cycle produces electron carriers NADH and FADH\(_2\) electron carriers. These carriers are oxidized at complexes I and III of the electron transport chain (ETC) located at the inner membrane. This process allows for protons to be pumped into the intermembrane space to create an electrochemical gradient that drives the ATP synthase to phosphorylate ADP. The ATP generated is used in various active cellular processes such as neurotransmitter synthesis, vesicle exocytosis, and ion transport in neurons. It is important for mitochondria to exist in regions of the cell with high ATP demands, such as the synaptic terminal in neurons.

The brain is an organ with considerable ATP demands. In many cells, mitochondria function at a basal rate that does not expend its full bioenergetic capacity. Thus there is a ‘leftover’ or reserve capacity available to serve other cellular systems with higher bioenergetic demands (57). However, mitochondrial dysfunction is possible, which has an immense
compromising impact on many cellular systems. While not fully understood, the dopaminergic neurons of the SNc are especially vulnerable to mitochondrial dysfunction as they have particularly high ATP requirements and thus have a greater need for a greater reserve capacity. These neurons have a higher basal rate of oxidative phosphorylation (OXPHOS) and consequently ROS production compared to non-SN dopaminergic neurons (58). Because of this however, they have low bioenergetic reserve capacity due to basal rates being near maximum. Immunolabeling and single neuron tracing determined that SNc neurons also have more mitochondria located in highly-arborized, thinly-myelinated axons compared to dopaminergic neurons of the ventral tegmental area (58). This may suggest that the extensive axonal arborization may require more ATP in order to support neurotransmission through these axons. Furthermore, as arborization allows dopaminergic neurons of the SN to create divergent and widespread networks, neurodegeneration of the SN in PD has a massive impact on the nigrostriatal system as a whole (59). SN dopaminergic neurons also have low ROS-clearing antioxidant species such as glutathione (GSH), which correlates with reduction in complex I activity and ATP production in GSH-deficient PC12 cells (60). As seen in PD, if mitochondrial respiration is reduced for a prolonged length of time, it will eventually compromise cell viability. Overall, susceptibility to ATP depletion and low reserve capacity in SN neurons may lead to ROS accumulation that may be involved in DNA damage, aggregate buildup, excitotoxicity, neuroinflammation, and impairment of degradation pathways. These factors are part of the reason why mitochondrial dysfunction is central to PD.
1.4.2 Reactive Oxygen Species and Oxidative Stress

1.4.2.1 Reactive Oxygen Species

ROS are a normal by-product of cellular metabolism under basal and stress conditions. In the process of ROS production, molecular oxygen is reduced to the superoxide free radical, which can be further reduced to the hydroxyl radical and hydrogen peroxide, and eventually water. These species can be generated during various cellular processes, with a major source being OXPHOS in the mitochondria. OXPHOS is essential for pairing NADH oxidation with ATP synthesis by generating a proton gradient in the mitochondrial intermembrane space (61). During OXPHOS, superoxide radicals are generated as by-products from normal redox reactions that transfer electrons from NADH and FADH$_2$ to oxygen at complexes I and III respectively. At these complexes, there is an electron leak that is not currently well understood, causing a univalent reduction of molecular oxygen, giving it an unpaired electron and forming superoxide (62). It is thus important that antioxidant systems exist to help maintain a balance between the production and degradation of ROS to prevent cellular injury.

1.4.2.2 Oxidative Stress

Oxidative stress specifies a situation in which the production and destruction of free radicals by antioxidant processes becomes imbalanced. This impinges on neuronal health if disturbances are not reversed, as seen in many age-related neurodegenerative diseases. Oxidative stress may involve the production and action of harmful byproducts such as ROS or reactive nitrogen species. Build up of these radicals can lead to DNA and macromolecule damage, changes in mitochondrial permeability and membrane potential, and disruption of calcium homeostasis. This will be discussed further in section 1.4.2.3-1.4.2.5. These changes compromise cell viability, the functioning of vital enzymes, and may potentially lead to disruption of cellular respiration and ATP production by ETC uncoupling or changes in redox state.
1.4.2.3 Dopamine Toxicity

Dopamine is also an origin of oxidative stress as ROS and toxic dopamine quinones are generated during its auto-oxidation process (63). Specifically, once dopamine is released from a neuron and metabolized, it may auto-oxidize into superoxide or neurotoxic, protein-altering compounds such as 3,4-dihydroxyphenylacetaldehyde (DOPAL) and dopamine-o-quinone. This may suggest that dopamine contributes to the downfall of the neuron that produced it.

It has been proposed that at neutral pH, harmful dopamine-o-quinones may react with DNA via a depurinating 1,4-Michael addition (63). This is a cyclization reaction that forms leukochrome, which is then further oxidized into aminochrome and superoxide. Aminochrome may then polymerize to form neuromelanin. However at pH levels lower than 6, the amino group on dopamine can instead be protonated and will undergo a slower 1,4-Michael addition with nucleophiles such as DNA. The result of this reaction is the formation of depurinating DNA adducts, which can lead to the generation of mutations associated with PD or cancer (63, 64).

Quinone formation has been linked to mitochondrial, proteasomal, and UPS dysfunction. For example, it was shown that when dopamine and DOPA are oxidized into aminochrome and dopachrome respectively, proteasomal inhibition occurs (65). This inhibition would result in accumulation of misfolded and aggregated proteins, but can be prevented with the addition of NADH and quinone reductase (65). Specifically, quinones target the sulfhydryl group of cysteine residues, often found at protein active sites to covalently modify and inhibit protein function or make them insoluble (66). Some proteins impacted by quinones include monomeric α-synuclein, parkin, DJ-1, and UCH-L1 predominantly in dopaminergic neurons and surrounding cells (67-69). This suggests that quinone formation may play a role in the aggregation of α-synuclein. In the mitochondria, there is evidence that dopamine itself can act on complex I of the ETC and inhibit respiration (70).
1.4.2.4 mtDNA Damage

Mitochondria contain a circular genome known as mtDNA containing genes encoding eleven mRNAs that produce thirteen proteins and their synthesis machinery (71). Because neurons of the SN have a high oxidative capacity and are sensitive to mitochondrial dysfunction, they are vulnerable to mtDNA mutations. mtDNA is more prone to oxidative damage than nuclear DNA because it is a direct target of ROS generated from mitochondrial OXPHOS. This results in mutations that may exacerbate mitochondrial dysfunction and ROS production in an inevitable downward spiral. For example, mtDNA mutations can lead to production of abnormal mitochondrial enzymes, which can greatly impact respiratory chain function and thus ATP production (72). In the SN, this can eventually lead to neurodegeneration associated with PD.

As mentioned previously, dopamine plays a role in DNA damage. It has been hypothesized that the mechanism of dopamine auto-oxidation can also trigger mtDNA damage, thus impacting genes and proteins associated the ETC (64). Recent research has also shown that dopamine and its precursor levodopa (L-DOPA) are able to enter the nucleus, where they reduce Cu(II) to Cu(I), a process that generates ROS and copper-dependent DNA breakage (73).

1.4.2.5 Apoptotic Mechanisms Involving the mPTPs

Apoptosis, or programmed cell death, is a process by which the cell is able to morphologically change and prepare for its own destruction. While apoptosis is crucial for regular functioning and survival of cells and cellular systems, an excessive amount of cell death due to mitochondrial dysfunction, oxidative stress, and ROS has detrimental biological consequences and implications in neurodegenerative diseases (74). Normally, mitochondria contain enzymatic and non-enzymatic antioxidants that are able to detoxify and thus minimize the impact of ROS. However, this becomes compromised when the production of ROS exceeds the capacity for detoxification processes. For example, loss of superoxide dismutase 1 (SOD1), a
detoxifying enzyme important for converting superoxide to hydrogen peroxide, leads to superoxide buildup and induction of apoptosis (74). Enhanced ROS levels may lead to a ROS-induced increase in mitochondrial membrane permeability, an early apoptotic event known as mitochondrial permeability transition (MPT). MPT involves an unwanted increase in permeability and thus lower selectivity of the inner mitochondrial membrane to larger molecules and cytoplasmic fluids via mitochondrial permeability transition pores (mPTPs) (75). This event may cause the release of cytochrome c and other apoptotic factors into the cytoplasm, which activates caspases that allow apoptosis to commence. Furthermore, the increased membrane permeability causes a reduction in mitochondrial transmembrane potential, which induces further oxidative stress and eventually apoptosis (74). The loss of transmembrane potential can no longer drive intermembrane protons to pass through the ATP synthase to assist in generating ATP, thus leading to uncoupling of the ETC. This uncoupling creates an ATP deficit, forcing the cell to work harder to generate ATP.

Also following the permeability transition, an increase in osmotic pressure in the mitochondria occurs due to the high influx of small molecules, water, and cytoplasmic fluids in attempts to return the system to homeostasis (75). However, this leads to mitochondrial swelling, which expands the inner membrane, matrix, and cristae folds, eventually causing outer membrane to rupture since it has a smaller surface area than the inner membrane (75). Otherwise, herniation of the inner membrane through the ruptured outer membrane can result due to the expanded matrix. Some swelling is normal in mitochondria when the MPT is transient, however when prolonged, matrix proteins and cytochrome c may be released into the cytoplasm (75).
1.4.3 Ubiquitin-Proteasome System (UPS)

The UPS is the principal mechanism by which cells are able to mark, transport, and catabolize soluble cellular waste such as proteins or unhealthy mitochondria that may harm the cell. Ubiquitin is a 76-amino acid protein that can be attached or removed from soluble proteins of the nucleus, cytosol, or ER as a posttranslational modification (PTM). The ubiquitin-activating enzyme (E1) links with ubiquitin in an ATP-dependent reaction, then interacts with the ubiquitin-conjugating enzyme (E2) to facilitate ubiquitin transfer to the substrate with substrate-specific ubiquitin ligase (E3). The ubiquitinated protein is then sent to the proteasome to be degraded. When neurons are under oxidative stress, the UPS is relied upon to clear unwanted ROS, protein aggregates such as α-synuclein, and damaged mitochondria. Normally, the monomeric, soluble form of α-synuclein can be degraded by the 20S proteasome whether or not it is ubiquitinated (76). However, when wild-type α-synuclein is overexpressed or proteasomes are inhibited, non-ubiquitinated soluble α-synuclein increases and intracellular inclusions form (76). In both sporadic and familial forms of PD, insoluble α-synuclein aggregates disrupt proteasome function and thus its degradation is impaired. For example, the half-life of A53T α-synuclein transfected into SH-SY5Y cells was 50% greater than wild-type α-synuclein (77). This result could suggest that α-synuclein aggregates accumulate in the cytoplasm when expressed at higher concentrations, which could reveal more about the roots of PD pathogenesis (77). Impaired degradation can create an imbalance in which more α-synuclein aggregates are being generated than are being broken down, generating more cellular stress.

The method by which the proteasome becomes inhibited remains elusive. There are theories that protein aggregates clog the proteasome directly or that the proteasomes are sequestered into cellular inclusions away from parts of the cell that they are needed. Other studies show that UPS impairment can occur prior to the sequestration of aggregates and UPS
components into cellular inclusions, suggesting that inclusions form as a protective or compensatory mechanism rather than a pathological mechanism (78). The presence of aggregates could also indirectly impair UPS function by taking up free ubiquitin in the cell and disturbing ubiquitin homeostasis (78), however ubiquitin measurements taken in cells with high levels of aggregation proves this theory wrong (79).

1.4.4 Lysosomal Dysfunction

The lysosome is a vesicle-like organelle with an acidic interior that is optimal for the function of the various hydrolytic enzymes it contains. The lysosome functions to breakdown various biomolecules for waste disposal and recycling by autophagy. For example, the monomeric form of α-synuclein can be degraded by chaperone-mediated autophagy (CMA), the UPS, or by macroautophagy (80, 81). However, it is important that dysfunctional proteins such as mutant α-synuclein and ubiquitinated cytoplasmic inclusions are also hydrolyzed. The proteolytic mechanisms required to do so depends on the load or burden α-synuclein creates, mutations, protein folding, cellular localization, PTMs of the protein, as well as other unknown factors (80, 81). When the load is heavy or certain mutations or PTMs are present, this bears a negative impact on the lysosomal system. However, mutants of α-synuclein can specifically impair CMA by blocking lysosomal translocation and preventing uptake of itself and other lysosomal substrates (82). One study showed that α-synuclein oligomers inhibit the Hsp70/Hsp40 chaperone system, which is involved with protein folding, disaggregation of misfolded proteins, signal transduction, and quality control (83). Overall in PD, when the functional integrity of the lysosomal system is compromised, α-synuclein turnover and protein homeostasis are disrupted. This contributes to further aggregation, impairment of lysosomal function and trafficking, and can eventually influence apoptosis and neurodegeneration.
1.4.5 Protein Aggregates & Effects

Proteins may become misfolded due to genetic mutations, errors that are bypassed during transcription or translation, or due to environmental or cellular factors acting upon them. These misfolded proteins act as an indicator of cellular health. They can be soluble or insoluble and in some cases may become incorporated into larger aggregates with other substances. In the case of PD and a wide range of other human neurodegenerative diseases, these aggregates can be neurotoxic. Normally, damaged or misfolded proteins are subject to homeostatic mechanisms that allow them to be degraded. However, there may be issues with cellular machinery that prevent degradation of these proteins, causing them to accumulate. Otherwise, the aggregates may overcrowd the cell and obstruct cellular processes such as molecular or vesicular trafficking or protein degradation (84). In the case of PD, there are two major types of proteinaceous aggregates that are of great concern.

1.4.5.1 Lewy Bodies

A histopathological component that coincides with neurodegeneration in both sporadic and familial PD is the accumulation of inert cytoplasmic proteinaceous aggregates known as Lewy bodies. Lewy bodies were discovered by Fritz Lewy and largely consist of α-synuclein, neurofilaments, and ubiquitin (85). Lewy bodies may form via the overlap of self-assembly and aggregation mechanisms, post-translational phosphorylation, and proteolysis. In the PD brain, Lewy bodies can be widely distributed, but are most concentrated in pigmented monoaminergic neurons of the SN and locus coeruleus. Lewy bodies found in brainstem regions tend to exhibit a dense core of filamentous and granular material with a surrounding corona of radially-oriented filaments. Those found in more cortical regions, although also made up of similar filaments, are more loosely-arranged and spherical with lack of a distinct core and halo (86).
PD may also involve the presence of Lewy neurites, which are abnormal amyloid fibrils that are composed of neurofilaments, α-synuclein, and ubiquitin similar to Lewy bodies. Interestingly, the amount of Lewy neurites exceeds Lewy bodies in initial stages of PD (87). Compared to Lewy bodies, which tend to accumulate in the soma, Lewy neurites accumulate in the axon, and may interfere with normal axonal transport, thus jeopardizing the function and survivability of neurons (88). Their function remains unknown due to lack of neuronal models on this subject.

1.4.5.2 α-Synuclein

There are three known synucleins including α-synuclein, β-synuclein, γ-synuclein, as well as a synuclein-like protein called synoretin. These molecules have a highly-conserved repeat at the amphipathic N-terminal region, a hydrophobic centre, and a negatively-charged acidic C-terminus. In particular, α-synuclein is highly expressed in neurons. Thus, it is a focal point for neurodegenerative disorders known as α-synucleinopathies, which involve an intracellular aggregation of α-synuclein.

α-synuclein is a 19kd protein of 140 amino acids coded by the α-synuclein gene SNCA on chromosome 4q21. It was first discovered in electric fish and named due to its apparent location in the cytosol, axon terminals, and the nuclear envelope of neurons in the brain (89). It was determined by nuclear magnetic resonance (NMR) spectroscopy that the structure of α-synuclein is naturally unfolded until it interacts with lipid membranes at its N-terminal repeats to take on an α-helical structure, while its C-terminus remains unfolded (90, 91). Under pathological conditions, soluble α-synuclein monomers form insoluble aggregates by interacting with themselves or other proteins. These aggregates are capable of inducing cytotoxicity and interrupting cellular processes such as protein degradation, axonal transport, and
neurotransmission (77, 83). A large portion of the α-synuclein molecule is made up of seven imperfect repeats of eleven amino acids. The imperfect repeats are non-identical fragments with a conserved six amino acid core sequence KTK(E/Q)GV that is involved in the α-helix arrangement (89). Each repeat is separated by five amino acids except for repeats four and five, which are separated by nine amino acids. It is within this nine amino acid region where the known α-synuclein A53T mutation associated with PD occurs (as discussed in section 1.2.1). Since many of these repeats occur near the N-terminus, the A53T mutation could impact α-synuclein structure, self-oligomerization, aggregation, and interactions with other proteins.

The synucleins all undergo PTMs, but α-synuclein in particular may undergo phosphorylation, sumoylation, nitrification, oxidation, addition of 4-hydroxy-2-nonenal, and alternative splicing. These modifications may be associated with α-synuclein aggregation, neurotoxicity, and Lewy body formation. A study showed that 90% of insoluble α-synuclein was phosphorylated compared to soluble α-synuclein in the cytosol, suggesting the role of phosphorylation in α-synuclein oligomerization (92). Furthermore, it has been suggested that ubiquitination of lysine residues occurs on phosphorylated α-synuclein rather than monomeric 19kDa α-synuclein. Since α-synuclein is normally degraded in a ubiquitin-independent manner, PTMs and poly-ubiquitination may interrupt α-synuclein recognition by the proteasome, overwhelming the proteolytic system, leading to aggregation associated with PD pathogenesis (93). Additionally, alternative splicing can result in four different isoforms of α-synuclein known as SNCA98, SNCA112, SNCA126 and SNCA140, which differ in function, structure, aggregation ability, and aggregate structure. Electron microscopy in vitro shows that the SNCA140 isoform aggregates significantly more than the other three isoforms (94).
The function of wild type α-synuclein remains debatable. It is proposed to play a role in the transport of synaptic vesicles to axon terminals by enhancing SNARE function and complex formation. For example, α-synuclein is able to directly facilitate SNARE complex formation in transgenic SNARE-deficient mice (95). Therefore when there is a loss of endogenous α-synuclein such as when it incorporates into Lewy bodies in PD, age-dependent loss of neuronal function is observed that may be related to SNARE complex formation (95). It was also shown that absence of α-synuclein does not lead to changes in expression of other synaptic vesicle proteins, synaptic terminal morphology, or dopamine release and reuptake (96). More recently, overexpressed and endogenous α-synuclein have been associated with acceleration of exocytotic kinetics and dilation of the exocytotic fusion pore. This occurs by inhibition of the kiss-and-run event whereby vesicles begin to fuse with the cell membrane, but immediately retract to reform the vesicle (97). However, A53T and A30P α-synuclein mutations prevent fusion pore dilation and thus exocytosis, which may prevent normal neurotransmission and thus function of the SN (97).

There is also evidence that α-synuclein is involved with dopamine production. Co-immunoprecipitation experiments in rat striata and cell lines demonstrate that α-synuclein interacts with TH, where it inhibits TH from removing the hydroxyl group from L-tyrosine (98). The same study showed that overexpression of either wild-type or mutant A53T α-synuclein reduces dopamine synthesis because it impacts phosphorylation of TH that is required for its activity (98). Another study showed that at the presynaptic membrane, the NAC hydrophobic segment of α-synuclein forms a complex with the C-terminus of dopamine transporters (DATs). This causes the DATs to cluster, leading to increased uptake of dopamine and possibly dopamine-induced neurotoxicity and apoptosis (99).
Lastly, it is possible that \( \alpha \)-synuclein plays a role in locations other than the synaptic terminal. A recent study claims that \( \alpha \)-synuclein does not localize to the mitochondria, but may be present in a subdomain of the ER known as the mitochondrial-associated membrane of the ER (MAM), where it can regulate mitochondrial morphology and influence calcium transfer between the ER and mitochondria (100, 101). A53T and A30P mutants interact less with MAMs, leading to reduction in MAM function and mitochondrial fission/fusion dynamics, causing mitochondrial fragmentation (100). In addition, \( \alpha \)-synuclein has nuclear localization where it forms complexes with histones. This occurs to inhibit histone acetylation, however this nuclear targeting can also lead to acceleration of its pathological aggregation and promote neurodegeneration (102). While some functions of \( \alpha \)-synuclein have been identified, more research is required to confirm its roles in the cell.

1.4.5.3 Aggregation of \( \alpha \)-synuclein

\( \alpha \)-synuclein aggregation can be triggered by various factors. It can begin to aggregate independently in high concentrations, as a result of genetic mutations, or due to PTMs. \( \alpha \)-synuclein has also been shown to associate with other proteins and form cytoplasmic aggregates such as Lewy bodies. For example, \( \alpha \)-synuclein generates cytoplasmic inclusions when co-transfected with synphilin-1 (103). This may indicate that synphilin-1, a protein of unknown function, accelerates the aggregation of \( \alpha \)-synuclein and formation of Lewy bodies, and thus plays a role in PD pathogenesis. In addition, cytochrome c and \( \alpha \)-synuclein co-incubation provokes \( \alpha \)-synuclein aggregation (104). Furthermore, \( \alpha \)-synuclein and cytochrome c co-localize in Lewy bodies of PD patients, suggesting that the ETC protein and apoptotic regulator cytochrome c may contribute stress-induced \( \alpha \)-synuclein aggregation (104).
α-synuclein aggregates have been proposed to display prion-like propagation and intercellular transport from diseased to healthy neurons (105). This is further supported by the fact that the disease appears to spread from the olfactory bulb in early stages of PD through to the limbic system, midbrain, and brainstem in later stages (106). The actual spread can occur if α-synuclein escapes the cell by exocytosis or after cell lysis due to neuronal death. This release is followed by its uptake into other neurons by endocytosis or passive uptake by breaching the plasma or endosome lipid membrane, allowing it to enter the cytosol where it may ‘seed’ or trigger aggregation of native α-synuclein monomers (107, 108).

The mechanism by which α-synuclein leads to oxidative stress and PD still remains uncertain. There is support for the ideas that α-synuclein aggregation precedes and triggers mitochondrial dysfunction, or that its aggregation is the result of mitochondrial dysfunction. For example, the α-helical structure that α-synuclein can take on is characteristic of mitochondrial targeting sequences, which allows α-synuclein to be imported into the mitochondria (109). The result of this is disruption of complex I activity in the ETC and increases in ROS (109). Another study showed that α-synuclein can lead to complex I inhibition and increased mitophagy in dopaminergic neurons under proteasomal stress, suggesting that α-synuclein targets the mitochondria as a compensatory mechanism (110). As previously mentioned, there is also the possibility that ROS production or cytochrome c release by dysfunctional mitochondria may also trigger α-synuclein aggregation, which would suggest that aggregation follows mitochondrial dysfunction. Moreover, based on its localization and function in axon terminals, it makes sense for aberrant α-synuclein to also impact events such as axonal transport, microtubule formation, and exocytosis of neurotransmitters. Lastly, because α-synuclein interacts with TH, its
aggregation can interrupt dopamine synthesis and homeostasis that may contribute to loss of dopamine in the PD brain as well perpetuate toxic dopamine metabolite and ROS formation (98).

Based on the above, it is clear α-synuclein can directly affect the mitochondria. However, there is also evidence that α-synuclein may also have an indirect effect on the mitochondria via impairment of other cellular systems such as the UPS, CMA, or ER-Golgi trafficking. For example, α-synuclein aggregates may influence seeding of α-synuclein monomers or aggregation of other proteins such as tau and β-amyloid, thus disrupting protein homeostasis or “proteostasis” (111). Not only could this lead to overloading the UPS and CMA and impairing ROS removal and mitophagy, the impact on a protein such as tau may lead to impaired microtubule formation that can affect many other cellular systems including the mitochondria.

While a significant amount of knowledge has been obtained surrounding the impact of α-synuclein in PD pathology, it remains uncertain whether its aggregation is the source or result of mitochondrial dysfunction and oxidative stress. It is clear that further research is required in order to clarify the perplexity of the roles of α-synuclein in PD pathogenesis.

1.5 Sirtuins

The sirtuins or silent information regulator 2 (SIR2) family consist of seven proteins existing in various subcellular localizations with biological functions such as enhancing cellular energetic mechanisms and regulation of metabolism and gene expression (112). Sirtuins have been linked to the increase in longevity, energy efficiency, stress resistance, and cytoprotection. Sirtuins were first discovered in Saccharomyces cervisiae yeast and identified as the SIR2 gene, which regulates the type of mating yeast express via transcriptional silencing (112). This process involves the recruitment of the SIR complex by DNA sequences, which then deacetylate histones at lysine residues. This causes the chromatin to compact in a way that is inaccessible to
transcriptional machinery, hence the term ‘silencing’ (113). It has been suggested that the silencing process is involved in cell senescence. For example, when yeast replicate there are extrachromosomal ribosomal DNA circles (ERCs) that are generated that have an effect similar to telomere shortening in humans and other higher eukaryotic organisms. SIR2, however, can slow ERC accumulation and thus senescence by compacting the heterochromatin (114).

Sirtuins are expressed ubiquitously in human tissue, with some sirtuins being expressed in greater levels in different regions of the brain. For example, the brainstem has high expression of sirtuins 1, 3, and 7, the striatum expresses high sirtuins 3, 5, 6, and 7 expression, sirtuins 2, 4, 5, and 7 are highly expressed in the spinal cord, and sirtuins 5-7 are highly expressed in the olfactory bulb (115). Each sirtuin possesses an nicotinamide adenine dinucleotide (NAD+) -dependent domain and have ADP-ribosyltransferase activity or deacylase activity. Sirtuin activity is promoted by NAD+ and inhibited by nicotinamide (NAM), thus the activity of sirtuins depends on a combination of the NAD:NADH ratio and NAM levels (116). Deacetylation is an important PTM that allows for the activation or inactivation of a multitude of proteins, which can have a large impact on various cellular processes. In particular, sirtuin-mediated deacetylation occurs on lysine residues and is coupled to NAD+ hydrolysis to yield NAM and ADP-ribose (117). The acetyl group is then removed from the lysine residue following NAD+ hydrolysis and consequently transferred to the ADP-ribose.

The sirtuins have specific subcellular localizations where they target a wide range of substrates involved in many cellular pathways (117, 118). Sirtuins 1-3 may be found in the nucleus, cytoplasm, and mitochondria respectively and function as protein deacetylases (119). Sirtuins 4 and 5 are located in the mitochondria, where sirtuin 4 functions as an ADP-ribosyl transferase and deacetylase while sirtuin 5 functions as a demalonylase and desuccinylase (119, 120). Sirtuin 6 is located in the heteronucleus and functions as an ADP-ribosyl transferase and
deacetylase, while sirtuin 7 acts as a deacetylase in the nucleolus where it also interacts with RNA polymerase I (119, 121). Together, the sirtuins play a role in metabolism, aging, and DNA repair.

1.5.1 Sirtuin 3

Sirtuin 3 (SIRT3) is a protein encoded by chromosome 11 in humans that is targeted and translocated to the mitochondrial matrix by a 142-residue N-terminal MLS (122). At first, SIRT3 is synthesized into an inert 44kDa protein. However, once SIRT3 enters the mitochondria, it is cleaved into its active 28kDa form by a matrix enzyme known as mitochondrial processing peptidase (MPP) (123). SIRT3 has over 700 mitochondrial substrates that it acts upon to control a range of mitochondrial processes (124). As mentioned above, SIRT3 exhibits NAD$^+$-dependent protein deacetylase activity within the mitochondria and works to regulate the ratio of acetylated and deacetylated mitochondrial proteins (125). Deacetylation is considered a type of PTM of lysine residues that can affect enzyme activity, stability, and active site properties. Enzyme acetylation in the mitochondria can thus play a role in the regulation of metabolism, antioxidation, and apoptosis. One study showed that only SIRT3 KO and not SIRT4 or SIRT5 KO in mice leads to mitochondrial enzyme hyperacetylation (125). Another study showed that SIRT3 deacetylated and thus activated acetyl CoA synthetase 2 at lysine-642 in the presence of NAD$^+$, whereas SIRT5 did not (126). These studies suggest SIRT3 as the main controller of mitochondrial acetylation.

SIRT3 deacetylates enzymes involved in Kreb’s cycle, the ETC, antioxidation, fatty acid β-oxidation, and amino acid metabolism. For example, SIRT3 directly associates with and deacetylates complex I of the ETC at NADH dehydrogenase 1 α subcomplex 9 (NDUFA9) (127). In the ETC, NDUFA9 deacetylation is crucial for the functioning of complex I to facilitate
proton gradient production and drive ATP synthesis. SIRT3 also deacetylates and activates acetyl CoA synthetase 2, which combines acetate and coenzyme A to form acetyl CoA (126). This means that SIRT3 helps target acetate towards energy production. The role of SIRT3 in metabolism is possible because of its capacity to respond to levels of NAD\(^+\) and acetyl CoA intermediates associated with vital mitochondrial metabolic pathways. During the deacetylation reaction, SIRT3 consumes NAD\(^+\) provided by complex I as it oxidizes NADH. NAD\(^+\) is converted to NAM, which along with the ATP/ADP ratio, can impact the NAD\(^+\)/NADH ratio in the mitochondria. Maintenance of the NAD\(^+\) pool is vital for regulating sirtuins, especially in cells with higher OXPHOS requirements such as neurons. In times of stress, the NAD\(^+\)/NADH ratio decreases, which is not optimal for SIRT3 activity. Taken together, this information suggests that regulation of mitochondrial activity relies on mechanisms of reversible protein acetylation, whereby SIRT3 is a large contributor.

In addition to its role in metabolism, SIRT3 has been proposed as a cytoprotective agent. For example, SIRT3, but not SIRT4 or SIRT5, inhibits mPTP formation and opening, an event that may otherwise trigger mitochondrial swelling, mitophagy, and apoptotic events (and thus neurodegeneration). This is done through deacetylation of lysine 166 of a protein component of the mPTP known as cyclophilin D (CypD) (128). Specifically, it was shown that SIRT3-deficient cardiomyocytes in mice develop an age-dependent increase in mitochondrial swelling due to the opening of mPTPs, leading to cardiac dysfunction (128). Furthermore, maintenance and proper function of mitochondria partially depends on lowering the rate of production and levels of ROS. In times of cellular stress when ROS levels are high, there is a compensatory increase in SIRT3 transcription and thus mitochondrial SIRT3 levels in attempts to restore cellular homeostasis. This increase in SIRT3 promotes deacetylation and activation of superoxide dismutase 2 (SOD2), an enzyme that scavenges ROS in the mitochondria (129). SIRT3 also acts to regulate
the glutathione (GSH) antioxidant system via deacetylation of isocitrate dehydrogenase 2 (IDH2), an enzyme involved with the Kreb’s cycle. IDH2 decarboxylates isocitrate to form α-ketoglutarate and NADPH. α-ketoglutarate continues through cellular respiration pathways while NADPH can be used to reduce the oxidized form of GSH known as GSSG (130). In this way, GSH can be generated to scavenge hydrogen peroxide in the mitochondria. As discussed previously, ROS also has the ability to damage mtDNA. SIRT3, however, is capable of deacetylating enzymes such as OGG1 and Ku70 involved with mtDNA repair, thus assisting in maintaining the integrity of the mitochondria and preventing apoptosis (131, 132).

1.5.2 Relationship of Sirtuin 3 to aging and mitochondrial function

As discussed in section 1.4.1, mitochondria are cellular organelles that have significant roles in maintenance of cellular homeostasis, metabolism, ROS production, and apoptosis. Dysfunction of the mitochondria is crucial in PD pathogenesis due to the susceptibility of SNC neurons to depletion of energy carriers, DNA damage, Ca^{2+} imbalances, and ROS accumulation. Therefore, mitochondria may be a suitable and effective therapeutic target for PD.

The various roles of SIRT3 in metabolism, oxidative stress, and apoptosis through regulation of mitochondrial acetylation levels suggest that it may play a role in cytoprotection and aging. In other words, SIRT3 may be implicated in age-related diseases such as PD. Studies have shown that SIR2 and related homologs have the ability to influence lifespan. For example, SIR2, SIR3, and SIR4 null alleles introduced into yeast resulted in a reduction in lifespan, especially in the case of SIR2, since the null allele prevented suppression of ERC generation (as discussed in section 1.5) (114). Appropriately, based on the information discussed thus far in section 1.5 as well as current investigations, elevating SIRT3 levels exogenously could potentially reinforce mitochondrial health, leading to a reduction in neurodegeneration and the
progression of PD. For example, overexpression of SIRT3 in HEK293 cells is shown to increase oxygen consumptions, glycolysis, and mitochondrial substrate oxidation, albeit by unknown mechanisms (133). On the other hand, SIRT3 KO mice are observed to develop hyperacetylation of SOD2 and CypD, as well as increased neuronal vulnerability to mitochondrial dysfunction and death when exposed to metabolic stress (134). The hyperacetylation of a multitude of mitochondrial enzymes in absence of SIRT3 also causes a significant decrease in ATP production (127, 134). When expressed normally, SIRT3 is able to deacetylate SOD2 to allow for ROS clearance as well as CypD to inhibit mPTP formation and apoptosis compared to SIRT3 KO neurons (134). Based on these studies, it is reasonable to consider that overexpression of SIRT3 may have neuroprotective and neurorestorative abilities with respect to PD.

1.6 Current PD Research

Although a vast amount of research has been done on PD, the pathogenic mechanisms by which PD occurs are yet to be understood. Moreover, there are no disease-modifying treatments available that are able to slow, reverse, or halt the progression of PD. The treatments and therapies that exist are only capable of managing motor and non-motor symptoms. Various in vitro and in vivo models have been created in attempts to recapitulate the classic neuropathological and behavioural aspects of PD. Ideally, a PD model should exhibit age-dependent and slowly-progressing PD-like dopaminergic neurodegeneration, motor dysfunction, rigidity, resting tremor, α-synuclein and ubiquitin contained within Lewy bodies, oxidative stress, and can be reliably replicated across subjects. However, even the best models that exist tend to exclude some important features of PD. Thus it is important to choose a model that best encompasses the manifestations of PD to generate valid results. While in vitro models are useful for examination of simpler mechanisms, genes, proteins, or neurotoxicity in a controlled
environment, *in vivo* models allow for experimental observation in a living subject. For example, the efficacy of a candidate drug may provide more relevant or valid results in a live organism. Although many *in vivo* PD models exist, two recent α-synuclein rat models will be the focus of this section.

1.6.1 *in vivo* Parkinson’s Disease Models

1.6.1.1 Preformed Fibrils (PFF)

As mentioned in section 1.4.6, α-synuclein does not fold into a secondary structure upon translation. However, under pathological conditions, α-synuclein monomers interact and bind with each other to form insoluble aggregates, or interact with lipid membranes to acquire an α-helical structure that is susceptible to oligomerization (90, 91). The basis of the preformed fibril (PFF) model is the inoculation of fibrillized α-synuclein aggregates to induce PD-like pathology in healthy cells or organisms. Initially, it was hypothesized that cells would uptake the PFFs, which would then propagate through neurons via interactions with endogenous α-synuclein (135). One study demonstrated a similar effect by introducing crude homogenates from 6-month old mice expressing human mutant tau into the hippocampus ALZ17 mice, which express a long non-fibrillized form of tau. This led to recruitment of ALZ17 tau proteins into filaments in the cerebral cortex and hippocampus, and eventually spreading to more distant brain regions including the non-injected hemisphere (136). This seeding mechanism was demonstrated in a PD cell model, where α-synuclein fibrils influenced formation of intracellular inclusions by transforming soluble endogenous α-synuclein into insoluble, hyperphosphorylated, and ubiquitinated aggregates (135). Other studies demonstrate that α-synuclein can pass from neuron to neuron via endocytosis or to brain regions with direct neural connections to form Lewy body-
like inclusions (137). Furthermore, this seeding mechanism is possible under physiological, wild type conditions, as recombinant human wild-type α-synuclein PFFs can recruit endogenous α-synuclein into pathological aggregates similar to Lewy bodies in primary hippocampal neurons (137, 138). Current investigations are examining whether the seeding of PFFs can take place in vivo under wild type, physiological conditions in young Sprague-Dawley rats, which may potentially become another suitable model for in vivo study of PD.

1.6.1.2 Adeno-Associated Virus (AAV)

The adeno-associated virus (AAV) is a vector used as a method for gene delivery into a host cell. The AAV has the ability to reliably integrate into the host genome at a specific site to allow for gene expression (139). The virus lacks pathogenicity and involves very little immune response since much of the viral genome is removed (139). AAV delivery is also known to have long-term expression in the cells it transfects (139). In addition, there are twelve serotypes of AAV that exist, all of which possess the same size, non-enveloped, single stranded genome, but differ in their tropism, or the types of cells they infect (139). The transduction of AAV can also be confined to a single hemisphere and certain cell types, unlike transgenic mice brains which are affected more diffusely, allowing the contralateral hemisphere to act as a control (140). Therefore, it is these features of the AAV make it useful for developing models of disease, often in rats and non-human primates.

One of the uses of AAV is for overexpression of proteins. Thus in PD, the AAV can be used to express mutant PD-related proteins such as A53T α-synuclein to induce parkinsonian deficits. For example, a PD study was done on rats using a recombinant AAV to overexpress mutant α-synuclein in nigrostriatal dopaminergic neurons. This led to progressive α-synuclein aggregate formation, dystrophy of neurons, and eventually cell death in dopaminergic neurons (140). Another study was performed by injecting mutant A53T rAAV into the SN of marmosets,
which similarly led to generation of α-synuclein aggregates, motor impairment such as head-position bias, and dystrophic neurons (141).

In the central nervous system, AAV1 and AAV2 are optimal serotypes for transduction of neurons (142). Tissue distribution may also rely on factors such as delivery route, while properties of each serotype may depend on kinetics, level and length of expression, localization, and capsid domains and structure (139). These serotypes can also be pseudotyped, where the capsid and genome from different serotypes are mixed. Pseudotyping improves AAV transduction efficiency, distribution, or transgene expression and help tailor the vector to fit gene transfer requirements by combining features of different serotypes. For example, Gao et al. (2003) showed that AAV2/1 and 2/5 had a wider distribution and retrograde transport than AAV2/2 and was able to efficiently transduce cells not normally targeted by AAV2/2 (143). McFarland et al. also found that nigral neurons were transduced most by rAAV2/1 compared to rAAV 2/5 and 2/8, which had 2-3 times greater transduction than AAV2/2 (144). Moreover, it is believed that pseudotypes can generate more neurodegeneration and motor deficits in a shorter time span with less variability, compared to the AAV2 serotype (145). It was also shown that transduction of human mutant A53T α-synuclein using the AAV1/2 pseudotype allowed for precise targeting of the SN due to the 1:1 ratio of serotype 1 and 2 capsid proteins, which led to quickly progressing dopaminergic neurodegeneration and synucleinopathy (146). Therefore, the use of AAV for overexpression, specifically chimeric AAV pseudotypes, could form the bases for new and improved models of PD.

1.6.2 Choice of Virus for overexpression of proteins in vivo

In addition to provoking dopaminergic cell death and oxidative stress that is comparable to other methods of inducing parkinsonism such as injection of 6-OHDA, MPTP, or rotenone,
the AAV viral vector approach exhibits progressive cytopathological aggregate formation, cell death, and behavioural and motor abnormalities as observed in humans with PD (146, 147). For these reasons, in this thesis study, AAV1 was used to administer empty vector (EV) and SIRT3-myc into the SNc of rodents. The chimeric AAV1/2 pseudotype was used to introduce human α-synuclein mutant A53T into the SNc of rats, in which the genome of the AAV2 serotype and a 1:1 ratio of serotype 1 and 2 capsid proteins are combined. The purpose of this is to improve transduction efficiency and brain tissue penetration towards that of the AAV1 serotype and alter tissue tropism towards that of the AAV2 serotype. This would impact neurons of the SN that might not necessarily be transduced as effectively by AAV1 or AAV2 serotypes alone in vivo (142). Therefore, this model should be able to closely mimic human PD in rodents and is a practical approach in PD research for examining stages of pathology and potential disease-modifying agents. To date, AAVs have been used in various clinical trials for several diseases, including PD (148). Clinical trials involving the use of AAV to administer glutamic acid decarboxylase to the STN of PD patients have been tested, showing no adverse effects or intolerance to the virus, along with improvements on motor tests and reductions in brain metabolism (149). Thus if successful, this study using AAVs to examine SIRT3 as potential a disease-modifying agent could possibly be used in clinical trials in the future.

1.7 Rationale

Given its ability to regulate mitochondrial protein acetylation levels to contribute to the maintenance of metabolism and antioxidative processes, SIRT3 has multi-faceted positive effects on mitochondrial health and aging. In PD, mitochondria are vulnerable to dysfunction and oxidative stress, especially in SN dopaminergic neurons. Furthermore, neurodegenerative diseases have been linked with mitochondrial dysfunction. For this reason, SIRT3 may be
useful in modifying neurodegenerative diseases impact in cells, specifically at the
mitochondrial level. As discussed in section 1.5.2, there are a modest number of studies that
have focused on the role of SIRT3 in neuroprotection, suggesting its function in disease
modification. At the same time, other studies have shown that the presence of SIRT3 is
detrimental. Novgorodov et al. (2015) showed that after ischemia, SIRT3 directly deacetylates
and thus increases activity of the ceramide synthase, leading to ceramide build up, which then
inhibits complex III of the ETC (150). Further exploration of the effects of SIRT3 in vivo is
required to elucidate its benefits. It would be valuable to investigate the impact of SIRT3
overexpression on mitochondria before and during oxidative stress conditions arise. In a
disease model, this would allow for the determination of the possible protective and remedial
roles of SIRT3.

The overall goal of this study was to determine whether SIRT3 had disease-modifying
effects in the most relevant model of parkinsonism. Whilst there are pros and cons to all
models, the AAV over-expression of human mutant α-synuclein (A53T) rat model was chosen
as it exhibits a similar disease progression to that observed in PD patients. The A53T AAV
model of PD is much more rapid in that changes in striatal dopamine metabolism and the
presence of aggregates occur around the same time as motor impairment, with death of
dopaminergic nigrostriatal neurons occurring later. Despite this, it is the best rodent equivalent
to the pathological timeline of humans with PD. Furthermore, this is one of the few non-toxin
rodent models that show a behavioural phenotype as well as tyrosine hydroxylase (TH)-labeled
cell loss. While the toxin models are useful for studying changes in neuronal circuitry
downstream of the SN, they do not mimic the pathological mechanisms underlying PD as well
as the AAV model. In order to better understand the role of SIRT3 in the mitochondria and in
the pathogenesis of PD, its impact before and after the induction of parkinsonism in vivo needs to be evaluated.

1.8 Hypothesis and Aims

Hypothesis

It was hypothesized that SIRT3 would have disease-modifying effects in a rodent model of parkinsonism. Since SIRT3 is a protein, an rAAV was generated to overexpress SIRT3.

Aims

1. Neuroprotective role of SIRT3

   To evaluate the neuroprotective ability of SIRT3-myc AAV. SIRT3-myc was intranigrally infused to naïve rats, 7 days prior to induction of parkinsonism, to ensure that SIRT3 had reached maximum expression prior to induction of PD.

2. Neurorestorative role of SIRT3

   To assess the neurorestorative potential of SIRT3-myc AAV, SIRT3-myc was administered intranigrally 18 days following administration of A53T. This time point for SIRT3 infusion was chosen, as neurons show clear signs of stress, such as α-synuclein aggregation, changes in striatal metabolism and behavioural impairment.

3. SIRT3 localization and function in the mitochondria

   To examine and confirm whether SIRT3-myc delivered via AAV localizes to the mitochondria where it should function as a deacetylase, similar to endogenous SIRT3.
1.9. Proposal

Validating the neuroprotective and neurorestorative capabilities of SIRT3 using the A53T rAAV model in rats would strengthen current data that propose SIRT3 as a disease-modifying agent with the ability to protect and restore dopaminergic neurons in PD patients. If SIRT3 is able to slow or halt the progression of neurodegeneration and restore damaged neurons back to a pre-PD state, it may prove to be a valid and efficacious life-extending therapy for PD patients compared to currently existing treatment. Overexpression of SIRT3 in the SN will enhance mitochondrial function. When administered before A53T, SIRT3 will prevent neurodegeneration and cellular stress in SN neurons and when administered after A53T, SIRT3 will reverse the effects of cellular stress in SN neurons that could potentially lead to neurodegeneration. If SIRT3 is able to display disease-modifying effects in parkinsonian and pre-parkinsonian rats, there is potential to introduce SIRT3 by AAV to PD patients in clinical trials in the future.
2. SIRT3 as a Disease-Modifying Agent: Neuroprotection and Neurorestoration

Dr. Jacqueline Gleave contributed to behavioural testing (section 2.1.2), conducted the optimization study (section 2.1.3), performed neuroprotection surgeries (section 2.1.5), contributed to perfusions (section 2.1.6.1), contributed to cryosectioning (section 2.1.6.2), contributed to IH (2.1.6.3), and prepared HPLC samples (section 2.1.6.5). Dr. Sherri Thiele conducted the optimization study (section 2.1.3). James Barber performed surgeries for the mitochondrial isolation study (section 2.1.6.6). Dennison Trinh contributed to mitochondrial isolation (section 2.1.6.6), contributed to western blots/SDS-PAGE and performed blot imaging (section 2.1.6.7), and performed statistical analyses for the mitochondrial isolation study (2.1.7).

2.1 Materials and Methods

2.1.1 Animal Care

All studies were performed in accordance with the University of Toronto and Canadian Council on Animal Care (CCAC) guidelines. Sprague-Dawley male and female rats (250-300g) (Charles River) were pair housed in a 12-hour light/dark cycle with access to food, water, bedding, and environmental enrichment.

2.1.2 Assessment of Forelimb Asymmetry

To determine the impact of parkinsonism on motor function, the cylinder test was used to examine forelimb asymmetry (151). To eliminate bias or skewing of results due to limb preference, baseline behaviour for each rat was tested one week prior to the first stereotaxic surgery (see section 4.2.4). To maintain motivational states during the assessment period, rats were deprived of food the evening before testing, which was conducted in the morning at the same time each testing day before dopamine levels drop in accordance with the rodent circadian rhythm. During the test, rats were placed individually into a clear glass cylinder (dimensions), where they were videotaped using a video camera (SONY Handycam® HDR-
CX405, The Source, Scarborough). The assessment period ended either when the rat made a total of 25 forelimb contacts with the wall of the cylinder or when 6 minutes had passed. Each rat completed a total of three non-consecutive trials on the same day. Videos were analyzed post Hoc in a single-blind manner to reduce bias. Forelimb asymmetry was assessed by counting the number of left, right, or both forelimb contacts for a maximum of twenty contacts. Forelimb asymmetry was assessed three and six weeks following intranigral infusion of SIRT3 for the neuroprotection study and A53T for the neurorestoration study.

2.1.3 SIRT3-myc Optimization and Expression Stability in vivo

Before surgeries were performed, the concentration of the SIRT3-myc and A53T rAAV vectors were optimized to determine a concentration. Sprague-Dawley rats were stereotaxically infused in the right SN with one of four titres: $3.37 \times 10^8$ GC, $5.18 \times 10^8$ GC, $6.74 \times 10^8$ GC, $22.50 \times 10^8$ GC. The brains were removed for SDS-PAGE and Western blot analysis or IHC fourteen days after injection. A final concentration of $5.18 \times 10^8$ GC (genome copies) for the SIRT3-myc rAAV was determined and $5.10 \times 10^9$ GC for the A53T rAAV was used as determined previously (146).

2.1.4 Vectors

The SIRT3-myc AAV1, SIRT3$^{H248Y}$-myc AAV1, empty AAV1, A53T AAV1/2, and empty AAV1/2 were the vectors used to create the experimental groups (Table 1). AAV serotypes differ in tropism or the cells they are able to infect. This may depend on their route of delivery or capsid surface domains and structures (143). The AAV2 serotype is known to have 80% transduction efficiency while AAV2/1 exhibits a large transduction volume in SNc neurons (152). The chimeric AAV1/2 vector contains the ITRs of the AAV2 serotype and the
capsid of the AAV1 and AAV2 serotypes in a 1:1 ratio. This gives the virus tissue tropism (the type of cells it can infect) associated with the AAV1 serotype (Ex. neurons). Also, the AAV1/2 chimeric vector is known to be highly efficient at transducing the SNc compared to other brain regions (146, 152). The AAV1/2 vector is also capable of causing rapid degeneration of nigrostriatal dopaminergic neurons with α-synuclein aggregates (146). Therefore, the AAV1/2 serotype was chosen for expressing human mutant A53T. The AAV1 serotype was chosen for expressing SIRT3-myc and SIRT3$^{H248Y}$-myc, which were produced at Penn State Vector Core facility (Pennsylvania, USA). The empty vectors used corresponded to the serotypes of the non-control vectors.
Table 1.

Outline of the rodent groups used for the neuroprotection and neurorestoration studies.

<table>
<thead>
<tr>
<th>Neuroprotection Groups</th>
<th>Neurorestoration Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Naïve</td>
<td>1. Naïve</td>
</tr>
<tr>
<td>2. EV AAV1 + EV AAV1/2</td>
<td>2. EV AAV1 + EV AAV1/2</td>
</tr>
<tr>
<td>3. EV AAV1 + A53T AAV1/2</td>
<td>3. EV/Control AAV1 + A53T AAV1/2</td>
</tr>
<tr>
<td>4. SIRT3-myc AAV1 + A53T AAV1/2</td>
<td>4. SIRT3-myc AAV1 + A53T AAV1/2</td>
</tr>
<tr>
<td>5. SIRT3^{H248Y}-myc AAV1 + A53T AAV1/2</td>
<td></td>
</tr>
</tbody>
</table>

**Naïve** no treatment

**EV** empty vector

**A53T** human mutant alpha synuclein

**SIRT3-myc** myc-tagged sirtuin 3

**Control** empty vector or PBS injection

**SIRT3^{H248Y}-myc** deacetylase-deficient SIRT3
2.1.5 Stereotaxic Surgery for Intranigral AAV Infusion

Each rat underwent two rounds of stereotaxic surgery, beginning one week after baseline behavioural testing was completed. In the neuroprotection study, rats received SIRT3-myc AAV one week prior to A53T AAV infusion. In the neurorestoration study, rats received A53T AAV eighteen days prior to SIRT3-myc AAV. The experimental design is outlined in Figure 1. The AAV was diluted the morning of surgery to the optimal concentration in a biosafety cabinet with cold 1x PBS and kept on ice until use. Rats were anaesthetized via inhalation of 5% isoflurane in an enclosed chamber, then shaved, weighed, injected with a dose of an analgesic ketoprofen (10mg/kg s.c) (Anafen, BD 1mL syringe, 26G x3/8 needle, CDMV, Toronto) and swabbed with topical lidocaine/prilocaine (EMLA 5%, CDMV, Toronto, Ontario). The rats were then transferred to the stereotaxic frame and the isoflurane was set to 3%. The head of the rat was secured with the ear and incisor bars so that the top of the skull was flat with no movement of the skull. Isoflurane was reduced to 2.5% and antiseptic isopropyl alcohol wipes and betadine were alternately swabbed twice each to sterilize the incision site. The rat was monitored for breathing and its temperature was maintained using a heating pad. A toe pinch was performed to ensure the animal was unconscious before creating the incision. The incision was made and the Bregma landmark on the skull was located. The coordinates of Bregma were recorded and used to calculate the drilling and injection site (AP -5.2mm, ML -2.0mm, DV -7.5mm from Bregma). A dental drill with a round-tip drill bit was used to create a small hole in the skull (Dremel, USA). After drilling, the loaded needle (10µL, Hamilton, USA) was then primed and lowered into the calculated coordinates of the SN with the needle bevel facing the anterior brain. The AAVs were infused into the SN at a rate of 0.2µl/min over ten minutes (final volume 2µl) using a microinjector (Stoelting, USA). During
infusion, animals were injected with 2-3mL 3.3% dextrose 0.3% sodium chloride s.c to replenish lost fluids. The nails of the rat were also trimmed to prevent wound opening, excessive irritation from scratching, or breaking of stitches post-surgery. After the infusion, the needle was raised slightly out of the SN region (90° counterclockwise turn DV direction) to allow diffusion of the AAV over the SN region for five minutes. The needle was removed and the animals were sutured using an interrupted suture technique (SOFSILK Non-absorbable Wax Coated, Braided Black Silk, Sterile Surgical Suture #4-0, Medstores, University of Toronto). The animal was then placed in a clean recovery cage with a supplemental diet (Jello, Walmart, Scarborough, Ontario). Autoclaved surgical tools were used for a maximum of five surgeries, and sterilized between surgeries using a Germinator 500 hot glass bead dry sterilizer (VWR, USA).

For two days post-surgery, animals received systemic administrations of ketoprofen (10mg/kg, s.c). Extra days of injections were provided as needed for animals with slower healing, wound complications, or those requiring re-suturing. Sutures were removed between ten and fourteen days post-surgery.

**Neuroprotection Surgeries**

One week following baseline behavioural testing, rAAV expressing SIRT3-myc or vehicle (EV) was stereotaxically infused unilaterally into the right SN. Seven days later, a second surgery was performed where A53T or empty rAAV was infused unilaterally into the right SN.

**Neurorestoration Surgeries**

One week following baseline behavioural testing, rAAV expressing A53T or EV was infused unilaterally into the right SN. Eighteen days after A53T was injected, a second surgery
was performed where SIRT3 rAAV, empty rAAV or phosphate buffered solution (PBS) as a control, were infused unilaterally into the right SN.
Figure 2: Diagram outlining the timeline of the neuroprotection and neurorestoration methods.

**Neuroprotection**

- **Day -7**
  - Behavioural testing (Forelimb asymmetry test)

- **Day 0**
  - SIRT3-myc or EV intranigral AAV administration

- **Day 7**
  - Mutant A53T α-synuclein or EV intranigral AAV administration

- **3 Weeks**
  - Behavioural testing (Forelimb asymmetry test)
  - Striatal DA metabolism (HPLC)
  - Dopamine cell loss (stereology in SNc)

- **6 Weeks**
  - Behavioural testing (Forelimb asymmetry test)
  - Striatal DA metabolism (HPLC)
  - Dopamine cell loss (stereology in SNc)

**Neurorestoration**

- **Day -7**
  - Behavioural Testing (Forelimb asymmetry test)

- **Day 0**
  - Mutant A53T α-synuclein or EV intranigral AAV administration

- **Day 18**
  - SIRT3-myc or EV intranigral AAV administration

- **3 Weeks**
  - Behavioural testing (Forelimb asymmetry test)
  - Striatal DA metabolism (HPLC)
  - Dopamine cell loss (stereology in SNc)

- **6 Weeks**
  - SN Mitochondrial isolation and analysis of acetyl-lysine levels in SN
  - Behavioural testing (Forelimb asymmetry test)
  - Striatal DA metabolism (HPLC)
  - Dopamine cell loss (stereology in SNc)
Figure 2: Diagram outlining the timeline of the neuroprotection and neurorestoration methods.

For the neuroprotection study, rats acclimated for one week and then underwent baseline behavioural testing. One week following behavioural testing, the rats underwent stereotaxic surgery for administration of SIRT3-myc or Empty AAV1. Seven days later, the rats underwent a second stereotaxic surgery for administration of A53T or Empty AAV1/2. At three and six weeks post SIRT3-myc surgery, behavioural testing was performed. Half of the rats had their brain removed at three weeks and the other half at six weeks after behavioural testing for post-mortem analyses. For the neurorestoration study, rats acclimated for one week and then underwent baseline behavioural testing. One week following behavioural testing, the rats underwent stereotaxic surgery for administration of A53T or Empty AAV1/2. Eighteen days later, the rats underwent a second stereotaxic surgery for administration of SIRT3-myc, SIRT3^{H248Y}-myc, or Empty AAV1. At three and six weeks post SIRT3-myc surgery, behavioural testing was performed. Half of the rats had their brain removed at three weeks and the other half at six weeks after behavioural testing for post-mortem analyses. An additional group of brains were extracted for a mitochondrial isolation protocol to analyze of SIRT3-myc function and mitochondrial localization.
2.1.6 Post-Mortem Analyses

2.1.6.1 Perfusion and Dissection

Three or six weeks following the first AAV administration, the male and female Sprague-Dawley rats underwent three or six-week behavioural testing respectively. On the same day immediately after their behavioural testing, rats were transcardially perfused with ice cold 1X PBS and inhalation of 5% isoflurane/oxygen anaesthetic. After the animal had been perfused, the brain was extracted and coronally bisected on ice posterior to the striatum. The rostral portion of the brain was fixed in 4% paraformaldehyde (PFA) and stored at 4°C. The ipsilateral striatum of the caudal portion of the brain bisection was placed in a cryovial and flash frozen in liquid nitrogen. These ipsilateral striata were then sent to CMN/KC Neurochemistry Core Lab (Vanderbilt University, USA) for HPLC analysis or fixed for cryosectioning.

2.1.6.2 Cryosectioning

Before cryosectioning, the fixed brains were submerged in a 30% sucrose solution until the brains sunk (approximately two days) to indicate absorption of enough sucrose to become isotonic and cryoprotect the brains. The caudal portion of the brains were cryosectioned at 40µm using the Leica CM1900 UV Cryostat model to prepare thin slices for immunohistochemistry (IH) (section interval of six, all sections saved, but every 6th section used). Sections were taken from approximately Bregma AP -4.3 to AP -6.5 to capture the SNc. The brain sections were stored in cryoprotectant (30% glycerol, 30% ethoxyethanol, 40% PBS) and frozen at -20°C until IHC was performed.
Table 2.
List of antibodies used for immunohistochemistry (IH) and Western blot (WB) (154).

<table>
<thead>
<tr>
<th>Antibody</th>
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<td></td>
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<td>Immunostar (22941)</td>
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<td>Tyrosine Hydroxylase(rabbit)</td>
<td>Millipore (AB152)</td>
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</tr>
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</table>
2.1.6.3 Immunohistochemistry (IH)

The brain slices were stained either for α-synuclein/TH myc-TH or TH/NeuN in a four day process. Sections were washed three times for 5 minutes in 0.2% Triton-X 100, quenched with endogenous peroxidase in 3% H₂O₂ for 3 minutes, then washed three more times for 5 minutes in 0.2% Triton-X 100. Sections were then blocked for 1 hour in 5% normal goat serum (NGS, Jackson Immunoresearch Laboratories, USA) in 0.1% Triton-X 100. The sections were incubated at room temperature with the primary antibody rabbit anti-NeuN (ABN78, Chemicon, USA) 1:1000 diluted in 0.2% Triton-X 100 and 1% NGS overnight (~16 hours). Sections were washed three times for 5 minutes in 0.2% Triton-X 100 and incubated with the secondary antibody goat anti-rabbit biotinylated (BA-1000, Vector Labs, USA) 1:400 diluted in 0.2% Triton-X 100 and 1% NGS for 2 hours at room temperature. Sections were then washed three times for 5 minutes in 0.2% Triton-X 100 followed by an incubation in ABC Elite (made 30 minutes prior, 2.5mL ddH₂O, 1 drop A, 1 drop B) for 1 hour at room temperature protected from light. The sections were then washed three times for 5 minutes in PBS, then DAB staining was added (3mL diluent, 3 drops DAB; ImmPACT DAB, Vector Labs, USA) and incubated for 30 seconds. Sections were then moved to cold PBS to stop the reaction. The sections were washed again three times for 5 minutes in 0.2% Triton-X 100 then incubated overnight (~16 hours) at room temperature in primary antibody mouse anti-TH (22941, Immunostar, USA) 1:1000 diluted in 0.2% Triton-X 100 and 1% NGS. The sections were washed three times for 5 minutes in a mixture of tris-buffered saline and Tween 20 (TBS-T). The sections were then incubated in secondary antibody alkaline phosphatase conjugated goat anti-mouse (Alkaline Phosphatase Substrate Kit III; 115-055-166, Vector Labs, USA) 1:250 in 1% NGS and TBS-T for 2 hours at room temperature. This was followed by three 5-
minute washes in TBS-T and then incubation in Vector Blue (2.5mL 100mM Tris HCl pH 5.2, 1 drop 1, 1 drop 2, 1 drop 3; SK5300, Vector Labs, USA) for 6 minutes. The sections were washed three times for 5 minutes in PBS. The sections were mounted onto Superfrost® Micro Slides (48311-703, VWR, USA) with PBS, labeled, and dried covered over night for approximately 24 hours. The slides were submerged in a series of ddH2O for 3 minutes then 70%, 95% and 100% ethanol for 1 minute each, followed by Histoclear twice for 3 minutes each to clean the slides and enhance clarity. The coverslips were added with VectaMount (Vector Labs, USA) mounting medium to help preserve histochemical staining in the tissue sections.

### 2.1.6.4 Stereology

Following completion of IH, the number of TH-positive cells were counted using stereology with a Zeiss Axioimager M2 Microscope System (Carl Zeiss, Toronto, Ontario) in conjunction with the StereoInvestigator Version 9 program (MBF Bioscience, USA). Using the optical fractionator workflow set to a section thickness of 40µm and section interval of 6, brain sections were traced at the SNc and counted in 240µm gaps (1:6 section interval). The user manually determined the tissue thickness at each counting site and a TH marker was used to mark dopaminergic cells labeled with TH/NeuN. Once counting was completed, a Microsoft Excel file was then generated by StereoInvestigator outlining the parameters as well as the final cell count estimate and related results. This was counted single blind to reduce bias. Counts were only included if Schmitz-Hoff error estimate was <0.1.

### 2.1.6.5 HPLC Analysis of Striatal Dopamine and Dopamine Metabolites

High performance liquid chromatography (HPLC) is a technique that allows separation of a solution containing various molecular compounds for identification and quantification.
purposes. The substances in a mixture are pumped through an HPLC column with a solvent and separated based on their attraction to the mobile phase within the column. A detector then determines the order of elution of substances in the mixture and measures their concentration. Therefore, HPLC method was chosen to quantify dopamine and dopamine metabolite levels in the ipsilateral striatum of six-week SIRT3-treated and parkinsonian rats. Both the ipsilateral and contralateral (control) striatal sections from the caudal portions of the coronal bisections were frozen in liquid nitrogen and sent to CMN/KC Neurochemistry Core Lab (Vanderbilt University, USA) for HPLC analysis. The sections were homogenized in trichloroacetic acid (TCA) (0.01M sodium acetate, 0.0001M EDTA, 5ng/mL isoproterenol, 10.5% methanol) and then centrifuged. An Antec Decade II electrochemical detector (at 33°C) and a C₁₈ HPLC column (100 x 4.60 mm) was used to analyze the resulting supernatant for biogenic amines. A mobile phase consisting of 89.5% 0.1M TCA, 10.5% methanol, 0.01M sodium acetate, 0.0001M EDTA, at a pH of 3.8 was used to elute the biogenic amines.

2.1.6.6 Mitochondrial Isolation and Purification

Mitochondria were isolated in order to allow for verification that SIRT3-myc has similar function and localization to that of endogenous SIRT3. To do this, the SN was dissected at a six-week time point from perfused neurorestoration rats. An isolation protocol was run (adapted by Chinopoulos et al., 2011)(153), involving a series of centrifugations with various buffers. This method allows for the acquisition of purified mitochondria, in this case for Western blot to determine acetylation, without the need to pool tissues from multiple animals (153). The SN region of the brains were first individually homogenized in 1mL MSEGTA-BSA (225mM mannitol, 75mM sucrose, 5mM HEPES (pH 7.4), 1mM EGTA, dissolved in water) with a 1mL manual homogenizer for approximately thirty to thirty-five
strokes. The homogenate was transferred into two 1.5mL tubes, and MSE GTA-BSA was added to fill each tube to yield a total volume of ~1.5mL each. Both tubes were then mixed by inversion and centrifuged at ~500g for five minutes. The pellet, which contains cellular debris at this stage, was discarded. The collected supernatant was transferred into new 1.5mL tubes and centrifuged again at 14,000g for ten minutes. The supernatant was decanted and the remaining pellets for a given animal were resuspended and combined together in 0.2mL 12% Percoll-MSEGTA (100% Percoll™-MSEGTA buffer made from 225mM mannitol, 75mM sucrose, 5mM HEPES (pH 7.4), and 1mM EGTA, all dissolved in 100% Percoll™, then diluted to 12% using MSEGTA made from 225mM mannitol, 75mM sucrose, 5mM HEPES (pH 7.4), and 1mM EGTA all dissolved in H2O). This resuspension was layered on top of 1mL 24% Percoll-MSEGTA in a new 1.5mL tube and centrifuged at 18,000g for fifteen minutes. The resulting sample appeared to have a transparent band in the middle with cloudy portions on either side. The cytosolic fraction consisted of the top 0.5mL cloudy portion of the centrifuged sample, thus was aspirated off and saved. To the remaining fraction, 1mL of MSEGTA was added and mixed by inversion, then centrifuged at 18,000g for five minutes. The pellet remaining was resuspended in buffer, then 1.3mL of MSEGTA was added, inverted, and centrifuged at 14,000g for five minutes. The resulting supernatant was decanted and 0.1mL of MSEGTA was added to resuspend the pellet, yielding the isolated mitochondrial fraction.

2.1.6.7 SDS-PAGE and Western Blot

SDS-PAGE was used to separate the samples obtained during the mitochondrial isolation. Western blotting was used to quantify SIRT3-myc and the deacetylation levels of acetyl-lysine in the isolated mitochondrial fraction from six-week neurorestoration rats. A volume of 11.25µL 4X Laemmli Sample Buffer (161-0737, BIO-RAD, Medstores, University
of Toronto) was used to prepare the samples. This was followed by addition of the corresponding amount of 1X Tris Buffered Saline (TBS) to yield 30µg of protein out of 45µL total volume. A 10% acrylamide gel was used to load the samples for SDS-PAGE along with 5µL of BLUeye Prestained Protein Ladder (GeneDireX, USA) for monitoring separation, protein weight referencing, and verifying Western blot efficiency of transfer. The gels were run at 65V through the stacking layer and 125V through the separating layer. Primary antibodies (See Table 1 for WB antibodies) were stored overnight at 4°C following Western transfer and blocking. Blot imaging was done using Image Lab software (BIO-RAD, USA) and ImageJ was used for analysis.

2.1.7 Statistical Analysis

Differences between the various animal groups of the neuroprotection and neurorestoration study for AAV optimization, behavioural analysis, stereology, and HPLC were analyzed by a nonparametric, one-way ANOVA (p < 0.05). This analyzes differences between group means to determine whether the samples stem from the same distribution, without assuming the data follows a specific distribution. A Tukey post-hoc test was done wherever results were significant to confirm where the significance occurred between groups.

Comparisons made for the Western blots of the mitochondrial acetylation study were done using a Mann-Whitney U test. The Mann-Whitney U test is a test of the null hypothesis to compare means between two groups without assuming a normal distribution (non-parametric).

All analyses were performed and graphed on GraphPad Prism 6.0 and results are expressed as mean ± standard error of the mean (SEM) with probability (p).
3. Results

3.1 SIRT3-myc AAV Optimization in vivo

Before surgeries were performed, the concentration of the SIRT3-myc rAAV was optimized in order to determine dose and to reduce the chance of a false-negative result (154). The optimal concentration should have no impact on endogenous SIRT3 levels, the contralateral hemisphere, the neuron number, or the dopamine phenotype while raising SIRT3-myc levels at least twofold above endogenous SIRT3 levels. Fourteen days after stereotaxic injection with one of four titres (3.37x10^8 GC, 5.18x10^8 GC, 6.74x10^8 GC, 22.50x10^8 GC) in the right SN, the brains were analyzed for SN SIRT3-myc expression and neuron number in both hemispheres. At all titres, SIRT3-myc was highly expressed in the ipsilateral SN compared to endogenous SIRT3 with little expression on the contralateral side (Figure 3Ai). The titre of 5.18x10^8GC appeared to be the most similar to endogenous SIRT3 in terms of optical density. Furthermore at all titres, SIRT3-myc was expressed at least three times greater than endogenous SIRT3, with titres 3.37x10^8 and 5.18x10^8 displaying the highest expression (Figure 3Aii). In addition, no titre had a significant impact on NeuN-positive cell counts in the SN relative to the uninjected hemisphere compared to EV rats (Figure 3B). The titre of 5.18 x 10^8 GC was chosen as it met all optimization criteria best and had a 90% transduction efficiency in the SN. This titre was also similar to the titre used for AAV1 by McFarland et al (2009)(144).
Figure 3: Optimization of SIRT3-myc rAAV titre for overexpression studies (154).

A)

i. % loading control

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<tr>
<th>[SIRT3 (x10^8 GC)]</th>
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<th>5.18</th>
<th>6.74</th>
<th>22.5</th>
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</thead>
<tbody>
<tr>
<td>operated side</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unoperated side</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

SIRT3-myc (31 kDa)
endo SIRT3 (28 kDa)

β-actin (~43kDa)

ii.

<table>
<thead>
<tr>
<th>SIRT3-myc : endo SIRT3</th>
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</thead>
<tbody>
<tr>
<td>[rAAV SIRT3-myc (x10^8 GC)]</td>
</tr>
<tr>
<td>3.37</td>
</tr>
<tr>
<td>EV</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>% unoperated hemisphere</th>
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<tbody>
<tr>
<td>[rAAV SIRT3-myc (x10^8 GC)]</td>
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<tr>
<td>3.37</td>
</tr>
<tr>
<td>EV</td>
</tr>
</tbody>
</table>
Figure 3: Optimization of SIRT3-myc rAAV titre for overexpression studies

To determine the optimal titre of SIRT3-myc rAAV, rats were stereotaxically injected in the right SN with one of four titres (3.37x10^8 GC, 5.18x10^8 GC, 6.74x10^8 GC, 22.50x10^8 GC). Fourteen days later, their brains were removed for SDS-PAGE and Western blot analysis. (Ai) Western blot showing the expression of SIRT3-myc (31kDa) and endogenous SIRT3 (29kDa) with β-actin as the loading control. Lanes 1, 3, 5, 7, and 8 show the injected hemisphere while lanes 2, 4, and 6 show the uninjected hemisphere. The graph represents the percentage of SIRT3-myc expression relative to β-actin ± SEM (Aiii) Graphical representation of SIRT3-myc OD as a percentage of endogenous SIRT3 OD (mean ± SEM), showing that all titres cause at least a three-fold increase in SIRT3 levels. (B) Graphical representation of the effect of SIRT3-myc titres on NeuN cells counts in the SN, expressed as a percentage of NeuN in the unoperated side (mean ± SEM). There was no significant difference between EV and SIRT3-myc treated animals in terms of NeuN cell counts compared to the uninjected side.
3.2 Assessment of the Neuroprotective Effect of SIRT3 in A53T rats

As previously discussed, to assess the impact of SIRT3 in the SNc neurons of A53T rats, an rAAV of serotype 1 expressing SIRT3 was acquired. To help identify between injected SIRT3 and endogenous SIRT3 during post-mortem analyses, SIRT3-myc was encoded in the AAV. In the neuroprotection study, SIRT3-myc AAV or empty vector (EV) was unilaterally injected into the SNc seven days before the injection of A53T AAV also into the SNc. Three weeks post A53T injection, there was a significant 13.1% increase in forelimb asymmetry between the EV+EV and EV+A53T groups, but not between EV+EV and SIRT3+A53T groups (EV+EV 8.3 ± 2.9% versus EV+A53T 21.3 ± 2.9%) (Figure 4A). Also at three weeks, there were no stereological differences between any groups (Figure 5A), but HPLC results show a significant change in striatal dopamine metabolism represented by the increase in dopamine metabolite to dopamine ratios in the EV+EV group compared to the EV+A53T group (HVA/DA EV+EV 103.5 ± 4.1 EV+A53T 127.6 ± 8.1, DOPAC/DA EV+EV 96.4 ± 3.8 EV+A53T 125.5 ± 8.2) but not significant compared to the SIRT3+A53T group (HVA/DA 116.3 ± 5.1, DOPAC/DA 114.5 ± 4.3) (Figure 6A, C, E). Dopamine production and metabolism is outlined in Figure 12. At six weeks post A53T injection, there was a significant difference in forelimb asymmetry between the EV+EV (1.9 ± 5.3% asymmetry) and SIRT3+A53T (10.5 ± 2.1 % asymmetry) groups each compared to the EV+A53T group (33.4 ± 6.8% asymmetry) (Figure 4B). There was also a significant decrease in TH-positive neurons in the SN of EV+A53T rats compared to the EV+EV group (1933 ± 246 cells and 3598 ± 542 cells respectively) but no significance between the EV+A53T and SIRT3+A53T groups (3439 ± 510 cells) (Figure 5B). At six weeks, significant change in striatal dopamine metabolism was observed based on the dopamine to dopamine metabolite ratios in parkinsonian rats (HVA/DA...
135.2 ± 10.7; DOPAC/DA 143.8 ± 11.7) compared to EV+EV rats (HVA/DA 106.7 ± 3.7; DOPAC/DA 105.5 ± 3.1) and SIRT3+A53T rats (HVA/DA 100.1 ± 7.5; DOPAC/DA 100.8 ± 7.0) (Figure 6B, D, F). Overall, when SIRT3-myc rAAV was injected seven days prior to A53T rAAV, parkinsonian deficits normally induced by mutant A53T were diminished. In other words, forelimb asymmetry, loss of dopaminergic neurons, and changes striatal dopamine metabolism were not observed in parkinsonian rats due to SIRT3 overexpression.
Figure 4: SIRT3 reduces motor deficits when administered before manifestation of A53T parkinsonism (154).

A)

3 Week
Forelimb Asymmetry

B)

6 Week
Forelimb Asymmetry
Figure 4: SIRT3 reduces motor deficits when administered before manifestation of A53T parkinsonism.

To assess motor impairment, the cylinder test was used to measure forelimb asymmetry at three weeks (A) and six weeks (B) post SIRT3 AAV-injection. The control consists of animals that were injected with empty vector (EV) during both surgeries. The significant difference between EV+EV and parkinsonian animals at three and six weeks displays success of the A53T mutant in generating motor deficits. ANOVA (A) 3-weeks: F = 3.87, p = 0.025, n = 23, 28, 29; (B) 6-weeks; F = 8.80, p = 0.0013, n = 10, 11, 7. *p < 0.05, **p < 0.005, where n value represents EV+EV, EV+A53T, and SIRT3+A53T respectively. All neuroprotection data is expressed as mean ± SEM.
Figure 5: The A53T model causes a decrease in neuron number that SIRT3 does not protect at 3 or 6 weeks (154).

A) 

3 Week
Dopamine Cell Count

B) 

6 Week
Dopamine Cell Count
Figure 5: The A53T model causes a decrease in neuron number that SIRT3 does not protect at 3 or 6 weeks

To assess the impact of SIRT3 on neuron number, stereology was used to quantify the number of TH-positive neurons in the SNc at three weeks (A) and six weeks (B) post SIRT3 AAV-injection. TH is an enzyme found in dopaminergic neurons responsible for the conversion of amino acid tyrosine to L-DOPA, a precursor of dopamine. The control consists of animals that were injected with empty vector (EV) during both surgeries. ANOVA (A) 3-weeks: F = 1.07, p = 0.37, n = 6, 6, 4; (B) 6-weeks; F = 4.52, p = 0.022, n = 10, 10, 7. *p < 0.05, where n value represents EV+EV, EV+A53T, and SIRT3+A53T respectively. All neuroprotection data is expressed as mean ± SEM.
Figure 6: SIRT3 lowers the ratio of dopamine metabolite/dopamine at 6 weeks post SIRT3 injection (154).
Figure 6: SIRT3 lowers the ratio of dopamine metabolite/dopamine at 6 weeks post

SIRT3 injection

Striatal dopamine turnover and dopamine metabolite to dopamine ratios were assessed using HPLC at three and six weeks post initial SIRT3 AAV injection. Both ipsilateral and contralateral hemispheres to the AAV injection site were assessed. Striatal dopamine turnover refers to the ratio of production and breakdown of dopamine at SN dopaminergic projections at the striatum. Similarly, ratios of dopamine metabolites relative to dopamine determine whether dopamine is being produced or broken down into its metabolites.

Dopamine production and metabolism is outlined in Figure 12. Striatal dopamine turnover changes were not significant between any groups at both three and six weeks. Striatal dopamine turnover ANOVA (A) 3-weeks: F = 3.36, p = 0.066, n = 6, 6, 4; (B) 6-weeks; F = 2.18, p = 0.1360, n = 10, 10, 7. SIRT3 significantly reduced both HVA and DOPAC dopamine metabolite/dopamine ratios at six weeks. Dopamine metabolite (HVA) to dopamine ratio (HVA/DA) ANOVA (C) 3-weeks: F = 4.09, p = 0.042, n = 6, 6, 4; (D) 6-weeks; F = 5.72, p = 0.0096, n = 10, 10, 7. Dopamine metabolite (DOPAC) to dopamine ratio (DOPAC/DA) ANOVA (E) 3-weeks: F = 6.31, p = 0.0122, n = 6, 6, 4; (F) 6-weeks; F = 8.64, p = 0.0016, n = 10, 9, 7. *p < 0.05, **p < 0.01, where n value represents EV+EV, EV+A53T, and SIRT3+A53T respectively. All neuroprotection data is expressed as mean ± SEM.
3.3 Assessment of the Neurorestorative Effect of SIRT3 in A53T rats

In the neurorestoration study, A53T rAAV or empty vector (EV) were unilaterally injected into the right SNc. Eighteen days later, SIRT3-myc or SIRT3<sup>H248Y</sup> AAV were also injected into the right SNc to determine whether SIRT3-myc could restore parkinsonian deficits induced by injection of mutant A53T. At three weeks following the A53T AAV injection, forelimb asymmetry was significantly higher in the EV+A53T group (31.9 ± 3.9% asymmetry) but not the SIRT3<sup>H248Y</sup>+A53T group (25.3 ± 9.8% asymmetry) compared to the EV+EV group (10.8 ± 4.5% asymmetry (Figure 7A). There was also no significant difference between the SIRT3+A53T group (19.7 ± 4.9% asymmetry) compared to the EV+A53T or SIRT3<sup>H248Y</sup>+A53T groups. There were no differences between groups with respect to three-week stereology (Figure 8A), or dopamine metabolite to dopamine ratios (Figure 9A, C, E). At six weeks post A53T injection, forelimb asymmetry was again significantly higher in the EV+A53T group (35.6 ± 5.6% asymmetry) but not the SIRT3<sup>H248Y</sup>+A53T group (40.0 ± 9.2% asymmetry) compared to the EV+EV group (9.7 ± 4.8% asymmetry) (Figure 7B). In addition, the percent asymmetry was amplified in the EV+A53T group compared to the three-week behavioural data, as expected. There was also no significant difference between the SIRT3+A53T group (11.8 ± 4.6% asymmetry) compared to the EV+A53T or SIRT3<sup>H248Y</sup>+A53T groups. Stereological data from the six-week time point showed a significant increase in TH-positive SN neurons in the SIRT3+A53T group (5684 ± 578 cells) compared to the EV+A53T group (2718 ± 420 cells) and the SIRT3<sup>H248Y</sup>+A53T group (2987 ± 309 cells) (Figure 8B). There was also a significant difference between the EV+EV and A53T+control groups with respect to striatal dopamine levels as well as dopamine metabolite to dopamine ratios. However, there was no significance between parkinsonian rats and SIRT3-
treated parkinsonian rats (Figure 9B, D, F). Overall, when SIRT3-myc rAAV was injected eighteen days after A53T rAAV, parkinsonian deficits were restored such that forelimb asymmetry was comparable to the EV+EV group (SIRT3+A53T 11.8 ± 4.6% asymmetry versus EV+EV 9.7 ± 4.8% asymmetry), loss of dopaminergic neurons was not observed (SIRT3+A53T 5684 ± 578 cells versus EV+EV 3820 ± 306 cells), and changes in striatal dopamine metabolism were reversed (HVA/DA SIRT3+A53T 110.9 ± 4.0 versus EV+EV 102.1 ± 2.4; DOPAC/DA SIRT3+A53T 112.0 ± 4.8 versus EV+EV 102.1 ± 2.0). In addition, animals injected with SIRT3$^{H248Y}$, a deacetylase deficient form of SIRT3, experienced forelimb asymmetry (40.0 ± 9.2% asymmetry) and dopaminergic cell loss (2987 ± 309 cells) similar to EV+A53T rats. This was significantly unlike data obtained from SIRT3+A53T rats (Figure 7B, 8B). However, forelimb asymmetry for both three and six-week time points between SIRT3+A53T and SIRT3$^{H248Y}$+A53T groups was unexpectedly not significantly different (but was close to significance, p = 0.06).
Figure 7: SIRT3 does not reduce motor deficits when administered after the manifestation of A53T parkinsonism (154).

A) 3 Week Forelimb Asymmetry

B) 6 Week Forelimb Asymmetry
Figure 7: SIRT3 does not reduce motor deficits when administered after the manifestation of A53T parkinsonism.

To assess motor impairment, the cylinder test was used to measure forelimb asymmetry at three weeks (A) and six weeks (B) post A53T AAV-injection. The control consists of animals that were injected with empty vector (EV) during both surgeries. An additional control for SIRT3 was included, which was a deacetylase-deficient SIRT3^{H248Y}-myc. Although SIRT3 appears to restore motor deficits back to levels similar of EV+EV animals at both three and six weeks, results were not significant. However, SIRT3^{H248Y}-myc parkinsonian animals displayed motor deficits similar to untreated parkinsonian animals, as expected, due to its lack of deacetylase function. The significant difference between EV+EV and parkinsonian animals at three and six weeks displays success of the A53T mutant in generating motor deficits. ANOVA (A) 3-weeks: F = 3.21, p = 0.028, n = 20, 25, 16, 13; (B) 6-weeks; F = 5.16, p = 0.0035, n = 12, 22, 8, 11. All neurorestoration data is expressed as mean ± SEM.
Figure 8: SIRT3 prevents dopaminergic neuron loss in the SNc at six weeks post A53T injection (154).

A)

B)
Figure 8: SIRT3 prevents dopaminergic neuron loss in the SNc at six weeks post A53T injection

To assess the impact of SIRT3 on neuron number, stereology was used to quantify the number of tyrosine hydroxylase (TH) positive neurons in the SNc at three weeks (A) and six weeks (B) post SIRT3 AAV-injection. TH is an enzyme found in dopaminergic neurons responsible for the conversion of amino acid tyrosine to L-DOPA, a precursor of dopamine. The control consists of animals that were injected with the empty vector (EV) during both surgeries. SIRT3 was able to prevent dopaminergic neuron loss at six weeks, with cell counts higher than that of EV+EV and naïve (red dotted line) levels. SIRT3\textsuperscript{H248Y}-myc parkinsonian animals expectedly displayed significant neuron loss compared to SIRT3-myc treated animals, similar to untreated parkinsonian animals due to its lack of deacetylase function. ANOVA (A) 3-weeks: F = 0.21, p = 0.82, n = 9, 5, 9; (B) 6-weeks; F = 8.97, p < 0.0001, n = 12, 19, 10, 12. *p < 0.05, **p < 0.005, **** p < 0.0001, n value represents EV+EV, A53T+Control, SIRT3+A53T, and SIRT3\textsuperscript{H248Y}-myc+A53T respectively. All neurorestoration data is expressed as mean ± SEM.
Figure 9: SIRT3 does not impact striatal dopamine turnover or dopamine metabolite/dopamine ratios at three and six weeks post A53T injection.
Figure 9: SIRT3 does not impact striatal dopamine turnover or dopamine metabolite/dopamine ratios at three and six weeks post A53T injection

Striatal dopamine turnover and dopamine metabolite to dopamine ratios were assessed using HPLC at three and six weeks post initial SIRT3 AAV injection. Both ipsilateral and contralateral hemispheres to the AAV injection site were assessed. Striatal dopamine turnover refers to the ratio of production and breakdown of dopamine at SN dopaminergic projections at the striatum. Similarly, ratios of dopamine metabolites relative to dopamine determine whether dopamine is being produced or broken down into its metabolites. Dopamine production and metabolism is outlined in Figure 12. Striatal dopamine turnover changes were not significant between any groups at both 3 and 6 weeks. Striatal dopamine turnover ANOVA (A) 3-weeks: F = 1.82, p = 0.39, n = 3, 12, 4; (B) 6-weeks; F = 3.83, p < 0.04, n = 5, 16, 5. Dopamine metabolite (HVA) to dopamine ratio (HVA/DA) ANOVA (C) 3-weeks: F = 0.002, p = 0.99, n = 3, 12, 4; (D) 6-weeks; F = 5.39, p < 0.01, n = 3, 16, 2. Dopamine metabolite (DOPAC) to dopamine ratio (DOPAC/DA) ANOVA (E) 3-weeks: F = 0.87, p = 0.44, n = 3, 12, 4; (F) 6-weeks; F = 5.42, p < 0.01, n = 6, 16, 5. *p < 0.05, n value represents EV+EV, A53T+Control, and SIRT3+A53T respectively. All neurorestoration data is expressed as mean ± SEM.
3.4 SIRT3-myc functions as a deacetylase in the mitochondria

At the six-week time point following A53T and SIRT3 AAV injections, the rats were transcardially perfused, brains removed and dissected. IHC was performed in order to take images, which showed that there was co-localization of SIRT3-myc with translocase of the outer mitochondria (TOM20) in SN cells (performed by Dennison Trinh). To determine if SIRT3-myc was localized to the mitochondria and functioning as endogenous SIRT3 would in vivo, the mitochondria of the SNC were isolated and assessed for acetyl-lysine levels. A mitochondrial isolation protocol was used to determine its function. The dissected SN was homogenized and used to isolate the mitochondrial fraction so that acetyl-lysine levels could be quantified by Western blotting. First, it was determined that TOM20 was located solely in the mitochondrial fraction rather than in the cytosolic fraction, indicating that the mitochondria were successfully isolated (Figure 10A). From examining the Western blots, it was determined that the active form of SIRT3 was expressed in both EV+A53T and SIRT3+A53T groups, which further lends support to results from the optimization study: addition of SIRT3-myc AAV has no impact on endogenous SIRT3 levels. In addition, two bands were observed in the SIRT3+A53T group at 31kDa and 47kDa, which coincides with 28kDa active short form and 44kDa inactive long form of SIRT3 each with the addition of the 3kDa myc tag (Figure 10B). Lastly, the acetyl-lysine levels in the mitochondria were assessed using antibodies in both the EV+A53T and SIRT3+A53T groups (Figure 10C). The resulting Western blot displayed five separate bands positive for acetyl-lysine at 42kDa, 45kDa, 57kDa, 65kDa, and 71kDa. However, densiometric analysis determined that only the 57kDa and 71kDa bands were significantly different between groups. Currently, the identities of these bands are unknown. More specifically, the level of acetylation, represented by the levels of acetyl-lysine, were
approximately three times greater in the EV+A53T group compared to the SIRT3+A53T group. This indicates that the overexpressed SIRT3-myc in the SIRT3+A53T group functions to deacetylate mitochondrial proteins at lysine residues \textit{in vivo}.
Figure 10: SIRT3-myc is expressed in the mitochondria from the ipsilateral SN of SIRT3-myc treated rats (154).

A) **TOM20**

<table>
<thead>
<tr>
<th></th>
<th>A53T + EV</th>
<th>A53T + SIRT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td></td>
<td>16 kDa</td>
</tr>
<tr>
<td>Cytosol</td>
<td>16 kDa</td>
<td></td>
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B) **SIRT3**

<table>
<thead>
<tr>
<th></th>
<th>A53T + EV</th>
<th>A53T + SIRT3</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>47 kDa</td>
<td>31 kDa</td>
</tr>
<tr>
<td></td>
<td>28 kDa</td>
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C) **Protein Acetylation**

<table>
<thead>
<tr>
<th></th>
<th>A53T + EV</th>
<th>A53T + SIRT3</th>
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<tbody>
<tr>
<td></td>
<td>71 kDa</td>
<td>65 kDa</td>
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<tr>
<td></td>
<td>57 kDa</td>
<td>45 kDa</td>
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<tr>
<td></td>
<td>42 kDa</td>
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[Bar graphs showing OD values for TOM20, SIRT3, and Protein Acetylation]
Figure 10: SIRT3-myc is expressed in the mitochondria from the ipsilateral SN of SIRT3-myc treated rats

At six weeks following A53T injection, neurorestoration parkinsonian and SIRT3-myc treated rat brains were removed to determine the localization of SIRT3-myc within ipsilateral SN neurons and examine its function. Each lane in the Western blots represents a single animal. A) Mitochondrial isolation. Mitochondria were isolated from the ipsilateral SN, which was followed by SDS-page and Western blotting. TOM20 expression is represented as a 16kDa band in mitochondrial and cytosolic fractions. The graph represents the optical density (OD) mean ± SE of TOM20 in the mitochondrial and cytosolic fractions. B) Mitochondrial SIRT3-myc and endogenous SIRT3 expression. The 47kDa, 31kDa, and 28kDa bands represent the uncleaved myc-tagged, and cleaved-myc tagged, and the cleaved endogenous forms of SIRT3 respectively. The graph represents the OD mean ± SEM of each of the three forms of SIRT3 relative to TOM20 in both parkinsonian and SIRT3-myc treated animals. This suggests the localization of exogenous SIRT3-myc to the mitochondria. C) SIRT3-myc deacetylase function. The Western blot displays the acetyl-lysine levels in the mitochondria for five different protein bands, for which only the 57kDa and 71kDa bands were significant. The graph was generated from these two significant bands and represents the OD mean ± SEM of mitochondrial acetyl-lysine levels relative to TOM20 in both parkinsonian and SIRT3-myc treated animals. This result suggests that overexpression of SIRT3 decreases acetylation in the mitochondria. The identities of the proteins with the acetylated lysine residues in the blot are unknown. Lane 8 was removed due to sampling from the animal in lane 9. Lane 6 was a statistical outlier and was thus also removed from the study. •p < 0.05.
4. Discussion

4.1 Assessment of vector and model use

The purpose of this study was to validate SIRT3 as a neuroprotective and neurorestorative disease-modifying agent in the mutant human A53T α-synuclein rat model of PD. The AAV is a method used to deliver a gene into a host cell where the gene can then be incorporated and expressed long-term along with host genes (139). Furthermore, this expression can be confined to one hemisphere of the brain, unlike transgenic animal models, which display a more diffuse gene expression. This allows for the use of the unaffected hemisphere as a control. The human mutant A53T rAAV model of PD was thus chosen for this study due to its ability to express a mutant form of α-synuclein. This model also progressively induces typical parkinsonian phenotypes such as dopaminergic cell death, oxidative stress, aggregate formation, and motor and behavioural deficits. In comparison, toxin models involving rotenone, MPTP, or 6-OHDA are not capable of producing results that fully mimic PD progression in humans, nor do they exhibit high reproducibility. With the A53T rAAV model, by three weeks post-infusion there is evidence of forelimb asymmetry and α-synuclein aggregation. By six weeks, dopaminergic cell loss is observed in the SNc (146, 155).

AAV was also the method chosen for the delivery of myc-tagged SIRT3 into the neurons of the SNc. Using this method, the efficacy of the SIRT3 as a disease-modifying agent could be determined by injecting it prior to and after the manifestation of A53T parkinsonism in rats. According to previous studies, the transduction efficiency of AAV1 in SNc dopaminergic neurons of rats is high (144, 156). For this reason, the rAAV of serotype 1 was used to transfect neurons with SIRT3. In our study, SIRT3 was myc-tagged on its C-terminus in order to help differentiate it from endogenous SIRT3 during post-mortem analyses. The
uncleaved, inactive form of SIRT3 is 44kDa in size, which is then cleaved to an active 28kDa form by mitochondrial processing peptidase (MPP) in the mitochondrial matrix. The added myc tag however possesses a weight of approximately 3kDa, giving SIRT3-myc a slightly higher weight at 47kDa, which is important to note when examining Western blot results. The structure of the human SIRT3 protein is illustrated in Figure 11.

In addition to the AAV serotype 1, the chimeric AAV1/2 pseudotype for human mutant A53T was also used to improve transduction efficiency and alter tissue tropism towards SN neurons. Specifically, the AAV1 serotype can penetrate brain tissue well, while AAV2 has strong neuronal tropism and can be used to produce high concentrations. Therefore, it is these features of AAV1 and 2 serotypes that make the AAV1/2 pseudotype useful for developing models of disease, especially in rats and non-human primates. According to McFarland et al. (2009), injection of rAAV 2/1 serotype resulted in an intense, widespread expression throughout the SN, globus pallidus, and striatum (144). In the present study, it may be possible that results would have been more comparable if a single serotype was used for SIRT3, A53T, and empty vectors. However in attempts to control for this, wherever a serotype 1 AAV was used, the animal also received a pseudotype 1/2 injection. For example, a neurorestoration rat receiving A53T AAV1/2 during its first surgery would then receive SIRT3 or empty AAV1 during its second surgery. In this way, each animal received an AAV1 and an AAV1/2 injection.

Prior to surgeries, the concentration of the SIRT3 rAAV was optimized in order to determine dose. The optimal concentration should have had no impact on endogenous SIRT3 while raising the SIRT3-myc levels at least two-fold over that of endogenous SIRT3 levels solely in the injected hemisphere. There is a possibility that raising SIRT3 levels may lead to an excess of deacetylation reactions in the mitochondria. In turn, this could produce high levels of
mitochondrial NAM while reducing the levels of NAD$^+$. This could be recognized by the cell, which would then react homeostatically by expressing less endogenous SIRT3. However, it was noted that the chosen titre had no impact on endogenous SIRT3 levels while raising SIRT3-myc three times greater than endogenous SIRT3. Furthermore, it was important that the chosen titre would not impact the contralateral side. If the administration of SIRT3-myc somehow diffused or migrated into other brain regions, especially into the contralateral hemisphere, the results obtained would have been invalid and the contralateral hemisphere would have no longer served as a valid control. Additionally, the optimal concentration had no impact on the dopaminergic phenotype of the SN neurons or the neuron number. Perhaps if the injected titre was too high, it could have a toxic or damaging effect on mitochondrial function that leads to cell death. Or, there is the possibility that a high titre may lead to excessive amounts of deacetylation in the mitochondria by SIRT3. This could lead to activation of unnecessary enzymes and bring the mitochondrial environment out of homeostasis. This could impact the neuron numbers and stereological results would not provide an accurate representation of the neuron count. The SIRT3 rAAV was optimized to $2.59 \times 10^{11}$ GC/mL (2 µL injected, $5.18 \times 10^8$ GC final concentration) as this amount met all of the optimization criteria and was capable of infecting 90% of SN neurons. In the 2009 study by McFarland et al., rAAV pseudotypes 2/1, 2/2, 2/5, and 2/8 were generated by transfecting HEK293A cells with transgenes, packaging, and helper plasmids, then isolating the viruses for stereotaxic use at a similar concentration of $6.20 \times 10^8$ GC (144). However, this was applied to an AAV2/1 serotype that did not encode SIRT3. The concentration of the A53T rAAV1/2 we used had been optimized in a previous study by Koprich et al. (2010) to $2.55 \times 10^{12}$ GC/mL (2 µL injected, $5.10 \times 10^9$ GC final concentration) (146). In this study, the A53T AAV1/2 vector was used to produce a PD rat model. A high titre of $5.10 \times$
$10^{12}$ GC/mL was injected into the SN, where it took three weeks for pathology to arise with A53T α-synuclein expression spanning the SN and traversing the nigrostriatal pathway to the striatum (146).
Figure 11: Diagram of human myc-tagged SIRT3 protein structure

SIRT3-myc (47kDa)

N-Terminus 1

MLS Approx. MPP Cleavage Site

248 Active Site

366 NAD⁺ Binding Site

126-382 Deacetylase Domain

16kDa 28kDa 3kDa

C-Terminus 399

Myc tag
Figure 11: Diagram of human myc-tagged SIRT3 protein structure

The diagram represents the full-length, inactive form of the human SIRT3 protein (44kDa) with a myc-tag at the C-terminus (3kDa). The mitochondrial localization sequence (MLS, dark blue) targets SIRT3 to the mitochondria, where it enters via TIM and TOM complexes. Once in the mitochondrial matrix, MPP cleaves SIRT3 approximately at the location indicated in red. This gives rise to the 28kDa active form of SIRT3 (light blue), which can then exhibit NAD\(^+\)-dependent deacetylase activity at its deacetylase domain between residues 126 and 382. The myc tag allows for differentiation between injected and endogenous SIRT3 in the present study.
4.2 Efficacy of the A53T AAV1/2 rodent model of PD

A previous study by Koprich et al. (2010) showed that injection of a 2µL titre of $5.10 \times 10^{12}$ GC/mL leads to observable pathology after three weeks (146). Expression of A53T α-synuclein was also observed throughout the SN and striatum, which was determined by performing immunofluorescence to examine co-localization of TH and GFP each with A53T α-synuclein (146).

Based on *in vivo* behavioural data for the neuroprotection study, administration of the A53T AAV1/2 vector during the second surgery caused a significant increase in forelimb asymmetry at both three and six weeks in rats that were administered EV during the first surgery. This is representative of motor deficits due to the rat having a bias towards use of the forelimb ipsilateral to the injection site. This bias may indicate that mutant α-synuclein is compromising neuronal function in the SN, a brain region that plays a role in movement. In addition, these same rats displayed a decrease in dopaminergic SN neurons at six weeks. It is possible that by this time point, the A53T α-synuclein aggregates were impairing normal cell function and leading to cell death. As discussed previously, α-synuclein aggregates can impair ER-Golgi trafficking and axonal transport, microtubule formation, macromolecule degradation systems such as the UPS, trigger mitochondrial fragmentation, and cause DAT clustering that may lead to dopamine toxicity (99). Furthermore, it is possible that the α-helical structure of α-synuclein functions similar to a MLS, allowing it to localize to the mitochondria where it can contribute to ROS production, disruption of complex I, and release of cytochrome c to trigger apoptosis (18). It is in these ways that α-synuclein aggregation may have led to the downfall of SN neurons.
Furthermore, EV+A53T rats displayed an increase in HVA/DA and DOPAC/DA ratios at both three and six weeks. Dopamine is converted to a metabolite known as DOPAL by monoamine oxidase, which is then converted to DOPAC by aldehyde dehydrogenase. HVA is then derived from DOPAC by the catechol-\(\alpha\)-methyltransferase (COMT) enzyme. A general pathway of dopamine synthesis and metabolism has been outlined in Figure 12. These metabolites are typically measured as an indicator of oxidative stress and dopamine metabolism. Greater levels of dopamine metabolites such as HVA and DOPAC relative to dopamine may indicate that dopamine is being degraded more than it is being produced. In other words, this may infer that neuronal health and activity is being compromised, as it reflects the rate of dopamine synthesis. Furthermore, the conversion of dopamine to DOPAL releases hydrogen peroxide as a by-product, meaning that dopamine metabolite production leads to increased levels of free radicals. In addition, DOPAL is able to induce oligomerization of \(\alpha\)-synuclein \textit{in vitro}, which can directly damage the structure of synaptic vesicles by permeabilizing the membrane, affecting its ability to store dopamine (157). This could be a potential issue in PD patients who take L-DOPA, as dopamine derived from the drug would not be stored properly under these circumstances. Moreover, L-DOPA and dopamine can also be oxidized into toxic quinones, as discussed in section 1.4.2. Neurons under stress have increased levels of ROS such as hydrogen peroxide. Thus, high DOPAC ratios suggest that dopamine breakdown contributes to further deterioration of the cell and interruption of normal dopamine storage and neurotransmission. Mutant \(\alpha\)-synuclein also has the ability to interact with and inhibit TH, disrupting dopamine homeostasis as well as causing a decrease in vesicular monoamine transporter 2 (VMAT2) (98, 158). VMAT2 is responsible for uptake of dopamine and sequestration of dopaminergic neurotoxins into storage vesicles (98, 158).
Taken together, our results suggest that dopamine neuropathology and oxidative stress may begin to arise at three weeks and are present and exasperated at six weeks. This confirms the three-week time span for parkinsonian neuropathology to arise, as proposed by Koprich et al. (2010) (146).

For neurorestoration experiments, the cylinder behavioural test resulted in a significant increase in forelimb asymmetry at both three and six weeks post A53T AAV injection. Like the neuroprotection study, this is indicative of motor impairment due the bias in use of the forelimb ipsilateral to the injection site. Similarly, this suggests that communication is being impaired between neurons of the SN and neurons in brain regions they project to. Again, this is possibly due to the effects of the build up of mutant α-synuclein associated with this model. Moreover, the A53T AAV injection had no significant impact at either three or six weeks on the dopaminergic neuron count in the SN. However, in the published version of this study by Gleave et al. (2017), it was found that dopaminergic neuron count was significantly lowered in untreated parkinsonian rats at three weeks (154). It is possible that the variance in significance may be due to a difference in subjects included or some difference in statistical calculation. It was also noted that the dopamine cell count at three weeks for parkinsonian rats in neuroprotection versus neurorestoration studies appeared quite different (1700 cells vs 5104 cells, respectively). However, there are a low number of subjects in these groups and the stereology users also may have been different. Both of these factors could have contributed to the overall accuracy (or lack thereof) in these results. HPLC data regarding striatal dopamine turnover and dopamine metabolite:dopamine ratios showed that EV+A53T rats displayed a decrease in striatal dopamine turnover and an increase in both HVA/DA and DOPAC/DA ratios at six weeks post A53T injection. This indicates that the A53T rAAV model of PD that
is used here has a negative impact on the production of dopamine in SN neurons that project to the striatum. If dopamine is not being produced, either due to compromised cellular function or neuron loss, this would also lead to the observed increase in dopamine metabolites relative to dopamine levels.

Overall, while results for EV+A53T animals differ between the neuroprotection and neurorestoration studies, there appears to be a trend that demonstrates a α-synucleinopathy-related decline. In particular, both neuroprotection and neurorestoration animals exhibit significant motor impairment, which manifests as a forelimb use bias by three weeks post initial injection. Furthermore, neuroprotection and neurorestoration animals display an increase in the dopamine metabolite:dopamine ratios, suggesting a slowing or lack of dopamine synthesis in SN neurons relative to its metabolism, which may be indicative of cellular dysfunction or neuron loss. For these reasons, it would appear that this rodent model of PD may be useful for future research. Through the optimization step in this study and that by Koprich et al. (2010), it was determined that the chosen concentration of AAV had no impact on neuron number (146). Therefore, the AAV vector itself was considered neutral and was not beneficial or toxic to the neurons. Because the vector method of delivery has been shown to be neutral in vivo, this model surpasses previous toxin-based models. However, it has been expressed that previous in vivo models of PD such as the rotenone or MPTP model are either toxic, difficult to replicate, or both. It is important that A53T AAV model of PD can diminish or eliminate these problems seen in other models. The replicability of this experiment will be discussed further in section 4.6.
Figure 12: General outline of the synthesis and metabolism of Dopamine

L-Phenylalanine

L-Tyrosine

Levodopa (L-DOPA)

DOPA Decarboxylase

Dopamine

Phenylalanine Hydroxylase

Tyrosine Hydroxylase (TH)

Monoamine Oxidase (MAO)

Dopamine Hydroxylase

H$_2$O + NH$_3$

3,4-Dihydroxyphenylacetaldehyde (DOPAL)

3,4-Dihydroxyphenylacetic Acid (DOPAC)

Homovanillic Acid (HVA)

Methylation

Phenylethanolamine N-methyltransferase

Epinephrine

Norepinephrine

Catechol-o-methyltransferase (COMT)
Figure 12: General outline of the synthesis and metabolism of dopamine

Synthesis of dopamine begins with amino acids L-phenylalanine or L-tyrosine. Phenylalanine, an essential amino acid, can be converted into tyrosine by phenylalanine hydroxylase. Tyrosine is then converted to L-DOPA by tyrosine hydroxylase, an enzyme that is often targeted in staining of catecholaminergic neurons. L-DOPA is then decarboxylated by DOPA decarboxylase to form dopamine. From here, dopamine can be hydroxylated to form norepinephrine and eventually epinephrine. In an alternate pathway, dopamine is converted to DOPAL by monoamine oxidase, where its amino group is replaced with a carbonyl aldehyde group. In this reaction, hydrogen peroxide is generated as a by-product. The carbonyl group of DOPAL is then converted to carboxyl group by aldehyde dehydrogenase to form DOPAC. DOPAC is then converted to HVA by COMT. HVA and DOPAC ratios relative to dopamine are often used to make inferences about dopamine turnover and metabolism, which may also be an indicator of cellular stress. This is because the metabolism of dopamine contributes to the overall increase in ROS levels, as ROS is generated in its conversion to DOPAL. In addition, situations may arise where L-DOPA and dopamine are oxidized to form toxic quinones that can have damaging effects on the neuron.
4.3 SIRT3 overexpression demonstrates neuroprotective effects when injected prior to the development and manifestation of parkinsonian neuropathology, respectively.

SIRT3 is an NAD$^+$-dependent protein deacetylase found in the mitochondria that plays a role in metabolism, stress resistance, and longevity. In PD, death of dopaminergic neurons means there is a lack of dopamine required for communication with neighbouring neurons. This leads to loss of function in the SN, which is normally responsible for controlling movements. Thus, if SIRT3 overexpression is capable of improving cell functioning in a way that prevents or slows cell death, it may prove to be an effective agent in PD pathology. In this study, it was found that a 2µL injection of SIRT3 is neuroprotective when injected into a healthy rat SN before A53T rAAV administration, when oxidative stress is not yet apparent.

During the cylinder test, when there is unilateral damage to the right SN, rats will display a biased use of the right forelimb on the walls of the cylinder. Results showed that SIRT3 is capable of preventing and reversing motor deficits (forelimb asymmetry) at six weeks post SIRT3-myc administration (Figure 4B). The significant result here indicates that SIRT3 may contribute to the prevention (or at least the reduction) of motor deficits at six weeks that typically arise by three weeks in an A53T parkinsonian rat. This suggests that SIRT3 has a beneficial impact on the cell, likely due to its function in the mitochondria. For example, SIRT3 can directly deacetylate MnSOD, increasing its antioxidant activity to lower mitochondrial ROS (159). Furthermore, this study also showed that increasing MnSOD expression did not result in higher antioxidant activity, suggesting that SIRT3 is required for activation of MnSOD in the mitochondria (159). As discussed in 1.5.1, SIRT3 also indirectly increases GSH antioxidant activity by deacetylating and thus activating a Kreb’s enzyme IDH2 that generates NADPH from isocitrate. NADPH is a reducing agent functions with glutathione
reductase to reduce GSSG to GSH (130). GSH is then able to scavenge ROS such as hydrogen peroxide. This supports the idea that the reduction of ROS via the action of SIRT3 may prevent damage to DNA, overload of the UPS, and triggering of events such as MPT that induce apoptotic mechanisms. If cell death occurs due to these circumstances, loss of normal motor function becomes apparent. It must be noted, however, that the sample size of our SIRT3-myc treated rats at six weeks was small while the range of data in untreated A53T parkinsonian animals was large. These characteristics of the data may have contributed to result significance, however such a result should be confirmed by increasing subject numbers.

As outlined in Figure 4A and B, impending nigral neurodegeneration associated with parkinsonism was not significantly impacted by the administration of SIRT3 both at three and six weeks. A significant result here would have further confirmed the impact of SIRT3 on preventing cell death associated with parkinsonian neuropathology. However, the low sample sizes may not have provided the statistical power to achieve a significant result. In addition, there is variability in EV+EV dopamine cells counts at three and six weeks. It is possible that this difference is due to user error, which will be discussed further in section 4.6. However, if such variability exists between the EV+EV stereology results, there is also a chance that variability exists between other groups as well. This may also account for the lack of significance in the effects of SIRT3 on dopamine cell numbers. In the case that the results are indeed accurate, there is a possibility that the surgery performed on SIRT3-myc rats were unsuccessful, causing SIRT3 expression levels in the SN to be below expected levels. Otherwise, it is possible that the staining applied during IH was not strong enough to allow for a clear staining of cells with TH/NeuN overlap.
HPLC data (Figure 6D and F) demonstrates that SIRT3-myc overexpression was able to reduce the ratio of both HVA/DA and DOPAC/DA at the six-week time point compared to untreated parkinsonian rats. A low ratio of dopamine metabolites relative to dopamine indicates that dopamine turnover is reduced. Otherwise, the remaining dopamine would be broken down into its metabolites, thus increasing this ratio along with ROS levels. As discussed, dopamine metabolism leads to the generation of hydrogen peroxide that can induce quinone formation and prevent dopamine storage in the axon terminal. A study by Ahn et al. (2008) in mouse embryonic fibroblasts claims that hydrogen peroxide causes a dissociation of SIRT3 from complex I, which would cause a reduction in complex I activity and thus ATP synthesis downstream (127). This raises the notion that overexpression of SIRT3 may protect the ETC from the impact of ROS such as hydrogen peroxide. This in fact was demonstrated in a later study, which showed that SIRT3 overexpression in HEK293 cells is protective against hydrogen peroxide and leads to an increase in cell viability, especially when IDH2 is also overexpressed (130). This would increase activation of IDH2 that would eventually lead to generation of more GSH to eliminate ROS buildup. Taken together and applying these ideas to our in vivo model, this suggests that ectopic overexpression of SIRT3 contributed to the prevention of cellular stress build up that would overwhelm the cell and lead to ATP deficits, dopamine toxicity, overload of the UPS, and eventually cell death. SIRT3 overexpression would then make the mitochondria the origin of this increase in viability in SN neurons. SIRT3 influencing stabilization of ATP production and lowering of ROS in the mitochondria would then impact other regions of the cell. For example, this could allow SN neurons to continue carrying out normal, ATP-dependent, ROS-generating processes such as production and release of dopamine, thus maintaining normal ratios between dopamine and its metabolites.
While other studies on PD, Alzheimer’s disease, Huntington’s disease, and stroke have shown SIRT3 to be neuroprotective in vitro, this is the first set of evidence showing that SIRT3 displays neuroprotective capacity in an in vivo model of PD. In a rotenone model of PD in SH-SY5Y human neuroblastoma cells, lentiviral overexpression of SIRT3 prevented accumulation of α-synuclein, decreased ROS generation, prevented reductions in SOD and GSH, suppressed alterations in the mitochondrial membrane potential, and improved overall cell viability. Not surprisingly, knockdown of SIRT3 had the opposite effect (160). Conversely, Novgorodov et al. (2015) suggest that SIRT3 deacetylates and thus activates the ceramide synthase. This may lead to inhibition of complex III of the ETC, causing eventual ROS accumulation and mitochondrial dysfunction (150). It was shown that SIRT3 gene ablation in a rodent model of stroke prevents ceramide synthase deacetylation, and thus rescues complex III activity and ceramide accumulation (150). Because SIRT3 has over seven hundred substrates that it acts on, this result could suggest that the deacetylase function of SIRT3 can impact mitochondrial events in different ways. Moreover, it could be that there is an optimal level of SIRT3 or acetylation/deacetylation ratios required for healthy mitochondrial function.

4.4 SIRT3 overexpression demonstrates neurorestorative effects when injected following the development and manifestation of parkinsonian neuropathology

SIRT3 overexpression successfully prevented motor deficits and increased the ratio of dopamine relative to its HVA and DOPAC metabolites when administered before parkinsonian deficits developed. In addition, we also wanted to determine if overexpressing SIRT3 would have the same impact when administered after parkinsonism had developed. If possible, then SIRT3 may prove to be an effective treatment in PD patients that have reached a point in the
disease where symptoms are present and cell death has occurred. It was found that a 2µL injection of SIRT3 is neurorestorative when injected into the SN after A53T rAAV administration, when oxidative stress is already apparent. Based on optimization experiments, it takes three days for SIRT3 to reach optimal levels in the brain. Because the effects of the A53T do not become apparent until three weeks post-infusion (146), the SIRT3-myc (or EV, or PBS control, or SIRT3^{H248Y}-myc) AAV1 was injected eighteen days post-A53T rAAV administration. This would allow SIRT3 to reach optimal levels when parkinsonian deficits and oxidative stress start to arise. It was thought that SIRT3 overexpression would thus be most beneficial to SN neurons at this time point.

Results of the cylinder test at three and six weeks (Figure 7) show that SIRT3 had no significant impact on reducing motor abnormalities in parkinsonian rats to levels similar to that of the EV+EV group. If significant, this would have shown that SIRT3 is able to improve mitochondrial function to the point where the cell is able to carry out its normal activities, such as dopamine neurotransmission. This would permit normal communication with neighbouring neurons, allowing the SN as a whole to carry out its role in movement. However, if the obtained result is reflective of the potential of SIRT3, then it is possible that the oxidative damage in neurons had already reached a point that SIRT3 could not compensate for. In analyzing the data at three and six weeks (Figure 7A and B), it seems that there were quite a few data points that could be considered “outliers” based on their distance from the mean. These values may skew the results in a way that makes the overall group mean appear higher than it is, which could affect the results of the ANOVA. As for the six-week SIRT3+A53T group (Figure 7B), there are fewer subjects compared to the other groups, which may also influence how representative the graph is of the effects of SIRT3. Furthermore, there is a great
range of asymmetry values in A53T parkinsonian animals in both directions. While this could be attributed to error during surgery or behavioural test analysis, it could also mean that the A53T rAAV impacted the contralateral hemisphere or that the brain was using compensatory mechanisms to make up for the right SNc lesion. In cases where the asymmetry is closer to zero, this may be a result of reduced activity or habituation of the rat during repeated trials. While there visually appears to be a decreasing trend in asymmetry in the SIRT3 group compared to untreated parkinsonian animals, it is possible that all of these characteristics of the data do not allow for a significance to be observed.

Neurorestoration stereological results show that administration of SIRT3 had a significant impact on preventing dopaminergic cell death in SN neurons compared to untreated rats at six weeks (Figure 8B). At three weeks, the subject numbers in each group were somewhat small and contained a wide range of data points (Figure 8A). This causes the graph to appear as though all groups had similar cell counts after three weeks. While this may be due to error, if this data is in fact accurate then it indicates that dopaminergic neurodegeneration is not significant or observable after three weeks. However, this would be contrary to what was described by Koprich et al. (2010) (146). Similarly at six weeks, there is also a wide range of data points that may have also influenced the data. Another unexpected aspect of this data is that the SIRT3-myc treated rats at six weeks have a significantly higher cell count compared to EV+EV and naïve animals. It would be expected that the SIRT3-myc group would have an equal if not slightly lower cell count to EV+EV and naive animals, since they have been injected with the A53T AAV. In other words, SIRT3+A53T animals would be expected to display some parkinsonian-related neuron stress or loss due to the presence of mutant α-synuclein aggregation. If SIRT3 was capable of fully restoring stressed neurons, we would
expect to see neuron counts equal to that observed in EV+EV animals. Otherwise, if SIRT3 was partially capable of restoring stress neurons, we would then expect cell numbers slightly lower than that observed in the EV+EV and naïve groups. Furthermore, the EV+EV cell counts were quite lower than naïve cell counts, although it would have been expected that these groups would have similar cell counts. While it is possible that the counts are due to differences in counting between stereology users, the naïve group also only contains four subjects. The low subject number may not be representative of actual cell counts in naïve animals, and could have led to low statistical power and high variation.

It would be easier to assume that the cell numbers in Figure 6B are due to errors made by stereology users or a consequence of surgery execution. Another explanation for the TH levels could be due to the fact that TH is a highly regulated enzyme. Therefore, the expression of TH could vary not only between subjects, but over the course of the experiment, generating the variation in stereology results. TH activity is increased when dopamine is needed and decreased when it is not. Perhaps SIRT3 has an indirect affects on the cell that leads to increased TH or dopamine, which is why six-week neurorestoration SIRT3+A53T rats have such high cell counts. It seems unlikely that SIRT3 would be able to trigger this much neuron proliferation and neurogenesis in rats past the embryonic stage. There are two brain regions involved in neurogenesis, the dentate gyrus and the subventricular zone. Other brain regions are currently not known to generate new neurons. However, if SIRT3-myc itself or a SIRT3 AAV are capable of migrating into neighbouring brain regions in a way that allows it to reach these neurogenic regions, it is possible that SIRT3 could influence cell numbers. Whether dentate gyrus or subventricular-derived neurons could migrate and thus specifically increase cell numbers in the SN remains a question. A study by Jiang et al. (2017) showed that
overexpression of SIRT3 reduces oxidative stress and cytotoxicity of neural stem cells induced by microglia activation caused by β-amyloid accumulation (161). SIRT3 was able to prevent this stress by inhibiting mPTP opening and cytochrome c release through lowering CypD activity and expression, all of which are events associated with apoptosis (161). This supports the idea that SIRT3 could behave similarly in response to A53T-induced oxidative stress used in our model. Furthermore, the impact of SIRT3 on neural stem cell survival could mean that it helps regulate proliferation, which could potentially play a role in repair mechanisms against neurodegenerative disorders such as PD. In other words, maybe SIRT3 can spread to and positively impact neurogenic regions, where neuronal migration could then replenish the SN as a mechanism to compensate for neurons lost due to PD.

Neurorestoration HPLC data representing the striatal dopamine turnover and ratio of dopamine metabolites relative to dopamine show that SIRT3 overexpression has no significant impact at both three and six weeks in all cases (Figure 9). This suggests that SIRT3 is not able to restore cells under stress to a point where they can continue normal dopamine production. In addition, because the ratio of dopamine metabolites HVA and DOPAC relative to dopamine were not lowered to levels comparable to the EV+EV group, this suggests that as dopamine production ceases, its catabolism exceeds its production and its metabolites begin to build up.

This is indicative of a cell that is under stress, potentially due to the impact of mutant α-synuclein build up. As discussed in section 1.4.2, mutant α-synuclein is capable of clustering the DAT, causing excessive dopamine uptake that can lead to dopamine toxicity such as quinone and ROS production (99). In addition, α-synuclein can impact axonal transport. It is possible that transport of substrates and machinery required at synaptic terminals for dopamine production is being hindered. Otherwise, dopamine toxicity could be contributing to
dysfunction related to dopamine production in the synaptic terminal. Although SIRT3 was overexpressed, it is possible that the stress resulting from the initial A53T AAV injection had become too great for SIRT3 to manage at this point.

Lastly, in the neurorestoration study at six weeks, an additional group was tested as a control for SIRT3. This group included parkinsonian animals that were injected during their second surgery with a catalytically-inactive (or deacetylase-deficient) mutant form of SIRT3 known as SIRT3\(^{H248Y}\)-myc. According to Hirschey et al. (2010), SIRT3\(^{H248Y}\) has to ability to associate with, but not deacetylate, a mitochondrial protein and SIRT3 substrate known as long-chain acyl CoA dehydrogenase (162). The cylinder test results show an increasing, yet non-significant trend in forelimb asymmetry, but with a large range of values. If significant in comparison to EV+EV or SIRT3-myc treated animals, this would suggest that administration of SIRT3\(^{H248Y}\)-myc has no impact on improving motor impairment, similar to untreated parkinsonian rats. As for stereological analyses, SN dopaminergic cell counts in rats that received SIRT3\(^{H248Y}\)-myc were quite significantly lower than SIRT3-myc treated rats, as expected. Furthermore, SN cell counts in SIRT3\(^{H248Y}\)-myc rats were similar to cell counts of untreated parkinsonian rats. This indicates that injection of the SIRT3\(^{H248Y}\)-myc mutant had no impact on ameliorating neurodegeneration. This is very likely due to its lack of deacetylase activity. Furthermore, SIRT3\(^{H248Y}\)-myc acts as a competitive inhibitor of SIRT3, thus can weaken or eliminate the effects expected from endogenous SIRT3-myc. If this had occurred, however, it would have been expected that SIRT3\(^{H248Y}\)-myc would reduce cell counts to levels lower than that of A53T parkinsonian rats. This is because A53T rats possess endogenous SIRT3 that is not being inhibited or competed with by SIRT3\(^{H248Y}\)-myc. Further testing would be required to analyze this phenomenon.
4.5 SIRT3 targets the mitochondrion, where it functions as a protein deacetylase

There is conflicting information about the exact localization of SIRT3 in the cell. Some studies suggest that SIRT3 only localizes to the mitochondria due to its N-terminal MLS, while other studies suggest SIRT3 also plays a role in the nucleus. In one study, HEK293T cells transfected with human SIRTs 1, 2, and 3 vectors were generated and used to obtain whole-cell and mitochondrial lysates for immunoprecipitation (123). Whole cell lysates were found to contain SIRT3 in its long 44kDa and cleaved 28kDa form, while mitochondrial lysates only contained SIRT3 in its cleaved form. Only cells transfected with SIRT3 showed an increase NAD$^+$-dependent deacetylase activity specifically in the mitochondria. Immunofluorescence showed that SIRT3-GFP localized exclusively to the mitochondria and this was not observed when its MLS was deleted. A later study by Scher et al. (2007) also used human SIRT3 in 293F TREX cells to determine that its N-terminal domain plays a role in localizing SIRT3 to the mitochondria. However, unlike Schwer et al (2002), they determined that the long form is catalytically active and can be found in the nucleus where it functions to deacetylate histones (163). They also claimed that cells under stress or overexpression of SIRT3 led to the migration of SIRT3 from the nucleus to the mitochondria (163). These studies did use human SIRT3, which unlike mouse SIRT3, contains the MLS. However, different cell lines and plasmids may have affected transduction, localization, or produced other confounding variables leading to these variable results. The relationship between the mitochondrial and nuclear localizations of SIRT3 needs to be clarified to better understand its role in the cell.

In the present study, to determine the function and localization of the injected SIRT3-myc, mitochondria from the ipsilateral SN of six-week rats were isolated. Following the mitochondrial isolation, the mitochondrial fraction was compared to the cytoplasmic fraction
via Western blot to ensure a successful isolation. This was done using a mitochondrial membrane protein known as TOM20. It was expected that the mitochondrial fraction, rather than the cytoplasmic fraction, would contain TOM20 when run on a Western blot if properly isolated. By obtaining a mitochondrial fraction, it was possible to determine whether SIRT3-myc was localized to the mitochondria similar to endogenous SIRT3. The mitochondrial isolation was confirmed by the strong 16kDa band (TOM20) strictly present in the mitochondrial fraction and not the cytosolic fraction (Figure 10A). However, based on the high speed at which the fractions were centrifuged, it is possible that more than just mitochondria were collected into this fraction that was meant to be isolated mitochondria. Furthermore, no cytoplasmic, nuclear, or other organelle markers were used to ensure the quality of the mitochondrial isolation was valid. This will be discussed further in section 4.6.

Western blot and optical density measurements showed significant presence of SIRT3-myc in its long (47kDa) and cleaved (31kDa) forms in the mitochondria of parkinsonian rats that received SIRT3-myc, but not in the untreated parkinsonian group (Figure 10B). Furthermore, this result provides evidence that SIRT3-myc not only solely targeted the mitochondria as endogenous SIRT3 does, but it was cleaved to its shorter, active form. The cleaved, active form of SIRT3-myc existing in the mitochondrial isolate suggests that this location is where the cell requires it to function as a protein deacetylase. Therefore, it makes sense that the mitochondrial localization allows SIRT3-myc to present its cytoprotective effects via mediating the deacetylation of various mitochondrial substrates. While this data does provide further evidence of the mitochondrial localization of SIRT3, it does not support the results of the study by Schwer et al. (2002), which claims that the long form of SIRT3 was undetectable in the mitochondria because it is an inactive precursor that is synthesized in (and
thus exists within) the cytoplasm (123). Once the long form of SIRT3 reaches the mitochondria, it is proteolytically processed by MPP to cause its enzymatic activation (123). However, it cannot be said that our results support or invalidate other subcellular localization of SIRT3 because no immunofluorescence was used to show co-localization of SIRT3 with organelles other than the mitochondria.

In addition, both A53T+EV and A53T+SIRT3 groups displayed 28kDa bands with similar optical density representing the cleaved form of endogenous SIRT3 (Figure 10B). This proves that the injection of SIRT3-myc had no impact on endogenous SIRT3 expression, which was one of the preferred stipulations of finding an optimal concentration to inject. This could have been a potential issue if the overexpression of SIRT3-myc acted in a negative feedback loop to prevent expression of endogenous SIRT3. However, as this was not the case, the injection of SIRT3-myc allowed for an approximate three-fold increase in overall SIRT3 levels.

The function of SIRT3-myc was demonstrated through the lower ratio of acetylated lysine residues relative to TOM20 in the mitochondria of the SIRT3-myc treated rats compared to the untreated parkinsonian group. However, it was noted that five separate protein bands appeared on the Western blot (Figure 10C). The identities of these bands currently are unknown and thus are a topic of future studies, as will be discussed in section 6. Of the five bands, only the 57kDa and 71kDa bands were found to have significantly lower acetylation levels compared to the untreated parkinsonian group and thus were used for optical density comparison in the graph in Figure 10C. It is also possible that the unknown bands are artifacts that may be created by factors such as high levels of antibodies used, excess lysate loaded onto the gel or other methodological issues. Although a Lowry assay was implemented to control
for overall protein loading, no controls were used to account for other possible issues. Further study in this area is required in order to reinforce this data and identify the unknown protein bands.

4.6 Research Limitations

The limitations of a study are the attributes of the methods or model that may negatively influence the results. It is possible that some limitations can be avoided while others cannot. It is crucial to address the limitations in order to fully understand the interpretation of results. While the degree to which these limitations reduce the quality of the research may be limited, it is also important that they are discussed so that changes can be made in future experiments.

An important factor in choosing a model in many cases of research is replicability. An issue with previous models of PD such as the toxin models was that they were not able to be easily replicated. This may be due to either variability between researchers or because of a genetic or environmental variance that creates a differential impact in animals of the same species. In this study, it can be said that there is some degree of replicability. However with the wide range of techniques and number of individuals that were involved in this study, replicability may be questioned and cannot be fully confirmed. For example, in the behavioural and stereological data in Figures 4, 5, 7, and 8, the individual data points are displayed. In most cases, there is a wide range of data points while in other cases they are very few data points. It is possible that the range in data is due to the way the researcher conducted an experiment or confounding variables that were present. For example, there were multiple stereology users. While they were blinded to the study, each person could have responded differently to training,
resulting in some samples being counted more conservatively or liberally. In the EV+EV groups for the neuroprotection study, there was great variability in SN dopaminergic cell count between three and six-week animals (2102 cells versus 3598 cells respectively). It seems as though this difference is too great to be due to the long-term impact of the vector or surgery. To account for this issue in future, it may be beneficial to have specific individuals carry out a particular role or set of roles in the study, if possible. This would help to maintain consistency throughout the entire experiment.

As mentioned in section 4.3, because some animals were excluded from the study, some groups were left with very low sample sizes. This could have skewed the results to some degree or prevented a significant result from existing (type II error). For example, the stereology results at three weeks for both the neuroprotection and neurorestoration studies have small sample sizes. Perhaps results would have been different or more accurate if more subjects were included. It is important to not only have samples sizes that would improve the statistical power and thus precision of results, but also to ensure consistency across groups within the experiment while also displaying external validity. As a result, the smaller samples sizes could have made it difficult to find significant relationships in the data that may have existed (or vice versa) had the sample size been larger. Despite this, smaller samples sizes can be overcome by carrying out experiments on additional subjects, which is something that could be achieved in the future.

Another area where variability could have existed would have been during the stereotaxic surgeries for AAV administration. Different surgeons could have been trained differently by different individuals, used slightly different methods or equipment, have different levels of experience and proficiency, or provide different post-operative care for the
animals. For example, a new method of sterilization of surgical tools was introduced to the University of Toronto Scarborough Campus vivarium in 2016. However, some surgeries had been carried out with older methods of sterilization prior to this change. It is possible that post-operative issues such as infection or irritation could have impacted the survivability or behaviour of the rodent. As the surgeries were carried over the span of a few years, this naturally resulted in various surgeons contributing to this part of the study. As previously suggested, it may have been beneficial to give fewer individuals the role of surgeon, if possible, to achieve a higher degree of consistency.

Lastly, another limitation that was noted was that the isolation of the mitochondrial fraction was not confirmed to its fullest extent. TOM20, a mitochondrial membrane protein, was used to ensure that the mitochondrial fraction and not the cytosolic fraction was expressing this protein. However, it would have been beneficial to confirm this further by using markers for a protein of the cytoplasm or other organelles. If impurities were present in the mitochondrial fraction, these markers may have shown up on the Western blot. This is also a concern because the centrifuge speed used was 18000g, a high speed that may pull down other components of the cell into the mitochondrial fraction. Because there are studies that show SIRT3 localization in the nucleus is possible, it remains unknown if components of the nucleus were pulled into our mitochondrial fraction as it was not controlled for or tested. Thus, our results could possibly be representative of SIRT3 localizing to both the mitochondria and the nucleus where it functions as a deacetylase.
5. Summary and Conclusion

SIRT3 is an NAD⁺-dependent protein deacetylase that is targeted and translocated to the mitochondrial matrix by an N-terminal MLS. SIRT3 natively exists as an inactive 44kDa protein, which is then cleaved into its active 28kDa form by MPP. Through its deacetylase activity, SIRT3 is capable of regulating the activity of a number of mitochondrial enzymes involved in metabolism and cytoprotection.

To examine its role in cytoprotection and aging in vivo, SIRT3 was overexpressed in an A53T α-synuclein rat model of PD. This was the first study that presents SIRT3 as a neuroprotective disease-modifying agent. The rAAV A53T model used was one that had been previously determined as an effective method for analyzing disease-modifying agents. SIRT3 was overexpressed in the right SN before and after the manifestation of parkinsonism using an AAV1 vector. We then tested to confirm the mitochondrial localization and deacetylase function of SIRT3 in SN neurons. Results show that SIRT3 is able to prevent motor dysfunction and reduced dopamine relative to its metabolites when expressed before parkinsonian deficits develop. SIRT3 is also capable of restoring dopaminergic neuron count in the SN after cellular stress and parkinsonian deficits had become apparent. Lastly, we found that SIRT3 was located in the mitochondria rather than the cytoplasm where it functioned as a protein deacetylase. These results suggest that the efficacy of SIRT3 is due to its deacetylase function in the mitochondria, as without this function, parkinsonian deficits are unmitigated. This supports the hypothesis that SIRT3 has cytoprotective, disease-modifying abilities in vivo.

As mentioned, SIRT3 has over 700 mitochondrial substrates. Thus, it is possible that when SIRT3 is overexpressed, it acts on many of these substrates to have a widespread effect on lowering bioenergetic demands and oxidative stress. Evidence of the neuroprotective
capabilities of SIRT3 in a range of neurodegenerative disorders, *in vivo* or *in vitro* may suggest its usefulness as a therapeutic agent in human cases. For example, in Alzheimer’s disease, SIRT3 expression is decreased and tumor suppressor protein p53 is upregulated. Not only does p53 translocate to the mitochondrial matrix where it complexes with Bax protein to trigger cytochrome c release, it also reduces expression of NADH dehydrogenase genes ND2 and ND4. This leads to ROS build up and reduction of oxygen consumption, which SIRT3 is able to restore via deacetylation of p53 (164). Overall, our findings suggest that SIRT3 may slow or halt the progression of PD. In patients, this may protect them from reaching more advanced stages of the disease and possibly extending their life expectancy.
6. Future Directions

In future studies, it may be possible to test the neuroprotective abilities of SIRT3 in other models of PD or other neurodegenerative disorders. For example, our lab has been testing the efficacy of SIRT3 in the MPTP and PFF in vivo models. Since there is a large range of studies that have focused on in vitro experimentation, moving to in vivo studies may also help reveal results that may apply to human medicine. The AAV1 has already been used as an effective method of gene delivery in human clinical trials, therefore its use is not farfetched. However, these trials were carried out via intramuscular injections in a muscular dystrophy study and percutaneous administration in a heart disease study (156, 165). It would be beneficial to determine a noninvasive method of intracranial administration or a way by which the AAV may cross the BBB for diseases of the brain such as PD. It has been found that the AAV9 serotype can cross the BBB after being injected intravenously (166). Thus, it may be possible to construct an AAV pseudotype that may cross the BBB and transduce neurons efficiently. However, this method would likely result in a widespread neural transduction of the CNS. Therefore, another consideration to be made would be to look into how to target a particular brain region.

Our study has shown that SIRT3 is able to prevent the damaging effects of cellular stress due to mitochondrial dysfunction to improve cell viability. However, it would also be interesting to see if SIRT3 in the mitochondria has an indirect impact on the actual aggregation of mutant α-synuclein in the cytoplasm. We have shown how SIRT3 can ameliorate the oxidative stress created as a result of α-synuclein aggregation. There is a possibility that SIRT3 treated rats accumulated less mutant α-synuclein, through deacetylation of a substrate
that can influence cytosolic events. However no assays or imaging methods were used to
determine this, but is an area that is open to study in the future.

In the present study, the identity of the five acetylated protein bands in the Western blot
were not determined and thus remain unknown. Future plans have been discussed about
looking into mass spectroscopy in order to determine the identities of these proteins, which are
possible substrates of SIRT3 in the mitochondria. Once known, it is possible to look further
into these proteins to elucidate their function in PD pathology. Furthermore, the mitochondrial
isolation was only carried out in six-week neurorestoration rats. To complete the analysis, it
would be beneficial to carry out this experiment in three-week animals as well as in
neuroprotection animals. This would provide the information needed to make comparisons
between three and six-week time points, as well as between neuroprotection and
neurorestoration studies. The mitochondrial isolation protocol also did not take into account
the use of a cytosolic marker to assess the quality of the isolation. This is something that could
be tested in future to ensure results are being analyzed in a true mitochondrial fraction. In
addition, the SIRT3\textsuperscript{H248Y}-myc group was only tested within the neurorestoration paradigm in
six-week animals, where SIRT3\textsuperscript{H248Y}-myc was administered after the induction of
parkinsonism by A53T rAAV. There are plans to apply SIRT3\textsuperscript{H248Y}-myc to a neuroprotection
paradigm in order to complete and further confirm the results of this study. Additionally, it is
possible that this study could be applied to higher organisms with more evolutionary
relationship to humans compared to rats, in order to bring us closer to the possibility of
clinically testing SIRT3 overexpression in human PD patients.
7. References


