PCR Analysis on the Effects of Alpha Synuclein Treatment on C6 Rat Glioma Cells

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PCR ANALYSIS ON THE EFFECTS OF ALPHA SYNUCLEIN TREATMENT ON C6 RAT GLIOMA CELLS

by

Michael J. Helmus

A thesis submitted to the Graduate College in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences Western Michigan University December 2019

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Ide for taking me on as his last student before retiring. Your gentle guidance is admirable and your mentorship during my thesis project has contributed to helping me become a more successful scientist. Additionally, being able to work on MSA would not have been possible without you and it has been both thrilling and a pleasure to contribute towards MSA research. I am thankful to have Dr. John Spitsbergen and Dr. Robert Eversole on my committee for the three of you together allowed me to make my thesis project my own and helped to keep me on track when the path forward waivered at times. Thank you to Dr. Silvia Rossbach for pushing me to further my education; I would not have attempted this without your positive reinforcement of my capabilities and scientific mind.

I would also like to thank MPI Research for financial support and the use of laboratory space. A special thank you goes out to my sister, Alison Helmus, for always listening to me talk about MSA and the few “eureka” moments those one-sided conversations created.

Michael J. Helmus
PCR ANALYSIS ON THE EFFECTS OF ALPHA SYNUCLEIN TREATMENT ON C6 RAT GLIOMA CELLS

Michael J. Helmus, M.S.
Western Michigan University, 2019

Multiple System Atrophy (MSA) is a rare neurological disease that mainly implicates the neuronal protein, alpha synuclein, as being centrally involved in MSA pathology. Despite synucleinopathy dogma, little is known about alpha synuclein's role in MSA pathogenesis. The Ide laboratory previously conducted an Affymetrix gene expression study utilizing the post-mortem pons tissue from eight individuals with MSA and five individuals without any known neurological disorders. One conclusion from that study was that downregulation of numerous mitochondrial homeostasis related genes had occurred. It is hypothesized that alpha synuclein can perturb mitochondrial homeostasis through direct interaction with mitochondria. This study utilized Reverse Transcriptase PCR and Droplet Digital PCR to confirm downregulation of the mitophagy related gene, \textit{Bnip3}, that was observed in the MSA Affymetrix study, when C6 rat glioma cells were treated with a single dose of human wild type alpha synuclein. qPCR and ddPCR was then used to assess the impact of alpha synuclein treatment on mitochondrial genome copy numbers, which served as a surrogate measure of mitochondrial number, and the single copy nuclear-encoded gene, Beta Actin. Detection of mtDNA and Beta Actin was decreased at all time points assessed. This study demonstrates that a single treatment of alpha synuclein resulted in decreased detection of mitochondrial genomes per cell, which offers potential insight into synucleinopathy pathogenesis.
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CHAPTER 1

INTRODUCTION

Overview and History

Multiple System Atrophy (MSA) is a rare neurological disease that is poorly understood by the medical and scientific community. Typically occurring in an individual’s sixth decade of life, MSA progresses rapidly and presents with a variety of autonomic failures and eventually, complete loss of motor control after only a few years after onset. MSA is one of the lesser known synucleinopathies and shares many similarities with Parkinson’s Disease and Dementia with Lewy Bodies. The synucleinopathies implicate involvement of the neuronal protein, alpha synuclein, and the toxic accumulation of aggregated and fibrillated alpha synuclein in different cell types and regions of the brain give rise to these pathologically distinct diseases. In MSA, alpha synuclein traffics to the cytoplasm of oligodendrocytes, disrupting their essential role in neuronal maintenance and homeostasis. Ultimately, it has not been elucidated as to whether alpha synuclein abnormalities of any kind are the etiological cause of MSA or simply an observed symptom of this disease.

The first known clinical occurrence of MSA was published in 1900 (1). It was reported that two individuals suffered from adult-onset sporadic ataxia with extrapyramidal, urinary and hypotensive symptoms. Autopsy on one of these individuals revealed gross degeneration of the olives, pons and cerebellum. It is in this report where the term olivopontocerebellar atrophy was first used. Twenty-five years later, Bradbury
and Eggleston reported a similar occurrence in three patients with idiopathic postural hypotension (2) and in 1960, Shy and Drager described a case study of an individual with severe parkinsonism, multiple autonomic systems failure, atrophy and postural hypotension (3). Gross degeneration of the inferior olives, caudate nucleus and the intermediolateral columns of the spinal cord was observed upon autopsy. One year later, autopsy of three individuals with pontine, olivary, cerebellar and striatonigral degeneration was reported. Shy and Drager would define these combinatorial symptoms as “Shy-Drager Syndrome”.

Less than a decade later, Graham and Oppenheimer coined the term “Multiple System Atrophy” (4). Prior to this, MSA had been categorized as numerous distinct diseases, however, Graham and Oppenheimer recognized a key feature that MSA subtypes had in common. Specifically, they noted that neuronal atrophy was present in all MSA cases, leading them to posit that MSA symptoms result from combinatorial dysfunction of different brain regions that stemmed from neuronal atrophy. This observation would come to be a defining feature of MSA.

Pathology

Several subtypes of MSA are used to define key characteristics of this multifactorial disease (5). MSA-P is used to characterize MSA with Parkinson’s-like symptoms and predominantly includes extrapyramidal features with striatonigral degeneration. MSA-C is used to characterize MSA with predominant cerebellar dysfunction with progressive ataxia. Shy-Drager Syndrome is still used today, albeit less frequently, and is
characterized by features of Parkinsonism with pronounced autonomic nervous system failure. MSA subtypes attempt to categorize the numerous pathological symptoms that can occur in MSA.

Those with MSA-P predominantly experience striatonigral degeneration (SND) of dopaminergic neurons in the substantia nigra pars compacta, GABAergic neurons of the caudate-putamen and medium-spiny neurons. Those with MSA-C predominantly experience olivopontocerebellar atrophy (OPCA) which includes degeneration of Purkinje neurons in the cerebellar cortex as well as neurons of the pontine nuclei and inferior olivary complex. Those with either MSA-P and MSA-C subtypes also experience degeneration of the brainstem and spinal cord as well as numerous other regions in the brain.

Approximately 60-82% of MSA patients have developed akinetic-rigid parkinsonism at the time of diagnosis and these individuals respond poorly to L-Dopa treatment. Approximately 10-30% of MSA patients have cerebellar features at the time of diagnosis (6). MSA-P is the most common form of MSA; 87-98% of those with MSA will develop some feature of parkinsonism (7). Multiple features of parkinsonism, which occur in 33-64% of patients, include irregular, jerky postural tremors and rest tremors. Approximately 8% of MSA patients experience pill-rolling tremors that are typically indicative of PD. One fifth of MSA patients report an increased rate of falling during their first year of disease progression, however, falling is typically indicative of Progressive Supranuclear Palsy (PSP), which is the rarest synucleinopathy known to date. Cerebellar features predominantly include gait ataxia in 90% of patients, limb ataxia in 87% of
patients and nystagmus in 38% of patients. Additionally, 47% of patients will develop kinetic ataxia, 69% will develop scanning dysarthria, 23% will develop cerebellar oculomotor dysfunction and 24% will develop intention tremors.

The first reported MSA symptom for most patients is speech impairment. Those with MSA-C present with scanning dysarthria while those with MSA-P tend to have high-pitched, quivery and croaky intonations while speaking. Ultimately, most of those with MSA lose their ability to speak. Dysphagia is a prevalent MSA symptom and there are numerous incidences of MSA patients choking to death.

Erectile dysfunction is thought to be the first MSA symptom for men (8), and both men and women experience sexual deficits. 73% of both men and women have urinary dysfunction with urge incontinence and 48% lack the ability to completely empty the bladder during urination (9). One third of patients will experience both urge incontinence and urinary retention. Micturition is also an early MSA symptom and is generally considered more severe than in those with PD.

Orthostatic hypotension (OH) occurs in about 50% of MSA cases. Clinically, MSA diagnostic criterion is met when a minimum of 30 mmHG drop in blood pressure and a 15 mmHG drop in diastolic blood pressure occurs. Syncope after a sudden postural change is quite common in MSA and is a known symptom that dates back to Shy and Drager’s initial clinical report from 1960. Other cardiovascular autonomic failures occur and often include dizziness, nausea, palpitations, tremulousness, headaches and an overall general weakness (10).
Respiratory dysfunction is predominantly marked by inspiratory stridor in 9-69% of MSA cases and is thought to occur primarily from the pathological adduction of the vocal cords. Degeneration of the nucleus ambiguus culminates in the paralysis of the vocal cord abductor muscles (11) and deep, involuntary gasps or sighs occurs in 43% of patients. Several sleeping disorders are associated with the observed respiratory dysfunction in MSA patients. Sleep apnea is reported in 40% of patients (12) and likely results from degeneration of the pontomedullary respiratory centers. Prolonged cardiac ventricular arrhythmias and hypoxemia from sleep apnea and laryngeal patency can result is nocturnal sudden death (13). Nocturnal sudden death ultimately claims the lives of most individuals who have fully progressed MSA.

Most individuals with MSA have gastrointestinal dysfunction. This includes chronic constipation, upper and lower gastrointestinal dysfunction, anhidrosis and hypohidrosis. Those with MSA-C will typically have pyramidal features with hyperreflexia and Babinski signs.

By the third year of disease progression, 68% of patients will have severe postural instability. Recurrent falling episodes can happen to MSA patients while they are still able to walk, though this is less common in MSA and is typically indicative of PSP. Abnormal posture occurs in all MSA cases; camptocormia is more pronounced when an individual is walking and decreases while sitting (14). One hallmark of MSA is the eventual inability to unbend their neck or back without aid; individuals with progressed MSA tend to spontaneously lean forward and will remain in that posture until they are physically
moved back into their normal resting posture. Additionally, Pisa Syndrome and disproportionate antecollis are symptoms of MSA.

Epidemiology

Few epidemiological studies have been conducted to date and such efforts have been hampered by MSA’s rarity of occurrence. Despite this, some data have been collected. One 14-year cohort study in Minnesota estimates incidence rates to be at 0.6 per 100,000 people. This rate increased to 3 per 100,000 people in those who are aged fifty and over (15). A study conducted in Iceland produced data with comparable rates to that obtained from the Minnesota study (16). Incidence rates obtained from Russian and Swedish populations differed slightly when compared to the other two studies (17, 18), though not appreciably. The World Health Organization has estimated European populations to have prevalence rates of 3.4 per 100,000 people which rises to 7.8 per 100,000 for individuals over 40 years of age (16). The highest estimated prevalence rate is 4.9 per 100,000 people (19).

Alpha Synuclein

The hallmark of the synucleinopathies is the aggregation, fibrillization and subsequent accumulation of alpha synuclein in specific cell types and regions of the central nervous system. Accumulation of alpha synuclein is so severe that it is used in postmortem histological analysis of brain tissues where the locations of alpha synuclein aggregates are used to confirm which type of synucleinopathy the patient had. In MSA, alpha synuclein aggregation occurs primarily in the cytoplasm of oligodendrocytes and is
termed Glial Cytoplasmic Inclusions (GCI's). Glial Nuclear Inclusions (GNI's), Neuronal Cytoplasmic Inclusions (NCI's) and Neuronal Nuclear Inclusions (NNI's) can also be found, albeit less predominantly. As such, alpha synuclein aggregation and deposition occurs in neurons and glial cells. It is well characterized, however, that cell death of oligodendrocytes occurs first, followed by neuronal cell death (20).

Alpha Synuclein is a small intrinsically disordered protein consisting of 140 amino acids. Coded by the gene, SNCA, alpha synuclein is a prevalent neuronal protein, accounting for approximately 1% of total cytosolic proteins in neurons (21). Alpha synuclein is mainly localized to presynaptic termini, however, as will be discussed later, it can also be found localized to mitochondria and within the nucleus of neurons.

Alpha synuclein is expressed in a wide variety of tissues and is more abundantly expressed in neurons and platelets when compared to any other cell type (22). Data on alpha synuclein expression in oligodendrocytes has provided mixed results. Until recently, most studies concluded that alpha synuclein was not expressed in oligodendrocytes (23). More recent studies utilizing techniques such as laser-capture microdissection have established that oligodendrocytes do express alpha synuclein and moreover, that alpha synuclein expression is higher in oligodendrocytes in MSA brain when compared to control brains (24). Other studies, for example, have established that oligodendrocyte lineage cells express alpha synuclein and expression significantly decreases during oligodendrocyte maturation (25). Given this information, oligodendrocytes do express alpha synuclein but this is minimal when compared to
neuronal expression and as such, the source of pathogenic alpha synuclein is likely predominantly derived from neurons.

The N terminal half of alpha synuclein is well characterized and is composed of two amphipathic alpha helices that are separated by a small hinge region. The C terminal half of alpha synuclein is poorly characterized and studies on this region are greatly hampered given that it is intrinsically disordered. X-Ray Crystallographic structural analysis has not been successful to date given the inability of wild type alpha synuclein to form crystals. A single structure has been obtained by NMR structural analysis and importantly, this structure was only able to be obtained by stabilizing human wild type alpha synuclein onto synthetic micelles (26). Cryogenic Electron Microscopy studies have also yielded a stable structure of fibrillated human wild type alpha synuclein (27).

Numerous small nucleotide polymorphisms (SNP’s), duplications and triplications have been identified in SNCA, however, none of these SNP’s have been correlated to MSA pathology (28). For example, the A53T, A30P, E46K, and H50Q mutations are associated with familial PD and the A18T and A29S mutations are associated with sporadic PD (29). These mutations have been shown to abrogate membrane binding due to non-conservative amino acid substitutions in the amphipathic helical domains. Additionally, duplications and triplications of SNCA, though rare, have also been associated with early onset PD. This information demonstrates that perturbing alpha synuclein’s membrane binding function can give rise to PD and its associated symptoms. Overexpression of alpha synuclein through upregulation of SNCA, aside from duplications or triplications, can also give rise to PD. Knockout of alpha synuclein in mouse embryos results in loss of
dopaminergic neurons of the substantia nigra (30) and silencing of SNCA results in nigral degeneration on par with alpha synuclein overexpression (31). Though SNCA is not mutated or overexpressed in MSA, studies on alpha synuclein in PD allow one to elucidate alpha synucleins normal function which is necessary towards understanding its potential relevance and contribution towards MSA pathogenesis.

Despite decades worth of research, the function of alpha synuclein has yet to be elucidated, however, the identification of alpha synuclein’s membrane binding motifs led to a slew of functional studies that began to shed light on alpha synuclein’s function (32-34). Alpha synuclein lays down into phospholipid bilayers in a parallel manner (35). Numerous studies have demonstrated that alpha synuclein can influence the curvature of membranes upon membrane binding and this is due in part to the flexibility of the amphipathic helices imparted by the hinge region that separates the two alpha helical motifs (36). Importantly, the hydrophilic C terminus of alpha synuclein weakly and transiently binds to lipid membranes (37) demonstrating that the C terminus does not significantly contribute towards membrane binding, which likely holds a different function altogether.

Alpha synuclein has a predisposition towards binding to anionic phospholipids including phophatidylserine and phospatidylglycerol (38, 39). Furthermore, alpha synuclein has a preference for binding to lipid rafts (40) and some of the SNCA mutations, namely A30P, contributes to decreased membrane binding efficiency (41). Numerous conformations of membrane-bound alpha synuclein have been proposed that not only allow for influencing curvature of membranes but these conformations can also
impact the formation of vesicles and the resulting size of the vesicles. Alpha synuclein can form single elongated alpha-helix, broken alpha-helix and elongated helix conformations (42). Importantly, alpha synuclein has predisposition for binding to membranes with pronounced curvature and then secondarily influences further changes to membrane curvature. As such, alpha synuclein may preferentially bind to vesicles including exosomes, endosomes and synaptic vesicles.

Alpha synuclein’s normal function in neurons is predominantly thought to revolve around neurotransmitter release and recycling (43-45). Numerous studies implicate alpha synuclein as having a central role in maintenance and regulation of synaptic vesicles and the synaptic vesicle pool, overall. Natively, unfolded alpha synuclein exists in monomeric forms at the synapse. Alpha synuclein then adapts to the alpha helical structure upon membrane binding and may form helical multimers upon binding to synaptic vesicles. This binding event stabilizes the target membrane and protects alpha synuclein from aggregation and potentially oxidative stress as well (46).

**Mitochondria**

Mitochondrial health and homeostasis are essential towards normal cellular health, and deficits in mitochondrial function have taken a central role in the pathology of numerous neurological diseases, including PD and MSA (47, 48). Mitochondria are dynamic organelles; in neurons, mitochondria show dynamic localization throughout complex neuronal architecture (49, 50). Kinesins and dynein, in part, facilitate movement of mitochondria up and down axons (51) and actin-facilitated movement has been
observed as well (52). Mitochondrial morphology is altered depending on where mitochondria are located. Mitochondrion width tends to remain fixed at 1 µm whereas mitochondrial length can vary greatly; in axons, mitochondria are typically between 1-3 µm in length and mitochondria in dendrites tend to be longer (53). Given the high energy requirements of neurons, especially at the synapse, mitochondrial localization throughout the neuron is essential, albeit, poorly understood.

Each mitochondrion contains 2-10 genomes and the number of mitochondrion per cell depends on the overall energy requirements of the cell (54). For example, it is estimated that 1000-2000 mitochondria are in each liver cell, composing one fifth of the entire cell mass. Considering that the brain uses ~20% of all oxygen when the brain itself is only 2% of the overall body mass (55), it is likely that mitochondrial numbers are equally as high, if not higher, in neurons as they are in liver cells.

The Warburg Effect was postulated in 1924 and describes the observation that immortalized in vitro cells predominantly use glycolysis over aerobic respiration (56). The inverse Warburg Effect was postulated several decades later and expanded on the Warburg Effect; healthy neurons predominantly utilize oxidative phosphorylation whereas healthy glia predominantly utilize glycolysis. Important to the inverse Warburg Effect is the observation that Kreb’s cycle derived lactate, produced by glia, is secreted as part of the complex neurotropic support glia provide to neurons (57, 58). As such, lactate secretion by glia is currently thought to supplement neuronal energy production.
It is well established that mitochondrial genomes become heteroplasmic with increasing age (59, 60). Kang et al found that iPSCs derived from numerous tissue sources contain low levels of heteroplasmic point mutations and the frequency of these mutations increase with age (61). Importantly, many of these mutations occurred in RNA coding genes that can lead to defects in respiratory functions, especially with consideration that the mitochondrial genome encodes for protein subunits found in complex's I, III, IV and V. The occurrence of mitochondrial DNA heteroplasmy in neurological diseases has been under investigation for decades (62-64). Numerous labs have directly implicated mitochondrial DNA mutations, including substitutions and deletions, and subsequent heterogeneity of the mitochondrial pool to be directly correlated to a slew of neurological diseases (65, 66).

Mitochondrial DNA mutations have been described in MSA. One study was conducted on a family with known familial MSA of the cerebellar subtype. Sequencing of mitochondria isolated from blood and a muscle biopsy from this patient provided data that an exceptionally large mitochondrial DNA deletion of 12,806 bp was present in the mitochondria from the muscle biopsy, but not the mitochondria isolated from blood cells (67). This mutation spanned all genes encoding oxidative phosphorylation related proteins. Morphologically, the mitochondria from the muscle biopsy were enlarged and included mitochondria with abnormal shapes, including elongated and branched mitochondrion.

Genomic DNA mutations have also been described in MSA. The best studied genomic mutation is in the gene, COQ2, and as such further implicates mitochondrial
deficits as a potential cause of MSA (68). COQ2 is a gene that codes for the enzyme parahydroxybenzoate-polyprenyltransferase which catalyzes one of the final reactions in the biosynthesis of ubiquinone (CoQ). Ubiquinone is essential in the electron chain transport, and mutations to COQ2 result in loss of function and ultimately, deficits in ubiquinone synthesis. Importantly, COQ2 mutations have only been linked to familial inheritance of MSA in those with Asian ancestry.

**Alpha Synuclein and Mitochondria**

It has been well established that alpha synuclein has a high propensity towards binding mitochondrial membranes as it contains a mitochondrial localization signal in the N-terminal domain. Nakamura et al demonstrated that overexpression of alpha synuclein was sufficient to drive mitochondrial fission and that the rate of fission exceeded the rate of fusion (69). Morphological changes to other organelles did not occur nor was the mitochondrial membrane potential perturbed, however, following mitochondrial fragmentation, a decrease in respiration was observed which culminated in neuronal death. Other labs report similar findings; overexpression of alpha synuclein in *in vivo* and *in vitro* neurons results in mitochondrial fragmentation (70).

Alpha synuclein has been reported to directly bind to mitochondrial proteins in addition to binding to mitochondrial membranes. Di Maio et al reported that alpha synuclein bound to TOM20 when *in vitro* dopaminergic neurons were treated with rotenone. Binding of TOM20 to its coreceptor, TOM22, was impaired as a result (71). This result was recapitulated in rats injected with AAV2-SNCA constructs and in postmortem
PD brain tissues. The authors also found that import of proteins into mitochondria was drastically reduced which holds significance considering some 1500 nuclearly encoded proteins are present within mitochondria. Complex I activity was assessed and was found to be diminished as well, presumably as a result of loss of protein import.

Given that the hallmark of the synucleinopathies is the accumulation of aggregated and fibrillated alpha synuclein, Ludtmann et al identified normal and pathological differences when in vitro neurons/astrocytes in a coculture system were treated with either alpha synuclein monomers or oligomers. The authors found that monomeric alpha synuclein improved ATP synthase efficiency whereas alpha synuclein oligomers localized to the vicinity of ATP synthase but impaired complex I-dependent respiration (72). They also found that ATP synthase beta units were oxidized and that mitochondrial lipids were peroxidated. Ultimately, the mitochondrial permeability transition pore (mPTP) was aberrantly opened causing mitochondrial swelling and eventually cell death.

Wang et al performed similar experiments using primary mouse or rat cortical neurons. Alpha synuclein amyloid fibrils were preformed and then treated to either source of cortical neurons. Using serine 129 phosphorylated alpha synuclein as a measurement of the exogenously added pathogenic alpha synuclein, the authors found that aggregated alpha synuclein bound to mitochondria with a higher propensity than endogenous monomeric alpha synuclein (73). These findings were confirmed in animal models and in postmortem PD and MSA brains. The rate of oxygen consumption was
measured in *in vitro* experiments and cells incubated with aggregated alpha synuclein had significantly lower rates of respiration.

**Mitophagy**

In recent years, interest in mitophagy has taken center stage in the pathogenesis of numerous synucleinopathies, including PD and MSA. Typically considered a form of selective macroautophagy that specifically removes damaged or superfluous mitochondria, recent interest in mitophagy revolves around the possibility of aberrant activation of mitophagy machinery or conversely, defects in mitophagy processes.

Mitochondrial metabolism is intimately coupled with the generation of Reactive Oxygen Species (ROS) that inherently damage components of the mitochondrion, including lipids, proteins and nucleic acid. Cellular homeostasis demands that damaged components of mitochondria be repaired or if damage is excessive, that the mitochondrion be degraded in its entirety. Lack of repair or degradation of damaged mitochondria may lead to further generation of ROS and the potential for proapoptotic proteins to be released into the cytosol, ultimately resulting in cellular death (74).

Mitophagy can be classified into three distinct variants (75). Type I mitophagy occurs primarily during nutrient deprivation. Mitochondria and mitochondrial fission products are engulfed by preautophagic structures (PAS) forming mitophagosomes. Following mitochondrial sequestration, the mitophagosome acidifies leading to mitochondrial depolarization and subsequently, hydrolytic digestion by lysosome derived proteolytic proteins. Type II mitophagy occurs primarily with physical damage to

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mitochondria. For example, adequate photodamage to mitochondria may result in mitochondrial depolarization and the recruitment of LC3 to the mitochondrial outer membrane. LC3 then recruits the mitophagosome leading to acidification and hydrolytic digestion by lysosome derived proteolytic proteins. Type III mitophagy is technically a form of micromitophagy and is marked by the formation of mitochondria derived vesicles (MDV’s) from mitochondria and at the expense of the mitochondria. MDV’s are often enriched in oxidized mitochondrial proteins and often localize to multivesicular bodies. MDV’s contained within multivesicular bodies are then either degraded by the autolysosome or are released into the extracellular environment.

Mitophagy type II and type III are currently viewed as being the most pertinent to the synucleinopathies. Under normal physiological conditions, PINK1 continuously localizes to mitochondria where it is rapidly degraded by matrix processing peptidases. PINK1 is then further degraded in the cytosol by the proteasome (76). Damaged mitochondria or depolarized mitochondria leads to a reduction of PINK1 cleavage and PINK1 subsequently accumulates on the mitochondrial outer membrane through interaction with TOM. PINK1 then autophosphorylates itself where it then ubiquitinates its own serine 65 residue. Ubiquitination of serine 65 recruits Parkin to the mitochondrial outer membrane where it is then activated by phosphorylation. Parkin then polyubiquitinates numerous mitochondrial substrates (77) including VDAC1, MFN 1/2 and Miro 1 which induces mitochondrial fission and mitophagy (78) through eventual recruitment of LC3 adapters. PINK1 and Parkin are two genes that are mutated in
Parkinson’s Disease, therefore, deficits in mitophagy are central to the pathology of some variants of PD.

LC3 may also act on mitochondria in a ubiquitin-independent manner through binding to LC3 receptors. One such receptor is Bnip3 (BCL2/Adenovirus E1B 19 kDa Interacting Protein 3). Bnip3 homodimerizes on the mitochondrial outer membrane and is activated by phosphorylation at Ser17 and Ser24. Bnip3 suppresses cleavage of PINK1 (79) thereby activating mitophagy mechanisms as previously discussed.

Bnip3 contains an atypical BCL-2 homology domain 3-only (BH3-only) motif that typically allows for binding to antiapoptotic BCL-2 family members. Bnip3 is unique, however, since it interacts with Bcl-2 and other family members through its transmembrane domain instead of the BH3-only domain (80). Early studies identified Bnip3 to be an apoptosis-inducing protein that became functionally active after overcoming Bcl2 suppression. In this regard, Bnip3 interacts with Bax and Bak to induce apoptosis (81). Bnip3 also contains a LC3-Interacting Region (LIR) domain allowing for direct binding to LC3 proteins and it also contains a transmembrane domain for integration into the mitochondrial outer membrane (82). Under the control of Hypoxia Inducible Factor 1 (HIF1), Bnip3 was primarily thought to induce cell death through apoptosis and necrosis during hypoxic conditions. For example, it has been reported that Bnip3 can induce autophagy mediated cell death in gliomas in response to hypoxia, ceramide or arsenic trioxide treatment (83). Conversely, it has also been reported that Bnip3 can contribute to cell survival. Bnip3 was found to be upregulated in solid tumors and specifically in cells that were still viable yet were in a hypoxic region of the tumor
It is hypothesized that mitophagy mechanisms occur during hypoxia so as to prolong cell survival at the expense of a portion of the mitochondrial pool.

*Bnip3* is significantly upregulated in numerous tumor cells whereas it is minimally expressed in normal cells due to its ability to cause cell death. In tumors where upregulation occurs, tumors tend to be more aggressive. Interestingly, it has also been found that *Bnip3* expression is silenced by methylation in some tumor cells that are undergoing an epithelial to mesenchymal transition (85).

In 2007, Dr. Ide’s lab published a paper on gene expression changes from the rostral pons of Multiple System Atrophy patients (86). Out of tens of thousands of genes that were assessed for differential expression, 254 genes were found to be differentially regulated; 180 genes were downregulated and 74 genes were upregulated. Genes that were downregulated included genes involved with mitochondrial function, ubiquitin-proteasome function, protein modification, glycolysis/metabolism and ion transport. Genes that were upregulated included genes involved with transcription/RNA modification, inflammation, immune system function and oligodendrocyte maintenance and function.

The aim of this master’s thesis is to build upon the information provided in the Ide laboratory’s 2007 publication. Specifically, prior students have developed a MSA cell culture model using the C6 rat glioma cell line. Using this cell line, exogenously added human wild type alpha synuclein is added to culture media and then incubated for variable amounts of time. Prior students have focused on establishing that alpha
synuclein is endocytosed and that mitochondrial membrane potentials are perturbed in an alpha synuclein dose dependent manner. The aim of my study is to utilize various PCR methodologies to identify genes that respond to alpha synuclein treatment with the primary objective of focusing on gene candidates that were found to be differentially regulated from the Ide laboratory’s 2007 publication.
CHAPTER 2

PCR ANALYSIS ON THE EFFECTS OF ALPHA SYNUCLEIN TREATMENT ON C6 RAT GLIOMA CELLS

*Exploratory PCR Array and Bnip3/Hprt1 ddPCR*

Considering alpha synucleins probable role in MSA pathogenesis, an exploratory PCR array was purchased to assess if alpha synuclein treatment could result in the differential regulation of any oxidative stress related genes. Genes on the rat oxidative stress PCR array create proteins that are involved with mitochondrial health, homeostasis and function. Furthermore, several genes on the rat oxidative stress PCR array were identified by the Ide laboratory as being differentially regulated in those with MSA. The C6 cell culture model that the Ide laboratory developed was utilized so as to identify differentially regulated genes in glial cells when they were treated with a single dose of alpha synuclein. Identification of any gene that was differentially regulated after a single treatment with alpha synuclein may hold significance in MSA pathogenesis.

ddPCR was then used to fully assess the effects of alpha synuclein treatment at multiple time points following identification of the *Bnip3* candidate gene. ddPCR was chosen as it is the most sensitive PCR technique described to date and the particular strength of ddPCR is the absolute quantification of mRNA transcripts without the use of an external calibration curve or housekeeping gene. Additionally, the sensitivity and accuracy of ddPCR is particularly important considering that cell culture experiments were not replicated in this pilot study.
Materials and Methods

Cell Culture

C6 rat glioma cells were procured from the ATCC (ACTT© CCL-107™) and cultured in F-12K medium containing 15% horse serum and 2.5% fetal bovine serum. Cells were maintained in T25 flasks and utilized for experiments prior to cells reaching confluency. Briefly, cells were washed once with 1x HBBS and then trypsinized at 37°C until cells were detached from the cell culture flask. Trypsin was then quenched with complete medium and cell counts were obtained using a hemocytometer. Cells were then plated at a density of 4x10^4 cells/mL per 24-well culture plate with each well containing 1 mL media. Three days after plating, cells that were approximately 90% confluent were treated with 1 mL of 32 µg/mL alpha synuclein (rPeptide, Cat# S-1001-1) in complete medium for 1 hour, 2 hours or 4 hours in duplicate. Each time point included duplicate control wells that received complete medium and the identical volume of water used for alpha synuclein dosing.

At a later date, cells were plated as described above, however, cells were treated with 1 mL of 32 µg/mL alpha synuclein (rPeptide, Cat# S-1001-1) in complete medium for 12 hours, 24 hours or 48 hours in duplicate one day after plating. All wells were harvested prior to cells reaching confluency. Each time point included duplicate control wells that received complete medium and the identical volume of water used for alpha synuclein dosing.
RNA Extraction and Purification

Cells were harvested for RNA at the time points above using QIAGen’s RNeasy Fibrous Tissue Mini Kit (Cat# 74704) per the manufacturer’s specifications and with minor modifications. Briefly, cells were washed once with 1X HBBS and then 320 µL Buffer RLT supplemented with βME (10 µL βME per 1 mL Buffer RLT) was pipetted into each well on ice. Following a short incubation on ice, lysates were collected and stored frozen at -20°C. On the day of purification, cell lysates (300 µL) were thawed at RT and then 590 µL nuclease-free water and 10 µL Proteinase K were pipetted to each lysate and mixed by pipetting. Lysates were then incubated at 55°C for 10 minutes in a VorTemp™ 56 with 1000 rpm shaking. Following this, 450 µL 100% EtOH was pipetted to each lysate and then incubated for 5 minutes at RT after mixing by pipetting. Lysates were then pipetted to individual columns and centrifuged at 13,000 rpm for 1 minute. The columns were then washed with 350 µL Buffer RW1 and centrifuged as described above. Columns were then treated with DNase I for 15 minutes per the manufacturers specifications and then columns were washed again with 350 µL Buffer RW1 and twice with 500 µL complete Buffer RPE. Following a 1 minute centrifugation to remove residual wash buffer, RNA was then eluted in 40 µL nuclease-free water. Purified RNA was then aliquoted and stored at -80°C.

RNA Quantification and cDNA Synthesis

RNA was quantified using the NanoDrop 8000. The means of duplicate measurements were used for downstream applications. cDNA was synthesized using the High-Capacity
cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Cat# 4374967) per the manufacturer’s specifications. Briefly, 1000 ng of each RNA sample was pipetted into a 20 µL total reaction and then the reverse transcription reaction was performed at 25°C for 10 minutes, 37°C for 120 minutes and then 85°C for 5 minutes with an infinite hold at 4°C on a GeneAmp 9700. Following completion of the reverse transcription reaction, 80 µL of nuclease-free water was pipetted to each reaction. cDNA was then aliquoted and stored at -80°C.

2100 BioAnalyzer

RNA integrity was assessed by following the manufacturer’s specifications.

RT-qPCR

The Rat Oxidative Stress PCR Array (ROSL-I) was purchased from Real Time Primers, LLC and used per the manufacturer’s specifications with some modifications. Briefly, the master plate was thawed at RT and then centrifuged for 5 minutes at 1500 rpm to drop the liquid down to the bottom of the plate. Final PCR array conditions per well were 0.1 µM forward and reverse primer, 1x Power SYBR™ Green PCR Master Mix (Applied Biosystems, Cat#4367659) and 10 ng cDNA in a final reaction volume of 20 µL. Once plated, the PCR plate was centrifuged for 5 minutes at 1500 rpm to drop liquid down to the bottom of the plate. The following cycling conditions were used: 50°C for 2 minutes, 95°C for 10 minutes and 50 cycles of 95°C for 10 seconds and 58°C for 45 seconds. The PCR array was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using the 96000 Emulation mode. Data was manually analyzed using delta delta Ct methodology.
**ddPCR**

Each ddPCR reaction consisted of 0.1 µM forward and reverse primer from the Rat Oxidative Stress PCR Array (ROSL-I), 1x QX200™ ddPCR™ EvaGreen Supermix (BioRad, Cat#186-4034) and 2 ng cDNA in a final reaction volume of 20 µL. Briefly, the complete ddPCR reaction was pipetted into the QX200 Automated Droplet Generator to generate droplets. The PCR plate was then placed into the C1000 Touch Thermal Cycler with 96-Deep well Reaction Module and cycled as follows: 95°C for 5 minutes and 40 cycles of 95°C for 30 seconds and 58°C for 60 seconds. The reaction products were then cooled to 4°C for 5 minutes, raised to 90°C for 5 minutes and then lowered to 4°C with an infinite hold. Ramp rates of 2°C/second were used. Reaction products were then read on the QX200 Droplet Reader. Bnip3 and Hprt1 primer sets were used. Each sample was assayed in duplicate.

**Results**

**Exploratory PCR Array**

The specific aim of the exploratory PCR Array was to identify any differentially regulated genes when C6 rat glioma cells were treated with a single dose of 32 µg/mL alpha synuclein. Previous students in Dr. Ide's laboratory had developed the C6 rat glioma cell model to mimic MSA pathology, therefore, the secondary aim of the exploratory PCR Array was to recapitulate any differential gene expression observed in MSA patients. C6 rat glioma cells were treated with 32 µg/mL alpha synuclein, in duplicate cell culture wells, for 1 hour, 2 hours or 4 hours. Purified RNA was of similar
concentration and high purity (Figure 1) and RNA was highly intact as assessed by the BioAnalyzer (Figure 2). One treated sample and one control sample from the 4 hour time point was used for the PCR Arrays to identify any potential gene candidates.
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Figure 1. Nanodrop™ 8000 RNA Concentration and Purity Data. Nanodrop™ 8000 data shows that RNA is highly pure and free of PCR inhibitors.
Figure 2. Bioanalyzer Data Showing Highly Intact Total RNA. Samples from left to right are: Ladder (L), 1 hr Control_1 (1), 1 hr Control_2 (2), 2 hr Control_1 (3), 2 hr Control_2 (4), 4 hr Control_1 (5), 4 hr Control_2 (6), 1 hr 32 µg/mL Alpha Synuclein_1 (7), 1 hr 32 µg/mL Alpha Synuclein_2 (8), 2 hr 32 µg/mL Alpha Synuclein_1 (9), 2 hr 32 µg/mL Alpha Synuclein_2 (10), 4 hr 32 µg/mL Alpha Synuclein_1 (11) and 4 hr 32 µg/mL Alpha Synuclein_2 (12).

Data was analyzed by delta delta Ct methodology. Ct values of less than 25 that resulted in fold changes of 2.0 and greater were considered promising gene candidates that would be analyzed further using mRNA from the full time course. 88 genes that are involved in oxidative stress related pathways were analyzed and a single gene, *Bnip3* met the above criteria for additional testing. *Bnip3* was downregulated by a fold change of 2.01. Bnip3 was also identified as being downregulated in MSA patients.
Bnip3 and Hprt1 ddPCR; Short Time Course

Having potentially identified a gene that responds to alpha synuclein treatment, it was decided to run the short time course experiment using Bnip3 and Hprt1 primer sets to assess the early effects of alpha synuclein treatment on these genes. The Hprt1 housekeeping gene was not necessary to run considering that ddPCR allows for absolute quantification without an external calibration curve or housekeeping gene for normalization, however, it was included as a control to account for technical implementation of the ddPCR. Hprt1 was also the most stably expressed housekeeping gene from the PCR Array pilot experiment, having provided identical Ct values to the hundredth of a decimal.

The short time course was conducted on cells that were approximately 90% confluent at the time of dosing and this experimental methodology was implemented so as to provide the highest total RNA yield as possible. This was also the source of total RNA used in the PCR Array pilot experiment. Importantly, C6 cells were dosed when cells were subconfluent.

For Bnip3 gene expression by ddPCR absolute quantification, the 1 hour, 2 hour and 4 hour time points had a 14.74% increase, 9.45% decrease and a 10.35% decrease, respectively (Figure 3) when treated with alpha synuclein. Mean copy numbers for the 1 hour, 2 hour and 4 hour control samples are 6445, 6140 and 5650, respectively. Mean copy numbers for the 1 hour, 2 hour and 4 hour alpha synuclein treated samples are 7395, 5560 and 5065, respectively.
For Hprt1 gene expression by ddPCR absolute quantification, the 1 hour, 2 hour and 4 hour time points had a 10.60% increase, 4.93% decrease and a 1.57% decrease, respectively (Figure 4) when treated with alpha synuclein. Mean copy numbers for the 1 hour, 2 hour and 4 hour control samples are 3138, 3340 and 3593, respectively. Mean copy numbers for the 1 hour, 2 hour and 4 hour alpha synuclein treated samples are 3471, 3175 and 3536, respectively.

Figure 3. Bnip3 mRNA Gene Expression by ddPCR (Short Time Course). C6 Cells were treated with 32 µg/mL alpha synuclein for 1 hour, 2 hours or 4 hours. RNA was extracted, purified and analyzed by ddPCR. Data are represented as mean copy numbers from two cell culture wells that were analyzed in duplicate PCR’s.
Figure 4. Hprt1 mRNA Gene Expression by ddPCR (Short Time Course). C6 Cells were treated with 32 µg/mL alpha synuclein for 1 hour, 2 hours or 4 hours. RNA was extracted, purified and analyzed by ddPCR. Data are represented as mean copy numbers from two cell culture wells that were analyzed in duplicate PCR’s.

*Bnip3 and Hprt1 ddPCR; Long Time Course*

Data from the short time course experiment described in the previous section suggested that *Bnip3* expression decreased when C6 cells were treated with alpha synuclein over a short amount of time, therefore, a longer time course experiment was conducted to assess if *Bnip3* was more substantially downregulated after up to two days post treatment with alpha synuclein. The long time course utilized the same experimental
methodology as described in the previous section, however, the long time course experiment was conducted on cells that were allowed to grow up to 90% confluency by the second day after treatment; this methodology was implemented so that cells would not reach confluency during the course of the experiment. As such, cells were cultured in the presence of alpha synuclein, as opposed to the short time course experiment where subconfluent cells were dosed and harvested no more than four hours after dosing.

For *Bnip3* gene expression by ddPCR absolute quantification, the 12 hour, 24 hour and 48 hour time points had a 3.63% decrease, 16.75% decrease and a 29.26% decrease, respectively (Figure 5) when treated with alpha synuclein. Mean copy numbers for the 12 hour, 24 hour and 48 hour control samples are 3400, 5015, and 89150, respectively. Mean copy numbers for the 12 hour, 24 hour and 48 hour alpha synuclein treated samples are 3276, 4175 and 63065, respectively.

For *Hprt1* gene expression by ddPCR absolute quantification, the 12 hour, 24 hour and 48 hour time points had a 7.71% increase, 3.48% decrease and a 3.74% decrease, respectively (Figure 6) when treated with alpha synuclein. Mean copy numbers for the 12 hour, 24 hour and 48 hour control samples are 3528, 4455 and 33255, respectively. Mean copy numbers for the 12 hour, 24 hour and 48 hour alpha synuclein treated samples are 3800, 4300 and 32010, respectively.

The exploratory PCR array identified one candidate gene as being differentially regulated when C6 cells were treated with a single dose of alpha synuclein. ddPCR analysis confirmed that *Bnip3* was consistently downregulated over time. *Bnip3*’s essential
role in mitophagy offered a promising lead into how MSA pathogenesis may occur and reflects some of the current hypotheses researches have pertaining to synucleinopathy pathogenesis.

Figure 5. Bnip3 mRNA Gene Expression by ddPCR (Long Time Course). C6 Cells were treated with 32 µg/mL alpha synuclein for 12 hours, 24 hours or 48 hours. RNA was extracted, purified and analyzed by ddPCR. Data are represented as mean copy numbers from two cell culture wells that were analyzed in duplicate PCR's.
Figure 6. Hprt1 mRNA Gene Expression by ddPCR (Long Time Course). C6 Cells were treated with 32 µg/mL alpha synuclein for 12 hours, 24 hours or 48 hours. RNA was extracted, purified and analyzed by ddPCR. Data are represented as mean copy numbers from two cell culture wells that were analyzed in duplicate PCR’s.

*mtDNA and gDNA Analysis by ddPCR and qPCR*

The *Bnip3* ddPCR results indicated that the *Bnip3* gene was downregulated over time when C6 cells were treated with alpha synuclein. Considering Bnip3’s essential role in mitophagy, it was hypothesized that alpha synuclein treatment was leading to clearance of mitochondria from C6 cells. Furthermore, it was hypothesized that *Bnip3* was
downregulated in order to preserve the remaining mitochondrial pool lest Bnip3 mediated mitophagy result in cellular death.

In order to test this hypothesis, qPCR was utilized to quantify mitochondrial genome copy numbers through the surrogate measurement of a single mitochondrial encoded gene, Cytochrome C Oxidase subunit 1 (Mtco1). Detection of Mtco1 relies upon intact mitochondrial DNA (mtDNA), therefore, there would be reduced detection of Mtco1 if alpha synuclein treatment was causing mitochondrial clearance. Importantly, mitochondria targeted for degradation would experience rapid degradation of mtDNA and therefore, the template used for PCR.

ddPCR was also used to quantify Beta Actin (Actb) which is a single copy gene that is nuclearly encoded. This experiment was originally conducted to act as a surrogate measurement of cell number. Considering that Beta Actin is a single copy gene, the copy number from ddPCR would directly correlate to the number of cells that contributed towards the weight of DNA in each PCR reaction. Ultimately, this was meant to be used to normalize mtDNA copy number to cell number so that an estimate of mitochondrial genome copy numbers per cell could be ascertained.

Materials and Methods

Cell Culture

C6 rat glioma cells were procured from the ATCC (ACTT© CCL-107™) and cultured in F-12K medium containing 15% horse serum and 2.5% fetal bovine serum. Cells were maintained in T25 flasks and utilized for experiments prior to cells reaching confluency.
Briefly, cells were washed once with 1x HBBS and then trypsinized at 37°C until cells were detached from the cell culture flask. Trypsin was then quenched with complete medium and cell counts were obtained using a hemocytometer. Cells were then plated at a density of 4x10⁴ cells/mL per 24-well culture plate with each well containing 1 mL media. One day after plating, cells were treated with 1 mL of 32 µg/mL alpha synuclein (rPeptide, Cat# S-1001-1) in complete medium for 1 day, 2 days or 4 days in duplicate. Each time point included duplicate control wells that received complete medium and the identical volume of water used for alpha synuclein dosing.

**DNA Extraction and Purification**

Cells were harvested for DNA at the time points above using QIAGen’s QIAamp DNA Mini Kit (Cat# 51304) per the manufacturer’s specifications and with minor modifications. Briefly, cells were lysed in the culture dish with 200 µL Buffer AL. Following a short incubation at RT, 200 µL PBS was pipetted to lysates. Lysates were then collected and stored frozen at -80°C. On the day of purification, cell lysates (400 µL) were thawed at RT and then 20 µL Proteinase K were pipetted to each lysate and mixed by pipetting. Lysates were then incubated at 56°C for 20 minutes in a VorTemp™ 56 with 1000 rpm shaking. Following this, 230 µL 100% EtOH was pipetted to each lysate and incubated for 5 minutes at RT after mixing by pipetting. Lysates were then pipetted to individual columns and centrifuged at 13,000 rpm for 1 minute. The columns were then washed with 500 µL Buffer AW1 and centrifuged as described above. Columns were then washed with 500 µL Buffer AW2 and centrifuged as described above. Following a 1 minute centrifugation to
remove residual wash buffer, DNA was then eluted in 100 µL nuclease-free water. Purified DNA was then aliquoted and stored at -80°C.

**DNA Quantification**

DNA was quantified using the NanoDrop 8000. The mean concentration of duplicate measurements was used for downstream applications.

**2100 BioAnalyzer**

DNA integrity was assessed by following the manufacturer’s specifications for the DNA Laddering Assay.

**qPCR**

A 500 bp gene fragment containing both rat Cytochrome C Oxidase amplicons was synthesized by IDT. Briefly, the DNA concentration was measured by the NanoDrop 8000 and then diluted so as to make single use aliquots that were 1e8 copies/µL. Two primer sets for rat Cytochrome C Oxidase subunit 1 (Mtco1)(Table 1) were designed using Primer Express (Applied Biosystems). Final PCR array conditions per well were 0.1 µM forward and reverse primer, 1x Power SYBR™ Green PCR Master Mix (Applied Biosystems, Cat#4367659) and 50 ng total DNA in a final reaction volume of 50 µL. Once plated and mixed, the PCR plate was centrifuged for 5 minutes at 1500 rpm to drop the liquid down to the bottom of the plate. The following cycling conditions were used: 50°C for 2 minutes, 95°C for 10 minutes and 50 cycles of 95°C for 10 seconds and 58°C for 60 seconds. The qPCR was performed on an Applied Biosystems QuantStudio 7 Real-Time PCR
System using a ramp rate of 1.6°C/second. Data analysis was performed using the QuantStudio 7 Real-Time PCR System software.

**ddPCR**

Each ddPCR reaction consisted of 0.1 µM Beta Actin (Actb) forward and reverse primer (Table 1), 1x QX200™ ddPCR™ EvaGreen Supermix (BioRad, Cat#186-4034) and 50 ng DNA in a final reaction volume of 20 µL. Briefly, the complete ddPCR reaction was pipetted into the QX200 Automated Droplet Generator to generate droplets. The PCR plate was then placed into the C1000 Touch Thermal Cycler with 96-Deep well Reaction Module and cycled as follows: 95°C for 5 minutes and 40 cycles of 95°C for 30 seconds and 58°C for 60 seconds. The reaction products were then cooled to 4°C for 5 minutes, raised to 90°C for 5 minutes and then lowered to 4°C with an infinite hold. Ramp rates of 2°C/second were used. Reaction products were then immediately read on the QX200 Droplet Reader. Each sample was assayed in duplicate.

Table 1. Sequences of Custom Designed Primers for qPCR and ddPCR.

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<tr>
<td></td>
<td>Actb-R1 TTG GCC CAA TAG AGG GGA GG</td>
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Results

Cytochrome C Oxidase Subunit 1 qPCR

Treatment with alpha synuclein led to the downregulation of Bnip3, a gene whose protein product is involved with mitophagy. In order to assess mitochondrial integrity and quantity, two qPCR primer sets were designed against Cytochrome C Oxidase subunit 1, a gene that is encoded by the mitochondrial genome. qPCR of mtDNA is meant to serve as a surrogate measure of mitochondrial copy numbers through measuring mitochondrial DNA copy numbers. C6 rat glioma cells were treated with 32 µg/mL alpha synuclein, in duplicate cell culture wells, for 1 day, 2 days or 4 days. Purified DNA was of acceptable concentration and purity (Figure 7) and no appreciable DNA degradation was detected by the BioAnalyzer (Figure 8). A single standard curve was prepared for use with both primer sets. The highest concentration started at 1e8 copies/well and was serially diluted by 1 log increments to a final concentration of 1e2 copies/well. Primer set one generated a standard curve (Figure 9 and Figure 10) with efficiency of 91.5% and R²=1.000 and primer set two generated a standard curve (Figure 11 and Figure 12) with efficiency of 97.5% and R²= 1.000. No template controls (NTC’s) were not detected for either primer set. Sample quantities are the mean of two samples performed in triplicate wells and both primer sets used the same sample DNA with weight input of 50 ng/well. For Cytochrome C Oxidase subunit 1 primer set 1, 1 day, 2 day and 4 day samples had decreased mitochondrial copy numbers of 39.7%, 35.4% and 30.2% (Figure 13), respectively, when cells were treated with alpha synuclein. Mean copy numbers for the 1 day, 2 day and 4 day control samples are 193653, 467747 and 978220, respectively. Mean copy numbers for the 1
day, 2 day and 4 day alpha synuclein treated samples are 116689, 302326 and 682381, respectively. For Cytochrome C Oxidase primer set 2, 1 day, 2 day and 4 day samples had decreased mitochondrial copy numbers of 24.4%, 35.4% and 39.4% (Figure 14), respectively, when cells were treated with alpha synuclein. Mean copy numbers for the 1 day, 2 day and 4 day control samples are 278893, 470021 and 1007939, respectively. Mean copy numbers for the 1 day, 2 day and 4 day alpha synuclein treated samples are 210943, 303867 and 611068, respectively.

Two important observations were identified with this experiment. First, mitochondrial genome copy numbers greatly increase over time. This result is independent of the weight of total DNA loaded per reaction since DNA weight input per reaction is normalized to 50 ng. Additionally, the increase in mitochondrial genome copy number is independent of treatment with alpha synuclein.

The more critical observation from this experiment is that alpha synuclein treatment led to loss of detection of a considerable portion of mitochondrial genomes at each time point. This is a particularly intriguing result considering that this occurred after only a single dose of alpha synuclein. The importance of this result will be discussed in greater detail later in this manuscript.
<table>
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Figure 7. NanoDrop™ 8000 DNA Concentration and Purity Data. Nanodrop™ 8000 data shows that DNA yield is consistent between time points. DNA is highly pure and suitable for PCR.
Figure 8. 2100 BioAnalyzer DNA Laddering Assay Results. Samples from left to right are: Ladder (L), 1 day Control_1 (1), 1 day Control_2 (2), 2 day Control_1 (3), 2 day Control_2 (4), 4 day Control_1 (5), 4 day Control_2 (6), 1 day 32 µg/mL Alpha Synuclein_1 (7), 1 day 32 µg/mL Alpha Synuclein_2 (8), 2 day 32 µg/mL Alpha Synuclein_1 (9), 2 day 32 µg/mL Alpha Synuclein_2 (10), 4 day 32 µg/mL Alpha Synuclein_1 (11) and 4 day 32 µg/mL Alpha Synuclein_2 (12). The DNA laddering assay did not detect any discernable degradation products.
Figure 9. Mtco1 Primer Set 1 Standard Curve Amplification Plot. The amplification plot of the standard curve generated using synthetic dsDNA shows high specificity and sensitivity. The No Template Controls (NTC’s) were not amplified.
Figure 10. Mtco1 Primer Set 1 Standard Curve Plot. The standard curve plot generated using synthetic dsDNA is linear ($R^2 = 1.000$) and efficient ($E = 91.5\%$).
Figure 11. Mtc01 Primer Set 2 Standard Curve Amplification Plot. The amplification plot of the standard curve generated using synthetic dsDNA shows high specificity and sensitivity. The No Template Controls (NTC’s) were not amplified.
Figure 12. Mtco1 Primer Set 2 Standard Curve Plot. The standard curve plot generated using synthetic dsDNA is linear ($R^2 = 1.000$) and highly efficient ($E = 97.5\%$).
Figure 13. Mtc01 Primer Set 1 Sample Data. C6 Cells were treated with 32 µg/mL alpha synuclein for up to 1 day, 2 days or 4 days. DNA was extracted, purified and analyzed by qPCR. Data are represented as mean copy numbers from two cell culture wells that were analyzed in triplicate PCR's.
Figure 14. Mtc01 Primer Set 2 Sample Data. C6 Cells were treated with 32 µg/mL alpha synuclein for up to 1 day, 2 days or 4 days. DNA was extracted, purified and analyzed by qPCR. Data are represented as mean copy numbers from two cell culture wells that were analyzed in triplicate PCR's.

**Beta Actin ddPCR**

Beta Actin ddPCR was originally conducted so as to offer a secondary means of normalization for the mitochondrial DNA PCR data. The primary means of normalization for PCR is nucleic acid weight input into each reaction well, however, nucleic acid weight input only offers an estimate on how many cells contributed the nucleic acid being analyzed. Analyzing a single copy gene, such as Beta Actin, would theoretically allow for copies of mtDNA to be expressed as copies of mtDNA per cell.
ddPCR was performed as previously described using 50 ng DNA per reaction and by using the same source of DNA that was used for the mitochondrial DNA qPCR. For 1 day, 2 day and 4 day, Beta Actin detection was decreased by 25.9%, 33.8% and 21.2% (Figure 15) respectively, when cells were treated with 32 µg/mL alpha synuclein.

This result indicates that there is consistently reduced detection of Beta Actin DNA. Interestingly, the decreased detection of Beta Actin remains fairly consistent throughout the time course when assessing controls and alpha synuclein treated cells independently. This result implies that minimal degradation of nuclear DNA has occurred and can’t be used for normalization purposes.
Figure 15. Beta Actin ddPCR Sample Data. C6 Cells were treated with 32 µg/mL alpha synuclein for up to 1 day, 2 days or 4 days. DNA was extracted, purified and analyzed by ddPCR. Data are represented as mean copy numbers from two cell culture wells that were analyzed in duplicate PCR’s.

Multiple Sequence Alignment

The amplicon sequences for Mtc01 and Beta Actin amplicons were aligned against each other using T-Coffee software (87). Sequence alignment was conducted to assess if any of the PCR amplicons generated in this study had any sequence homology which may suggest that alpha synuclein is interacting with DNA in a site-specific manner and causing DNA damage that interferes with PCR. The Mtc01 primer set 1 amplicon was found to be 35.3% homologous to Mtc01 primer set 2 amplicon. The Mtc01 primer set 1
The amplicon was found to be 29.3% homologous to Beta Actin amplicon and the Mtco1 primer set 2 amplicon was found to be 36.3% homologous to the Beta Actin amplicon (Figure 16).

Multiple sequence alignment indicates that there is a fair degree of homogeneity in all aligned sequences. This offers supporting information that alpha synuclein may be interacting with DNA in a site specific manner.

![Multiple Sequence Alignment of PCR Amplicons](image)

Figure 16. Multiple Sequence Alignment of PCR Amplicons. Asterisks indicate identical nucleotides from all three amplicons.
Discussion

The hallmark of Multiple System Atrophy is the toxic accumulation of alpha synuclein in oligodendrocytes. Alpha synuclein found within oligodendrocytes exist as monomers, aggregates and fibrils that are predominantly located in the cytoplasm. Of importance is the current dogma that alpha synuclein is predominantly a neuronal protein with minimal to no expression occurring in glial cells or in any other cell type found in the brain. As such, MSA is currently viewed as a neuropathy whereby toxic species of alpha synuclein are secreted into the extracellular space by neurons and endocytosed predominantly by oligodendrocytes, presumably due to their proximity to neurons since other glial cells uptake alpha synuclein as well.

In 2007, the Ide laboratory published Affymetrix gene expression data from a MSA cohort study. This study was fortunate to have been conducted on pons tissue donated by eight individuals who were diagnosed with MSA and five age matched individuals without any known neuropathies. The resulting gene expression data that was obtained was one of the first data sets of its kind and proved to stand the test of time having broadly identified key features of MSA. Despite the success of that study, there are several caveats to consider. The first is that the gene expression data set was obtained from whole pons tissue and as such, there were numerous cell types within that tissue. The resulting gene expression changes are not likely representative of each cell type's transcriptome, but rather the mean gene expression change of the entire pons cell population as a whole. Secondly, it is well known that mRNA is not stable and degradation of mRNA can begin
occurring minutes after death. The earliest post-mortem collection of patient brain used was 8 hours with the latest post-mortem collection at just over 32 hours.

Despite the caveats stated above, at the time, there was currently no better way to conduct gene expression analysis on those with probable MSA, or anyone with a neurological disease for that matter. To this end, the development of in vitro cell culture or animal model systems are important towards elucidating disease pathogenesis since these systems can offer end point analysis that real patients cannot realistically offer. In addition, no patient can provide samples that show disease pathogenesis; at the time of donation most patients have died from their disease and well after their disease has fully progressed.

The main aim of my pilot study was to assess the use of the C6 rat glioma cell culture system that the Ide laboratory had previously developed. This would be accomplished by recapitulating gene expression trends from the MSA Affymetrix gene expression data sets and then to further study how gene products could be involved in alpha synuclein mediated pathogenesis.

The C6 rat glioma cell line has multiple phenotypes resembling oligodendrocytic and astrocytic lineages. The C6 rat glioma cell line is highly mutagenized. For example, Gao et al sequenced the C6 rat mitochondrial genome and found 229 indels and SNPs which accounts for 1.40% of the total mitochondrial genome being mutated (88). Both of these features are beneficial in a MSA cell culture system since MSA affects multiple glial cell types and a more genetically heterogenous mitochondrial genome pool may be more
comparable to that observed in older individuals. The main benefit of the C6 rat glioma cell culture system, however, is that a single high concentration dose of exogenous alpha synuclein can be applied to glial cells, thus allowing for RT-PCR analysis on how a single dose of alpha synuclein may affect the C6 transcriptome. Ultimately, my study sought to identify early events that may lead to MSA pathogenesis.

The C6 rat glioma cell line is an immortalized cell line that was cloned from a rat glial tumor that had been induced by treatment with N-nitrosomethylurea which accounts for the large number of mutations that are present in this cell line. Preliminary data from the Ide lab suggested that baseline gene expression and mitochondrial genome copy numbers greatly differed depending on the passage number of C6 cells utilized for experiments. Given this characteristic of the C6 rat glioma cells, experiments were not repeated. As such, statistics was not conducted on any data summarized in this pilot study.

The oxidative stress PCR array was chosen as a starting point for my study due to the well-established fact that oxidative stress is a component of the synucleinopathies. Though it has not been ascertained as to if oxidative stress is a prerequisite for synucleinopathy pathogenesis, alpha synuclein itself is well described to bind to mitochondrial membranes, and at high concentrations, can even form mitochondrial derived vesicles. This feature of alpha synuclein in of itself warranted an exploratory PCR study into how alpha synuclein may affect genes related to oxidative stress pathways as many of these proteins are intrinsically tied to mitochondrial health, homeostasis and
function. Additionally, several genes that were present in the MSA Affymetrix gene expression data set are included in the rat oxidative stress PCR array.

A Ct value cutoff of 25 was chosen so as to not include any low abundant mRNA species as it was deemed less likely that these would provide meaningful options for future experiments. Additionally, low abundant targets have a higher probability of producing larger fold changes but these fold changes may not be meaningful due to low copy numbers per cell and due to the experimental result approaching the sensitivity limit for PCR. A fold change of 2.0 was chosen as this is industry standard for potentially meaningful changes in gene expression.

*BNip3* was the only gene that met these criteria and importantly, this gene had statistically significant fold changes in the MSA Affymetrix gene expression data set that was produced in 2007 by the Ide laboratory. Therefore, total RNA from two time course experiments was used to assess how a single 32 µg/mL alpha synuclein dose affected *BNip3* expression over the course of up to two days post treatment. ddPCR allows for the absolute quantification of nucleic acid without the necessity of using a standard curve. It is also highly sensitive, even when compared to other PCR techniques. Also, unlike relative gene expression by RT-qPCR, it does not require the use of a house keeping gene for normalization. Regardless, Hprt1 was included in the ddPCR analysis so as to offer a secondary means to assess the quantity, quality and purity of RNA used in these experiments. The primary means to assess RNA quantity, quality and purity was the Nanodrop™ and the 2100 BioAnalyzer, and these instruments provided data that the RNA was highly pure and intact. RNA quantity was comparable between samples for the short
time course which was expected due to the short nature of the time course. For the longer
time course, total RNA yield increased over time which was expected since cells were
plated at a density that allowed them to grow to confluency in the presence of alpha
synuclein. RNA from both the short and long time course experiments was transcribed at
the same time and cDNA was analyzed on the same ddPCR plate so as to minimize inter
assay variability.

*Hprt1* was not found to be consistently expressed at each individual time point
assessed, with the exception of the 48 hour time point. The ATCC notes that the C6 cell
line upregulates the S100 family members nearly 10-fold when cells approach confluency
and it is probable that *Hprt1* expression follows a similar trend based on data presented in
this study. Consistent expression of *Hprt1* was anticipated and shows that RNA quality,
quantity and purity were adequate for ddPCR. More importantly, the result demonstrated
that treatment with alpha synuclein did not affect *Hprt1* gene expression. The result also
demonstrated that RNA was loaded equally for ddPCR.

*Bnip3* gene expression marginally increased after one hour of treatment with alpha
synuclein, however, expression was consistently decreased at every other time point
assessed. Similar to *Hprt1* expression, *Bnip3* expression also greatly increased at the 48
hour time point and was independent of treatment with alpha synuclein. Though the
ATCC does not characterize expression of *Bnip3* in C6 rat glioma cells, this gene too is
likely abundantly expressed as cells approach confluency. The largest decrease in *Bnip3*
expression of 29.26% occurred at the 48 hour time point which correlates to an
exceptional reduction in mRNA transcripts.
Bnip3 is a protein that is involved with numerous cell death processes. It is implicated in apoptosis as well as mitophagy and as such, the expression of Bnip3 is tightly regulated and activity of Bnip3 protein is regulated by localization and post translation modification. Interestingly, Bnip3 expression is exceptionally increased in some tumors and silenced by methylation in others. In some tumors, Bnip3 can be highly expressed yet little to no protein is translated. In the C6 rat glioma cell line, it is evident that Bnip3 expression significantly increases as the cells approach confluency. This also occurred with Hprt1 gene expression and it’s perhaps important to note that the source of the cells, the ATCC, makes mention that certain genes are upregulated 10-fold as cells approach confluency. A literature search yielded one paper that assessed Bnip3 protein abundance levels by western blot, however, no other sources of literature were found to have provided comparable data in the C6 cell line (89). Western blots were attempted in the Ide lab but no Bnip3 signal was ever detected.

Annamarie Valenti, a previous student in the Ide lab, conducted analysis on cell viability and cell number, using the same C6 cell culture model (90). She found no statistical differences in cell viability for cells treated with up to 64 µg/mL alpha synuclein vs. control. Interestingly, for the 2 day treatment, cell numbers were comparable whereas cell numbers were substantially reduced for the 4 day, 32 µg/mL alpha synuclein treatment; cell numbers were reduced by approximately 60%.

When considering previous data generated in the Ide laboratory, it is clear that the substantial reduction in cell number from treatment with 32 µg/mL alpha synuclein is not a result of cell death. As such, though the Bnip3 result is considered pilot data, the
reduction in total number of mRNA transcripts was intriguing enough to warrant a continuation of this study. It was surmised that a decrease in Bnip3 expression was a response due to the aberrant activation of mitophagy. Alternatively, it was deemed possible that other mechanisms that resulted in a clearance of mitochondria could result in a feedback loop that decreased expression of mitophagy related genes, including Bnip3, regardless of whether mitophagy was occurring or not. With this, the primary mechanism of consideration was that alpha synuclein mediated clearance of mitochondria was occurring.

Two qPCR assays were designed in order to investigate the possibility of mitophagy occurring when cells were treated with a single dose of alpha synuclein. Two primer sets were designed against the rat mitochondrial gene, Cytochrome C Oxidase subunit 1 with the intention of using qPCR as a surrogate measure of mitochondrial numbers. Both primer sets were designed so that amplicons were directly flanking each other with the intention of detecting any differential copy numbers that might result. This design was meant to control for other mechanisms of mitochondrial DNA degradation, such as activation of endonucleases (91). Both qPCR results were comparable to each other and both results indicate that there is a consistent decrease in the detection of mtDNA copy numbers.

A third PCR assay was designed in order to ensure that total DNA was input at the same weight and was originally meant to qualify the mitochondrial qPCR result since both experiments used the same source of total DNA. A primer set was designed against Beta Actin so as to assess copy numbers detected from this single copy gene. This
methodology is often used as a surrogate measure of cell number and can provide more accurate cell copy numbers than relying on DNA concentration alone. Interestingly, detection of the Beta Actin gene was also decreased at two of the three time points assessed.

The DNA concentration, purity and integrity was comparable for DNA harvested at individual time points and minimal differences in these samples do not account for either of the mitochondrial or Beta Actin PCR results. Furthermore, there was no statistical difference in DNA yields at each time point. Substantial differences in mitochondrial DNA copy numbers would not offer an appreciable difference in DNA concentration, however, the copy number differences in Beta Actin should have been apparent in the DNA concentration. The fact that there is no appreciable difference in DNA concentrations implies that cells are viable, yet DNA harvested from these cells has likely been damaged. DNA that has been damaged or nicked will not be detectable by PCR. Though the 2100 Bioanalyzer did not detect substantial differences in DNA integrity, it is likely that any damage is beyond the sensitivity of the 2100 Bioanalyzer to detect. Other labs report that alpha synuclein can bind to DNA in a site specific manner (92, 93) and can also cause DNA damage in the form of single strand breaks (94).

The main hallmark of alpha synuclein is that it is well known to bind to membranes, and it has a propensity for binding to mitochondrial membranes in particular. Prior studies by numerous labs have observed this feature of alpha synuclein, however, no literature searches yielded information on how this may affect mitochondrial genomes. As such, this may be the first PCR study to demonstrate that a single dose of
alpha synuclein can decrease mitochondrial genome copy numbers and also have an effect on nuclear genome integrity. Lastly, recurrent secretion of alpha synuclein by neurons may destabilize the local cellular transcriptome in MSA patients.

**Conclusion**

In conclusion, this study confirms the downregulation of the mitophagy related gene, *Bnip3*, that was observed in the human MSA Affymetrix study that was conducted by the Ide laboratory in 2007. Furthermore, this study further qualifies the use of the C6 rat glioma cell culture model in *in vitro* MSA research. Having confirmed downregulation of the mitophagy related *Bnip3* gene upon treatment with a single exogenous dose of alpha synuclein, it was then shown that detection of the mitochondrial encoded Cytochrome C Oxidase subunit 1 gene and the nuclearly encoded Beta Actin gene was decreased when assessed by qPCR and ddPCR. Previous studies conducted in the Ide laboratory demonstrate that there is no effect on cell viability when C6 rat glioma cells are treated with 32 µg/mL alpha synuclein. Lastly, it is shown that both amplicon sequences for Mtco1 and the Beta Actin amplicon are homologous to each other to a moderate degree.

The results of this study demonstrate that the mitophagy related gene, *Bnip3*, is downregulated in response to alpha synuclein treatment. The detection of mitochondrial genome copy numbers is decreased when assessed by qPCR which insinuates that mitochondrial clearance occurs when cells are exposed to a single dose of alpha synuclein. Alternatively, alpha synuclein has been shown to bind to DNA, causing DNA
damage that may also explain the decreased detection of mitochondrial genomes by qPCR. Additionally, detection of the Beta Actin gene is decreased and in conjunction with previous studies demonstrating no loss of cell viability, further suggests that transient DNA damage has occurred in a site specific manner; widespread genomic insults would result in loss of cell viability and proliferation. Ultimately, this study suggests that a single treatment of alpha synuclein can affect the integrity of the mitochondrial genome pool which holds profound implications for those with MSA and other synucleinopathies.

Future work will need to identify why there was decreased copy numbers for mitochondrial genomes and Beta Actin as this study did not identify why there was decreased copy numbers. Furthermore, this study did not identify if Bnip3 protein contributes to mitophagy related processes in conjunction with alpha synuclein treatment. Given the large body of scientific research into synucleinopathies, it is possible that alpha synuclein itself perturbs mitochondrial homeostasis and function which culminates in a feedback mechanism to decrease mitophagy related processes in order to preserve the remaining mitochondrial pool.
REFERENCES


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