Exogenous Alpha-Synuclein Induces Cell Death Related Proteins in C6 Oligodendrocyte-Like Cells Corresponding to Protein Expression Observed in Multiple System Atrophy

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EXOGENOUS ALPHA-SYNUCLEIN INDUCES CELL DEATH RELATED PROTEINS IN C6 OLIGODENDROCYTE-LIKE CELLS CORRESPONDING TO PROTEIN EXPRESSION OBSERVED IN MULTIPLE SYSTEM ATROPHY

by

Derrick Samuel Hilton

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Doctor of Philosophy
Department of Biological Sciences
Advisor: Charles Ide, Ph.D.

Western Michigan University
Kalamazoo, Michigan
August 2012
THE GRADUATE COLLEGE
WESTERN MICHIGAN UNIVERSITY
KALAMAZOO, MICHIGAN

Date June 14, 2012

WE HEREBY APPROVE THE DISSERTATION SUBMITTED BY

Derrick Samuel Hilton

ENTITLED Exogenous Alpha-Synuclein Induces Cell Death Related Proteins in C6 Oligodendrocyte-like Cells Corresponding to Protein Expression Observed in Multiple System Atrophy

AS PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF Doctor of Philosophy

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EXOGENOUS ALPHA-SYNUCLEIN INDUCES CELL DEATH RELATED PROTEINS IN C6 OLIGODENDROCYTE-LIKE CELLS CORRESPONDING TO PROTEIN EXPRESSION OBSERVED IN MULTIPLE SYSTEM ATROPHY

Derrick Samuel Hilton, Ph.D.

Western Michigan University, 2012

Multiple System Atrophy (MSA) consists of three disorders; Autonomic Dysfunction, Cerebellar Ataxia, and Parkinsonism. In MSA, the protein Alpha-Synuclein (SNCA) appears in the central nervous system as misfolded protein aggregates primarily in oligodendrocytes. This dissertation reports the results from studies examining the effect of exogenous SNCA has on a cell model: C6 oligodendrocyte-like cells. Treated cells were evaluated using western blot and DNA microarray. In addition the expression of proteins was evaluated using immunocytochemistry in MSA patient tissue.

C6 cells were shown to take up SNCA when added to the media. SNCA also underwent a truncation when taken up by C6 cells. SNCA induced cell death in C6 cells in a dose-dependent manner. SNCA also induced changes in expression of Caspase 3 (CASP3), a marker for apoptosis.

In C6 cells treated with SNCA, two apoptosis-related genes, Programmed Cell Death 4 (PDCD4) and Pancreatic Derived Factor (FAM3B) showed changes in protein expression. These changes appeared to
correspond to expression of CASP3. In MSA patients, FAM3B, PDCD4 and CASP3 appeared to be expressed in fibers containing myelin basic protein, a marker for oligodendrocytes, suggesting a relationship with the disease.

Cells treated with SNCA undergo changes in gene expression. In cells treated with SNCA, 3 genes were upregulated and 20 genes were downregulated. Many of these genes were related to microtubules, apoptosis and/or the AKT/PI3K/mTOR pathway.

One gene of interest, Hook Homolog 3 (HOOK3), was evaluated via western blot. After treatment with SNCA, HOOK3 protein showed a decrease in expression that confirmed the decrease in gene expression of HOOK3 observed in the DNA microarray. In MSA patients, HOOK3 co-localized with SNCA in the granule cell layer of control patients and decreased in granule cell layer of MSA patients. Interestingly, as HOOK3 and SNCA decreased in the granule cell layer, HOOK3 co-localized with SNCA in the white matter tracks of MSA patients.

Overall these results confirm that C6 cells treated with exogenous SNCA function as a model for MSA. The treatment of SNCA also appeared to influence proteins related to the AKT/PI3K/mTOR pathway, potentially regulating apoptosis.
ACKNOWLEDGMENTS

Five years ago, I did not know what Multiple System Atrophy was. After completing the work contained in this dissertation I have a greater appreciation for what it means to be human. I see the courage those afflicted with this disease and their caregivers, and I would like to thank them for being examples to us all of what it means to be heroic. Especially when completing this dissertation, I would think of Bob and Sue Summers to keep me motivated and working hard. Their lives have touched me, and I will carry that all the days of my life.

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Derrick Samuel Hilton
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CHAPTER I
AN INTRODUCTION TO MULTIPLE SYSTEM ATROPHY

Multiple System Atrophy (MSA) is a condition, similar to Parkinson’s Disease (PD), but consists of three disorders; autonomic dysfunction, cerebellar ataxia, and parkinsonism. MSA compromises functions such as balance, blood pressure, breathing, swallowing, and fine motor control. Aggregates of the protein α-Synuclein (SNCA, NACP, non A4 component of amyloid precursor) appear in the central nervous system (CNS) as cellular inclusions in MSA (Duda et al, 2000), as well as in other diseases, (e.g., PD, and Alzheimer’s disease with Lewy Bodies) referred to as synucleinopathies (Ueda et al. 1993, Spillantini et al. 1995, Polymeropoulos et al. 1997, Langerveld et al. 2007). In MSA, synuclein aggregates appear primarily in oligodendrocytes as glial cytoplasmic inclusions (GCI) (Tu et al. 1998). Both myelinating oligodendrocytes and associated neurons appear to die in MSA, but the role of SNCA in MSA is unknown.

Inclusion bodies in the brain of Alzheimer’s patients were first identified by isolating an unknown protein and generating an expression library. SNCA was identified as the non-Amyloid beta fragment (non-amyloid component, NAC) of the Amyloid protein found in Alzheimer’s. (Ueda et al. 1993) This fragment is thought to be the cause of aggregation in Lewy bodies (Iwia, et al., 1995).
Due to the occurrence of Lewy body aggregation in both Alzheimer’s and Parkinson’s disease, the DNA sequence of the NAC fragment (SNCA) was examined in PD patients. SNCA was found to be located on the 4q21 chromosome of PD patients (Ueda et al. 1993, Spillantini et al. 1995). Immuno-staining of SNCA showed accumulation in the Lewy bodies of PD patients (Spillantini et al. 1997). Identification of SNCA allowed for the comparison of DNA in families with PD, resulting in identification of three point and two duplication mutations in the SNCA gene. The first mutant A53T was shown to be an autosomal dominant point mutation occurring in families with a history of PD (Polymeropoulos et al. 1997). Following this discovery, two more point mutations, E46K and A30P were also identified to be heritable mutations in PD (Vaughan et al. 1998, Kruguer et al. 2001). In addition to point mutations, duplication of the normal SNCA gene in families with PD was also shown as a cause for Lewy bodies (Ibanez et al., 2004).

The SNCA protein contains 140 amino acids and has similar functional and structural homology to 14-3-3 class proteins. SNCA will also bind with 14-3-3 class proteins, BAD and Protein Kinase C proteins (Ostrerova et al, 1999). SNCA acts as a chaperone with synphilin 1 in the exocytosis of vesicles of the presynaptic region of axons (Shirakashi et al. 2005), and in the recycling of vesicles involved
in dopamine neurotransmitter release by binding to fatty acids and regulating phospholipase D2 (Lotharius and Brundin 2002). SNCA also binds with actin, reducing the rate of polymerization and increasing depolymerization (Sousa et al. 2009). Under normal conditions, SNCA is bound by lysosomal-associated membrane protein 2 (LAMP2a) and heat shock conjugate 70 (HSC70) for degradation by the lysosome (Cuervo 2010). The amino acid substitution that occurs in A53T results in a reduction in the transport of SNCA into the lysosome (Cuervo et al. 2004).

SNCA has been shown to induce cell death in a variety of experiments. Cultures of rat neuronal hippocampal progenitor (H19-7) cells show increases in cell death as treatment dose of SNCA increases (Sung et al. 2001). In a co-culture, SH-SY5Y cells transfected with the adeno/SNCA gene were shown to produce SNCA protein and supply rat primary cortical neurons and mouse cortical neuronal stem cells with SNCA through endocytosis. This transfer resulted in apoptosis via induction of Caspase 3 in cortical neurons (Desplats et al. 2009). Additionally in another study, cultured SH-SY5Y cells with overexpressed A53T mutant, or wild type SNCA, showed localization of A53T and SNCA WT with the mitochondrial membrane, releasing Cytochrome C, leading to oxidative stress-induced apoptosis (Parihar et al. 2008). Glial cells transfected with a gene overexpressing SNCA
were shown to increase apoptosis due to oxidative stress (Stefanova et al, 2001).

In other experiments the SNCA gene was transfected into oligodendrocytes to produce neuropathology (Shults et al., 2005, Tsuboi et al., 2005). Cultured oligodendrocytes transfected with SNCA and another gene, p25α, that induces SNCA aggregation, resulted in Caspase 3-induced apoptosis (Kragh et al, 2009).

In MSA, significant apoptosis has been shown to occur in oligodendrocytes, but not neurons (Probst-Cousin et al., 1998). This is different from PD where apoptosis primarily occurs in neurons. Oligodendrocytes may lack the ability to deal with the quantity of SNCA a neuron normally contains due to its role in transport of synaptic vesicles to distant terminals. In MSA, neurons and/or other cell types might make misfolded SNCA, which is then taken up by the oligodendrocytes resulting in cell death. Oligodendrocytes might also be modifying SNCA in an inappropriate manner resulting in aggregation of the protein.

Thus, the objectives of the present study were first to determine if oligodendrocyte-like cells can take up exogenous SNCA in vitro; second, to determine if uptake induces apoptosis and related gene expression. C6 cells are a rat glial cell line originally isolated from rat gliomas that expressed S100 protein. Depending on growing
conditions, these cells can be differentiated into astrocyte or oligodendrocytes-like cells. When treated with retinoic acid, C6 cells differentiate into oligodendrocyte-like cells that express myelin proteolipid protein (Lopez-Barahona et al. 1993, Zhang et al. 2001). Thus, C6 oligodendrocyte-like cells serve as a model system to study the effects SNCA has on Oligodendrocytes.

An additional objective of this study was to confirm the presence of proteins in MSA patients that were shown to change expression in C6 oligodendrocytes due to treatment with SNCA. To accomplish this I used paraffin embedded sections of cerebellum from patients that have passed on due to MSA. These tissue sections were analyzed using immunocytochemistry methods.

Chapter 2 examines if C6 cells uptake exogenous SNCA, how uptake is influenced by SNCA concentration, and if SNCA induces changes in a protein found in GCIs in MSA. Cells were treated with wild-type SNCA or a familial mutant SNCA A53T, to determine if C6 cells would take up both types of exogenous SNCA. Next I evaluated how the concentration of SNCA in media influenced the quantity of SNCA taken up by the cells. To confirm that SNCA induced changes in cell physiology, a protein (Heat shock conjugate 70, HSC70) known to occur with SNCA in GCIs was measured via western blot analysis.
Chapter 3 describes how SNCA induces cell death and apoptosis in C6 oligodendrocytes. Cells were treated with increasing concentrations over 2 and 4 days. After treatment, viability of the cells was tested using a trypan blue assay. To measure apoptosis, expression of Caspase 3 (CASP3), the effector protein in apoptosis, was measured using western blot analysis.

Chapter 4 describes how DNA microarray analysis was used to determine global gene expression patterns influenced by SNCA treatment of C6 cells. This experiment created a “roadmap” of gene expression changes related to SNCA uptake in C6 oligodendrocytes. Thus, C6 cells were treated at two concentrations of SNCA for 4 days. After treatment mRNA extracted from cells was hybridized to Affymetrix DNA microarrays and scanned. The results from the two SNCA treatments were compared to results from control cells. Messenger RNA from one gene of interest, Hook Homolog 3 (HOOK3), was regulated by SNCA treatment in the Affymetrix study. To confirm SNCA-induced changes in expression, HOOK3 protein was measured using western blot analysis.

Chapter 5 examines two genes of interest related to apoptosis. Programed Cell Death 4 (PDCD4) and Pancreatic-derived Factor (PANDER, FAM3B) were shown to occur in two separate DNA microarray studies of MSA patients (Ide et al., unpublished data,
Langerveld et al., 2007). Using western blot analysis I examined how the concentration of SNCA affected expression of these two proteins in C6 cells. To confirm the involvement of FAM3B in MSA, I used Immunocytochemistry (ICC) methods to stain for the presence of FAM3B with Myelin Basic Protein (MBP, a marker for oligodendrocytes) in the white matter tracts of MSA patients.

Chapter 6 examines the expression of HOOK3 and SNCA in control and MSA patient cerebellum. As discussed in chapter 3, HOOK3 was shown to be influenced by SNCA treatment of C6 cells. HOOK3, a member of the Hook family, binds to microtubules and golgi (Walenta et al., 2001). SNCA has been known to interact with Microtubule Associated Protein Tau (Spillantini et al., 1999) and Beta Tubulin 3 (Nakayma et al., 2009). As such, using ICC methods, I stained the granule cell layer and white matter tracts for HOOK3 and SNCA in MSA and control patients. Samples were photographed and the area of HOOK3 and SNCA contained in the granule cell layer was measured.

In conclusion, chapter 7 examines how C6 cells function as a model for MSA. In addition I summarize how many of the genes evaluated in this study have interactions with the pathways AKT and PKC. Using this data I formulate a theory of how SNCA influences apoptosis in Oligodendrocyte like cells. Understanding how apoptosis
is influenced by SNCA oligodendrocytes could be useful in preventing cell death of oligodendrocytes and associated neurons in MSA.
CHAPTER II

ALPHA-SYNUCLEIN UPTAKE BY C6 OLIGODENDROCYTE-LIKE CELLS INDUCES CHANGES IN EXPRESSION OF HEATSHOCK CONJUGATE 70, A PROTEIN KNOWN TO ACCUMULATE IN MULTIPLE SYSTEM ATROPHY

Introduction

As stated in chapter 1, MSA is a member of diseases referred to as synucleinopathies (Ueda et al. 1993, Spillantini et al. 1995, Polymeropoulos et al. 1997, Langerveld et al. 2007). Both oligodendrocytes and associated neurons die in MSA, but the role of SNCA is unknown. In MSA, SNCA aggregates appear primarily in oligodendrocytes as GCI (Tu et al., 1998). To date oligodendrocytes have not been shown to produce SNCA, suggesting that oligodendrocytes take up SNCA from an exogenous source.

Previous studies have shown that both neurons and glia have the capacity to take up exogenous SNCA through endocytosis (Sung et al. 2001, Ahn et al. 2006, Liu et al. 2007, Liu et al. 2009). In a co-culture experiment, neurons took up misfolded SNCA provided by other neurons overexpressing SNCA through endocytosis, resulting in Lewy body formation (Desplantes et al. 2009). Interestingly in co-culture, astrocytes (glial cells sharing a common lineage with oligodendrocytes) were shown to take up SNCA secreted by neurons, resulting in an inflammatory response by the astrocytes (Lee et al. 2010). Microglia
were also shown to take up SNCA through receptor-mediated endocytosis (Lee et al. 2008).

To date, no studies have examined if a PD familial variant of SNCA, such as A53T, is taken up by oligodendrocytes. However, A53T and other synuclein fibrils have been shown in PD models to form inclusion bodies and induce apoptosis (Parihar et al. 2008, Kragh et al. 2009). Thus, my first objective was to determine if C6 oligodendrocyte-like cells take up SNCA Wild Type (WT) and/or a PD familial mutant, SNCA A53T. Protein uptake was measured using western blot analysis of protein from cells treated with two concentrations of SNCA WT or A53T for 4 days.

My second objective was to determine if the amount of SNCA provided in media results in a change in the quantity of SNCA taken up by C6 cells. To evaluate protein uptake, cells were treated using 5 increasing concentrations of SNCA for 2 and 4 days. Following treatment, protein was extracted from C6 cells and analyzed for SNCA using western blot.

As discussed above, other studies have shown that glial cells can take up SNCA through endocytosis. SNCA taken up by C6 cells should induce changes in expression of a range of proteins inside the cell. If SNCA is taken up in C6 cells in a dose-dependent manner, then
proteins known to associate with SNCA should also change in a dose-dependent manner.

To confirm that SNCA has an effect on C6 cells, a protein known to act as a chaperone for SNCA and occurring in GCIs of MSA was evaluated for changes in expression. Heat Shock Cognate 70 (HSC70) is a constitutively expressed heat shock protein found to occur in GCIs of MSA patients (Kawamoto et al. 2007). It binds with LAMP2A to promote chaperone-mediated autophagy of SNCA (Cuervo et al. 2004). Downregulation of the HSC70 gene expression has also been correlated with SNCA aggregates in the pons of MSA patients (Langerfield et al. 2007). Thus HSC70 protein expression was evaluated using western blot analysis of protein from C6 cells treated for 2 and 4 days with 5 increasing concentrations of SNCA.

Methods
Preparation of C6 oligodendrocyte cultures
C6 rat glioblastoma cells obtained from ATCC (CCL-107™) were cultured in RPMI 1640 media (GIBCO©). Synuclein stocks were created from lyophilized protein (Sigma-Aldrich©). To induce an oligodendrocyte-like phenotype, 5 µl of retinoic acid (RA) from a stock solution (1 µg RA / 1ml 95% EtOH) was added to flasks for each ml of media (retinoic acid induces Myelin Proteolipid Protein in C6 cells.
resulting in oligodendrocyte-like cells (Lopez-Barahona et al. 1993, Zhang et al. 2001).

**Confirmation of Oligodenrocyte-like cells**

To confirm retinoic acid inducement of the oligodendrocyte marker Myelin Basic Protein (MBP), C6 cells were treated with RA in culture for 4 days and examined using Immunocytochemistry methods. Briefly, following treatment cells were washed with Ca, Mg-free tyrodes solution, and fixed for 4 hours in Bouin’s solution. After fixation, cells were washed in 70% EtOH to remove the Bouin’s solution. Cells were then washed 3 times with Tris-buffered saline, TBS (pH 7.2-7.4) and treated with 3% normal mouse serum (NMS) in TBS for 1 hour to inhibit cross reactivity of the secondary antibody. After blocking, cells were treated overnight with 1:500 Rabbit anti-MBP (Sigma-Aldrich) antibody. The following day, cells were washed 3 times with TBS and treated with biotinylated mouse anti-rabbit secondary (Chemicon) at a concentration of 1:200 for 30 minutes. Next cells were washed 3 times in TBS and labeled using the Vectastain ABC-AP kit (Vector©) with Vector© Red substrate per the manufacturer’s protocol.

Following staining, cells were coversliped and 4 fields (40x objective, 352 µM x 276 µM) per sample were photographed using a
Spot Cooled Color Digital Camera (Diagnostic Instruments, Inc, Sterling Heights, Mi) attached to a Nikon Eclipse E600 Microscope. The area of staining in cells was measured using ImageJ (nih.gov). Stained area was selected using the color deconvolution macro in ImageJ. The selection was then converted to a pure black and white image using the threshold tool. Total area stained was then measured. The same process was used to select for total cellular area. The percent of area stained in cells was then analyzed using a t-test from the statistical program Statview (version 5, from SAS institute). Analysis revealed that Retinoic Acid was shown to induce increased expression of MBP (Figure 1).
Figure 1. MBP staining in C6 cells treated with Retinoic Acid. A. RA-treated Cells B. Non-treated cells. C. No primary antibody. Cells treated with retinoic acid had a significantly higher expression of MBP protein. RA treated cells had staining for MBP in approximately 66 percent of the total cellular area in culture. RA- cells had MBP staining in approximately 34 percent of the total cellular area (p=0.027, standard bar = 20 µm, error bars = 1 S.E.)

Western Blot Analysis

Cells were extracted in RIPA buffer with protease inhibitor cocktail (Sigma ©). The concentration of extracted protein was calculated using a Bradford assay. Ten micrograms of protein was run with 4-20% NuPAGE® Tris glycine gels with NuPAGE® SDS running buffer per the manufacturers protocol (Invitrogen©). Briefly using the Bradford assay, samples were loaded into microtubes with 2 µl of reducing agent and 5 µl of 4x SDS glycine running buffer. Finally the
total volume in the micro tube was brought to 15 µl using dH₂O. After electrophoresis, protein was transferred for 2 hours to using NuPAGE® Tris-glycine transfer buffer onto nitrocellulose blot paper (Invitrogen©). Protein was resolved using a Western Breeze© kit per manufacturers protocol, with rabbit anti-SNCA antibody (Sigma ©) at 1:1000, or goat anti-HSC70 (Santa Cruz ©) at 1:500.

Statistics and Image Analysis

The nitrocellulose blots were scanned and saved in JPEG format. Band intensity was analyzed using ImageJ (nih.gov) gel measuring function. Statistics for the average area were calculated using Statview version 5 software, from SAS institute, using an ANOVA comparison.

Results

C6 cells take up extracellular SNCA

To determine if cells would take up SNCA, C6 cells were treated with SNCA WT or SNCA mutant A53T for 4 days. Using western blot analysis, protein extracted from C6 cells showed that both SNCA WT and A53T protein were taken up by C6 cells (Figure 2). Additionally truncated forms of the proteins were found for both types of SNCA after uptake. The stock bands for SNCA WT and A53T occur at an apparent molecular weight of 17 kDa. A second band found in C6 cell
extracts, was detected at 15 kDa below the typical band. The uptake of SNCA WT and A53T in C6 cells were not significantly different from each other (Figure 2).

Figure 2. Western Blot anti-SNCA A. Synuclein standard 0.325 µg, B. Untreated Cells, C. 2 µg/ml SNCA WT, D. 12 µg/ml SNCA WT, E. 2 µg/ml A53T, F. 12 µg/ml A53T. Additional lanes are SeeBlue® Plus2 protein standard for Myoglobin Red, Lysozyme and Aprotinin. SNCA and A53T bands occur at an apparent molecular weight of 17 kDa, with a second band detected at 15 kDa below the typical band (A). Uptake of wild type versus mutant (A53T) SNCA by C6 cells was not significantly different for both bands (p=0.101) (N=4).

The next objective was to determine if SNCA uptake is dependent on the concentration of SNCA in media and/or the duration of exposure. C6 cells were treated with SNCA at increasing concentrations for 2 and 4 day replicates to determine if dose and time had an influence on the uptake of the protein. For both the 2 and 4 day treatments, SNCA uptake was significantly higher in the 6, 8.5 and
11 µg/ml treatments groups, compared to the control, 1.5 and 3 µg/ml treatment groups (Figure 3).

![SNCA uptake after 2 days](image1)

![SNCA uptake after 4 days](image2)

Figure 3. Western Blot of C6 cells treated with SNCA for 2 or 4 days. Untreated C6 cells, 1.5 µg/ml, 3 µg/ml, 6 µg/ml, 8.5 µg/ml and 11 µg/ml SNCA). Concentrations above 6ug/ml were statistically different from control, 1ug and 3ug/ml treatments of SNCA for both 2 and 4 days. N=4, Error bars = 1 S.E.

Expression of HSC70 in C6 cells changes with SNCA treatment concentration

To evaluate if SNCA induces changes in expression of proteins known to occur in GCIs, HSC70 expression was evaluated using western blot (Kawamoto et al., 2007). After 2 days of treatment, cells treated with 6 µg/ml of SNCA had significantly higher expression of HSC70 than control cells, 1.5 and 11 µg/ml treatments (Figure 4).
Cells treated with 11 µg/ml additionally had a lower expression than the 3 and 8.5 µg/ml treatment groups. After 4 days, cells treated with 3 µg/ml SNCA had significantly higher levels of HSC70 than the control cells, 6, 8.5 and 11 µg/ml treatment groups.
Figure 4. HSC70 expression after treatment with SNCA. C6 oligodendrocyte-like cells were treated with SNCA for either 2 or 4 days. Following treatment, western blot analysis of protein extracted from the cells showed an increase in HSC70 expression, followed by a decrease at higher concentrations of SNCA. This occurred in both the 2 and 4 day treatments at different concentrations. N=4, Error bars = 1 S.E.

**Discussion**

In this study, I demonstrate that C6 oligodendrocyte-like cells can be used as a model system for determining the effects of exogenous SNCA on oligodendrocyte-like cells. I found for both WT and A53T SNCA treatments appear to be taken up in a dose-dependent manner. A band was detected at approximately 17 kDa that
corresponds to the band observed when stock protein is electrophoresed. A second band was shown to occur in protein extracted from C6 cells at approximately 15 kDa. Inhibition of the lysosome was shown to abolish the truncated band in one study (Muntane et al. 2012). This second band suggests that the protein is taken up and modified by the C6 cells.

Truncation of SNCA has been shown to occur in Lewy bodies and oligodendrocytes of MSA patients (Fujiwara et al. 2002, Visanji et al. 2011, Muntane et al. 2012). The apparent modification of the protein observed in this study could be a truncated SNCA fibril resulting in a smaller apparent molecular weight than full length SNCA (Anderson et al. 2006, Luk et al. 2009, Muntane et al. 2012). The truncated SNCA protein could cause intercellular changes due to interaction with BAD, PKC-delta and 14-3-3 proteins (Ostrerova et al., 1999). Additionally overexpression of truncated SNCA has been shown to promote aggregation of SNCA (Liu et al. 2005, Visanji et al. 2011).

HSC70 is a constitutively expressed protein involved in the degradation of SNCA through the lysosome (Cuervo et al. 2004), and has been shown in GCIs of MSA patients (Kawamoto et al. 2007). In C6 cell studies, the highest expression of HSC70 occurred at different concentrations depending on the amount of time cells were exposed to SNCA. HSC70 expression was highest after treatment with 6 µg/ml
SNCA for 2 days, compared to 3 µg/ml after 4 days of treatment. This increase could be an example of the C6 cells attempting to cope with the increased amount of SNCA. It has been shown that overexpression of HSC70 can inhibit SNCA toxicity in Drosophila (Auluck et al. 2002). However the C6 cells appear to show a reduction in HSC70 expression. This could signify a failure of the cells to compensate for the increased SNCA. Interestingly, a decrease in HSC70 mRNA occurred in global gene expression measured in terminal MSA patients (Langerveld et al. 2007).

Regulation of HSC70 expression can be achieved through several processes. Expression can be increased with the treatment of insulin in diabetes resulting in an activation of the AKT pathway (Chen et al. 2006). Secondly, oxidative stress has also been shown to increase HSC70 expression as a mechanism to facilitate removal of unwanted protein into the lysosome (Kiffen et al. 2004, Saftig et al. 2009, Li et al. 2011). These pathways of activation could be compromised as the level of SNCA increases resulting in a loss of HSC70 activation.

Lastly, HSC70 also functions in uncoating of clathrin-coated vesicles (Rapoport et al. 2008). Downregulation of HSC70 prevents clathrin removal and reduces endocytosis. This could be an attempt by the cell to reduce uptake of SNCA by reducing endocytosis of the protein.
Conclusion

In conclusion, C6 cells take up SNCA when added to the media. This uptake does not appear to be influenced by the A53T mutation known to induce aggregation of SNCA. The amount of SNCA available for uptake does appear to influence the amount of SNCA absorbed. The pattern of SNCA uptake at 2 and 4 day treatments appears similar with significant amounts of protein being absorbed at the three highest concentrations tested.

SNCA appears to undergo a truncation when taken up by C6 cells. Truncations of 2-3 kDa smaller than full length SNCA, similar to those observed in this study, have also been seen in other studies of SNCA (Anderson et al. 2006, Luk et al. 2009, Muntane et al. 2012). Truncations in these studies have been shown to be intracellular in nature resulting in aggregated SNCA (Liu et al. 2005, Luk et al. 2009). Due to the observation of a truncated protein in C6 oligodendrocyte-like cells, this suggests that SNCA is being taken up by C6 cells.

Lastly, HSC70 is influenced by the concentration and amount of SNCA in culture. Changes in a protein known to rescue cells from SNCA treatment suggests C6 cells are responding to SNCA after uptake. Interestingly time appears to influence the expression of HSC70. At 2 days, 6 µg/ml of SNCA treatment shows high expression of HSC70, but at 4 days of treatment C6 cells show reduced
expression of 6 µg/ml compared to controls. If GCIs in MSA patients represent an initial increase of HSC70 to facilitate the removal of extra SNCA into the lysosome, then this could explain the initial increase in HSC70 due to SNCA treatment.

Overall this model demonstrates the probability of C6 oligodendrocyte-like cells to function as a model for SNCA uptake by oligodendrocytes in MSA. Use of this technique allows for further examination of SNCA-induced gene and protein expression changes in oligodendrocyte-like cells.
CHAPTER III
SNCA INDUCES TRYPAN BLUE-DEFINED CELL DEATH AND CHANGES IN EXPRESSION OF CASPASE 3 (A MARKER FOR APOPTOSIS)

Introduction

Programed cell death in multicellular organisms is referred to as apoptosis. It is involved in the removal of unwanted cells during development or from disease. Initiation of apoptotic cell death results in chromatin condensation, chromosome fragmentation, cell blebbing and shrinkage (Kerr et al. 1972, Savill et al. 1990, Brown et al. 2002). The cell breaks into fragments containing the intercellular components. Phagocytic cells then proceed to engulf these fragments clearing the debris (Krysko et al., 2006).

Caspase 3 (CASP3) is a protease that is activated during programed cell death (Porter and Jaenicke 1999). It is involved in the cleavage of proteins, condensation of chromatin and DNA fragmentation. CASP3 is expressed constitutively as Pro-Caspase 3 and is activated by proteolytic cleavage resulting in two subunits forming an active protein.

Two types of pathways are involved in apoptosis, intrinsic and extrinsic. Intrinsic pathways involve activation of apoptosis due to DNA damage and mitochondrial stress. Induction of the intrinsic pathway results in activation of CASP3 through Caspase 9. The extrinsic pathway involves extracellular signals through the Fas Associated
Death domain activating Caspase 8, which then activates CASP3 (Wlodkowic et al. 2011).

Trypan blue is a vital stain used for measuring viability of living cells in culture. Living cells exclude trypan. Non-viable cells are unable to exclude the dye and turn a blue color. To determine if SNCA, taken up by C6 cells, results in cell death, cells were treated with different doses of SNCA for 2 and 4 days. After treatment, cells were treated with trypan blue, and blue cells were counted to determine the percent of cells in culture that were non-viable.

As discussed above, CASP3 is expressed during apoptosis. To determine if C6 cells treated with SNCA were undergoing apoptosis, cells were treated with different doses of SNCA over 2 and 4 days. After treatment, protein was extracted and stained via western blot for CASP3.

**Methods**

**Trypan Blue Cell Viability Assay**

As described in chapter 2, C6 cells were cultured in 24 well plates for 2 and 4 days with SNCA. After treatment, cells were washed using PBS solution (pH 7.2-7.3) and trypsinized. After trypsin application for approximately 30 seconds to loosen adhered cells, 1ml of stock media was added to each well. Next, 0.1 ml of a 0.4% solution of trypan blue
in PBS was added to each well. After 5 min of exposure to trypan blue, 10 µl of cells were added to a hemocytometer. A picture of each quadrant on the hemocytometer was taken using a Spot Cooled Color Digital Camera (Diagnostic Instruments, Inc, Sterling Heights, Mi) attached to a Nikon Eclipse E600 Microscope. Cell counts were tabulated using the cell counter tool included in the ImageJ software package (nih.gov). Raw numbers were corrected for the dilution of cells in each well using the equation: Number of viable cells $\times 10^4 \times 1.2 = \text{cells/mL culture}$. Then the percent dead cells in culture were calculated using the ratio of non-viable/viable cells.

**Western Blot**

Briefly, as described in chapter 2, C6 cells were cultured for 2 and 4 days with SNCA. After treatment protein was extracted using RIPA buffer and electrophoresed. CASP3 protein was then resolved in western blots using rabbit anti-active-casp3 (Sigma ©) at 1:500.

**Statistics**

An ANOVA comparison was used to evaluate differences in the percentage of non-viable cells in culture as a function of SNCA treatment. Statistics were completed using Statview version 5 software, from SAS institute. In addition the same ANOVA method
was used to compare the differences in CASP3 western blot expression.

**Results**

**SNCA Induces Cell Death in C6 Oligodendrocyte-like Cells**

To determine if SNCA induces cell death, cells were treated with SNCA at different doses for 2 or 4 days. After 2 days cells treated with SNCA at 3 µg/ml or higher had a significantly higher percentage of cell death compared to controls (untreated cells) (Figure 5). Additionally cells treated with 1.5 µg/ml showed significantly lower cell death than cells treated with 6, 8.5 or 11 µg/ml. Cells treated with 3 µg/ml also showed significantly less cell death than cells treated with 11 µg/ml. After 4 days all treatment groups had significantly higher percentages of cell death than controls. Cells treated with 1.5 µg/ml showed significantly less cell death than 8.5 and 11 µg/ml treatment groups.
Figure 5. Trypan Blue Cell Counts. C6 cells were treated with SNCA for 2 or 4 days. After treatment, the percentage of non-viable cells was measured using Trypan Blue staining. The percentage of cells undergoing cell death in culture. After 2 days of treatment, 3, 6, 8.5 and 11 µg/ml treatment groups were significantly higher than control cells and cells treated with 1.5 µg/ml SNCA (a). Additionally cells treated with 11 µg/ml had significantly higher cell death than the 3 or 8.5 µg/ml treatments (b). After 4 days of treatment all treatments had significantly higher cell death than controls (c). Additionally, cells treated with 6, 8.5 and 11 µg/ml had significantly higher cell death than 1.5 µg/ml treated cells (d). N=4 Error Bars = 1 S.E.

SNCA Induces Changes in Caspase 3 Expression in C6 Oligodendrocyte-like Cells

To determine if cells were undergoing apoptosis, an antibody to active Caspase 3 was used to stain for the CASP3 Protein in western blots. After 2 days of treatment, cells treated with 6 µg/ml SNCA had significantly higher expression of CASP3 than control cells and 1.5 µg/ml SNCA treated cells (Figure 6). Additionally cells treated with 11 µg/ml SNCA of had higher expression of CASP3 than controls. All other groups showed a trend to higher expression of CASP3, although not significantly. In contrast, after 4 days, the lower dosage group treated
with 1.5 µg/ml SNCA had significantly higher expression of CASP3 than control, 8.5 and 11 µg/ml SNCA groups. Additionally cells treated with 8.5 µg/ml SNCA were significantly lower than 3 or 6 µg/ml treatments.

Figure 6. Caspase 3 protein expression after treatment with SNCA. C6 cells were treated with SNCA for 2 or 4 days. After 2 days, C6 cells treated with SNCA showed significantly increased expression of CASP3 protein with 6 and 11 µg/ml treatments, compared to controls (a). Casp3 protein expression was also higher in cells treated with 6 µg/ml SNCA compared to the 1.5 µg/ml treatment (b). After 4 days, C6 cells treated with 1.5 µg/ml SNCA showed an increase in CASP3 expression compared to control cells and cells treated with 8.5 or 11 µg/ml SNCA (c). In addition, cells treated with 3 and 6 µg/ml had significantly higher CASP3 expression than cells treated with 8.5 µg/ml SNCA (d). N=4, Error bars = 1 S.E.
Discussion

SNCA causes cell death in a dose-dependent manner of C6 oligodendrocyte-like cells after treatment. At 2 days, cells treated with 3 µg/ml SNCA had a significantly higher percent of dead cells in culture than controls. Cells treated with 1.5 µg/ml SNCA however were not significantly different from controls. After 4 days of treatment, the cell death threshold appears to shift to a lower concentration. Cells treated with 1.5 µg/ml SNCA for 4 days had a significantly higher percent of dead cells in culture.

This suggests that the uptake of SNCA may not be the only regulating factor of apoptosis. Phosphorylation of SNCA at Ser-129 (Visanji et al. 2011), or truncation of SNCA described in chapter 2, could induce changes to protein-protein interactions of SNCA with PLD2, BAD, 14-3-3, PKC-Delta (Jenco et al., 1999, Ostrerova et al., 1999, Bennett et al., 1998). These changes could result in a feedback loop of the IRS/PI3K/pAKT pathways resulting in a decrease pro-apoptotic factors BAD and Caspase 9 (Tiwary et al., 2011).

Cells treated with a lower dose of SNCA may need more time to accumulate SNCA inside the cell, to induce cell death. This would account for the lower dose group of 1.5 µg/ml SNCA having a significant difference from controls only after 4 days of treatment. Secondly the rate of cell death appears to be constant for the higher
dose groups. If more SNCA was required to induce cell death, it would be expected that SNCA at 8.5 or 11 µg/ml SNCA would increase the number of dead cells from 2 to 4 days.

**Conclusion**

SNCA induces cell death in C6 cells in a dose-dependent manner. Cells treated with SNCA at higher concentrations had significantly higher cell death than lower concentrations of treatment. This occurred at both 2 and 4 days of treatment.

After 2 days, SNCA induced an increased Caspase 3 expression in the 6 and 11 µg/ml treatment groups. However Caspase 3 expression after 4 days of treatment was highest in cells treated with 1.5 µg/ml SNCA. This also is reflected in the typan blue result where after 2 days of treatment, cell death at 1.5 µg/ml was insignificant. Then after 4 days of treatment cell death increased in this group resulting in a significant amount of apoptosis compared to control cells.

However, these results have some contradiction between the rate at which cells die and the expression of CASP3. After 2 days of treatment tyrpan blue staining and CASP3 expression are elevated due to SNCA. After 4 days of treatment CASP3 expression is increased in the lower concentration groups, but decreased in cells treated with the
higher concentrations of SNCA. HSC70 and/or other chaperone proteins could be reducing the amount of CASP3 expression.
CHAPTER IV
SNCA INDUCES CHANGES IN MESSENGER RNA EXPRESSION IN C6 OLIGODENDROCYTE CELLS, SUGGESTING NEW TARGETS OF INTEREST FOR STUDY IN MSA

Introduction

DNA microarrays are a genomics tool that allows for rapid screening of a sample for a large number of changes in gene expression. Oligonucleotides corresponding to specific genes are adhered to a fabricated chip (Maskos and Southern 1992). Samples of mRNA isolated from cellular tissue are hybridized to the chip and read with a laser scanner to evaluate expression of mRNA (Langerveld et al., 2007).

DNA microarrays have been used in many neurological studies of diseases (Mandel et al., 2003). Samples of mRNA have been taken from blood and brain cells of deceased patients (Glatt et al., 2005). Samples have also been taken from mouse models of disease (Bonin et al., 2004) and tissue culture models (Dimcheff et al., 2006).

Microarrays have also been useful in identifying genes previously unknown in MSA. Nineteen genes related to apoptosis had changes in expression in blood samples, including a regulator of HSC70 discussed in chapter 2 (Ide et al., unpublished data). In a DNA microarray study of human pons tissue, four other genes related to apoptosis were also
identified (Langerveld et al. 2007). Additionally, HSC70 was also found to be down-regulated and correlated with SNCA expression in the white matter tracts of the pons.

However, in evaluating the gene expression in patients with MSA, correlating the amount of SNCA influencing mRNA expression is not easily accomplished. With whole tissue, such as blood or sections of brain, the composition of the sample has a mixture of cell types. Thus, DNA microarray analysis of C6 cells was used in this study to better understand if SNCA could influence gene expression specifically of oligodendrocyte-like cells.

As such, C6 cells were treated with SNCA for 4 days, and mRNA was extracted and analyzed for changes in gene expression. To follow up on genes identified by the array, one gene of interest, Hook Homolog 3 (HOOK3) was evaluated using western blot of C6 oligodendrocyte-like cells treated with SNCA for 2 or 4 days at increasing concentrations.

Methods

DNA Microarray

As previously described in chapter 2, a control and two treatment groups (5 and 10 µg/ml SNCA) of C6 cells were grown for 4 days. After 4 days, total RNA was isolated from C6 tissue using
Qiagen RNEasy Mini kits following the manufacturer’s protocol (Qiagen, Valencia, CA).

Extracted RNA was analyzed for purity by calculating the absorbance ratio of A260/A280. RNA was also electrophoresed to ensure quality of the sample through examination of the 28S and 18S ribosomal bands. Having passed these checks, samples were hybridized to Affymetrix Rat 230 2.0 DNA microarrays per manufacturer’s protocol using an aliquot containing 5 μg RNA.

Arrays were evaluated using the Gene Array Scanner (Agilent Technologies). Expression of each gene was measured using Affymetrix Microarray Suite v5.0. Gene expression differences were analyzed using a t-test between either control vs 5μg/ml or control vs 10μg/ml treated cells (p-value < 0.05). All genes reported were selected using a cut-off criteria of 1.41 fold change from control cell gene expression.

**Western Blot**

As described in chapter 2, C6 cells were treated with SNCA for 2 and 4 days at increasing concentrations of 0, 1.5, 3, 6, 8.5 and 11μg/ml. Following treatment, protein was extracted and electrophoresed for western blot. HOOK3 protein was resolved using goat anti-HOOK3 (Santa Cruz ©) at 1:500.
Results

DNA microarray analysis shows changes in gene expression with treatment with SNCA

To evaluate changes in cell response, cells were treated with either 5 or 10 µg/ml SNCA for 4 days and expressed RNA was extracted for Affymetrix gene chip analysis (Table 1 & 2). Microarray analysis shows that several structural and metabolic genes were influenced by treatment. The highest fold change was found in the downregulation of Echinoderm microtubule associated protein like 4 (EML4) at the 10 µg/ml treatment. HOOK3 had the next greatest fold change difference and was downregulated at the 5µg/ml treatment. Interestingly the only three genes that were upregulated in either treatment of SNCA have links to apoptosis in other models. Tubby mouse homolog (TUB) was upregulated at 5 µg/ml. Insulin receptor substrate 2 (IRS2) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ERBB3) were upregulated at 10 µg/ml.

Using the DAVID bioinformatics resource database (Huang et al. 2009), the genes listed in table 1 and 2 were compared for similarities in GO function annotation. IRS2, NIFA, TUB and ERBB3 were classified as being a response to organic substance. Additionally, IRS2 and ERBB3 were also regulators of glucose import. HOOK3, PTPN4 and ARPC5L were classified as having cytoskeletal protein binding function.
RAD18, GPD2, PRPS2, ZFP-62 and ZFP131 were classified as having cation binding properties. PRPS2, SMC4 and ERBB3 were classified as having Nucleotide binding function. GPD2, PTPN3 and ERBB3 are also classified in phosphate metabolic processing. RAD18, PTPN3, ARPC5L and SMC4 have association with non-membrane-bounded organelles consisting of ribosomes and cytoskeleton.

DAVID only identified one disease common to the genes discussed in tables 1 and 2. ERBB3, IRS2 and GPD2 have a relationship with noninsulin-dependent, type 2 Diabetes mellitus. THOC2, EML4 and ANKRD11 were not reported as having similar function to any other genes reported. THOC2 is a component of the THO Complex that binds with mRNA post translation. ANKRD11 is a nuclear receptor substrate that modifies microtubules to become longer and more dynamic.
Table 1. DNA microarray analysis of C6 cells treated with 5 µg/ml SNCA for 4 days. Genes listed were statistically different from controls in treated cells (p<0.05).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Probe Set ID</th>
<th>Fold Change</th>
<th>Regulation</th>
<th>Gene Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tub</td>
<td>1369283_at</td>
<td>1.454974</td>
<td>up</td>
<td>tubby homolog (mouse)</td>
</tr>
<tr>
<td>Nfia</td>
<td>1369679_a_at</td>
<td>1.509205</td>
<td>down</td>
<td>nuclear factor I/A</td>
</tr>
<tr>
<td></td>
<td>1375562_at</td>
<td>1.492197</td>
<td>down</td>
<td>Transcribed Locus</td>
</tr>
<tr>
<td>Prps2</td>
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<td>down</td>
<td>phosphoribosyl pyrophosphate synthetase 2</td>
</tr>
<tr>
<td>RGD1560191</td>
<td>1379029_at</td>
<td>1.651205</td>
<td>down</td>
<td>similar to Zinc finger protein 62 homolog (Zfp-62) (ZT3)</td>
</tr>
<tr>
<td></td>
<td>1379130_at</td>
<td>1.539181</td>
<td>down</td>
<td>Transcribed Locus</td>
</tr>
<tr>
<td>Smc4</td>
<td>1383008_at</td>
<td>1.500074</td>
<td>down</td>
<td>structural maintenance of chromosomes 4</td>
</tr>
<tr>
<td>Hook3</td>
<td>1385931_at</td>
<td>1.739286</td>
<td>down</td>
<td>hook homolog 3 (Drosophila)</td>
</tr>
<tr>
<td>Thoc2</td>
<td>1386525_at</td>
<td>1.410694</td>
<td>down</td>
<td>THO complex 2</td>
</tr>
<tr>
<td>Ptpn4</td>
<td>1391757_at</td>
<td>1.426900</td>
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</tr>
<tr>
<td>Rad18</td>
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<tr>
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<td>zinc finger protein 131</td>
</tr>
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Table 2. DNA microarray analysis of C6 cells treated with 10 µg/ml SNCA for 4 days. Genes listed were statistically different from controls in treated cells (p<0.05).

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<th>Gene Symbol</th>
<th>Probe Set ID</th>
<th>Fold Change</th>
<th>Regulation</th>
<th>Gene Title</th>
</tr>
</thead>
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<tr>
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<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 3</td>
</tr>
<tr>
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<td>1.5204533</td>
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<td>Insulin receptor substrate 2</td>
</tr>
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<td>1.5183513</td>
<td>down</td>
<td>nuclear factor I/A</td>
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<td>Transcribed locus</td>
</tr>
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<td>echinoderm microtubule associated protein like 4</td>
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<td>down</td>
<td>glycerol-3-phosphate dehydrogenase 2, mitochondrial</td>
</tr>
<tr>
<td>Prps2</td>
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<td>down</td>
<td>phosphoribosyl pyrophosphate synthetase 2</td>
</tr>
<tr>
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</table>

Expression of HOOK3 in C6 cells decreases with the treatment of SNCA

To determine if changes in gene expression are reflected in protein expression, HOOK3 was evaluated using western blot after 2 and 4 days of treatment with increasing concentrations of SNCA. After 2 days of treatment, control cells and 3 µg/ml treated cells had significantly higher expression of HOOK3 than 11 µg/ml treated cells. After 4 days of treatment, control cells had significantly higher expression of HOOK3 than all treatment groups. Additionally, cells
treated with 1.5 and 3 µg/ml SNCA had significantly higher expression than 11 µg/ml treated cells.

Figure 7. HOOK3 expression in C6 cells treated with SNCA. Cells were treated with SNCA for 2 or 4 days. Both treatments showed a decrease in HOOK3 protein expression. After 2 days only cells treated with 11 µg/ml were significantly lower in HOOK3 protein expression. However, after 4 days, all cells treated with SNCA had significantly lower HOOK3 expression than controls. In addition cells treated with 11 µg/ml showed significantly lower expression than 1.5 and 3 µg/ml treatments. N=4, Error bars = 1 S.E.
Discussion

Changes in gene expression can be induced by treatment with exogenous SNCA. HOOK3, a member of the Hook family, binds to microtubules and Golgi (Walenta et al., 2001), is involved in protein endocytosis, clearing of macrophage scavenger receptors (MSR1) (Sano et al., 2007), and interacts with an apoptosis regulating gene (AKTIP) (Xu et al., 2008). Western blot analysis of HOOK3 showed a decrease in protein expression with treatment of SNCA at both 2 and 4 days. HOOK3 decrease appears to be greater at 4 days. This supports the DNA microarray data collected from the C6 cells showing a decrease in gene expression after 4 days of treatment.Interestingly, in a neural cell model testing the effect of prefibrillar SNCA, the Golgi was shown to become fragmented (Gosavi, et al., 2002). Due to the relationship HOOK3 has with SNCA this could explain the change in expression.

Several other genes of interest were identified using DNA microarray. Microtubule-related genes, EML4 and ARPC5L, were downregulated in the 10 µg/ml treatment. SNCA has been shown to interact with tubulin and other microtubule proteins (Alim et al., 2002). EML4 is responsible for stabilization of microtubules and influence growth rates in cells (Houtman et al., 2007). ARPC5L is believed to have similar functionality to ARPC5 as part of the actin 2/3
complex in regulation of actin polymerization (Humphray et al., 2004). Finally, TUB was upregulated in the 5 µg/ml treatment but not at 10 µg/ml. Interestingly TUB has been shown to interact with MAP1A (microtubule-associated protein 1A) a protein commonly found in protein aggregates (Ikeda et al., 2002).

Upregulation of Erbb3 has been shown to be involved in C6 oligodendrocyte survival as a receptor of neuregulins (Flores et al., 2000). Deactivation of ERBB3 has been shown to inhibit apoptosis (Sithanandam et al., 2005). ANKRD11, which has been shown to coactivate P53 in breast cancer (Neilson et al., 2008), was shown to be downregulated at 10 µg/ml. Interestingly P53 has been shown to occur in many neurological diseases resulting in apoptosis, including MSA (de la Monte et al., 1998). IRS2, shown to inhibit apoptosis (Ueno et al., 1999, Lingohr et al., 2003), was upregulated at 10 µg/ml.

Many genes of interest also have relationships with the PI3K-AKT pathway. ERBB3 has been shown to activate the PI3K-AKT pathway (Soltof, et al., 1994, O’Merra et al., 2011). IRS2 has also been shown to regulate the expression of the AKT pathway (Russo et al., 2007) Additionally, HOOK3 interacts with AKT through the FTS/HOOK/p107FHIP complex (FTS is also called AKTIP).

It is possible that the cells are attempting to cope with the increased SNCA. The changes in gene expression of ERBB3 IRS2 and
ANKRD11 suggest an inhibition apoptosis. In the previous chapter we see CASP3 apoptosis peaks in expression at different concentrations based on the concentration of treatment. This could be explained by the increased expression of ERBB3, IRS2 and the decrease in ANKRD11 if they are playing a role in regulating apoptosis.

**Conclusion**

Cells treated with SNCA appeared to undergo changes in gene expression. In cells treated with 5μg/ml SNCA, 1 gene was upregulated and 12 genes were downregulated. In cells treated with 10μg/ml SNCA, 2 genes were upregulated and 13 genes were downregulated. Many of these genes are related to microtubules, apoptosis and/or AKT.

To test if changes in gene expression match protein expression, C6 cells were treated with SNCA at increasing concentrations and evaluated via western blot analysis for HOOK3 protein. After 2 days of treatment with SNCA, HOOK3 protein showed a decrease in expression at the highest concentration of SNCA. After 4 days of treatment with SNCA, HOOK3 protein showed a decrease in expression at all concentrations of treatment. This result confirms the decrease in gene expression of HOOK3 observed in the DNA microarray.
CHAPTER V
TWO PROTEINS INVOLVED IN APOPTOSIS, PROGRAMMED CELL DEATH 4 AND FAMILY WITH SEQUENCE SIMILARITY 3, MEMBER B, APPEAR TO BE MARKERS IN MULTIPLE SYSTEM ATROPHY

Introduction

As discussed in chapter 3 CASP3, a marker for apoptosis, showed changes in expression due to the treatment with SNCA. Based on these data, I next wanted to determine if apoptotic-related genes previously identified using DNA microarrays, could be influenced by treatment with SNCA. Microarrays have also been useful in identifying genes previously unknown in MSA. Nineteen genes related to apoptosis had changes in expression in blood cells of MSA patients compared to controls (Ide et al., unpublished data). In a DNA microarray study of human tissue, four other genes related to apoptosis were also identified (Langerveld et al. 2007). Programmed Cell Death 4, PDCD4 had upregulated expression and was associated with the amount of misfolded SNCA in brains of patients with MSA (Langerveld et al., 2007). PDCD4 is a tumor suppressor gene that binds to eukaryotic translation initiation factor 4 alpha (EIF4A), and is thought to play a role in apoptosis (Lankat-Buttgereit and Göke 2003). TGF-B1 has been shown to induce PDCD4 apoptosis through the Smad Pathway (Nakashima et al., 2009). It is regulated through
the expression of cytokines IL-2 and IL-12 secreted by T-cells and Natural killer cells (Azzoni et al, 1998).

PDCD4, in addition to interacting with EIF4A, has some sequence homology with eukaryotic translation initiation factor 4 gamma (EIF4G). This results in regulating how EIF4G functions in transporting RNA to the ribosome for translation (Yang, et al, 2003; Suzuki, et al, 2008). This interaction inhibited CAP-dependent translation of protein in cells (Jansen et al., 2005). PDCD4 has also been shown to shuttle between the cytoplasm and nucleus when bound to RNA suggesting a role in cell growth and transformation (Bohm et al., 2003).

A second gene of interest, the killer cytokine, pancreatic-derived factor (FAM3B) was upregulated in the blood of MSA patients (Ide et al, in preparation). FAM3B was shown to induce apoptosis in pancreatic β-Cells (Cao, et al, 2003). FAM3B has also been shown to induce apoptosis in SH-SY5Y neuroblastoma cells (Narayan et al., 2006).

This experiment had three objectives. First, to determine if these two proteins are influenced by SNCA treatment, I treated C6 oligodendrocyte-like cells for 2 and 4 days with SNCA. Following treatment, protein was extracted and analyzed using western blot analysis. The second objective was to confirm that oligodendrocytes express Caspase 3 in patients with MSA. Using immunocytochemistry,
sections of cerebellar tissue from MSA patients and controls were double labeled for CASP3 and myelin basic protein (MBP), a marker of oligodendrocytes. Lastly, to determine if FAM3B and PDCD4 are expressed in MSA, sections of cerebellar tissue from the brains of MSA patients and controls were evaluated using immunocytochemistry to determine if these two proteins co-localized with MBP.

**Methods**

**Western Blot**

Briefly, as described in chapter 2, C6 cells were cultured for 2 and 4 days with SNCA. After treatment protein was extracted using RIPA buffer and electrophoresed. FAM3B protein was resolved in western blots using rabbit anti-FAM3B (Santa Cruz©) at 1:500. PDCD4 protein was resolved in western blots using rabbit anti-PDCD4 (Santa Cruz©) at 1:500.

**Immunocytochemistry**

Human cerebellum slides were obtained from the New York Brain Bank at Columbia University. Slides were de-paraffinized and rehydrated using a series of xylene and alcohol washes followed by TBS. Next heat antigen retrieval was used to unmask proteins. Slides were placed in 10mM Sodium Citrate buffer (pH 6.0) for 40 min at 80 ºC. After antigen retrieval slides were washed in 3% H₂O₂ for 5 min
followed by and two washes in TBS. Slides were next blocked for 1 hour in 3% normal goat serum. After blocking slides were incubated at 4 °C overnight in either rabbit anti-FAM3B (Santa Cruz©) at 1:50, rabbit anti-PDCD4 (Santa Cruz©) at 1:50 or rabbit anti-CASP3 (Sigma©) at 1:100. Slides were then washed in TBS and incubated in biotinylated goat anti-rabbit secondary antibody (Santa Cruz©) at 1:100 for 30 min. Slides were then washed three times in TBS. To detect biotin labeled secondary, a Vector ABC-DAB kit was used following the manufactures protocol (Vector© Labs). Slides were washed three times in TBS. DAB substrate was added to Tris-HCl buffer (pH 7.5) and placed on slides for 10 min. Next to double label MBP, slides were rinsed in dH₂O and washed three times in TBS. Slides were next blocked for 1 hour in 3% normal goat serum. After blocking slides were incubated at 4 °C overnight in anti-MPB at 1:50 (Santa Cruz©). Slides were then washed in TBS and incubated in biotinylated goat anti-rabbit secondary antibody (Santa Cruz©) at 1:100 for 30 min. Slides were then washed three times in TBS. To detect biotin labeled secondary, a Vector ABC-AP kit was used following the manufacturer’s protocol (Vector© Labs). This was followed by Vector© AP substrate kit using manufactures protocol (Levamasole (Vector) was added to quench endogenous AP per the manufacturer’s recommendations). Slides were washed three times in TBS then rinsed
in H₂O and counterstained for 1 min in Hematoxylin 1 (ThermoFisher). Next slides were rinsed in H₂O and placed in clarifier (ThermoFisher) for 1 min. Slides were rinsed in H₂O for 30 seconds with agitation, and then placed into bluing agent (ThermoFisher) for 1 min. Slides were then washed for 1 min in H₂O and dehydrated through a series of xylene-alcohol washes and cover slipped using Permimount (ThermoFisher).

Photographs were taken using the Spot Cooled Color Digital Camera system as described in chapter 2. Image analysis for DAB (brown) and AP (red) were completed using the ImageJ (nih.gov) color deconvolution tool.

The color deconvolution tool uses red, green, and blue (RGB) vectors input by the user to differentiate between red and brown colors. To establish these vectors I first used two images with singly labeled cells stained either with DAB or AP. Next I selected color deconvolution in ImageJ followed by the (Region of Interest) ROI function to measure and save the RGB vectors. After saving these vectors I opened the image of interest and selected the user input function in color deconvolution to input the RGB vectors from the single-labeled cells. After the function processed, red and brown colors were separated by the program into new images.
These images were then changed to 8-bit greyscale, and the threshold tool was used to convert the image to pure black and white. Using these images, I selected analyze particles in ImageJ and measured the amount of area stained in pixels. The area of stain was then converted to percent of the total area of interest. A t-test was then completed on this data using Statview 5.0 (version 5, from SAS institute).

**Results**

**Expression of PDCD4 in C6 cells decreases with SNCA treatment**

To determine if PDCD4 expression is influenced by SNCA treatment, cells were grown for 2 or 4 days and evaluated using western blot. After 2 days, cells treated with 6 µg/ml had significantly higher expression than control cells. After 4 days, cells treated with 3 µg/ml had significantly higher expression than controls, 6, 8.5 and 11 µg/ml groups. Cells treated with 1.5 µg/ml had significantly higher expression than 6, 8.5 and 11 µg/ml groups. Control cells also had significantly higher expression than 8.5 µg/ml treated cells.
Figure 8. PDCD4 expression after treatment with SNCA. After 2 days, 6 µg/ml was significantly higher than control cells (a) \( (p=0.0219) \). After 4 days, control cells were significantly higher than 8.5 µg/ml \( (b) \) \( (p=0.042) \). Cells treated with 1.5 µg/ml were significantly higher than 6, 8.5 and 11 µg/ml \( (c) \) \( (p=0.049, <0.001, 0.006) \). Cells treated with 3 µg/ml were significantly higher than controls, 6, 8.5 and 11 µg/ml groups \( (d) \) \( (p=0.023, 0.018, <0.001, 0.002) \). \( N=5 \), Error bars = 1 S.E.

Expression of FAM3B in C6 cells decreases with the treatment of SNCA

To determine if the cytokine FAM3B is influenced by treatment with SNCA, cells were grown for 2 or 4 days and evaluated using
western blot. After 2 days, cells treated with 1.5, 3, and 6 µg/ml had significantly higher expression than control cells. Cells treated with 6 µg/ml were also significantly higher than the 11 µg/ml treatment. After 4 days, cells treated with 3 µg/ml had significantly higher expression of FAM3B than the 8.5 or 11 µg/ml treatments.
Figure 9. FAM3B expression after 4 days of treatment with SNCA. After 2 days, 1.5, 3 and 6 µg/ml treatments were significantly higher than control cells (a) (p= 0.02, 0.03, 0.012). Additionally, 6 µg/ml was significantly higher than 11 µg/ml (b) (p=0.038). After 4 days 8.5 and 11 µg/ml were significantly lower than 3 µg/ml treatment (c) (p=0.023, 0.049). N=5, Error bars = 1 S.E.
Caspase 3 Co-localizes in the White Matter Tracts of MSA Patients

Caspase 3 (CASP3) a marker for apoptosis was also shown to be induced due to SNCA treatment (Chapter 3). To determine if CASP3 is expressed in oligodendrocytes of MSA patients, CASP3 was double labeled with MBP using ICC methods. Control patients expressed little active-CASP3 staining (2.3% of the total area) (Figure 1a). MBP staining was uniform through the white matter tracts (40.6% of the total area) (Figure 1b). In MSA patients, CASP3 staining increased to 17.5% of the white matter tract area and co-localized with MBP (Figure 1c). In addition, MBP staining appears to fragment in MSA patients. Staining of MBP decreased to 25.9% of the white matter tract area (Figure 1d). The area of cells double labeled with CASP and MBP increased from 0.1% in controls to 2.0% in MSA patients.
Figure 10. CASP3 immunocytochemistry of the white matter tracts in MSA patients. Red = MBP, Brown = CASP3. A. Control patient sections stain for background CASP3 expression in 2.3% of the total area. B. No apparent loss in MBP staining occurs in the white matter tracts of control patients. C. Staining of CASP3 increases in MSA patients to 17.5% of the area stained (p<0.001). D. MBP staining appears to double label with the fragmented CASP3. MBP showed a 14.7% decrease in the area stained (p=0.036) Lastly double labeled tissue for CASP3 and MBP increased from 0.1% in controls to 2.0% in MSA patients (p=0.012). N=5, Error Bars = 1 S.E
FAM3B Co-localizes in the White Matter Tracts of MSA Patients

To determine if FAM3B is present in MSA, tissue from patients and controls were evaluated using ICC methods. In control patients MBP was shown to stain uniformly in the white matter tracts (52.4% of the white matter tract area) (Figure 2a), while FAM3B did not appear to be highly expressed (2.5% of the area) (Figure 2c). In MSA patients MBP shows a fragmented pattern of staining and decreases to 29.2% in the white matter tracts (Figure 2b). FAM3B also showed a fragmented pattern of staining, that increased to 15.2% in the white matter tracts (Figure 2d). Double labeling of MBP and FAM3B increased from 0.7% in control patients to 7.7% of the total white matter tracts in MSA patients.
Figure 11. FAM3B immunocytochemistry of the white matter tracts in MSA patients. Red = FAM3B, Brown = MBP, Blue = Counterstain. A. The area stained for MBP in the white matter tracts of control patients appears to be uniform. B. The staining in patients with MSA shows fragmentation of the MBP in the white matter tracts. C. Little FAM3B staining occurs in the white matter tracts of control patients. D. FAM3B staining appears to double label with the fragmented MBP. Image analysis showed FAM3B staining increased from 2.5% in control patients to 15.2% of the white matter tracts in MSA patients (p<0.001). MBP staining decreased from 52.4% in control patients to 29.2% in of the white matter tracts in MSA patients (p<0.001). Lastly, double labeled tissue for FAM3B and MBP increased from 0.7% in control patients to 7.7% of the white matter tract in MSA patients (p=0.002). N=5, Error Bars = 1 S.E.
PDCD4 Co-localizes in the White Matter Tracts of MSA Patients

To determine if PDCD4 is present in MSA, tissue from patients and controls were evaluated using ICC methods. In control patients, MBP was shown to stain uniformly in the white matter tracts (60.45% of the area) (Figure 3a), while PDCD4 did not stain in large abundance (0.71% of the area) (Figure 3c). In MSA patients, MBP appeared to increase in fragmentation with the area of MBP staining decreasing to 35.16% in the white matter tracts (Figure 3b). PDCD4 increased in staining to 12.13% of the white matter tract. Double labeling of PDCD4 and MBP increased from 0.45% in control patients to 4.68% of the white matter tracts in MSA patients.
Figure 12. PDCD4 immunocytochemistry of the white matter tracts in MSA patients. Red = PDCD4, Brown = MBP. A. The area stained for MBP in the white matter tracts of control patients appears to be uniform. B. Little PDCD4 staining occurs in the white matter tracts of control patients. C. The staining in patients with MSA shows fragmentation of the MBP in the white matter tracts. D. PDCD4 staining appears to double label with the fragmented MBP. Image analysis showed PDCD4 staining increased from 0.71% in control patients to 12.13% of the white matter tracts in MSA patients (p=0.025). MBP staining decreased from 60.45% in control patients to 35.16% in of the white matter tracts in MSA patients (p=0.008). Lastly, double labeled tissue for PDCD4 and MBP increased from 0.45% in control patients to 4.68% of the white matter tract in MSA patients (p=0.013). N=5, Error Bars = 1 S.E.
Discussion

It appears that exogenous SNCA can induce proteins associated with cell death in C6 oligodendrocytes. PDCD4, FAM3B, CASP3 (shown in chapter 3), and HSC70 (shown in chapter 2) all change in expression due to SNCA treatment. In MSA patients, CASP3, FAM3B and PDCD4 were shown to double-label with MBP in the white matter tracts exiting the cerebellar folia. This corresponds to previous studies in MSA. MSA patients with olivopontocerebellar atrophy show loss of tissue in the white matter tracts, gliosis and axonal degeneration (Ozawa et al. 2004, Ahmed et al. 2012). Loss of oligodendrocytes and accumulation of GCIs in the cerebellar white matter tracts has been shown to precede loss of neurons (Papp and Lantos 1994). Expression of these apoptotic proteins in oligodendrocytes suggests a mechanism for cell death, resulting in a loss of myelin that would then lead to a loss of neurons.

As stated previously in chapter 2, HSC70 is involved with clearing SNCA. HSC70 expression patterns are similar to CASP3, PDCD4 and FAM3B. This suggests that the mechanism (HSC70/LAMP2a) clearing SNCA becomes overwhelmed and is no longer protecting the cell.

However, another possibility is that SNCA is influencing a common pathway to PDCD4, FAM3B, CASP3 and HSC70. In the
previous chapter, Insulin receptor substrate 2 (IRS2) was shown to be up-regulated in C6 cells treated with SNCA. Insulin has been shown to up-regulate expression of HSC70, preventing apoptosis (de la Rosa et al. 1998, Chen et al. 2006). Insulin regulation of the AKT/mTOR/PI3K pathway regulates apoptosis, survival, and homeostasis of cells (Morgensztern and McLeod 2005, Costa et al. 2012, Hsieh et al. 2012). Lastly, Insulin has also been shown to regulate expression of MAPK, PKC alpha and delta, and NF-kB (Martins et al., 2010).

FAM3B appears to regulate insulin sensitivity. HepG2 liver cells were isolated and pretreated with FAM3B. This pre-treatment reduced the sensitivity of insulin receptor and insulin receptor substrate 1 (IRS1) to insulin (Yang et al. 2009). This inhibition also reduced insulin-induced expression of PI3K and pAKT. In pancreatic beta cells treated with glucose, FAM3B showed an increase in expression. When treated with glucose and inhibitors of PI3K, CREB and PKC-alpha, all were shown to inhibit expression of FAM3B (Wang et al. 2008). This suggests a negative feedback loop regulating expression of PI3K and FAM3B.

As shown in the previous chapter IRS2 was upregulated in C6 cells treated with SNCA. IRS2 has been shown to regulate cell size and apoptosis in pancreatic beta cells through the homeobox transcription factor Pdx1 (Withers et al. 1998, Kushner et al. 2002, Linghor et al.
Interestingly Pdx1 was shown to bind to the promoter region of FAM3B regulating glucose stimulated expression of FAM3B (Burkhardt et al. 2008).

As stated previously, PDCD4 interacts with EIF4A and EIF4G. Insulin has been shown to up-regulate the interaction of EIF4G with EIF3 through the AKT/PI3K/mTOR pathway (Harris et al., 2006). Up-regulation of phosphorylated AKT also down-regulates PDCD4 in cancer cells (Fasan et, al. 2011). Overexpression of HSC70 was also shown to increase the solubility of EIF4G (Cuesta, et al., 2000). This relationship may play a role in MSA as EIF4G1 has been implicated in familial PD (Chartier-Harlin, et al., 2011).

Inhibition of PKC-delta increased expression of PDCD4 in Huh7 hepatoma cells (Nakashima et, al. 2010). It is possible SNCA is directly responsible for changes in PDCD4 expression. PKC-delta binds to and is down-regulated by SNCA (Jin et, al. 2011). This down-regulation of PKC-delta could result in the increase in PDCD4 expression observed in C6 cells treated with SNCA.

Conclusion

PDCD4 and FAM3B change in expression due to the treatment with SNCA in C6 Oligodendrocyte-like cells. This change appeared to be dose and time dependent. Protein expression also appeared to
correspond to the CASP3 protein expression in C6 oligodendrocyte-like cells described previously in chapter 3 and HSC70 described in chapter 2. In MSA patients, CASP3, PDCD4 and FAM3B were expressed in fibers containing MBP, suggesting a relationship between these proteins and demyelination due to oligodendrocyte apoptosis.

To date, little is known about PDCD4 and FAM3B and their roles in apoptosis. Although the studies discussed have shown that these two genes are involved in apoptosis, specific mechanisms have yet to be resolved. The data presented here represent the first time FAM3B has been stained for in a patient with a synucleinopathy. Understanding how PDCD4 and FAM3B are regulated and involved in apoptosis and regulated by the mTOR/AKT/PI3K pathway in MSA could generate new targets of treatment for the disease.
CHAPTER VI
PROTEIN HOOK HOMOLOG 3 LEVELS DECREASE IN THE GRANULAR
CELL LAYER OF THE MULTIPLE SYSTEMS ATROPHY CEREBELLUM

Introduction

As discussed above, HOOK3 is a member of the Hook family of proteins. HOOK family proteins bind to microtubules and are responsible for endocytic trafficking (Kramer et al. 1999). HOOK3 has been shown to be involved in defining the conformation of the Golgi complex (Walenta et al. 2001).

HOOK3 also has been shown to be involved with the innate immune system. HOOK3 regulates the expression of scavenger receptor A (SR-A, MSR1). Blockage of HOOK3 by the Samonella SpiC protein results in the disruption of the Golgi complex (Shortland et al. 2003). Additionally this disruption interferes with the phagosome-lysosome network.

Based on the DNA microarray and western blot data discussed in chapter 4, there appears to be a relationship between SNCA and HOOK3. As stated previously, SNCA has been shown to occur in glial cytoplasmic inclusions (GCI) of the white matter tracts in MSA. However oligodendrocytes do not produce SNCA. As such there must be a source in proximity to the white matter tracts of SNCA.

To look for the influence of HOOK3 in MSA, I examined the cerebellum for changes in protein expression. The cerebellar folia
 consist of 4 layers: molecular layer, Purkinje cell layer, granular cell layer and white matter tracts. The granule cells account for about half of the neurons in the central nervous system. Excitatory output from the pontine nuclei travel through myelinated axons called mossy fibers in the white matter tracts to the cerebellar granule cells. Excitatory granule cell output travels along parallel fibers through the Purkinje layer into the molecular layer where they synapse on the dendrites of the Purkinje cells. In MSA, patients appear to lose a percentage of several cerebellar cell types as the disease progresses. Myelinated fibers in the white matter tracts and, Purkinje cell numbers are reduced and the granule cell layer also loses a significant number of cells.

This experiment had three objectives. First, I sought to confirm SNCA is normally expressed in the cerebellum. Second, SNCA was shown to decrease expression of HOOK3 in C6 oligodendrocyte-like cells. If SNCA is involved in regulation of HOOK3 then SNCA and HOOK3 should be co-localized in the cerebellum. Lastly, if HOOK3 protein is expressed by oligodendrocytes, then expression of HOOK3 should be co-localized with Myelin Basic Protein (MBP), a marker for oligodendrocytes. Using Immunocytochemistry (ICC) methods cerebellar tissue from control and MSA patients was evaluated to determine the expression of these proteins.
Methods

Immunocytochemistry

Human cerebellum slides were obtained from the New York Brain Bank at Columbia University. Slides were de-paraffinized and rehydrated using a series of xylene and alcohol washes followed by TBS. Next heat antigen retrieval was used to unmask proteins. Slides were placed in 10mM Sodium Citrate buffer (pH 6.0) for 40 min at 80 °C. After antigen retrieval slides were washed in 3% H₂O₂ for 5 min followed by two washes in TBS. Slides were next blocked for 1 hour in 3% normal donkey serum. After blocking, slides were incubated at 4 °C overnight in goat anti-HOOK3 (Santa Cruz ©) at 1:100. Slides were then washed in TBS and incubated in biotinylated donkey anti-goat secondary antibody (Santa Cruz©) at 1:100 for 30 min. Slides were then washed three times in TBS. To detect biotin labeled secondary, a Vector ABC-DAB kit was used following the manufacturer’s protocol (Vector© Labs). Slides were washed three times in TBS. DAB substrate was added to Tris-HCl buffer (pH 7.5) and placed on slides for 10 min. Next to double label HOOK3 with either SNCA or MBP, slides were rinsed in dH₂O and washed three times in TBS. Slides were next blocked for 1 hour in 3% normal goat serum. After blocking slides were incubated at 4 °C overnight in either rabbit anti-SNCA (Sigma ©) at 1:200 or anti-MBP at 1:50 (Santa Cruz©). Slides were then washed
in TBS and incubated in biotinylated goat anti-rabbit secondary antibody (Santa Cruz©) at 1:100 for 30 min. Slides were then washed three times in TBS. To detect biotin-labeled secondary, a Vector ABC-AP kit in combination with Vector© AP substrate kit was used following the manufacturers protocol (Vector© Labs). Levamasole (Vector) was added to quench endogenous AP per the manufacturer’s recommendations. Slides were washed three times in TBS then rinsed in \(H_2O\) and dehydrated through a series of xylene-alcohol washes and cover slipped using Permimount (ThermoFisher).

Photographs were taken using the Spot Cooled Color Digital Camera system as described in chapter 2. Image analysis for DAB (brown) and AP (red) were completed using the ImageJ (nih.gov) color deconvolution tool. As described in the previous chapter, this tool allows the user to select red and brown tones in an image. After selection, red and brown colors were separated by the program and measured for the amount of area stained using the measure function of ImageJ. Area of stain measured was converted to percent area stained. A t-test was then completed using Statview 5.0 as described above.
Results

HOOK3 Staining in Control Patients Co-localizes with SNCA in the Granule Cell Layer and White Matter Tracts of the Cerebellum

To determine if HOOK3 expression was altered in MSA brain, cerebellar tissue from patients and controls were double labeled for HOOK3 and SNCA. Using a lower power magnification, it appeared that SNCA and HOOK3 stained in the granule cell layer of control patients but was reduced in patients with MSA (Figure 13). Additionally, it appeared that HOOK3 stains in the white matter tracts of control patients, but may double label with SNCA in MSA patients.

Using higher power magnification, staining of HOOK3 was found heaviest in the granule cell layer in the cytoplasmic spaces of control patients (Figure 14). Imagej (nih.gov) was used to measure the area of HOOK3 staining. Using a t-test, HOOK3 was found to decrease by approximately 24% in MSA patients compared to controls (P=0.012). SNCA also appears to decrease with HOOK3 in granule cells. Control patients had approximately 54% more area stained for SNCA than MSA patients.
Figure 13. Two examples of ICC-stained paraffin-embedded tissue from a control and MSA cerebellum. HOOK3 was resolved using DAB (brown) and SNCA was resolved using AP (red); PC = Purkinje Cell, ML = Molecular Layer, GCL = Granule Cell Layer, WM = White Matter Tract. A. Control patients show SNCA and HOOK3 protein in the granule cell layer. B. Texas red image of SNCA in the Granule cell layer of control patients. C. MSA patients show loss of HOOK3 and SNCA in the granular cell layer. D. Texas red image of MSA patients show a loss of SNCA in the granule cell layer and the presence of SNCA in the white matter tracts.
Figure 14. ICC analysis of HOOK and SNCA the Granule Cell Layer (GCL) in MSA cerebellum. HOOK3 was resolved using DAB (brown) and SNCA was resolved using AP (red); PC = Purkinje Cell, ML = Molecular Layer, GCL = Granule Cell Layer, WM = White Matter Tract. A. Control patient shows SNCA and HOOK3 protein in the granule cell layer. B. MSA patient shows loss of HOOK3 and SNCA in the granular cell layer. Staining was measured using Imagej and analyzed using a t-test. MSA patients showed a 54% reduction in SNCA staining ($p<0.001$). MSA patients also showed 24% reduction in HOOK3 staining ($p=0.012$), N=5, Error bars = 1 S.E.

White matter tracts were also evaluated for changes in expression of HOOK3 and SNCA. In control patients, only HOOK3 appeared in the white matter tracts (Figure 15a). In MSA patients SNCA and HOOK3 appear to co-localize in the white matter tracts (Figure 15b). ImageJ was used to separate the red and brown colors using the color deconvolution tool. In control patients, HOOK3 was
single labeled in 2.5% of the white matter tract. In MSA patients, the majority of HOOK3 double labeled with SNCA covering 2.6% of the white matter tract.

Figure 15. High magnification of two examples of ICC-stained paraffin-embedded tissue from a control and MSA cerebellum. HOOK3 was resolved using DAB (brown) and SNCA was resolved using AP (red); A. Control patients show HOOK3 protein in the white matter tracts B. MSA patients double labeling of HOOK3 and SNCA in the white matter tracts. Image analysis of singly labeled SNCA shows an increase in cells labeled with SNCA in patients (p=0.03). Cells singly labeled with HOOK3 had significantly higher expression in control patients (p<0.001). MSA patients show a majority of cells stained for HOOK3 and SNCA (p<0.001), N=3.

HOOK3 Co-localizes with MBP in the White Matter Tracts and the Granule Cell Layer of the Cerebellum

Using ICC methods, HOOK3 and MBP staining were evaluated in control and MSA patients. It appeared that HOOK3 stained in-between the granule cells and around Purkinje cells of control patients (Figure
Using Texas red fluorescence to better resolve MBP, it shows co-localization of MBP and HOOK3 in-between cells (Figure 16 C & F). Staining in MSA patients showed fragmentation of MBP and a loss of HOOK3 in-between granule cells. Using an overlay of HOOK3 in brightfield and MBP in Texas red, it appeared the remaining tissue was double labeled. MBP staining decreased from 21.3% in controls to 9.4% of the granule cell layer in MSA patients. HOOK3 staining decreased from 45.8% in controls to 32.9% of the granule cell layer in MSA patients. Cells double labeled with HOOK3 and MBP decreased from 3.8% in controls to 0.49% of the granule cell layer in MSA patients.
Figure 16. High power images of two examples of control and MSA granule cell layer.
Figure 16 - Continued

HOOK3 was resolved using DAB (brown) and MBP was resolved using AP (Texas red). PC = Purkinjie Cell, GCL = Granule Cell Layer. Control patients show diffuse labeling of MBP and HOOK3 protein in the granule cell layer (A,B & C). A. Brightfield highlights HOOK3 in-between the Granule cells and Purkinjie cells. B. Texas red identifies MBP in locations approximate to HOOK3 staining. C. Overlay of the brightfield and texas red images shows co-localization of MBP and HOOK3. MSA patients appear to lose both HOOK3 and MBP in the granular cell layer (D,E &F). D. Brightfield light shows a limited amount of HOOK3 in the granule cell layer. E. Texas red identifies MBP in locations approximate to HOOK3 staining. F. Overlay of the brightfield and texas red images shows co-localization of MBP and HOOK3 in the remaining tissue of patients. MBP staining decreased from 21.3% in controls to 9.4% of the granule cell layer in MSA patients. HOOK3 staining decreased from 45.8% in controls to 32.9% of the granule cell layer in MSA patients. Cells double labeled with HOOK3 and MBP decreased from 3.8% in controls to 0.49% of the granule cell layer in MSA patients.

Analysis of the white matter tracts showed a decrease in MBP and HOOK3 (Figure 17). In control patients, HOOK3 appeared best in rounded cell bodies. MBP stained the entire field including the areas of HOOK3. In MSA patients, the area of MBP staining decreased from a mean of 53% to 24% of the white matter tract (p=0.0002). HOOK3 staining also decreased from a mean area in control patients of 6.1% to 3.9% in MSA patients (p=0.043). Despite the decrease in staining, identification of HOOK3 and MBP was still possible.
Figure 17. High power images of two examples of control and MSA white matter tracts.
Figure 17 - Continued

HOOK3 was resolved using DAB (brown) and MBP was resolved using AP (Texas red). Control patients show cell bodies labeled with MBP and HOOK3 protein (A,B & C). A. Brightfield shows HOOK3 staining in cell bodies located in the white matter tracts. B. Texas red image shows diffuse staining for MBP in the white matter tracts. C. Overlay of brightfield and texas red images shows double labeling of HOOK3 and MBP in cell bodies located in the white matter tracts. MSA patients appeared to lose cell bodies containing HOOK3 and MBP (D,E & F). D. Brightfield showed HOOK3 located in the white matter tracts. E. Texas red shows fragmented expression of MBP. F. Overlay of brightfield and texas red images showed HOOK3 and MBP co-localize in the white matter tracts. Analysis of staining showed a significant decrease in MBP and HOOK3 (p=0.0002 & 0.043) N=4.

Discussion

HOOK3 appeared to double label with MBP confirming HOOK3 in Oligodendrocytes. Staining occurred in both myelinated fibers passing through the granule cell layer and in the white matter tracts. HOOK3 and SNCA staining in the granule cell layer of cerebellum in MSA patients decreased by 24% and 54% respectively. In control patients HOOK3 appeared to travel in long strands through the granule cell layer. SNCA appeared to be co-localized with HOOK3 along these strands in round formations. HOOK3 and SNCA staining in the white matter tracts of MSA patients appeared to be solely in cell bodies. Control patients only stained for HOOK3 in the white matter tracts.

HOOK3 is dependent on microtubules. As stated previously, HOOK3 uses microtubules to traffic endocytotic bodies in the cell, and to stabilize the Golgi complex. HOOK3 also uses microtubules for regulating neurogenesis and binding the centromere (Xuecai et al.,
2010). Given the ability of SNCA aggregation to hydrolyze microtubules, it is likely that HOOK3 normal functions are being disrupted. This could result in fragmentation of the Golgi, or problems with the proteasome-lysosome complex as seen in other diseases.

The reduction of HOOK3 in the granule cell layer and the decrease of HOOK3 protein with SNCA treatment in C6 cells suggest that the regulation of HOOK3 may be tied to the expression of SNCA. SNCA is already known to cause blockage of the lysosome when it is misfolded (Cuervo et al., 2004). Batten’s disease (juvenile neuronal ceroid lipofuscinosis) is a lysosomal storage disease causing the accumulation of lipofuscins in body tissues. Defects in this disease result in problems with the intercellular trafficking functions of HOOK1 (Luiro et al., 2004, Kama 2007). These observations suggest a common relationship with the lysosome, SNCA and HOOK3. Further research is necessary to determine if SNCA is interfering with normal HOOK3 function.

**Conclusion**

HOOK3 co-localizes with SNCA in the granule cell layer of control patients. Staining for HOOK3 appeared to occur in spaces between granule cells. HOOK3 and SNCA decrease in protein expression in the granule cell layer of MSA patients. Interestingly, as HOOK3 and SNCA
decrease in the granule cell layer, HOOK3 co-localizes with SNCA in the white matter tracts of MSA patients.

To determine what cell type HOOK3 occurs in, tissue was double labeled with HOOK3 and MBP. HOOK3 co-localizes with MBP in the granule cell layer of control and MSA patients. HOOK3 also co-localized with MBP in cell bodies located in the white matter tracts. Both HOOK3 and MBP decreased in expression in the white matter tracts of MSA patients.

Collectively this suggests that HOOK3 protein has a relationship with MSA. HOOK3 expression appears to change due do the presence of SNCA and the loss of oligodendrocytes. Further research is necessary to understand how HOOK3 interacts with SNCA.
CHAPTER VII
CONCLUSION

C6 cells treated with Retinoic Acid to induce an oligodendrocyte-like phenotype are useful in modeling synucleopathies. C6 cells were able to take up wild-type SNCA and a mutant SNCA known to PD, A53T. Uptake did not appear to change due to the amount of time cells were in culture. Cells also demonstrated changes in expression of HSC70, a protein known to accumulate in GCIs, in a dose and time dependent manner.

In addition to uptake of SNCA, cells appeared to truncate the SNCA protein. In a study using SNCA with a C-terminus truncation, apoptosis was shown to occur in glial cells (Stefanova et al., 2001). In the present study, treatment with SNCA showed an increase in trypan blue cell death. Changes in the protein expression of CASP3 and other apoptosis-related proteins, PDCD4 and FAM3B were also found to change in a dose and time dependent manner. These changes in apoptotic proteins appeared to have occurred faster at higher concentrations of SNCA treatment, followed by a decrease in expression. This change in apoptosis-related protein expression was similar to the change in expression of HSC70.

DNA Microarrays are useful in identifying expression changes related to SNCA treatment. Using our C6 cell model several genes not
previously described in MSA were identified. To follow up, one gene of interest, HOOK3 was examined for changes in protein expression. HOOK3 was found to decrease in a dose and time dependent manner when cells were treated with SNCA.

To determine if HOOK3 plays a role in MSA, cerebellar tissue was examined for SNCA and HOOK3 expression. HOOK3 and SNCA appeared to co-localize in the granule cell layer of control patients, and in the white matter tracts of MSA patients. The expression of HOOK3 and SNCA in the granule cell layer appeared to decrease in MSA patients when compared to control patients. When taken together with SNCA induced changes in C6 cell HOOK3 expression, it appears that HOOK3 may play a role in MSA.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is a database of direct and indirect protein interactions (STRING-db.org, Jensen et al. 2009). The data are compiled and scored for confidence in the interactions using textmining, curated databases, direct experiments, co-expression and gene fusions. Using this database to analyze the genes described in this paper, it appears that many are involved with the AKT pathway, PKC-delta and apoptosis (Figure 18).
Figure 18. Simplified interaction map of genes influenced by SNCA in C6 cells. STRING, a database for known and predicted protein-protein interactions, identifies potential interactions based on evidence collected from several databases. Genes identified in this dissertation are colored, linking genes are grey. Intensity of lines linking proteins represents the confidence associated with an interaction based on the curated databases. It appears that the genes of interest are clustered around the AKT pathway.

PI3K, Phosphatidylinositol 3-kinase, is known to accumulate in MSA (Wenning et al. 2008). It also has been shown to regulate expression of HSC70 (Bański et al. 2010). IRS2 identified in the DNA
microarray has been shown to regulate the expression of PI3K (Shaw, 2001). Upregulation of IRS2 could be one mechanism regulating HSC70.

IRS2 through PI3K could also regulate FAM3B. Beta cells treated with glucose showed an increase in FAM3B expression. Treatment with glucose and inhibitors of PI3K, CREB and PKC-alpha, also inhibit expression of FAM3B (Wang et al., 2008).

Control of PDCD4 can be accomplished through several mechanisms. The up-regulation of phosphorylated Akt appears to down-regulate PDCD4 in cancer cells (Fasan et al. 2011). PDCD4 expression appears to be up-regulated by PKC-delta (Nakashima et, al. 2010). It has been shown that PKC-delta is down-regulated in turn by SNCA (Jin et al. 2011).

Interestingly down-regulation of PDCD4 seen at the higher dosage and duration of treatment could explain the increase in expression of ERBB3. As shown in chapter 3, ERBB3 had increased expression at the higher concentration of SNCA treatment. ERBB3 has been shown to function as a regulator of the Smad pathway via SOS and GRB2 (Goodearl et al. 2001). PDCD4 has been shown to be involved with TGF-beta induced apoptosis via the Smad pathway (Davis et al., 2008). This suggests a potential relationship between these two proteins.
Intercellular trafficking by HOOK3 may also play a role in the AKT-mediated apoptosis. The HOOK family of proteins are known to function in the endocytic trafficking mechanism FTS/Hook/p107(FHIP) along with SNARE complexes (Xu et al. 2008). SNARE complexes with HSC70 and LAMP-2a for importation of SNCA into the lysosome (Cuervo et al. 2004). FTS (AKTIP) was shown to control phosphorylation of AKT and regulate apoptosis (Remy and Michnick 2004). However, expression of HOOK3 does not resemble the expression of CASP3, HSC70, PDCD4 or FAM3B. HOOK3 expression appeared to decrease in a dose and time dependent manner. The decrease in HOOK3 expression could be an attempt for the cell to shift traffic from the golgi to the lysosome complex increasing clearance of SNCA, rather than an activation of apoptosis.

However after 2 days of treatment the highest dosage of SNCA was the only group with a significant decrease in HOOK3, which is similar to the apoptosis-related proteins discussed above. Secondly a shift in the peak of CASP3 and related protein expression to the lower concentrations of SNCA may be related to the decrease of HOOK3 in the lower treatment groups of SNCA. This suggests that regulation of the AKT pathway may be dependent on not only the conditions of endocytic traffic but also expression of apoptotic proteins.
Overall this study establishes a new cell model for MSA. This model can be used to identify new target proteins of interest using various techniques. It also proposes how SNCA may influence cell death and intercellular trafficking. Further research on how SNCA is involved and disrupts intercellular trafficking is necessary to understand how apoptosis is chronically induced in MSA patients.
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