EFFECTS OF PRAMlintide ON MITOCHONDRIAL DYNAMICS AND HEALTH IN THE ALZHEIMER’S DISEASE APP/PS1 MOUSE MODEL

A thesis submitted to the Kent State University Honors College in partial fulfillment of the requirements for Departmental Honors

by

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<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>APPswe/PS1</td>
<td>Double transgenic Alzheimer’s disease mouse model</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>Aβ</td>
<td>Amyloid β</td>
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<tr>
<td>COX4</td>
<td>Cytochrome C oxidase subunit 4 (mitochondrial loading control)</td>
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<td>DLP1</td>
<td>Dynamin like protein 1 (mitochondrial fission)</td>
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<td>Fis1</td>
<td>Fission protein 1 (mitochondrial fission)</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3 phosphate dehydrogenase (tissue loading control)</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
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<td>MFN1</td>
<td>Mitofusion 1 (mitochondrial fusion)</td>
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<td>MMP</td>
<td>Mitochondrial membrane potential</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>NFTs</td>
<td>Neurofibrillary tangles</td>
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<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy protein 1 (mitochondrial fusion)</td>
</tr>
<tr>
<td>PRAM</td>
<td>Pramlintide (rodent derived analogue of amylin)</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>Super oxide dismutase</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes melitus</td>
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ACKNOWLEDGMENTS

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CHAPTER 1: INTRODUCTION

Background on Alzheimer’s Disease:

Alzheimer’s Disease (AD) is characterized by progressive neurodegeneration and cognitive decline and is the leading cause of dementia world-wide. It is estimated that 60-80 percent of all dementia cases are Alzheimer’s type (Alzheimer’s Association, 2016). Typically, AD is diagnosed by its characteristic cognitive symptoms including but not limited to, progressive memory loss, difficulty planning and completing everyday tasks, as well as disruptions in mood and sleep. Although the exact cause of AD is unknown, several risk factors have been associated with an increased chance of developing AD. These risk factors include but are not limited to, advanced age, obesity and diabetes, and several inheritable genomic mutations. Advanced age is currently the best-known risk factor for developing AD with most cases occurring in patients over the age of 65 (Alzheimer’s Association, 2016). Although most AD cases present later in life, there are a small percentage of AD cases which present early in life. Early onset familial AD has been linked to several different mutations to the amyloid beta precursor protein (APP) gene. The most notable of these mutations is the Swedish mutation which results in increased proteolytic cleavage of APP to form amyloid β (Marques et al., 2003) and the production and deposition of the classic pathological entity observed in the brains of AD patients, amyloid-beta plaques. Appearance of plaques and the other pathological entity,
tangles, typically begins in memory related areas such as the hippocampus and progresses throughout the limbic system and eventually the frontal lobe of the cortex (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011) The development and progression of pathology deposition tend to mimic the onset of symptoms and progression of the disease from affecting memory function to later affecting emotional regulation and executive function later in the disease (Serrano-Pozo et al., 2011). For this reason, plaques and tangles are hypothesized to be the main cause of cellular degeneration in AD (Hardy & Selkoe, 2002).

**Pathology:**

AD is characterized by the accumulation of extracellular amyloid β (Aβ) plaques, as well as intracellular neurofibrillary tangles (NFTs). Aβ is an aggregative protein fragment which is cleaved from the amyloid β precursor protein. Proteolytic cleavage of APP to form Aβ is mediated by the enzymes β and γ secretases. (Brien & Wong, 2011; Zhang, Thompson, Zhang, & Xu, 2011). NFTs are aggregates of the hyperphosphorylated protein tau which is a microtubule associated protein. Under normal circumstances, tau is involved in microtubule assembly and stabilization, however in AD and other neurodegenerative diseases hyperphosphorylation of tau prevents its interaction with tubulin destabilizing microtubules. (Iqbal, Liu, Gong, & Grundke-Iqbal, 2011).

Of relevance, while AD pathology is hypothesized to be the main cause of wide spread neuronal cell death and root cause of the functional deficits observed in this
disease, several lines of evidence suggest that pathology is not the only cause and could potentially be a result of a more fundamental insult rather than the cause of the disease (Ataya, Himohama, & Hiba, 2001; Smith, Perry, Rottkamp, Nunomura, & Raina, 2000). For example, epidemiological studies have shown increased risk of developing AD in patients with type 2 diabetes mellitus (T2DM), obesity or metabolic syndrome (Adler et al., 2014; Grizzanti, Lee, Camins, Pallas, & Casadesus, 2016). In addition, aging, a state highlighted by metabolic hypofunction is the main risk factor for AD. Following this line of evidence, several groups have implicated metabolic dysfunction in the pathogenesis of AD (Butterfield D., Domenico Di, & Barone, 2015) Importantly, metabolic dysfunction is closely tied to the major energy producing organelles, the mitochondria. Mitochondrial dysfunction is known to cause a state of oxidative stress in cells which we believe may be the fundamental insult that precedes pathology formation (Adler et al., 2014; Smith et al., 2000).

**Mitochondria and Oxidative stress:**

Cellular energy metabolism, and more specifically glucose metabolism, is the process of oxidizing glucose in the presence of oxygen to produce the energy carrying molecule adenosine triphosphate (ATP). During this process, energy is released from the breakdown of glucose by a series of enzymes in two major pathways called glycolysis, and the Krebs cycle. This energy is subsequently used to create an electrochemical gradient which then drives the production of ATP in the electron transport chain. This
process, called oxidative phosphorylation, is the main process of energy production and occurs in an organelle called the mitochondria.

Mitochondria have a structure which consists of an outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) separated by intermembrane space. Five major protein complexes (complexes 1-4, and ATP synthase) imbedded in the IMM make up the oxidative phosphorylation system. Complexes 1-4 transfer the electrons of intermediates from glycolysis and the Krebs cycle, NADH and FADH$_2$, through a series of redox reactions to pump protons across the IMM into the intermembrane space. This electrochemical proton gradient is known as mitochondrial membrane potential, and is harvested by ATP synthase to catalyze the phosphorylation of ADP to form ATP. Maintenance of mitochondrial membrane potential is critical for the function of these organelles and their ability to meet the energy demands of the cell (Hüttemann et al., 2008).

During the process of oxidative phosphorylation, oxidative byproducts known as reactive oxygen species (ROS) are also produced. ROS have many functions in the cell including acting as signaling molecules under controlled circumstances. Importantly, however, they are also capable of damaging cellular components lipids, proteins, and nucleic acids and impair cellular function if not properly neutralized by endogenous antioxidant enzymes or antioxidants found in the diet (Hussain et al., 2015; Wang et al., 2014). The direct byproduct of oxidative phosphorylation is the superoxide anion free radical. Superoxide anions are converted to hydrogen peroxide, a less reactive oxidative byproduct, by the enzyme superoxide dismutase (SOD). Mitochondrial membranes are
permeable to the small hydrogen peroxide molecule and it can diffuse into the cytosol. In the cytosol, hydrogen peroxide can react with transition metal centers to form the much more reactive hydroxyl ion free radical (Hussain et al., 2015). Thus, hydrogen peroxide must be neutralized by the enzymes catalase and glutathione peroxidase.

The generation of ROS and the antioxidant processes that clear it are normally kept in a balanced equilibrium preventing excessive damage to cellular structures. However, an increase in ROS production or a decrease in antioxidant defense can shift the equilibrium to favor ROS accumulation and cells enter a state of oxidative stress (Hussain et al., 2015; Wang et al., 2014). Interestingly, mitochondria are both the primary producers and targets of ROS. Superoxide anion and hydrogen peroxide produced in the mitochondria can oxidize lipids of the mitochondrial membrane and attack mitochondrial proteins and DNA (Andersen, 2004; Moreira, Nunomura, et al., 2009). When mitochondria are damaged in this way, their ability to produce ATP is diminished and they produce ROS at a higher rate creating a downward spiral eventually leading to cell death (Bolisetty & Jaimes, 2013). Changes in oxidative equilibrium produced by chronic exposure to free radicals and loss of mitochondrial function are known aspects of the aging process and hypothesized to be a major component of why aging is the principal risk factor for AD development (Smith et al., 2000; Zhu, Lee, Perry, & Smith, 2007).
Oxidative Stress in AD:

Oxidative stress has been implicated in AD and other neurodegenerative diseases as having an important role in cell death and degeneration. However, the ROS that are responsible for oxidative stress have very short biological half-lives and are difficult to measure directly, so to detect oxidative stress, more stable oxidized markers must be quantified (Wang et al., 2014). As previously stated, ROS can oxidize all major biomolecules and those oxidized lipids, proteins or nucleic acids can serve as markers of past oxidative stress events. (Wang et al., 2014) Indeed, tissue from AD patients has been found to have significantly higher levels of oxidative stress markers including lipid peroxidation, protein oxidation, nucleic acid oxidation, and reduced activity of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase than age matched control patients. (Wang et al., 2014) Additionally, multiple studies have shown that oxidative stress precedes the formation of hallmark pathologies amyloid β plaques, and NFTs, suggesting that oxidative stress may be a cause of pathogenesis rather than a result of it. (Andersen, 2004; Ataya et al., 2001; Wang et al., 2014) Under normal conditions, cells are kept in a healthy redox state by balancing the production of ROS and the antioxidant processes that clear them. In AD and other neurodegenerative diseases however, changes in cellular processes allow that balance to tip in the favor of ROS. The exact cause of this change is a subject of debate; however, several groups argue that mitochondrial dysfunction is involved. (Reddy & Beal, 2008; Wang et al., 2014)
Mitochondrial dysfunction in AD:

Given that mitochondrial dysregulation has a role in AD pathogenesis, the cause of such dysregulation has been a heavily studied subject. An aspect of mitochondrial structure that is unique is the presence of its own genomic DNA (mtDNA). Many mitochondrial proteins are in fact coded by mtDNA rather than nuclear DNA, and mtDNA, like any nucleic acid, is susceptible to mutation. Given that the most significant risk factor for developing AD is age, it has been hypothesized that mutations to mtDNA accumulated during aging result in dysfunctional mitochondrial proteins and increased production of ROS (Lin & Beal, 2006). As previously stated, increased production of ROS and oxidative stress may target mitochondria themselves leading to a downward spiral of mitochondrial dysregulation.

While mtDNA mutations may account for mitochondrial dysfunction associated with aging, they do not explain the mitochondrial dysfunction and oxidative stress reported in early onset familial AD (Wang et al., 2008). However, several studies suggest that Aβ targets mitochondria and alters their function in a way that promotes oxidative stress and metabolic dysfunction (Manczak et al., 2006; Reddy & Beal, 2008; Wang et al., 2008). It is known that Aβ localizes to the mitochondrial membrane interfering with mitochondrial function and the transport of mitochondrial proteins (Reddy & Beal, 2008). In vitro studies have shown that Aβ induced protein transport deficiencies are associated with decreased mitochondrial membrane potential and increased ROS production (Sirk et al., 2007). In human neuroblastoma cells expressing wild type and Swedish APP plasmids, Aβ overproduction was shown to be at least partially responsible for impaired
mitochondrial dynamics and the oxidative stress that results in early onset familial AD (Wang et al., 2008).

Mitochondria are dynamic organelles which continually fuse and divide to accommodate a cell’s energy demand and mitigate damage to mitochondrial structure (Youle et al., 2012). Fission events allow mitochondria to segregate damaged portions and eliminate them by the process of autophagy, while fusion events combine multiple mitochondria to improve their efficiency (Youle et al., 2012). These fission and fusion events exist in a dynamic equilibrium and are mediated by a family of large GTPase proteins. Fission events which divide mitochondria are mediated by the interaction between mitochondrial membrane protein Fis1 and dynamin like protein 1 (DLP1). Fusion events are mediated by optic atrophy protein 1 (OPA1) and mitofusion 1 and 2 (MFN1/2) (Yoon, Krueger, Oswald, & Mcniven, 2003; Youle et al., 2012). The majority of DLP1 is inactive and found in the cytosol, however, phosphorylation of DLP1 at serine residue 616 facilitates its recruitment to the mitochondrial membrane where it is actively involved in fission events (Wang et al., 2009).

Under normal conditions, mitochondrial fission and fusion dynamics are tightly regulated and balanced. In AD however, the mitochondrial dynamics equilibrium is shifted in favor of fission producing short fragmented mitochondria, as well as abnormal mitochondrial distribution (Eckert, Schmitt, & Götz, 2011; Wang et al., 2008, 2009). Several studies have confirmed that AD neurons are populated with short fragmented mitochondria and that synaptic areas are devoid of mitochondria compared to controls (Moreira, Carvalho, Zhu, Smith, & Perry, 2009). It is well documented that a balanced
equilibrium between fission and fusion is vital for mitochondrial function and
distribution. Lack of fusion or excessive fission leads to changes in mitochondrial
distribution and mitochondrial membrane potential causing cellular metabolism deficits
and increased ROS production (Chen, Chomyn, & Chan, 2005; Wang et al., 2009).

Following this line of reasoning, it is apparent that mitochondrial dysregulation,
specifically excessive fission and irregular distribution, have a role in the metabolic
deficits and oxidative stress observed in AD. Interestingly, it has also been noted that
insulin resistance associated with T2DM causes mitochondrial dysfunction and oxidative
stress. In vitro studies have shown that insulin resistance results in excessive
mitochondrial fission and increased ROS production (Anello et al., 2005).

**Metabolic dysfunction and Amylin in AD:**

Interestingly, aberrant insulin signaling associated with T2DM leads to excessive
mitochondrial fission and fragmentation in the liver, muscles, and brain of T2DM
patients (Grizzanti, Lee, Camins, Pallas, & Casadesus, 2016). In vitro studies have shown
that insulin resistance results in excessive mitochondrial fission and increased ROS
production (Anello et al., 2005). This fragmentation results in impaired glucose
metabolism, reduced ATP production, and increased oxidative stress, (Grizzanti et al.,
2016; Jheng et al., 2012) similar to what is observed in AD brains. As previously stated,
epidemiological studies have found T2DM to be a major risk factor for developing AD.
(Butterfield D. et al., 2015) Given this comorbidity, it is not surprising that AD and
T2DM share many commonalities that contribute to their pathogenesis. AD and T2DM are both associated with metabolic dysfunction, mitochondrial dysregulation, oxidative stress and advanced age. Also important, is the fact that abnormal insulin signaling in the brains of AD patients is very similar to the abnormalities observed in the periphery of T2DM patients (Adler et al., 2014). Because of the parallels in the pathogenesis of these two diseases and their common insulin signaling related metabolic dysfunction, AD has been considered by some a type 3 diabetes (de la Monte & Wands, 2008).

The exact cause of aberrant insulin signaling in AD is unknown, however recent studies show that in addition to dysregulation of insulin signaling and brain resistance, other metabolic hormones also show alterations (Adler et al., 2014; Grizzanti et al., 2016). In this regard, soluble amylin levels are reduced in mild cognitive impairment (precurso of AD) and in AD patients (Adler et al., 2014). Amylin is a peptide hormone that is blood brain barrier (BBB) permeable and is co-secreted with insulin by the pancreatic beta cells. Amylin functions to increase insulin sensitivity in target cells by inhibiting glucagon secretion, inducing satiety and slowing gastric bypass (Adler et al., 2014). As insulin resistance becomes progressively more severe in T2DM patients, the pancreas secretes increasing amounts of insulin and amylin as a compensatory mechanism resulting in a state of hyperinsulinemia and hyperamylinemia (Grizzanti et al., 2016). An interesting property of human amylin in addition to its BBB permeability is that like Aβ, it aggregates to form amyloid fibrils and plaques (Adler et al., 2014). In the state of hyperamylinemia that results from T2DM, a recent hypothesis is that human amylin aggregates in the brain and seeds future Aβ plaque formation (Grizzanti et al.,
A recent study did in fact find that the brains of AD patients, with and without diabetes, contained amylin plaques that were distinct from Aβ plaques with similar toxic properties (Jackson et al., 2014). However, like in AD, whether pancreatic cell loss and further worsening of T2DM is related to amylin plaque deposition/toxicity or the loss of native amylin function is still under debate (Akter et al., 2010). To this end, studies show that administration of recombinant (rat-derived) amylin, pramlintide (PRAM) improves insulin sensitivity and reduces T2DM insulin dependency and severity (Pullman, Darsow, & Frias, 2006). In addition, a few studies carried out in T2DM patients show that PRAM treated patients show reduced levels of oxidative stress (Ceriello, Lush, Darsow, Piconi, & Maggs, 2008). Importantly, the effectiveness of PRAM in T2DM is paralleled by its effectiveness in ameliorating cognitive function and oxidative stress parameters in animal models of aging and AD (Adler et al., 2014). However, how PRAM works to induce such improvements is completely unknown and the study goal of this thesis. In this regard, given the ability to reduce oxidative stress in both T2DM and AD and the tight relationship between metabolic status, mitochondrial function, and disease in both T2DM and AD, we hypothesize that pramlintide may function to rescue aberrant insulin signaling and ameliorate mitochondrial dysfunction associated with AD. To test this hypothesis, multiple aspects of mitochondrial function, which are known to be altered in the brains of AD patients, were assessed in animal and cellular models of AD.
CHAPTER 2: METHODS

AD mouse model, treatment, and tissue collection:

In this study, wild type (WT) mice were compared with PRAM treated and untreated transgenic mice. APPswe/PS1 double transgenic mice (Jackson Laboratories, ME), a well validated model of AD expressing mutant forms of APP and PS1 associated with early onset AD, and wild type mice were bred and housed in our animal facility (Kent State University, OH). Transgenic mice were administered either Pramlintide (GenWay Biotech, CA) at 6µg/day or saline via osmotic pump (Alzet, CA) infusion for 18 weeks. All mice were sacrificed at 8 to 10 months. Hippocampal tissue was collected and either frozen immediately or homogenized in a protease and phosphatase inhibitor cocktail buffer (Millipore, MA). PMSF (Cell Signaling, MA) was also added at a concentration of 1mM to the homogenization buffer as an additional protease inhibitor. Prior to analysis, all protein samples were assayed for total protein concentration using the BCA protein assay (Pierce, MA). All protein samples were stored at -80°C until use and thawed on ice before analysis.

Mitochondrial Isolation:

Mitochondria were isolated from frozen hippocampal tissue. Isolating mitochondria allowed a direct comparison of mitochondrial protein expression to
minimize error. Mitochondria were isolated using a mitochondrial isolation kit for tissue (Pierce, MA) per the manufacturers protocol. Buffers and reagents were scaled appropriately for the quantities of tissue used. Isolated mitochondria were collected as a pellet and snap frozen at -80°C to aid with homogenization. Mitochondrial pellets were then homogenized using the same homogenization buffer as whole tissue samples and stored at -80°C.

**Western blot analysis:**

Homogenized total hippocampal and mitochondrial protein samples were probed for various mitochondrial dynamics and function related proteins by western blotting. Total hippocampal protein homogenates were resolved using denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE). The gels used to resolve these samples ranged from 10% to 15% polyacrylamide depending on the size of the proteins to be quantified, and were run at 120 volts. 10% gels were used when probing for larger proteins (80-120 kDa), and 15% gels were used when probing for smaller proteins (10-50 kDa). Mitochondrial protein samples were resolved using 12% polyacrylamide gels or 10-20% precast gradient gels (BioRad, CA) at 100 volts. After SDS-PAGE resolution, proteins were transferred overnight at 30 volts and 4°C to immobilon PVDF immunoblot membranes (Millipore, MA).

After transfer, all immunoblots were blocked in 10% milk solutions made in tris buffered saline tween 20 (TBS-t for 1 hour at room temperature before placing them in
primary antibody solutions overnight at 4°C. Horseradish peroxidase (HRP) enzyme linked secondary antibodies were used at 1:1000 (antibody:TBS-t) dilutions and incubated at room temperature for 1 hour. After primary and secondary antibody incubations, immunoblots were washed in TBS-t three times (10 minutes each). All primary antibody solutions were prepared in TBS-t at dilutions ranging from 1:500 to 1:25,000 (antibody:TBS-t). Western blots were probed for the mitochondrial dynamics related proteins DLP 1 (1:2000, Abcam, UK), Fis 1 (1:1000, Santa Cruz, TX), OPA 1 (1:2000, Abcam, UK), Mfn 1 (1:500, Millipore, MA), which were normalized to either GAPDH or Cox 4 as loading controls. GAPDH (1:25,000, ), a housekeeping protein involved in glycolysis, was used as a loading control for full hippocampal protein homogenates. Cox4 (1:10,000, Millipore, MA), a subunit of the electron transport chain complex 4 and standard mitochondrial loading control (Wang et al., 2008), was used as the loading control for isolated mitochondrial protein samples.

After appropriate washing and incubation with primary and secondary antibodies, chemiluminescent images were obtained. To obtain chemiluminescent images, western blots were incubated in an enhanced chemiluminescence (ECL) HRP substrate (Millipore, MA) for 1 minute then developed using a chemiluminescence developer (Syngene, MD). All chemiluminescence images were quantified using the program ImageJ (open source, public domain). All western blots were repeated at least two to three times, unless otherwise stated, to confirm the results and representative images are reported in the results section.
Cell culture and treatments:

SHSY-5Y neuroblastoma cells (ATTC, VA) were grown and maintained in 10% FBS DMEM growth media (Gibco, CA) at 37°C and 5% carbon dioxide in a cell culture incubator. Neuroblastoma cells were split 1:5 when they were approximately 70-80% confluent. After 10-12 passages, cells were discarded and fresh SHSY-5Y cells were revived.

Mitochondrial membrane potential evaluation using JC-1 dye:

Mitochondrial membrane potential (MMP) was evaluated by performing a colorimetric assay using JC-1 dye (Cayman Chemicals, MI). The fluorescence emission of JC-1 is very specifically dependent on MMP which makes it an excellent probe for this experiment. JC-1 is a membrane permeable compound which is selectively taken up by the mitochondria of cells. In healthy cells with high MMP JC-1 is taken up by mitochondria where J-complexes form and fluoresce red. In cells with low MMP JC-1 remains in the cytoplasm and complexes do not form resulting in green fluorescence. In this study, 20,000 SHSY-5Y cells/well were seeded in a collagen coated black 96 well plate of good optical quality for fluorescence measurements. Cells were allowed to attach overnight before treatments began. Cells were divided into four treatment groups; untreated control, hydrogen peroxide insult only, and hydrogen peroxide insult with pramlintide treatment at two doses. Pramlintide treated cells received pramlintide at either 400nM or 1000nM for 24 hours. All cells were stained by adding JC-1 dye to the
existing media at a final concentration of 2.0µM and incubated for 20 minutes before reading the fluorescence signal. Red and green fluorescence emission signals were measured independently using a temperature controlled microplate reader at 37°C with excitation and emission wavelengths of \( \lambda_{Ex}/\lambda_{Em} = 545/620 \) and \( \lambda_{Ex}/\lambda_{Em} = 480/535 \) for red and green signals respectively. All treatment groups except control cells were treated with 5.7mM hydrogen peroxide for 5 minutes and a second fluorescence signal reading was performed. The ratio of red fluorescence signal to green fluorescence signal was calculated for both measurements and the change in red to green ratio from before and after hydrogen peroxide treatment was calculated. The change in red/green ratio was used to semi-quantitatively measure the change in MMP after hydrogen peroxide insult compared to control cells. A high ratio of red to green signal is indicative of healthy cells with high MMP and a low ratio of red to green signal compared to control cells is indicative of low MMP in unhealthy cells.

**Statistical analysis**

Differences between experimental groups were tested for statistical significance by performing either one way analysis of variance (ANOVA) where there were three experimental groups or the student’s T test for two experimental groups. Differences were considered statistically significant when \( p<0.05 \). Post hoc LSD analysis was also performed when one way ANOVA revealed significant differences between groups. The mean was calculated for each experimental group for western blot quantifications and
MMP calculations. Standard error was used as a measure of variance among these groups. Where appropriate, the grubbs test was used to determine if outliers were statistically significant.
CHAPTER 3: RESULTS

In this study, we demonstrated that several aspects of mitochondrial function and health that are known to be altered in AD are improved upon treatment with pramlintide. Western blot analysis by one way ANOVA revealed that mitochondrial expression levels of Fis1, Mfn1, and Dlp1 were significantly altered between groups. (P=0.034, 0.010, 0.020; F=4.556, 3.935, and 6.515; DF (between groups/within groups) =2/12, 2/8, and 2/14 for Fis1, Mfn1, and Dlp1 respectively). Post hoc analysis by LSD revealed that Fis1, Mfn1, and Dlp1 were significantly increased (p<0.05) in transgenic mice, and that Fis1 and Mfn1 were normalized to wild type levels (p<0.05) in pramlintide treated APP/PS1 mice. Interestingly, active DLP1 recruited to the mitochondria was significantly increased but cytosolic DLP levels were unchanged. No significant changes in Fis1, Mfn1, Dlp1, or OPA1 expression levels were observed in total hippocampal homogenates. Although not significant due to small sample size, Cox4 trended to be reduced in APP/PS1 mice compared to control and normalized in pramlintide treated animals compared to untreated transgenics. This suggests that total mitochondrial density is reduced in APP/PS1 mice and restored in pramlintide treated animals.
Figure 1: Mitochondrial protein expression in hippocampal homogenates relative to GAPDH

A. Fis1 17kDa
   GAPDH 37kDa

B. DLP1 – 82kDa
   GAPDH – 37kDa

C. OPA1 80kDa
   GAPDH 37kDa

D. PGC1 91kDa
   GAPDH 37kDa

E. Cox4 17kDa
   GAPDH 37kDa
Figure 1: **A.** Expression levels of Fis1 relative to GAPDH in hippocampal protein homogenates. Although not statistically significant due to high variability within groups, the trend was reduced Fis1 in hippocampal homogenates. N = 4-8/group. **B.** Expression levels of Dlp1 relative to GAPDH in hippocampal protein homogenates were not changed significantly between treatment groups. N = 4-9/group. **C.** Expression levels of OPA1 relative to GAPDH in hippocampal protein homogenates did not change significantly between experimental groups. N = 4-7/group. **D.** PGC1 expression levels were not significantly changed between treatment groups however the trend shows slightly decreased expression in transgenic mice which is restored to baseline in pramlintide treated transgenic animals. N =3-4/group. **E.** Although not significant due to small sample size, the trend is that Cox 4 expression relative to GAPDH in hippocampal homogenates increased in pramlintide treated APP/PS1 mice compared to untreated APP/PS1 mice suggesting increased total mitochondrial density. N =2-4/group.
Figure 2: Mitochondrial expression of dynamics related proteins in mitochondria isolated from hippocampal tissue relative to Cox4.

**Figure 2:** A. *Mitochondrial Fis1 expression is significantly elevated (P<0.05) in APP/PS1 mice compared to control animals and **significantly reduced (P<0.05) to wild type levels in pramlintide treated APP/PS1 mice. N = 4-6/group. B. *DLP1 expression was significantly (p<0.05) increased in transgenic animals compared to wild type,
however there was no change between transgenic and pramlintide treated animals. N = 5-6/group. C. *Mfn1 expression was significantly (p<0.05) increased in transgenic animals compared to wild type and **significantly reduced (p<0.05) to wild type levels in pramlintide treated animals. N = 3-4/group. D. OPA1 expression in mitochondria did not change significantly between treatment groups. N = 4-6/group.

Mitochondrial membrane potential analysis

Mitochondrial membrane potential, as represented by the relative ratio of red to green fluorescence signal, was evaluated using JC-1 dye in SHSY-5Y cells before and after an oxidative stress event in the presence and absence of pramlintide. In this study, we demonstrate that mitochondrial membrane potential is significantly reduced (p<0.05) by approximately 70% upon oxidative stress insult in SHSY-5Y cells compared to untreated control cells. Pramlintide treatment at 400 and 1000nM significantly protected against mitochondrial membrane potential loss upon oxidative stress insult (P<0.05). Due to time constraints, this experiment has not yet been repeated. Pending confirmation, these finding suggest that pramlintide promotes a healthier mitochondrial population in cells under oxidative stress conditions.
**Figure 3:** Change in mitochondrial membrane potential upon oxidative stress insult

![Graph showing change in mitochondrial membrane potential](image)

**Figure 3:** Percent change in ratio of red to green fluorescence signal in undifferentiated SHSY-5Y neuroblastoma cells after hydrogen peroxide insult. Control cells were untreated and did not receive hydrogen peroxide insult. H₂O₂ cells received only hydrogen peroxide insult, H₂O₂+PRAM400 or PRAM1000 cells were treated with 400nM or 1000nM pramlintide for 24hr prior to hydrogen peroxide insult. *MMP was significantly reduced (p<0.05) by approximately 70% in hydrogen peroxide treated cells compared to control. **MMP in Pramlintide treated cells was not significantly changed compared to control but was significantly increased (P<0.05) compared to hydrogen peroxide treated cells.
CHAPTER 4: DISCUSSION AND CONCLUSIONS

The purpose of this study was to explore a possible mechanism through which the synthetic rodent derived analogue of amylin, pramlintide, ameliorates cognitive deficits and oxidative stress associated with AD. Thus, various aspects of mitochondrial function that are important for mitochondrial health and efficiency were measured in this study. It is widely accepted that deviation from the healthy mitochondrial dynamics equilibrium or distribution can result in a downward spiral of mitochondrial and bioenergetic dysfunction (Youle et al., 2012). To this end, several groups have demonstrated that altered dynamics related protein expression levels dramatically influence these parameters. Wang et al. demonstrated that reduced expression of DLP1 results in elongated mitochondria while knock-down of OPA1 and Mfn1/2, as well as overexpression of DLP1 and Fis1 result in short fragmented mitochondria. Additionally, all of these manipulations, except overexpression of DLP1, resulted in abnormal mitochondrial distribution with decreased neuritic mitochondrial density and aggregation in the soma (Wang et al., 2009).

Given the dependence of mitochondrial dynamics on the expression levels of these proteins, we quantified various mitochondrial dynamics proteins as a measure of mitochondrial dynamics and morphology between pramlintide treated and untreated transgenic mice compared to wild type mice. In this study, we demonstrated that mitochondrial OPA1 expression is unchanged while mitochondrial Fis1, DLP1, and Mfn1
expression are significantly increased in APP/PS1 mice compared to wild type, and that PRAM restores Fis1 and Mfn1 to wild type expression levels. However, PRAM does not restore mitochondrial DLP1 to wild type levels. These findings agree with previous studies which show deviations from the normal fission/fusion equilibrium in AD (Wang et al., 2009). However, the exact changes in dynamics related protein expression is somewhat controversial. Some studies report increased expression of fission proteins but reduced expression of fusion proteins in the brains of AD patients (Wang et al., 2014) whereas others suggest overexpression of mitofusins play a key role (Wu, Zhu, Cao, & Sun, 2014). Interestingly, it has recently been reported that expression levels of fission and fusion proteins change with age in APP/PS1 mice suggesting that a complex system of compensatory mechanisms is at play (Xu et al., 2017). The discrepancies between studies in fission and fusion protein expression levels could be due to differences in the stage of AD represented. Regardless, our findings show a change in the fission/fusion equilibrium in APP/PS1 mice compared to control animals that is at least partially rescued by pramlintide treatment. Specifically, in this study a shift toward mitochondrial fission due to DLP1 and Fis1 upregulation in APP/PS1 mice possibly stimulates a compensatory upregulation of mfn1 to counteract excessive fission. An alternative explanation stems from the recent report that mitofusin upregulation is a necessary event in oxidative stress induced apoptosis (Wu et al., 2014). In this case upregulation of Mfn1 in APP/PS1 cells could indicate neuronal apoptosis in response to oxidative stress which pramlintide rescues. Future studies directed toward the functions of mitofusins in AD could be helpful in determining the exact mechanisms here. In either case, it is clear that
pramlintide restores some dynamics related protein expression levels to normal which likely results in a healthier population of mitochondria.

In addition to altered dynamics related protein expression in AD brain tissue, previous studies have shown abnormal mitochondrial morphology in APP/PS1 mice. It has been reported that mitochondria of APP/PS1 hippocampal neurons adopt what they describe as a “beads on a string” morphology, which appear to be fragmented mitochondria tethered together, as well as abnormal distribution patterns (Trushina et al., 2012). Given the dependence of morphology and distribution on mitochondrial dynamics, the changes in protein expression we observed are likely responsible for the morphological and distribution abnormalities observed by Trushina et al. (2012). Pramlintide may restore mitochondrial populations of APP/PS1 mice to normal morphological patterns by modulating fission and fusion. Mitochondrial health and efficiency is heavily impacted by their shape (Youle et al., 2012) and restoring normal morphologies in AD neurons would likely result in a healthier population of mitochondria. It would be interesting to directly observe mitochondrial morphology in PRAM treated APP/PS1 mice through microscopy studies and this provides an exciting avenue for future study.

Another aspect of mitochondrial health that is reportedly changed in AD neurons is their distribution. Mitochondria are transported within the cell to meet local energy demands and impaired transportation severely impacts the health of a cell. Interestingly, Wang et al. did not report a change in mitochondrial distribution after overexpression of DLP1 expression, but only morphological changes (Wang et al., 2009). Taken together,
this and our findings suggest that PRAM may rescue abnormal mitochondrial distribution as well as some morphological abnormalities by restoring normal levels of the fission and fusion proteins Fis1 and Mfn1. Restoring mitochondrial expression of Fis1 and Mfn1 to wild type levels may at least partially restore the fission/fusion balance to a healthy equilibrium. Again, morphological studies may shed further light into this aspect of mitochondrial health and function.

In addition to the widely reported morphological and distribution changes associated with mitochondrial populations in AD neurons, other aspects of mitochondrial health have also been shown to be altered in AD models. Multiple studies have found mitochondrial membrane potential, a general measure of mitochondrial health and function, to be reduced in AD models including APP/PS1 mice and neuroblastoma cells overexpressing APP and the Swedish mutation variant of APP (Eckert et al., 2011; Wang et al., 2008; Xie et al., 2013). Maintaining mitochondrial membrane potential is absolutely critical for ATP synthesis by oxidative phosphorylation and meeting the energy demands of the cell (Hüttemann et al., 2008). It has also been suggested that a drop in membrane potential is an early event in apoptosis (Gottlieb, Armour, Harris, & Thompson, 2003). Given that loss of mitochondrial membrane potential is almost universally reported in AD models, it could be one of the early factors that contributes to hypometabolism and massive cell loss in AD.

In this study, we demonstrate that under oxidative stress conditions, mitochondrial membrane potential is dramatically reduced and that pramlintide protects against this membrane potential loss. If this is also true in vivo, pramlintide may protect against
neurodegeneration by preventing hypometabolism and apoptosis associated with mitochondrial membrane potential loss. These findings give rise to another exciting avenue of future study. Future in vivo experiments may be conducted to directly measure functional parameters of mitochondria harvested from pramlintide treated and untreated APP/PS1 primary neurons.

Conclusions and Future Directions:

In this study, we demonstrate that pramlintide regulates several aspects of mitochondrial dynamics and health that are known to be impaired in AD and other neurodegenerative diseases. We found that pramlintide, at least partially, restores a proper balance of fission and fusion in these organelles as well as a healthy membrane potential. These findings support our hypothesis and suggest that pramlintide does in fact rescue abnormal mitochondrial dynamics in APP/PS1 mice. By improving mitochondrial dynamics and health, pramlintide may restore proper energy balance, reduce oxidative stress levels, and help prevent cell death associated with AD. The findings of this study along with previous studies suggest that pramlintide has therapeutic potential in the treatment and/or prevention of AD and this impressive therapeutic potential should be explored further in future studies.
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