TARGETING NEURODEGENERATION: IMPLICATIONS OF MITOCHONDRIA-DERIVED OXIDATIVE STRESS IN ALZHEIMER'S DISEASE AND THE USE OF MITOCHONDRIA–TARGETED THERAPEUTICS

by

MELISSA L. YOUNG

(Under the Direction of James L. Franklin)

ABSTRACT

The work presented herein illustrates the dynamic role of mitochondria in age-dependent neurodegeneration and adds to the growing body of work that supports mitochondria-targeted therapeutics as a viable option for oxidative stress-ridden neurological disorders, particularly Alzheimer's disease (AD). The results implicate mitochondria-derived oxidative stress as a prominent mediator of AD pathogenesis prior to and after disease onset. To further elucidate the role of mitochondria-derived oxidative stress in AD progression, the mitochondria-targeted antioxidant MitoQ was used to examine the effect of AD-like pathologies in an Alzheimer's disease mouse model aged well past the manifestation of AD-like pathologies and cognitive impairment. 3xTg-AD female mice were treated for five months with MitoQ *ad libitum* in drinking water starting twelve months after birth. Eighteen-month-old untreated littermate controls exhibited significant cognitive deficiency and AD-like pathology, while MitoQ improved memory retention compared to untreated 3xTg-AD controls. Additionally, MitoQ reduced brain oxidative stress, synapse loss, astrogliosis, microglial proliferation, Aβ

accumulation, caspase activation, and tau hyperphosphorylation. Furthermore, MitoQ increased the lifespan of 3xTg-AD mice compared to that of non-transgenic controls. These findings provide solid evidence of a role for mitochondria-derived oxidative stress in age-dependent neurodegeneration and further highlight the significant influence of mitochondria in AD. Consequently, these results further support the use of mitochondria-targeted therapies for AD treatment.

INDEX WORDS: Alzheimer's disease; oxidative stress; mitochondria; targeted antioxidants; learning and memory; 3xTg-AD mice; aging; MitoQ; neurodegenerative disease

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DEDICATION

I dedicate this work to my family and to my friends—who have become like family; without your support, none of this would have been possible.

Donte', my dear husband, you have been my rock. Thank you for enduring the ups and downs with me and most importantly for understanding. Graduate school is truly the hardest thing I have ever done, and you have been right there with me, supporting me, and being my own personal engineer/technology tech. Thank you.

To my family, especially my mom and sister, thank you for understanding the time I needed to be away to finish this degree. Often, people don't realize the time and dedication that graduate school requires and the mental and emotional toll that it takes. Thank you for your unwavering support.

To my dad, God rest his soul, thank you for being the kind of father who could raise a little girl to have the confidence and strength to endure all that graduate school encompasses. As a doctoral student, I have doubted myself and my ability too many times to count. Though you are no longer here to say it, I know you are proud.

To my grandmother, you have influenced my life more than anyone else. Your fierceness, caring heart, and natural knack for helping others in your own way inspired me to pursue a field that would allow me to do the same. You taught me that even though everyone isn't a people person, that doesn't mean one shouldn't do one's part to help others. While it certainly wasn't on purpose, you also inspired my research, which has

made this project both near and dear to my heart but also difficult at times. I am eternally grateful for our time together. Rest in peace.

It is said that no one does graduate school alone, and I couldn't agree more. Throughout this process, old bonds grew stronger and new bonds were forged. Arriana, Englann, and Rebekah, I love you all and even though we haven't been able to see each other as much as we'd like, all of you in your own way have impacted me and encouraged me throughout this process.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Dementia, as described in the *Diagnostic and Statistical Manual of Mental Disorders* (DSM), refers to a significant decline in cognitive abilities that interferes with one's independence in performing everyday activities. Alzheimer's disease (AD), the most prominent cause of dementia, accounts for roughly 80% of all dementia cases worldwide (Association, 2017). Symptomatically, patients experience extreme atrophy or deterioration of brain regions involved in learning and memory. The deterioration results in progressive memory loss, impaired judgment, and impaired reasoning. Remarkably, disease onset appears to follow one of two divergent pathways that both produce the same symptomatic phenotype but at a different age of onset. For the purposes of this review, I will briefly discuss Familial AD.

To our knowledge, humans develop AD in one of two ways, either via familial inheritance or sporadic development. The less common of the two is Familial Alzheimer's Disease (FAD), which accounts for roughly 1% of the total AD population. The remaining 99% of the AD population develop what is known as Late Onset Alzheimer's Disease (LOAD), a form often described as sporadic due to a lack of obvious disease triggers (Association, 2017). FAD follows the pattern of Mendelian inheritance and can be easily predicted; the genes involved have been identified and studied thoroughly. Consequently, the genes associated with FAD have been instrumental

in the development of model organisms used to study AD. As a result, a great deal of the data presented in this review come from models based primarily on FAD characteristics, despite its rarity.

Despite the divergent manifestation in disease onset, the characteristics of the diseases are remarkably similar. The hallmarks of the disease include the progressive accumulation of plaques, made up of aggregated amyloid beta (Aβ) peptide and neurofibrillary tangles consisting primarily of hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986b; Association, 2017). These lesions interfere with neuronal communication and nutrient transport within neurons, effectively contributing to several detrimental conditions that lead to neuronal loss. For many years, much of the clinical research that has been conducted focused on these hallmark pathologies. However, in addition to the major hallmark pathologies, mitochondrial dysfunction and oxidative stress are among the pathologies that have become more prominent in the quest to evaluate and further understand the etiology of the disease and elucidate new treatment therapies. The timeline of prevalence for associated pathologies coupled with the interactions of mitochondrial oxidative stress suggest a prominent role in either disease progression or symptom development throughout the disease state. Here I will explore the role that mitochondrial dysfunction and the subsequent oxidative stress play in AD progression, while also exploring targeted antioxidants as a potential therapeutic strategy for AD.

Mitochondria

Physiological function in the body is sustained primarily through energy-generating mitochondria. The central nervous system (CNS), in particular, depends heavily on the

efficiency of mitochondrial function, because the brain has a high energy demand, consuming 20% of the body's total energy expenditure (Mink et al., 1981; Raichle and Gusnard, 2002; Federico et al., 2012). The brain is highly susceptible to oxidative imbalance due to its substantial consumption of oxygen. Its composition rich in polyunsaturated fatty acids and the high content of transition metals contribute to an enhanced pro-oxidant state without proper antioxidant regulation (Pratico, 2008). As such, along with mitochondrial dysfunction, oxidative stress is also an early pathology in AD. Within the brain, neurons have limited glycolytic capacity, leading to a dependence on aerobic oxidative phosphorylation (OXPHOS) to meet energy demands and maintain operational function (Herrero-Mendez et al., 2009; Bolanos et al., 2010). An adequate energy supply from the mitochondria is central to the maintenance of several physiological functions including axonal transport, neuronal signaling, and ionic homeostasis (Picard et al., 2011). These organelles contain their own mitochondrial DNA (mtDNA) that encodes the subunits of the respiratory chain, providing a mechanism where electrons and oxygen combine to facilitate the flow of energy through the mitochondria (Picard and McEwen, 2014). The mitochondria generate energy through a process of shuttling electrons from respiratory complexes composed of low redox potential donors to high redox acceptors in the electron transport chain.

The process generates a proton gradient necessary for adenosine triphosphate (ATP) production, the main energy source in the body.

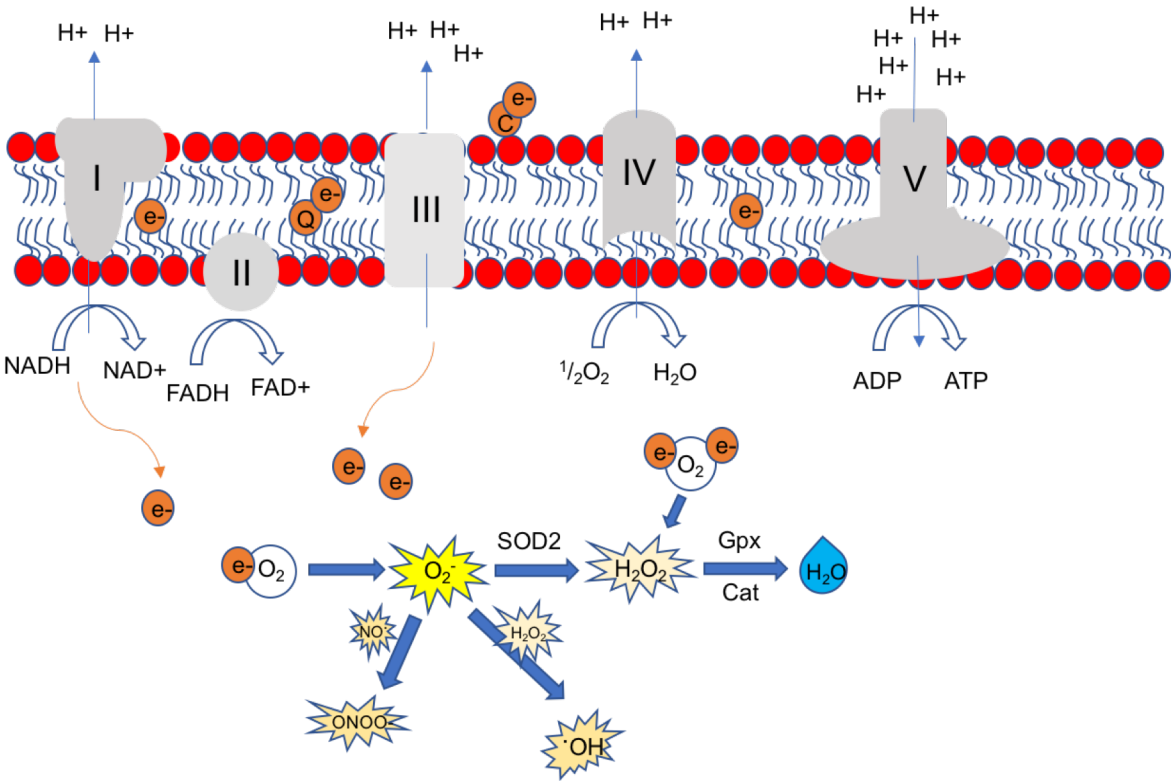

Figure 1.1 Origins of mitochondrial oxidative stress.

The TCA cycle reduces NAD+ and FAD+ to NADH and FADH2, which then donate the electrons from hydrogen to the chain via complex I and II, respectively. Coenzyme Q10 is reduced either by complex I or II, and ubiquinol is formed. Ubiquinol's main function is to transfer electrons to complex III. Complex III in turn reduces cytochrome c where it shuttles electrons to the complex IV. The chain is complete when all partially reduced intermediates result in a full, four-electron reduction of O_2 to H_2O. The energy produced from this process of favorable redox reactions is harnessed to pump protons from the matrix to the intermembrane space establishing a proton gradient over the IMM and creating the mitochondrial membrane potential ($\Delta\psi m$). The potential energy is converted to chemical energy in the form of ATP by the inner membrane H+- ATPase. Under normal conditions, 1-5% of the oxygen is converted to ROS via electrons leaked from the system providing evidence that a fair amount of intracellular ROS is caused due to the mitochondria.

Occasionally, electrons leak from the system, leading to the production of reactive oxygen species (ROS), thus reducing molecular oxygen to the superoxide radical (O_2^-) (Murphy, 2009). ROS, including non-radical oxidizing agents and non-oxygen centered radicals, referred to as reactive species (RS), are highly regulated through a tightly controlled antioxidant defense mechanism and are effectively detoxified. However, when the mitochondria are impaired, excessive leakage of electrons increases the formation of free radicals, thus increasing superoxide, the major reactive oxygen species (ROS). Superoxide, along with other downstream reactive species, propagate and activate each other further to cause oxidative damage to proteins, lipids, and DNA (Tritschler et al., 1994; Zhao and Zhao, 2013). In addition, the mitochondria themselves are susceptible to oxidative damage that also contributes to the damage of mtDNA. MtDNA are unique in that they do not have the same repair mechanisms prominent in DNA repair, and as a result mtDNA is far more susceptible to damage (Piko et al., 1988; Hirai et al., 2001; Lu et al., 2004; Zhu et al., 2006). MtDNA damage has been implicated in mitochondrial dysfunction and has been noted in both aging and neurodegeneration (Cortopassi and Arnheim, 1990; Sun et al., 2016; Lunnon et al., 2017).

In normal aging, somatic mitochondrial DNA acquires and accumulates a number of mutations hypothesized to contribute to age-related neurodegeneration (Lin and Beal, 2006). According to the Alzheimer's Association, aging is the number one risk factor for developing LOAD. This suggests that long-term physiological changes could set the stage for dysfunctional mitochondria that contribute to AD and other mitochondrial dysfunction-related diseases. Moreover, oxidative damage in normal aging has been cited as causative for neurodegeneration. In a transcriptional study looking at human brain

tissue aged 26 to 106 years old, researchers observed several age-based gene expression changes. In particular, after the age of 40 there were marked decreases in genes related to synaptic transmission and cellular transport, whereas genes related to antioxidant production and DNA repair increased. This phenomenon has been observed in humans and model organisms suggesting a compensatory mechanism initiated in response to age related damage (Loerch et al., 2008; Grimm and Eckert, 2017). Additionally, genes that were downregulated in particular showed evidence of oxidative damage, indicating a significant increase in RS despite increased expression of antioxidant mechanisms (Lu et al., 2004).

Despite the occurrence of mitochondrial dysfunction in normal aging and a number of disease states, its involvement in AD is of particular significance due to its intimate relationship with oxidative stress caused by ROS and interactions with hallmark pathologies of AD. The early manifestations of mitochondrial dysfunction in Mild Cognitive Impairment (MCI), a prodromal form of AD, suggest an influential role in AD progression (Pratico et al., 2002). Further, shifts in mitochondrial function, mitochondrial protein expression, and DNA are all associated with both aging and AD. Markers of oxidative damage are present at elevated levels in patients with MCI and animal models of AD. In addition, antioxidant levels as well as total antioxidant capacity are significantly lower (Halliwell, 2006; Sofic et al., 2006). Though the mitochondria operate as powerhouses of the cell, functions beyond the ETC, when impaired may contribute significantly to the overall AD etiology. Studies with nonhuman primates provide evidence that mitochondria may play a key role in synaptic transmission, contributing to the improvement of working memory and overall brain function (Hara et

al., 2014). Thus, providing another link between mitochondrial function and memory within the brain, and further suggesting that the brain is particularly susceptible to changes in mitochondrial function and equilibrium. These molecular changes lay the groundwork and prime the brain for mechanistic and pathological insults that lead to neuronal loss and eventually demented phenotypes.

Oxidative stress may occur primarily through the presence of dysfunctional mitochondria. In addition to the damage directly done to the mitochondria by excessive free radical leakage, the damage that occurs also exacerbates other AD pathologies, most notably the Aβ and tau pathologies. These interactions lead way to several negative feedback cycles that will be discussed further throughout this review. Markers of oxidative stress such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are not only elevated within the tissue but also are present in cerebral spinal fluid (CSF) in humans (Markesbery and Lovell, 1998; Galasko and Shaw, 2017; Rani et al., 2017). Additionally, mouse models harboring genes associated with APP and tau pathologies also exhibit markers of oxidation (LaFerla and Green, 2012; Webster et al., 2014). The Tg2576 mouse model of AD contains transgenes APP/PS1 and has a marked increase in lipid peroxidation compared to littermate controls (Massaad et al., 2009a). The data suggest that these pathologies and oxidative stress play a definitive role in the disease progression.

Amyloid precursor protein (APP) and Aβ have been documented to interact with mitochondria or mitochondrial proteins that also exacerbate the dysfunction (Cha et al., 2012). Excess Aβ in vivo leads to fragmentation, reduced density, and reduced length of mitochondria. Reports show Aβ accumulation in the mitochondrial membrane through a

mechanism dependent on outer mitochondrial transporter (TOM) translocase (Wang et al., 2008). Several studies have identified Aβ interactions within the mitochondrial membrane to lead to dysfunction (Reddy and Beal, 2008). Aβ peptide fragments have been reported to block the entry of nuclear-encoded proteins into the mitochondria resulting in decreased mitochondria membrane potential, increased ROS production, and altered mitochondrial morphology (Sirk et al., 2007; Wang et al., 2008; Zempel et al., 2010). The aforementioned evidence supports the amyloid hypothesis which suggests that Aβ is the culprit and initial insult for the development of AD. However, more recently, human-induced pluripotent stem cell-derived neuronal cells (iN Cells) from sporadic AD patients show aberrant mitochondria dysfunction, increased ROS, increased DNA damage, and increased OXPHOS complexes, not correlative to Aβ or tau levels (Birnbaum et al., 2018). Synaptic protein levels were also unaffected. This study not only highlights the fact that individuals who are prone to sporadic AD may have a metabolic change in neurons affecting pathology but also adds evidence that mitochondrial dysfunction occurs independently of amyloid pathology in sporadic AD. This study also further confirms the presence of mitochondrial dysfunction specifically in sporadic AD prior to Aβ and tau in the pre-symptomatic phase. Yet, also further strengthens the sentiment that mitochondria are central to AD etiology both independently and concurrently with other pathologies of AD.

Alzheimer's Disease Origins

On a molecular level, before atrophy occurs and neuronal death is evident, synaptic and metabolic dysfunction occurs in the neurons of AD patients. Remarkably,

the brain regions affected by this disease are highly specific. The dysfunction begins in the entorhinal cortex (ECII) brain region, specifically the lateral entorhinal cortex, where pyramidal cells in the lamina II are most heavily affected and eventually lost (Braak and Braak, 1985). From the ECII, pathogenesis spreads to the hippocampal network and eventually to the outer regions of the cortex, laying the groundwork for further disease progression (Khan et al., 2014). On a much broader scale, it has been established that the formation of memory requires an intact entorhinal-hippocampal circuit. The disruption of signal transmission between structures in the entorhinal-hippocampal circuit leads to cognitive deficits (Eichenbaum and Lipton, 2008; Morrison and Baxter, 2012).

Given the location of initial onset, the entorhinal-hippocampal circuit, the disease characteristics of memory impairment are quite apparent. More recently since pinpointing the brain regions initially affected, AD-associated markers and pathologies have been identified prior to obvious disease onset providing further clues on etiology. The accumulation of hyperphosphorylated tau protein has been identified in the locus coeruleus (LC) several years prior to AD-like pathogenesis. Braak and colleagues evaluated over 2000 post mortem brains from individuals spanning an entire lifetime and found that the accumulation in the locus coeruleus region occurs as early as 40 years of age (Braak et al., 2011). The combination of AD-associated brain regions affected early on, including the entorhinal cortex, the hippocampus, and the frontal cortex, further elucidate the origins of AD and provide a more complete picture of its pathogenesis.

Despite early detection of hyperphosphorylated tau, degeneration of the LC does not occur until mid- to late-stage AD. However, it has been suggested that the known functions of the LC make the brain more vulnerable to degeneration, as it innervates most

brain regions except the basal ganglia. As the LC is the main source of norepinephrine (NE), degeneration in this area of the brain contributes to a decrease in anti-inflammatory beta-adrenergic receptor-mediated Aβ microglia clearance, thus reducing clearance activation and further contributing to Aβ accumulation (Feinstein et al., 2016).

While the areas of the brain affected during AD pathogenesis have been clearly identified, what is lacking is the reason for disease onset in these brain regions. It has been suggested that AD begins in these areas because ECII neurons express higher levels of amyloid precursor protein (APP) even in cognitively normal patients (Harris et al., 2010). APP levels continue to increase with age and disease progression. Expression does not decline until later stages of AD (Roberts et al., 1993). Despite knowing where the disease originates, it is still unclear why sporadic AD occurs in elderly populations that lack the genetic mutations present in FAD. Additionally, it is also unclear whether EC dysfunction itself plays a singular role in cognitive deficits or whether it is the overall disruption of other cortical regions that ultimately leads to the decline (Harris et al., 2010).

The entire makeup of pathologies that occur in the ECII region, and subsequently in additional cortical regions of the brain, are not completely understood. Harris et al. provide evidence confirming that disease progression beginning solely in the ECII can spread and cause cognitive deficits and disease pathology with the development of the EC-APP mouse model that expresses the FAD APP selectively in the EC region of the brain (Harris et al., 2010). These data support several decades of hypotheses suggesting that early vulnerabilities in this brain area can lead to increased risk for AD(Braak and Braak, 1985; Devanand et al., 2007; Stoub et al., 2010; Braak et al., 2011; Khan et al.,

2014). While there aren't definitive markers for who will develop sporadic AD, several other elements have been identified as risk factors for AD. The most prominent being age, having a maternal family member who developed the disease, and the TREM2 and ApoE4 gene variants. However, despite the number of known risk factors and pathologies for sporadic AD, none has successfully led to the discovery of a definitive marker for early diagnosis.

While the search for dependable biomarkers is still ongoing, researchers have turned to conditions that often precede cognitive impairment and sometimes AD itself for answers. Many patients develop Mild Cognitive Impairment (MCI) before AD, and experts have argued that it may be an early stage of the disease (Pratico et al., 2002; Association, 2017; Lacour et al., 2017). MCI is characterized as mild, measurable changes in cognition that could include memory loss. Patients experiencing MCI are more likely to develop a form of dementia; however, some do not (Siemers et al., 2016; Association, 2017). Research involving those with MCI has shed light on potential mechanisms that lead to dementia. Particularly in the case of AD, MCI patients not only show atrophy in the ECII region of the brain similar to that seen in AD but also display pathologies that occur early on in the AD disease pathology (Masdeu et al., 2005). Mitochondrial dysfunction and increased oxidative stress are prevalent pathologies in MCI, and if MCI is truly a prodromal form of AD, these pathologies may indicate or contribute to later pathology. While not all patients who develop MCI go on to develop Alzheimer's disease, the specificity in the brain region affected compounded with early pathological similarities lends credence to the notion that MCI could be a precursor to

AD and explains why the symptoms of MCI and early stage AD are comparable (Masdeu et al., 2005; Harris et al., 2010; Khan et al., 2014).

As the leading form of dementia in the United States, Alzheimer's disease has garnered a great deal of attention and generated initiatives to find effective therapeutic agents. Still, the current FDA-approved drugs, acetylcholine esterase inhibitors and NMDA inhibitors, are only temporarily effective in treating the symptoms of the disease (Association, 2017; Glynn-Servedio and Ranola, 2017). Despite the lack of effective drug prospects for treating the disease, potential disease mechanisms have been uncovered that could lead to additional targets for disease treatment. Here we will discuss the potential mechanisms of AD as they relate to the amyloid hypothesis, mitochondrial dysfunction, oxidative stress, and the potential use of antioxidants as therapeutic agents based on known mechanisms of the disease.

Beta Amyloid (Aβ)

The leading hypothesis on AD progression, the amyloid cascade hypothesis, describes a pathogenic increase in amyloid precursor protein (APP) cleavage products that contribute heavily to disease progression (Hardy and Higgins, 1992). Those cleavage products, Beta Amyloid (Aβ) peptides, are produced through the process of sequential proteolytic cleavages by secretase enzymes of the transmembrane protein APP. The length of Aβ peptides produced depends heavily on the sequence of secretase enzymes that cleave APP (O'Brien and Wong, 2011; Chen, 2015). Two divergent pathways result in amyloidogenic and non-amyloidogenic phenotypes. Alpha secretase enzymes, the predominate pathway, cleave APP through the sequence of Aβ, preventing its formation

and resulting in a non-amyloidogenic product. In the amyloidogenic pathway, successive cleavage of two separate secretase enzymes results in the formation of Aβ peptides ranging from 38 to 43 amino acids long. Additional amino acids at the terminal end of the peptide result in increased aggregation (Tamagno et al., 2006; Seeman and Seeman, 2011).

The genetic makeup of these enzymes can affect APP cleavage and produce different peptide lengths (Chow et al., 2010). Beta site APP cleaving enzyme I (BACEI), also known as beta-secretase, contributes to the production of Aβ and cleaves just outside the sequence (Feng and Wang, 2012). BACE1 cleaves APP at the N-terminal, leaving a 99-amino acid product that is further cleaved by gamma secretase releasing Aβ peptides from the transmembrane protein. Gamma secretase enzyme is a complex of multiple proteins that influences the length of Aβ products produced from its cleavage. In FAD, genetics contributes to the pathogenic increase in APP cleavage, Interestingly, aberrant oxidative stress plays a similar role in the process even in the absence of genetic mutations (Misonou et al., 2000; Tamagno et al., 2005; Guglielmotto et al., 2010; Muche et al., 2017). Reactive species are intricately involved in the amyloidogenic pathway and perhaps serve as a bridge in understanding the divergence in sporadic AD and Familial AD. Recently, a direct link with ROS and the mechanistic increase of Aβ peptides in vascular endothelial cells demonstrated that the presence of ROS is not just an ancillary reaction to environmental factors (Muche et al., 2017). Muche and colleagues demonstrated not only that ROS increase the cleavage of APP to increase the formation of Aβ but also that the presence of ROS shifts the entire paradigm, creating a bias towards the increased formation of Aβ in the amyloidogenic pathway (Arimon et al.,

2015; Kanamaru et al., 2015; Muche et al., 2017). The presence of ROS mechanistically changes the environment to promote further Aβ production. The increase in ROS leads to a mechanistic increase in APP expression, thus priming the environment for additional peptide cleavage. ROS induces signaling pathways which lead to the activation of enzymes that are biased toward increasing the cleavage at residues that result in toxic peptides (Tamagno et al., 2005; Quiroz-Baez et al., 2009).

Initially, the increased occurrence of Aβ, was thought to be a function of beta and gamma secretase activity. However, the lack of therapeutic effectiveness in clinical trials with secretase inhibitors has raised additional concerns about this mechanism (Siemers et al., 2006; Doody et al., 2013; Chen, 2015). In contrast, perhaps instead of increased activity of the beta and gamma enzymes, there is rather a reduced activity in alpha secretase, the more dominant pathway (Chen, 2015). Aging is one mechanism that reduces the efficacy of the alpha secretase enzyme, allowing for non-specific enzymes (Beta & Gamma) to cleave in alternative areas, leading to increased production of neuropathic amyloid (Apelt et al., 2004; Nistor et al., 2007; Placanica et al., 2009). This argument proposes that alpha secretase enzymes are specific for the cleavage of APP and that this cleavage results in non-amyloidogenic cleavage products, while other enzymes cleave APP randomly. Although this theory provides alternative reasons as to why clinical trials targeting these enzymes have been unsuccessful, it does not explain why ratios of certain isoforms of Aβ peptide become more common in disease state progression if the selection is random. It is plausible that leaving oxidative stress out of the equation, a pathology closely related to aging and a pathology known to cause similar effects to genetic mutations present in AD has left AD research at a disadvantage. The

complex nature that reactive species (RS) play in physiology has made it difficult to pin point whether RS and subsequent oxidative stress are key triggers. However, with the advent of more advanced techniques and technology, we are now able to better understand the mechanistic effect aberrant oxidative stress has in diseases of aging.

Aβ is the most definitive pathology present in AD despite the fact that it does not correlate well with the major symptoms of AD: memory loss and cognitive decline. In fact, analysis of postmortem elderly brains detected the presence of Aβ, even when the person had not displayed symptoms of the disease (Pike et al., 2007; Maarouf et al., 2011). The presence of neurofibrillary tangles (NFT), however, does correlate to cognitive decline better than Aβ (Spires-Jones et al., 2009). Interestingly, Aβ, tau pathology, and oxidative stress appear to have an interrelated relationship in which the presence of one exacerbates the others, leading to aberrant tau formation, causing increased hyperphosphorylation and promoting NFT and increased amyloid toxicity (Rapoport et al., 2002; Ferrari et al., 2003).

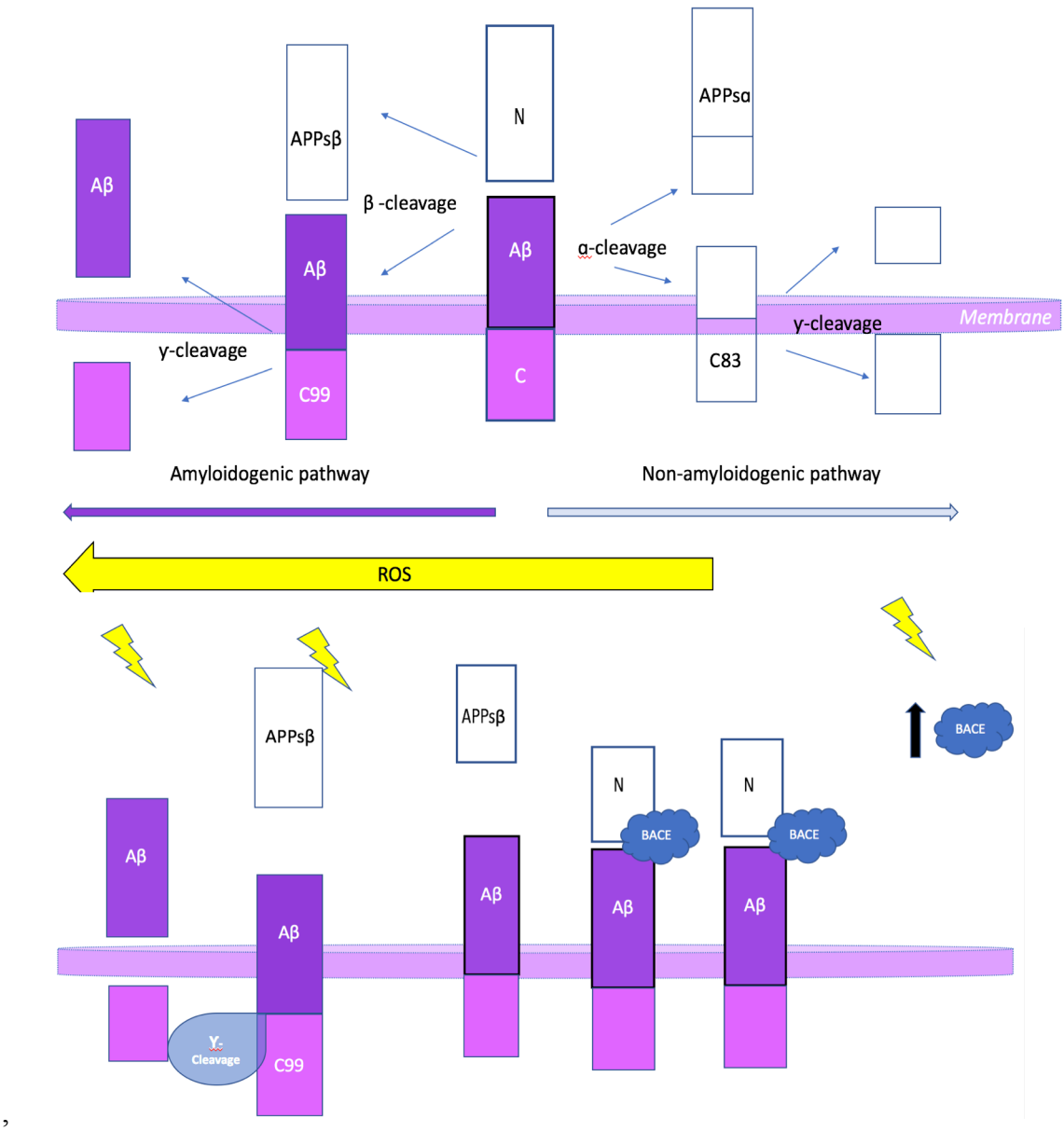

Figure 1.2 Oxidative Stress and APP Processing.

APP processing is modulated by the presence of RS, so much so that coordinating factors combine to create an environment that leans towards the amyloidogenic pathway. Introduction of RS induces up regulation of APP expression. The mechanism is not clear, but a decrease in alpha-secretase, a Ca+2-dependent protease has been observed to become inefficient with aging. In contrast, BACE expression and activity is increased by RS products. Gamma-secretase activity is increased by oxidative stress, mediated by the c-jun N-terminal kinase pathway.

Tau

Neurofibrillary tangles, characterized by the accumulation of aggregated protein within neuronal cells, were initially described in the early 1900s (along with Aβ); however, the main intracellular component of these tangles, tau, was not identified until the 1980s (Grundke-Iqbal et al., 1986b). Since its initial discovery, tau pathology has demonstrated a prominent role in the disease progression of AD despite its own occurrence outside of AD. Originally described as intracellular tangles, NFTs can now be further broken down into paired helical filaments (PHF) of twisted protein, primarily comprising hyperphosphorylated tau (Grundke-Iqbal et al., 1986b; Grundke-Iqbal et al., 1986a).

Tau is derived from the microtubule-associated protein tau (*MAPT)* gene, which is located on chromosome 17 and contains 16 exons (Andreadis et al., 1992). Splicing at various exons results in different isoforms of tau that change in relation to development, age, and disease state. In the mature human brain, alternative splicing at exons 2, 3, and 10 results in six isoforms of tau protein ranging from 352 to 441 amino acids long (Goedert et al., 1989; Andreadis et al., 1992). Changes in isoform prevalence have been associated with AD-related dementias (Association, 2017). In addition, tau has over 80 known phosphorylation sites, which under normal conditions are heavily regulated to maintain normal function. In the case of AD, phosphorylation is not as well controlled, resulting in a hyperphosphorylated state and making the protein vulnerable to tangling and dysfunction (Grundke-Iqbal et al., 1986a; Morris et al., 2011). As with Aβ, oxidative stress has been linked to mechanisms that further propagate aberrant tau pathology

(Goedert et al., 1997; Melov et al., 2007; Su et al., 2010). Since tau expression exists primarily in neurons, and is predominantly further localized in the axon, tangles can contribute to dysfunction in axonal transport in addition to disrupting vital functions of tau. Several transgenic mouse models overexpressing tau alone show several axonal alterations that result in disruptive changes to mitochondrial physiology and with age manifest dysfunctional ROS producing mitochondria (David et al., 2005; Stoothoff et al., 2009; Kopeikina et al., 2011). However, the role of aggregated tau protein during AD progression has further reaching consequences, impaired axonal transport of mitochondria between the cell nucleus and synapse also lead the energy imbalance and dysfunction further prompting the release of ROS effectively creating a negative feedback loop (Rapoport, 2003; Kopeikina et al., 2011). More recently in the htau mouse model, accumulation of human tau (htau) effectively disrupted mitochondria function and as a result decreased ATP levels and complex I activity. Further, mitochondria specific protein levels were altered due to the tau accumulation (Li et al., 2016). These studies taken together provide insights into the mechanisms by which tau pathology and mitochondria oxidative stress influence each other to further AD progression.

Tau plays several roles within cells, many of which are not well understood. The most well studied is its role in the stabilization of microtubules (Spires-Jones et al., 2009; Kadavath et al., 2015). Tau promotes polymerization and stabilization of microtubules that provide the framework for maintaining cellular morphology and trafficking. The process requires a binding mechanism at the C terminal domains facilitated by phosphorylation and a highly-regulated phosphorylation state allowing for rapid attachment and detachment of the microtubule. Together, all of these elements work to

moderate axonal trafficking of nutrients throughout the cell (Spires-Jones et al., 2009; Morris et al., 2011). In the presence of ROS, these functions are disrupted.

Tau binds to numerous elements beyond the cytoskeleton including lipids and signaling molecules. In light of this, it is important to recognize that despite stabilization and transport being the most well-known functions of tau, its additional interactions suggest that it could be a multifunctional protein (Morris et al., 2011). Likewise, tau may regulate signaling pathways in a mechanism in which it acts as a protein scaffold for signaling complexes.

One challenge to fully understanding AD etiology is the complexity of the associated pathologies. Tau pathology is present in several neurodegenerative disorders; however, differences in disease pathology have sparked questions regarding tau's function and its role in AD. In fact, while evidence of hyperphosphorylated tau in AD is well documented, it is not necessarily indicative of negative pathology. Tau in hyperphosphorylated states under non-disease state circumstances does not always appear to be pathogenic to cellular function. In fact, evidence suggest that hyperphosphorylated states may be important in synaptic plasticity and could be linked to neuroprotective mechanisms (Honson and Kuret, 2008). Hibernation of small mammals and "pseudo hibernation" induced in rodent models by lowering body temperature display hyperphosphorylation of tau similar to that which is present in neurodegeneration. This phenomenon is also followed by a reversible synapse loss which returns to normal after breaking hibernation (Arendt et al., 2003; Hartig et al., 2007; Stieler et al., 2009; Stieler et al., 2011; Arendt and Bullmann, 2013). In essence, this evidence suggests that tau

pathology may be linked to a protective mechanism that goes awry, further complicating our understanding of AD etiology.

Under pathogenic conditions, tau pathology is widely accepted as a key component of disease progression, but it is not exclusive to AD. Frontotemporal dementia (FTD), a related neurodegenerative disorder with a slightly younger age of onset than that of sporadic AD, features tau pathology in the absence of Aβ (Silva et al., 2004; Gasparini et al., 2007; Association, 2017). FTD features symptoms similar to those found in AD, including memory loss and the atrophy of affected brain regions. Related dementias such as FTD have been instrumental in our developing understanding of AD progression. However, these associated forms of dementia have also given rise to additional questions regarding which of the two major pathologies in AD, Aβ or tau, is driving the disease progression.

The hyperphosphorylation of tau is the major event that contributes to the formation of Neurofibrillary tangles (NFT). Neurofibrillary tangles (NFT) form as a result of a multistep process that begins with an imbalance in free unbound tau protein. As concentrations of unbound tau protein increase, so does the likelihood of misfolding and malfunction. Hypotheses attempting to explain these phenomena are subject to several factors. Mutations in the tau gene *MAPT* are common in FTD and lead to an increase in production of vulnerable tau isoforms; however, no such mutations have been identified in sporadic AD (Nacmias et al., 2014; Olszewska et al., 2016). As phosphorylation is the limiting factor in tau-microtubule interactions, imbalance and the aberrant activity of tau kinase and phosphatases could also play a detrimental role in increasing unbound protein concentrations. In addition to modulating pathological

cleavage of tau, oxidative stress also alters enzyme activity that disrupts the tightly regulated phosphorylation states needed for maintaining normal function. Kinase activity is altered in the presence of oxidative stress, particularly increasing glycogen synthase kinase-β (GSK-3B) activity that has been implicated in pathological tau phosphorylation (Zhang et al., 2005b). The relationship of chronic oxidative stress and tau pathology has been noted on numerous occasions *in vitro* and provides evidence that aberrant ROS is critical to tau pathology in vivo as well. Direct exposure to elements that promote oxidative stress results in increased levels of tau phosphorylation at the PHF-1 also increased. Increased activity of JNK and p38 coupled with decreased activity of PP2A also likely contribute to oxidative stress-induced tau phosphorylation (Su et al., 2010).

In contrast, oxidative stress also downregulates mechanisms that are essential to post-phosphorylation control, effectively inhibiting Isomerase PIN1 responsible for tau-phosphate removal (Lu et al., 1999; Sultana et al., 2006). Oxidative stress, well known for its ability to cause conformational changes in protein structure, often leads to less favorable protein binding with the addition of disulfide bridges and tyrosine nitration. Similar to kinases, inflammation and Aβ toxicity also interfere with tau and microtubule binding capacities by interfering with phosphorylation equilibrium (King et al. 2006). Tau is three to four times more phosphorylated in the sporadic AD brain than in the normal brain, and hyperphosphorylation increases at the site of microtubule binding (Wang et al., 2013; Eckert et al., 2014). What triggers hyperphosphorylation specifically in sporadic AD is not well understood; however, evidence strongly implicates mitochondrial oxidative stress in alterations responsible for NFT formation.

Further, tau pathology in the context of AD may be initiated through aberrant executioner caspase-3/7 activity that specifically cleaves tau at the ASP-421 residue (Rissman et al., 2004; Jarero-Basulto et al., 2013; Chu et al., 2017). Enzymatic cleavage at this location is linked to shifts in tau folding conformations associated with early neurofibrillary formation (Uboga and Price, 2000; Alonso et al., 2001; Rissman et al., 2004; Means et al., 2016). Both oxidative stress and Aβ elicit caspase activity *in vitro* and are well documented to induce tau pathology as well (Gamblin et al., 2003; Eckert et al., 2014). Moreover, caspase-cleaved tau has been shown to induced mitochondrial dysfunction in cortical neurons (Quintanilla et al., 2009). Providing further evidence of feedback mechanisms involving tau, Aβ, and mitochondria pathologies.

Disruption of tau phosphorylation regulation results in the hyperphosphorylated state that leads to NFT pathology in AD. Of the known phosphorylation sites on tau, several have been implicated in AD. After NFT assemble in neuronal cellular bodies, they form physical barriers to the transport of nutrients within the cells. In the disease state, physiological conditions cause a shift increasing hyperphosphorylated tau, resulting in increased accumulation in the somatodendritic compartment (Ittner and Gotz, 2011). This localization within the dendrite may play a significant role in AD pathogenesis.

Despite tau's ability to thrive and act as the sole (or main) pathological agent in numerous neurodegenerative disorders, in the context of AD, evidence suggests that a symbiotic interaction with Aβ mediates disease progression (Rapoport et al., 2002; Zempel et al., 2010; Frandemiche et al., 2014). Furthermore, the small population of hyperphosphorylated tau that accumulates in the dendrites suggest that tau may also act as a postsynaptic protein. Providing further context as to why tau pathology correlates

with cognitive decline better than amyloid pathology. In the dendrites, tau is accessible to tyrosine protein kinase FYN, and these interactions are implicated in mediating Aβ toxicity. The tau-FYN interaction initiates FYN targeting to postsynaptic sites where it is able to phosphorylate NMDA receptor subunit 2B (NR2B). Phosphorylation of the NMDA receptor subunit allows for the complex formation with postsynaptic density protein 95 (PSD95) (Ittner and Gotz, 2011). This interaction is essential for exotoxic downstream signaling and often leads to seizure, a symptom that occurs both in humans and animal models of AD. Additionally, in tau KO mice, this interaction is essential in inducing Aβ toxicity (Roberson et al., 2007; Ittner et al., 2010).

Taken together, major pathologies Aβ and tau both show dynamic interactions with mitochondria oxidative stress. Reduction of mitochondrial oxidative stress *in vivo* and *in vitro* have a domino effect and attenuate factors that exacerbate oxidative stress-mediated pathology, further strengthening the case for mitochondria-linked oxidative stress modulation in AD progression.

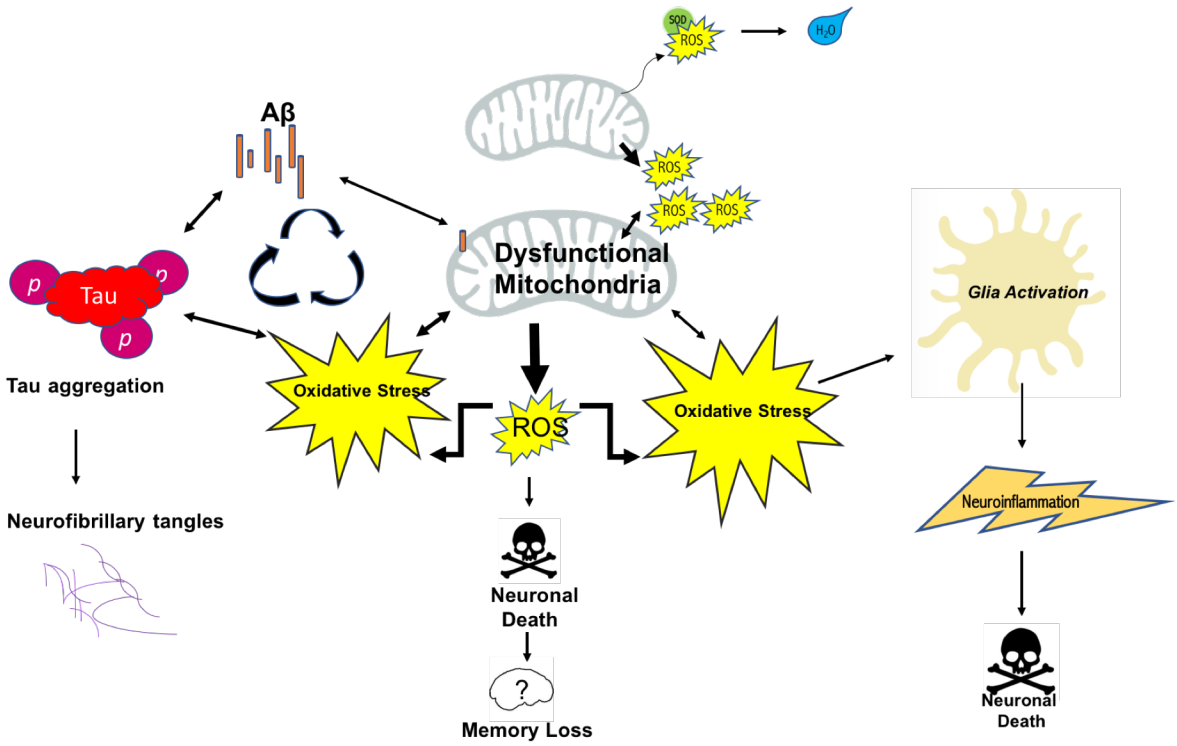

Figure 1.3: Mitochondrial dysfunction: Missing link in Sporadic AD pathogenesis. Controlled amounts of ROS are constantly made as a byproduct of cellular metabolism and inflammatory processes under physiological conditions. However, perturbations of the delicate equilibrium of ROS detoxification are suspected to play a crucial role in many neurodegenerative diseases. Mitochondria are the most susceptible to ROS toxicity. Mitochondrial proximity to ROS production and the structure make them particularly vulnerable targets for oxidative damage. The mitochondrial membranes are made up of long polyunsaturated fatty acid chains that are highly susceptible to oxidation and can result in depolarization of the mitochondrial membrane leading to additional impairments. MtDNA is localized close to ROS production sites, direct contact results in mtDNA mutation that have broad effect in the context of neurodegeneration. MtDNA encodes some unites of the ETC mitochondria complexes I, III, IV, ATP synthase where mutation can prime the system to increase ROS. Outside of the mitochondria, several AD pathologies have shown increase toxicity in the presence of ROS, further propagating pathology leading to neuronal death and memory loss.

To date, the current understanding of AD pathogenesis, particularly Aβ and tau, has produced numerous therapeutic strategies for treatment. Unfortunately, the theories behind those therapies have yet to manifest effective results. Despite the failure of clinical trials based on mechanisms focusing on misfolded proteins, one benefit has been a shift in the research and the direction of AD treatment. Aβ and tau pathologies have long been recognized as major contributors to AD; however, more recently, the complexity of these pathologies and their relationships with other pathologies such as mitochondrial dysfunction, oxidative stress, and inflammation have received much more attention in drug discovery.

Antioxidants in AD

Mitochondria are both a major source and a target of ROS, which can result in mitochondrial dysfunction and the propagation of other AD pathological factors. However, endogenous antioxidant defense mechanisms protect against oxidative damage under normal conditions. SOD, catalase, and glutathione peroxidase are the primary enzymes involved in the direct detoxification of ROS. Secondary enzymes, including glutathione reductase and glucose-6-phosphate dehydrogenase, function to decrease peroxide levels and maintain the supply of metabolic intermediates such as glutathione (GSH) and NADPH for proper functioning of the primary antioxidant enzymes (Vendemiale et al., 1999).

As previously mentioned, oxidative stress increases with normal aging and is not necessarily indicative of AD. However, in the case of AD, the compounding factors of age and damaging neuropathologies result in a notable elevated state of oxidative stress that has been linked to memory impairment (Fukui et al., 2001; Silva et al., 2004; Haddadi et al., 2014; Kruk-Slomka et al., 2016). Since mitochondrial dysfunction and oxidative stress are highly related to AD onset and progression, various antioxidants have been identified as plausible therapy alternatives that do not necessarily follow the amyloid hypothesis. Consequential success in decreasing oxidative stress and memory decline in mouse models via altering antioxidant capacity in the brain has garnered increased interest in using antioxidants as a possible treatment. Overexpression of mitochondria-specific superoxide dismutase (SOD2) in mouse models of AD reduced the occurrence of AD-like pathologies (Massaad et al., 2009a). The overexpression of the endogenous antioxidant SOD2 reduced deficits in memory and reduced Aβ load in the Tg2576 mouse model of AD. In addition, the development of a mouse model overexpressing mitochondria-targeted catalase (MCAT), an antioxidant not usually found within the mitochondria, resulted in an overall lifespan increase by 5 months (Mao et al., 2012). Further, supplementation of antioxidant compounds proved to be efficacious *in vivo*. Vitamin E supplementation lead to reduction in several markers of AD due to its chain breaking mechanism for lipid peroxides. Reductions were observed in markers for oxidative and amyloid pathology (Sung et al., 2004; Montiel et al., 2006; Devore et al., 2010; Wang et al., 2016). Additional antioxidant supplementation such Ginkgo biloba and lipoic acid also displayed similar effects and significantly improved cognitive performance in behavioral testing (Augustin et al., 2009; Siedlak et al., 2009; Devore et

al., 2010; Liu et al., 2015; Sinha et al., 2016). Together, these studies provide evidence that maintaining or increasing antioxidant capacity in mouse models is beneficial.

Table 1.1. Relevant antioxidants in Alzheimer's Disease research.

	Non-Targeted	Targeted
Natural Antioxidant	Green Tea Flavonoids Curcumin Ginkgo biloba Melatonin Selenium Omega-3 polyunsaturated fatty acid Caffeine Silibinin	MitoQ MitoVitE MitoTEMPOL SkQ1
Enzymatic Endogenous Antioxidant	SODs Catalase GSH-Px	Coenzyme Q10
Non-Enzymatic Endogenous Antioxidant	GSH Alpha-tocopherol (vitamin E) Vitamin C Beta-Carotene Lycopene Resveratrol Estrogen	Lipoic acid
Antioxidant Compounds	Idebenone Methylene Blue Monoamine oxidase-B inhibitor	SS (Szeto-Schiller) Peptides

Abbreviations: Mitoquinone Mesylate (MitoQ), Mitotocopherol (MitoVitE), Mito 4-hydroxy-2,2,6,6,-tetramethyl-piperidine-1-oxyl (MitoTEMPOL), (SkQ1), Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione (GSH)

However, success with antioxidants in animal models of AD has not translated well to antioxidant treatment in clinical trials. Agents with antioxidant properties such as

vitamin E, Ginko biloba, and melatonin have been introduced in the clinical setting with hopes of slowing disease progression (Huang et al., 2000; Saxena et al., 2010; Feng and Wang, 2012; Dysken et al., 2014). Thus far, only vitamin E has resulted in any improvement for patients with AD, and the effect was minimal (Dysken et al., 2014). One hypothesis suggested that perhaps the reason for this lack of success was that the therapeutic agents were not reaching the site of action (James et al., 2005). All of the aforementioned supplements have antioxidant ability; however, several barriers may prevent these agents from remaining active and sufficiently concentrated to produce a therapeutic effect. In particular, the blood-brain barrier (BBB) is a hurdle that must be crossed if a therapeutic drug is to reach the area in need of RS detoxification.

Despite the aforementioned problems associated with antioxidant therapy, mitochondrial associated-decline with aging and AD have continued to be targets for potential therapeutics. Mitochondrial free radical leakage, coupled with the prevalent and early occurrence of mitochondrial dysfunction in AD, has sparked further interest in targeted antioxidant therapy. Targeted antioxidants have been used to improve efficacy where non-targeting agents have fallen short. Many of these targeted antioxidants are triphenylphosphonium (TPP+) based, covalently bound to the lipophilic cation whose properties allow agents to cross through lipid bilayers and the blood-brain barrier and localize to the mitochondria. Among the most studied mitochondria-targeted antioxidants is Mitoquinone Mesylate (MitoQ), an antioxidant originally developed to protect the mitochondria from reactive species produced from excessive leakage of electrons from respiratory complexes (Kelso et al., 2001). MitoQ was studied for its ability to detoxify free radicals from the respiratory complexes and reduce oxidative stress in a number of

model systems. MitoQ is the combination of a ubiquinone moiety covalently bound to TPP+ with a ten-carbon alkyl chain. TPP+ absorbs to the apical side of the inner mitochondrial membrane, and ubiquinone absorbs to the matrix, where it is reduced to ubiquinol by respiratory complex II. Free radicals oxidize ubiquinol back to ubiquinone, resulting in regenerative properties for the compound. Endogenous ubiquinol in mammalian cells is able to shuttle electrons from both complex I and II to facilitate the movement of electrons between respiratory complexes. MitoQ, however, appears effective only at scavenging electrons from complex II (Kelso et al., 2001; James et al., 2005). Our published studies have shown that MitoQ treatment in young 3xTg-AD mice (2-4 months old) completely attenuated markers of oxidative stress compared to littermate controls. Additionally, in 3xTg-AD mice, early treatment halted disease progression altogether (McManus et al., 2011).

Reasonable success in mouse models suggests that targeted antioxidants such as MitoQ could be an effective therapeutic for AD. Though MitoQ may be among the most widely studied targeted antioxidants, there are other less-well-studied agents that show promise in the area of targeted-antioxidants. The SKQ1 (plastoquinonyl decyltripenylphosphonium) compound is similar to MitoQ in that the main antioxidant component is a quinone molecule. Plastoquinone is involved in the electron transport chain for light-dependent reactions during photosynthesis. SKQ1 reduces behavioral traits of aging in rats. The compound reduces oxidative stress due to mild uncoupling of the respiratory chain, thereby reducing ROS formation (Stefanova et al., 2010). Additionally, SS peptides use a sequence motif that targets them to the mitochondria. They are small and cell permeable, and they scavenge H_2O_2 and ONOO-, preventing lipid

peroxidation much like MitoQ (Szeto, 2006a). Endogenous and supplemental antioxidants are effective in reducing markers of oxidative stress and memory decline in mouse models of AD and aging. Despite how the experimental data suggest these mechanisms should be effective against AD progression the clinical trials have not matched what is seen at the bench. It reasonable to hypothesize that targeted antioxidants can succeed where untargeted antioxidants have failed.

Summary

A tremendous effort has been made to tackle the issues and mysteries of neurodegenerative diseases. Despite this effort, the fact remains that neurodegenerative diseases comprise a complex group of pathologies that are not fully understood. In the case of AD, not only is the pathogenesis complex and interrelated, but the focus on the amyloid hypothesis has resulted in years of disappointing clinical trials (Gilman et al., 2005; Doody et al., 2013; Le Couteur et al., 2016; Honig et al., 2018). In 2017 alone, several major pharmaceutical companies, including Novartis, Eli Lilly, Merck, and GSK, ended major trials due to lack of effectiveness. Further, while a more diverse group of disease modifying therapeutics is increasingly being studied in clinical trials, the vast majority of those agents focus on Aβ and tau pathology (Cummings et al., 2017; Atri et al., 2018; Bennett, 2018; Honig et al., 2018). This suggests a major need to shift the focus and increase the diversity of the pathologies targeted and explored at the clinical level. Here, I present work aimed at further uncovering the relationship between mitochondrial dysfunction, oxidative stress, and AD progression. We have adopted an approach that not only solidifies these pathologies in AD but also provides an additional framework and

proof of concept that targeting these pathologies is a viable option as a potential therapeutic strategy. This approach is not designed to discredit the results of amyloid hypothesis-driven research, but aims to point out that the amyloid-centric approach alone is not sufficient in finding effective treatments. Considering that, by the time Aβ is detectable, patients involved in clinical studies have experienced a significant amount of neuronal death. Taking advantage of the fact that mitochondrial oxidative stress appears to innervate from the beginning of onset and continues throughout the disease progression could benefit patients significantly. The evidence suggests mitochondrial oxidative stress initiation of disease pathology and involvement throughout the disease progression. This further contributes to neuronal loss directly and indirectly, regardless of the disease stage. All this together demonstrates the current potential for targeted therapeutics and use, even after definitive early detection biomarkers of AD are readily available.

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CHAPTER 2

MATERIALS AND METHODS

Reagents

Mitoquinone mesylate [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cycloheexadienl-yl) decyl triphenylphosphonium methanesulfonate], (MitoQ), complexed to beta-cyclodextrin was gifted from Michael Murphy via GlycoSyn Technologies (Lower Hutt, New Zealand). All other reagent were purchased from Sigma (St. Louis, MO) unless otherwise noted

Mice

The 3xTg-AD mouse model used in this study expresses three mutant human transgenes: amyloid precursor protein, APPswe; presenilin-1,PS1M146V; and four repeat tau, tauP301L (Oddo et al., 2003). Both of the mutations in amyloid precursor protein and presenilin-1 are associated with the familial form of AD, while the four repeat tau mutation is implicated in frontotemporal dementia. AD-like symptoms of the disease appear as early as 3-4 months of age and continue to progress with time. Beginning at twelve months of age female mice were administered 100 μM MitoQ complexed to cyclodextrin in a 1:4 ratio continuously in drinking water available *ad libtium* for 5 months. Littermate controls and non-transgenic controls with the same 129/C57BL6 genetic background were given access to drinking water that did not contain MitoQ. All mice were group housed in our animal facilities, given access to the same rodent chow,

bed-o-cob bedding and subjected to a 12 h light/dark cycle. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Spatial learning and memory retention

Following the five-month treatment period, mice were assessed for spatial memory retention using the Morris Water Maze behavioral test. For 8 consecutive days mice underwent acquisition trials where they were trained to find and escape onto a hidden platform within the water maze. The water maze consisted of a circular aluminum tank (4ft diameter) filled with opaque water and one slightly submerged plexiglass platform 14 cm in diameter. Water was maintained at 24 ±1°C and made opaque with nontoxic white tempura paint. Behavioral assessments were conducted as previously published with minor adjustments (McManus et al., 2011). Mice were placed on the hidden platform before the first acquisition trial for 10 seconds to reduce stress and establish existence of an escape platform. Acquisition trials followed where mice were placed in the water maze at one of four predetermined starting points and allowed a 60 second free swim to escape onto the platform. Mice unable to find the platform were manually guided there and allowed 30 s on the platform to become familiar with distinct spatial cues in the test area. Each mouse underwent four trials each day and was allowed to rest for 30 seconds in a holding cage with a warm towel in between trials. Trials were continued until mice met escape latency criterion defined as reaching the escape platform within 20 s or less. Spatial bias was determined in probe trials 1.5 and 24 h after the last acquisition trial. The platform was then removed and mice were allowed a 60 s free swim. Their swim paths

crossing the previous platform location were analyzed. To account for possible sensorimotor deficits, mice were subjected to a cued acquisition trial following the last probe trial. In the cued trials, the platform was replaced in the pool and visibly marked with a flag. Mice were placed in the maze at a novel position and allowed to find the newly placed platform. Times to reach the platform and swim speed were determined. Each mouse was handled and assessed for general health prior to cognitive assessment. All trials were recorded and analyzed using Ethovision Tracking software (Noldus Inc.) and Sigma Plot statistical software.

Tissue Acquisition

Mice were sacrificed in accordance to our animal use protocol with carbon dioxide followed by cervical dislocation. Brains were removed rapidly and split sagittally. Each cerebral hemi-sphere was either fixed in 4% paraformaldehyde, used fresh, or snap-frozen and stored at -80°C for biochemical assays.

Immunoblotting

Harvested brain tissues were homogenized in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris, 0.5% Sodium deoxycholate, 1% Triton X-100, 150 mM NaCl) supplemented with 100x protease inhibitor cocktail (Sigma). Samples were centrifuged at 13,000 rcf at 4°C for 15 minutes using an Eppendorff 5417R centrifuge. Equal amounts of protein were separated via SDS-PAGE, transferred to PVDF membranes for 1 hour on ice (Millipore) and blocked with 5% non-fat milk in TBS-T (10 mM Tris-HCL, 100 mM NaCl, and 0.1% Tween-20) for 30 minutes at room temperature. Afterwards, membranes

were incubated with primary antibodies glial fibrillary acid protein GFAP 1:1000 (Thermo Cat# PA3-16727, RRID: AB_2109795), synaptophysin 1:1000 (Millipore Cat# MAB525, RRID: AB_11214133), Nitrotyrosine 1:1000 (Invitrogen Cat# ab61392, RRID: AB_942087), and Tau 5 1:500 (Santa Cruz Cat# sc-58860, RRID: AB_785931) at 4°C overnight. Membranes were then washed for at least 20 minutes in TBS-T and incubated at room temperature in an anti-mouse or anti-rabbit HRP-linked secondary antibody 1:1000 (Cell Signaling Cat# 7076 and 7074, RRID: AB_330924 AB_2099233) followed by another 20 minute wash. β-actin 1:300 (GenScript Cat# A00730-40, RRID: AB_914100) and β-tubulin 1:1000 (Thermo Cat# PA1-41331, RRID: AB_2210397I) were used as loading controls. Proteins were detected using Chemiluminescent ECL Western Blotting Substrate (Pierce).

Lipid Peroxidation

Lipid peroxides were analyzed with the thiobarbituric acid reactive substances assay (TBARS). Brain tissue was homogenized in ice cold 1.15% KCL. Normalized volumes of protein adjusted for equal protein loading and equal volumes of 8.1% SDS and 20% Acetic Acid were added to a sample tube before adjusting the solution to pH 3.5 with 10 M NaOH. The samples were mixed well by vortex before adding an equal volume of 0.8% TBA in 0.25 M HCL and incubating at 95 °C for 1 h. After incubation, samples were cooled on ice and an additional equal volume of deionized water and 1:15 butanol/pyridine mixture was added to the sample and inverted several times. The formation of TBARS trapped in the organic phase of the extraction was measured by absorbance of colorimetric product at 532 nm by a spectra Max M2 microplate reader

(Molecular Devices). The amount of TBARS calculated in samples was determined from a standard curve produced by hydrolysis of tetraethoxypropane.

Immunohistochemistry

Individual cerebral hemispheres were fixed for 48 h in 4% paraformaldehyde, embedded in paraffin, cut into 12 um sections, and mounted on glass slides. Sections were deparaffinized and rehydrated through a series of incubations in xylene and ethanol. Following rehydration, antigen retrieval was achieved with heated 10 mM sodium citrate buffer pH 6.0 at 95 °C for 10 m in a humidity chamber. Antigen retrieved sections were then incubated for 30 m in 0.3% H_2O_2 in MeOH and blocked with VECSTAIN Universal blocking serum at room temp for 30 minutes. Following blocking, sections were incubated with AT8 (Thermo Cat# MN1020, RRID:AB_223647) TAU5 (Santa Cruz Cat# sc-58860, AB_785931), and AB42 (Bioss Cat# bs-0107R, RRID:AB_10858046) overnight at 4 °C Sections were visualized using an ABC immunoperoxidase kit from Vector Laboratories and diaminobenzidine substrate.

Amyloid Beta (1-42) Elisa

Soluble and Insoluble fractions of Aβ (1-42) were detected in both whole brain tissue and hippocampal tissue with minimum modification for BetaMark Colorimetric ELISA kit (Covance). Briefly, tissue was homogenized in an ice cold 0.6% SDS lysis buffer (50 mM Tris, 2 mM EDTA, 150 mM NaCl) supplemented with 100x protease inhibitor. Samples were centrifuged at 25,000 rcf at 4°C for 1 h. Supernatant containing soluble amyloid or amyloid peptide standards were added to ELISA plates in duplicate and

incubated overnight at 4 °C. The following day, the 96-well plate was thoroughly washed, incubated with tetramethylbenzidine substrate for 50 min while protected from light at room temperature, and the colorimetric product was determined by absorbance at 620 nm with a SpectraMax M2 microplate. The Bradford assay (Pierce) was utilized to ensure equal loading of protein.

Caspase 3/7 activity

Caspase 3/7 activity was measured using a Caspase-Glo 3/7 kit (Promega) and assessed following the manufacturer's instructions. Briefly, brain tissue from each treatment group was homogenized in ice-cold hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA) and centrifuged at 13,000 rpm for 15 minutes at 4 °C. Protein concentrations were determined with the Bradford Assay (Pierce) and then diluted with PBS to account for equal protein loading. Samples were incubated in a white-walled 96-well plate for 1 hour with equal volume Caspase-Glo reagent, and luminescence was measured by a SpectraMax M2 microplate reader. Luminescence was analyzed according to normalized relative light units (RLU).

Statistics

Graphical representations and statistical significance measures were determined with SigmaPlot 11.1 (Systat Software). Appropriate statistical analyses were conducted for experiments based on data distribution. Unless noted, statistical comparisons were made via one-way ANOVA and statistical significant indicates a p-value of 0.05 or less. Error bars represent ± SEM.

CHAPTER 3

THE MITOCHONDRIA-TARGETED ANTIOXIDANT MITOQ INHIBITS MEMORY LOSS AND NEUROPATHOLOGY IN AGED 3XTG-AD MICE

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Abstract

Oxidative stress is a confounding factor in the toxicity and accumulation of the overall neuropathology of Alzheimer disease. Evidence has shown that not only do oxidative stress, likely stemming from dysfunctional mitochondria, occur before major AD neuropathologies but also aggravate and promote AD etiology including aggregation of Aβ and the phosphorylation of tau. To further elucidate the contribution of mitochondrial oxidative stress in AD progression, we examined the ability of mitochondria-targeted antioxidant MitoQ (mitoquinone mesylate: [10-(4,5-dimethoxy-2-methyl-3,6 dioxo-1,4-cycloheexadienl-yl) decyl triphenylphosphonium methanesulfonate]) to inhibit AD pathology in triple transgenic mouse model of AD (3xTg-AD) after AD-like pathology onset. Following a treatment period of 5 months, female 3xTg-AD mice exhibited behavior indicative of an inhibition of cognitive decline. In addition, biochemical assessments of AD-like pathologies associated with our animal model revealed that MitoQ greatly reduced oxidative stress, inhibited synapse loss, astrogliosis, Aβ accumulation, caspase activation, along with tau hyperphosphorylation. Interestingly, MitoQ treatment also increased 3xTg survival similar to that of non-transgenic controls, suggesting that treatment also extends lifespan. The work we present herein supports a central role for the involvement of mitochondrial oxidative stress in AD and further supports the use of targeted antioxidants for a potential therapeutic.

Significance Statement

We provide evidence that supports previously published data that oxidative stress, produced specifically by the mitochondria, is a key component and perhaps a major

mediator for disease progression in the 3xTg-AD mouse model of AD. Reduction in oxidative stress results in the inhibition of AD-like characteristic changes in the brain. Supporting this, we show improvement of known cognitive decline in our model, along with inhibition of amyloid and tau pathologies. In addition, this approach aims to reignite interest and research for the use of targeted antioxidants as a viable therapeutic in the treatment of AD.

Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disorder characterized by the extensive atrophy of neuronal tissue resulting in gradual cognitive impairment and dementia. Evidence suggests that oxidative stress is a key mediator in the disease progression of AD (Gutteridge, 1994; Pope et al., 2008; Federico et al., 2012; Zhao and Zhao, 2013) that occurs well before and during the development of plaques and tangles (Pratico et al., 2002; Moreira et al., 2010a). Despite senile plaques, made up of beta-amyloid (Aβ) and neurofibrillary tangles (NFT) composed of hyperphosphorylated tau, being the most definitive hallmarks of AD the effects of mitochondria-mediated oxidative stress are intertwined throughout the disease and associated with its numerous pathologies.

Dysfunctional mitochondria result in an increase in free radical production and formation of reactive species (RS). At elevated levels these species overwhelm endogenous antioxidant capacity and go on to cause oxidative damage to DNA, proteins, and lipids. In the case of AD, oxidative damage promotes and aggravates known pathologies in *in vitro* studies and markers of oxidative stress have been shown to

increase *in vivo* with disease incidence (Zhao and Zhao, 2013). Perhaps the most detrimental of RS is formed when oxygen (O_2) is reduced to superoxide (O_2^-).

MitoQ (mitoquinone mesylate: [10-(4,5-dimethoxy-2-methyl-3,6 dioxo-1,4-cycloheexadienl-yl) decyl triphenylphosphonium methanesulfonate]) a mitochondria-targeted antioxidant is effective in reducing oxidative stress produced by O_2^- and other downstream RS. MitoQ is made up of the endogenous ubiquinone antioxidant moiety covalently bound to a triphenylphosphonium (TPP+) cation that acts as the targeting agent. MitoQ rapidly crosses the blood-brain barrier and concentrates in the mitochondrial membrane driven by the membrane potential (Kelso et al., 2001). Acting as an anchor TPP+ remains on the matrix side of the membrane allowing the ubiquinone to penetrate the membrane where is it reduced by respiratory complex II to ubiquinol and takes on its role as a antioxidant when oxidized back to ubiquinone by RS (James et al., 2005; Smith and Murphy, 2010). In a renewable fashion complex II reduces ubiquinone to repeat the cycle. Since MitoQ is a poor substrate for complex I and III it cannot substitute for endogenous ubiquinone, instead acting as a renewable antioxidant.

To further assess the role oxidative damage plays in disease progression, particularly in learning and memory retention, we treated female 3xTg-AD mice with MitoQ after AD pathology was present. The 3xTg-AD mouse model of AD is a well-characterized model of AD that develops AD-like pathology in an age dependent manner comparable to what is seen in humans (Oddo et al., 2003). Cognitive deficits are seen as early as four months of age (Billings et al., 2005), while markers of mitochondria dysfunction and the subsequent oxidative stress occur much earlier (Resende et al., 2008; Yao et al., 2009). By six months, the presence of synaptic dysfunction and extracellular

plaques are both highly detectable. Tau pathology, present in this model as neurofibrillary tangles, appears at 12 months, aligning with known progression in humans (Rissman et al., 2004). Previously we've evaluated the therapeutic potential of MitoQ on cognitive performance in young 3xTg-AD mice prior to disease onset (McManus et al., 2011). In that study, treating 3xTg-AD mice prior to disease onset with MitoQ prevents cognitive decline and associated AD-like pathologies. Preventative treatments for Alzheimer's are ideal. However, effective and reproducible biomarker standards for early detection of late-onset AD are still under study (Humpel, 2011; Sharma and Singh, 2016).

Due to the timeline of pathology development in our mouse model and the current lack of dependable biomarkers to identify sporadic AD well before onset, mice aged twelve months old were selected for treatment with MitoQ for a duration of five months. Here we present data showing that despite the disease-like onset in the 3xTg-AD model at twelve months old, MitoQ treatment much like our previous preventative study significantly improved spatial memory retention, inhibits markers of oxidative stress, astrogliosis, and Aβ accumulation. In addition, we demonstrate a decrease in tau pathology, that can be detected because of our age groups. Surprisingly, we also observed that MitoQ treated mice displayed a lifespan similar to that of our non-transgenic controls indicating that treatment also increases lifespan in our model of AD.

Results

MitoQ treatment enhanced the cognitive performance of aged 3xTg-AD mice.

After treatment, cognitive ability was assessed with the Morris water maze (MWM) to evaluate learning and memory retention (Morris, 1984). All mice were able to achieve a

baseline criterion defined as finding and escaping the MWM in 20 s or less during the training trials (Fig 1A). Eighteen-month old MitoQ treated 3xTg-AD mice reached criterion two days before age-matched littermate 3xTg-AD controls. Thus, MitoQ treatment significantly improved performance during acquisition trials. ($p<0.05$).

To determine the effect of MitoQ treatment on memory retention, the escape platform was removed and mice were allowed a 60 s free swim. Spatial bias was measured by the number of times the animals crossed the area where the platform was previously positioned. MitoQ significantly improved memory retention of the platform location over age-matched 3xTg-AD mice. Interestingly, eighteen-month old MitoQ - treated mice also outperformed age-matched nonTg mice (Fig. 1B). Following the last acquisition trial, mice were subjected to a cued trial where the platform was placed back into the MWM at a visible novel location marked with a flag. All mice escaped the MWM to the cued platform within the same period. No significant differences were seen among treatment groups, indicating that MitoQ had no effect on sensorimotor capabilities (p=0.107) (Fig. 1C). All mice were evaluated prior to the start of the training trials for general health, and swim speed was measured in the first trial. There were no discernable differences between the treatment groups (p=0.664), indicating that mice had equal ability to escape the maze (Fig. 1D).

Synaptic dysfunction is a likely contributing factor leading to cognitive deficits in 3xTg-AD mice. In fact, 3xTg-AD mice have a marked increase of age-related synaptic dysfunction (Oddo et al., 2003) manifesting as a significant decline of synaptophysin and contributing to the decline in spatial memory retention (Schmitt et al., 2009; Blanchard et al., 2010). Our behavior data suggest that MitoQ treatment may have a protective effect

on synaptic function shown through improved memory retention. To further investigate, we quantified synaptophysin levels in cortical tissue via immunoblots and showed that treatment beginning at 12 months of age inhibits synapse loss by 2-fold (Fig. 2).

MitoQ treatment inhibits oxidative stress in 3xTg-AD mice

Increased oxidative stress, a consequence of the overproduction of reactive species, precede most other neuropathology associated with AD. Lipid peroxidation products are among the biochemical modifications that occur due to excessive ROS and have been found in the brain, cerebrospinal fluid, and plasma of patients with AD (Montine et al., 2002; Galbusera et al., 2004; Bradley-Whitman and Lovell, 2015) . As early as three months of age, 3xTg-AD mice have elevated levels of brain lipid peroxidation compared to nonTg controls (Resende et al., 2008). Coupled with the increase of lipid peroxidation products, 3xTg-AD mice have a significant decrease of endogenous antioxidants such as vitamin E and glutathione (Pratico, 2008; Resende et al., 2008) that have chain breaking qualities to attenuate further free radical activity from lipid peroxides. MitoQ's antioxidant moiety, ubiquinone, is reduced by complex II into ubiquinol which effectively inhibits the formation of radical superoxide (Frei et al., 1990) effectively preventing further production of downstream reactive species. As such, MitoQ's antioxidant properties are extremely effective against lipid peroxidation (James et al., 2005). We investigated MitoQ's antioxidant capacity to decrease lipid peroxidation in late-stage AD-like pathology occurring in 3xTg-AD mice by measuring malondialdehyde (MDA) levels in brain tissue. MitoQ-treated mice displayed a two-fold decrease in MDA

levels compared to non-treated controls suggesting (*$p<0.05$), that MitoQ is capable of having a significant effect in blocking late-stage disease peroxidation (Fig. 3A).

In addition to lipid peroxidation, elevated oxidative stress causes damage to DNA and proteins that can lead to a buildup of toxic oxidation products when ROS are not properly detoxified due to decreased antioxidant capacity. Within this cycle, superoxide can react with nitric oxide producing another reactive species, peroxynitrite (ONOO-) resulting in major oxidative modifications that increase in both humans and mouse models of AD (Smith et al., 1997). However, our previous studies revealed that MitoQ attenuated the increase of ONOO- associated reactive species both *in vitro* with primary cells and *in vivo* in young 3xTg-AD female mice (McManus et al., 2011). To elucidate MitoQ's effect on nitro-oxidative modification in late-stage AD-like pathology, we measured nitrotyrosine levels in brain tissue via immunoblots (Fig. 3B). While MitoQ did not completely eliminate the presence of nitro-oxidative products, it does however, significantly reduce the presence of nitrotyrosine compared to non-treated 3xTg-AD mice.

MitoQ treatment inhibits astrogliosis and microglial cell proliferation

Tau pathology manifests in the 3xTg-AD mouse model in the form of NFT and is detectable in their brains by 12 months of age (Oddo et al., 2003). We used immunohistochemistry to investigate formation of NFT in the brains of 18-month-old female nonTg, 3xTg-AD, and 3xTg-AD mice that had received MitoQ treatment for the preceding five months. Figure 6*A* shows that MitoQ-treated 3xTg-AD mice had fewer NFT in their cortex than did untreated 3xTg-AD mice. No tangles were apparent in

nonTg mice. We quantified total tau protein expression and tau phosphorylation levels by immunoblot. Total tau levels were significantly elevated in the brains of 3xTg-AD mice compared to nonTg ones. MitoQ-treated mice had total tau levels similar to those of nonTg mice, indicating that MitoQ decreased the build-up of tau in the brain (Fig 6*B*). Tau phosphorylation levels, occurring at AD-associated phosphorylation residues Ser202/205, were about ten-fold higher in the 3xTg-AD brains than in the nonTg ones. MitoQ treatment reduced phosphorylated tau to a level that was indistinguishable from that of nonTg brains (Fig.6*C*).

Caspase-mediated cleavage of tau is involved in the development of NFT (Rissman et al., 2004; Rohn et al., 2008; de Calignon et al., 2010). Given that stabilization of oxidative stress in young 3xTg-AD mice inhibits elevated caspase activity (McManus et al., 2011), we evaluated MitoQ's effect on caspase activity in 18-month-old female 3xTg-AD mice that had received five months of MitoQ treatment. Caspase-3/7 activity was measured in brain tissue using a luminescent caspase activity assay. Caspase 3/7 activity was elevated about 3.5-fold in the 3xTg-AD brains as compared to the nonTg ones. Relative luminescent values demonstrated that MitoQ treatment blocked caspase activation by about 20% relative to 3xTg-AD mice (Fig. 7).

MitoQ treatment increases 3xTg-AD mice lifespan

The median lifespan of the 3xTg-AD mouse model is about 22-months, with mortality taking place as early as twelve-months old (Rae and Brown, 2015). The common time frame of NFT development and the occurrence of mortality in the 3xTg-AD mouse model suggests that tau pathology may play a role in shortening this mouse models'

lifespan (Hirata-Fukae et al., 2008). Although our end point was eighteen months of age, we saw significant mortality in untreated 3xTg-AD mice after twelve months. MitoQ-treated mice had a lifespan similar to nonTg mice (Fig. 8) that have median lifespans ranging from 25-34 months (D'Antona et al., 2010; Rae and Brown, 2015). Our data show that in addition to improved cognition and overall reduction in oxidative stress, MitoQ also extends the lifespan in this mouse model of AD.

Discussion

Evidence of oxidative stress is found in the brains of individuals who have died with AD and in the cerebrospinal fluid of patients with mild cognitive impairment, a condition that can be prodromal to AD (Nunomura et al., 2001, 2006; Praticò et al., 2002; Castellani et al., 2016). This stress precedes most of the neuropathological hallmarks of AD, suggesting that it may lie upstream from them. Mitochondrial dysfunction also occurs early in AD progression and is the likely source of the RS causing oxidative stress (Gutteridge, 1994; Pope et al., 2008; Moreira et al., 2010; Federico et al., 2012; Castellani et al., 2016). The appearance of oxidative stress in the development of AD has led to a number of clinical trials of antioxidants. These trials have largely been unsuccessful (Castellani et al., 2016). Possible reasons for these failures include poor ability of the antioxidants to cross the blood-brain barrier, inability to reach and/or enter mitochondria in sufficient concentrations, or that oxidative stress is merely associated with AD and is not critical for its etiology. The development of mitochondria-targeted antioxidants, such as MitoQ, that cross the blood-brain barrier and concentrate in mitochondria provide a better tool for determining whether mitochondria-associated

oxidative stress is important for the disease and, perhaps, may lead to new and novel therapies for treating it (Zhao et al., 2004; James et al., 2005; Murphy and Smith, 2007; McManus et al., 2011).

Oxidative stress and mitochondrial dysfunction are found not only in human AD but also in AD-like pathology in mouse models of the disease (Velliquette et al., 2005; Anantharaman et al., 2006; Butterfield et al., 2001, 2006, 2007; Resende et al., 2008; Yao et al., 2009; McManus et al., 2011). We previously demonstrated the ability of MitoQ to inhibit cognitive decline and AD-like neuropathologies in young 3xTg-AD mice (McManus et al., 2011). In that study, MitoQ treatment began two months after birth and continued for five months, a period during which the first AD-like pathologies become manifest. In the current study, we evaluated the effects of MitoQ treatment on cognitive decline and neuropathologies in these mice starting at 12 months after birth and continuing until 18 months of age. During this period, all of the known AD-like pathologies are present and are progressing in severity (Oddo et al., 2003). As in the younger 3xTg-AD mice, we found that MitoQ treatment of older mice was effective in improving spatial memory retention. Levels of the synaptic protein synaptophysin were significantly higher in the brains of MitoQ-treated mice than in the brains of untreated ones indicating that MitoQ treatment inhibited synapse loss and suggesting that this inhibition might underlie the observed cognitive improvement.

Oxidative stress, including lipid peroxidation, increases in the brain and other organs of mice and other animals with age (Rikans and Hornbrook, 1997; Floyd and Hensley, 2002; Navarro et al., 2004). The brains of young 3xTg-AD mice have significantly higher levels of lipid peroxides (MDA) than those of nonTg mice (Resende et al., 2008;

McManus et al., 2011). We found higher MDA levels in 18-month-old 3xTg-AD brains than we previously reported for the brains of seven-month-old animals, indicating that lipid peroxidation increases with age in the 3xTg-AD mice. However, we found that there was no difference in lipid peroxide levels in the brains of the 18-month old nonTg mice and the brains of age-matched 3xTg-AD animals. A possible explanation for the lack of a difference between the lipid peroxide levels in the older 3xTg-AD animals and the nonTg ones is that the lipid peroxide production had reached maximum levels in their brains. Regardless of the explanation, MitoQ treatment significantly decreased lipid peroxide levels in 3xTg-AD brains to below those in both the 3xTg-AD and the nonTg brains. Quantification of nitrosylation levels indicated that, at least by this measure, the brains of 18-month old 3xTg-AD animals were under considerably greater oxidative stress than nonTg brains. MitoQ treatment greatly reduced nitrosylation but not to the level found in nonTg animals.

Overexpressing mitochondria superoxide dismutase in transgenic mouse models of AD decreases Aβ production and inhibits cognitive decline (Li et al., 2004; Esposito et al., 2006; Massad et al., 2009). Similarly, treating young 3xTg-AD mice with MitoQ prevents increased levels of Aβ in their brains (McManus et al., 2011). Aβ peptides are produced by sequential cleavage of APP by β- and γ-secretases. Oxidative stress can increase Aβ production by increasing β- and γ-secretase expression and activity via stress-activated kinases (Tong et al., 2005; Karupagounder et al., 2009; Zhang et al, 2011; Tamagno et al., 2002, 2005, 2008). Oxidative stress can also lead to modification of caspase activity (Circu and Aw, 2010), and activated caspases can cleave APP (Banwait et al., 2008; Bredesen et al., 2010; Mukherjee and Williams, 2017). Aβ has toxic effects

on mitochondria (Lustbader et al., 2004; Sorrentino et al., 2018). Aβ can enter mitochondria via the TOM import machinery and induce mitochondrial dysfunction, at least in part, by inhibiting preprotein maturation (Peterson et al., 2008; Mossman et al., 2014). This inhibition imbalances the organelle proteome, causing decreased respiration and increased ROS production. Therefore, increased Aβ in AD may involve a positive feedback cycle in which mitochondria-derived oxidative stress increases Aβ production, and the Aβ then increases ROS via its effects on mitochondria. Consistent with such a cycle, MitoQ decreased both oxidative stress and Aβ levels in the 18-month-old 3xTg-AD brains.

Increased numbers of astrocytes and microglial cells are found in both human AD brains and the brains of 3xTg-AD mice (Oddo et al., 2003; McManus et al., 2011; Janelsins et al., 2005; Hansen et al., 2018). It is uncertain as to whether these inflammatory processes contribute to AD or are downstream of the neuropathological processes. There is evidence that microglial cells are involved in the etiology of the disease, as well as evidence that they are protective against it (Hansen et al., 2018). The ability of MitoQ treatment to reduce Aβ levels, reactive astrogliosis, and microglial cell proliferation is consistent with the proliferation of both astrocytes and microglial cells lying downstream of the Aβ pathology.

The amyloid hypothesis suggests that Aβ deposition causes NFT formation (Hardy and Allsop, 1991). Aβ deposition occurs earlier in human AD and the 3xTg-AD mouse model of AD than do NFT (Oddo et al., 2003; Selkoe and Hardy, 2016). Many studies have confirmed the interdependence of Aβ and tau pathology (Sato et al., 2018). Mutations in human APP that increase amyloid deposition also lead to increased levels of

tau in neurons (Bateman et al., 2012; Selkoe and Hardy, 2016). Introduction of Aβ into neuronal cultures can cause hyperphosphorylation of tau, necessary for formation of NFT (Jin et al., 2011). Experimental treatments that reduce Aβ burden also reduce NFT in 3xTg-AD mice (Oddo et al., 2004; Rasool et al., 2013; Dai et al., 2017). In the present study, MitoQ treatment reduced Aβ accumulation, NFT formation, and both total amounts of tau and hyperphosphorylated tau in the 3xTg-AD brains. Cleavage of tau by caspases can lead to tau aggregation and phosphorylation (Rissman et al., 2004; Rohn et al., 2008). Thus, MitoQ decreased two events thought to lie upstream of tau pathology, Aβ accumulation, and caspase 3 activity.

The increased lifespan in the MitoQ-treated 3xTg-AD mice was intriguing. The reason that these animals die earlier than nonTg ones has not been reported, and we did not do necropsies of dead animals. The most likely explanation for the extended survival was the reduction in neuropathologies associated with the disease. Further investigation of the means of death of these mice and the effect of MitoQ treatment on mortality will be needed to determine the mechanism.

In summary, our findings show that the mitochondria-targeted antioxidant MitoQ reduced cognitive and AD-like neuropathological symptoms in old 3xTg-AD mice. In humans, amyloid plaques can occur in the absence of cognitive deficits, and there is a poor correlation between the plaques and neuropathology (Maarouf et al., 2011; Wirth et al., 2013; Altman et al., 2015). Those findings, combined with the plethora of failed clinical trials targeting Aβ (Hardy and De Strooper, 2017), lends credence to the idea that these pathologies may contribute to AD but are perhaps not the singular cause of the disease. Evidence that oxidative stress and mitochondrial dysfunction precedes most of

the major AD pathologies and likely contributes to them suggests that targeting them may be more effective in treating the disease than targeting Aβ or tau. This idea is further supported by recent work showing that enhancing mitochondria proteostasis in an AD mouse model reduces Aβ-associated proteotoxic stress (Sorrentino et al., 2018). We and others have shown that antioxidants targeted directly to mitochondria, the source of most RS, are effective in reducing AD pathology in animal models of AD (Szeto, 2006; McManus et al., 2011; Mao et al., 2012). Because of their novel mechanism of action MitoQ, or other mitochondria-targeted antioxidants, may be effective therapies for treating human AD.

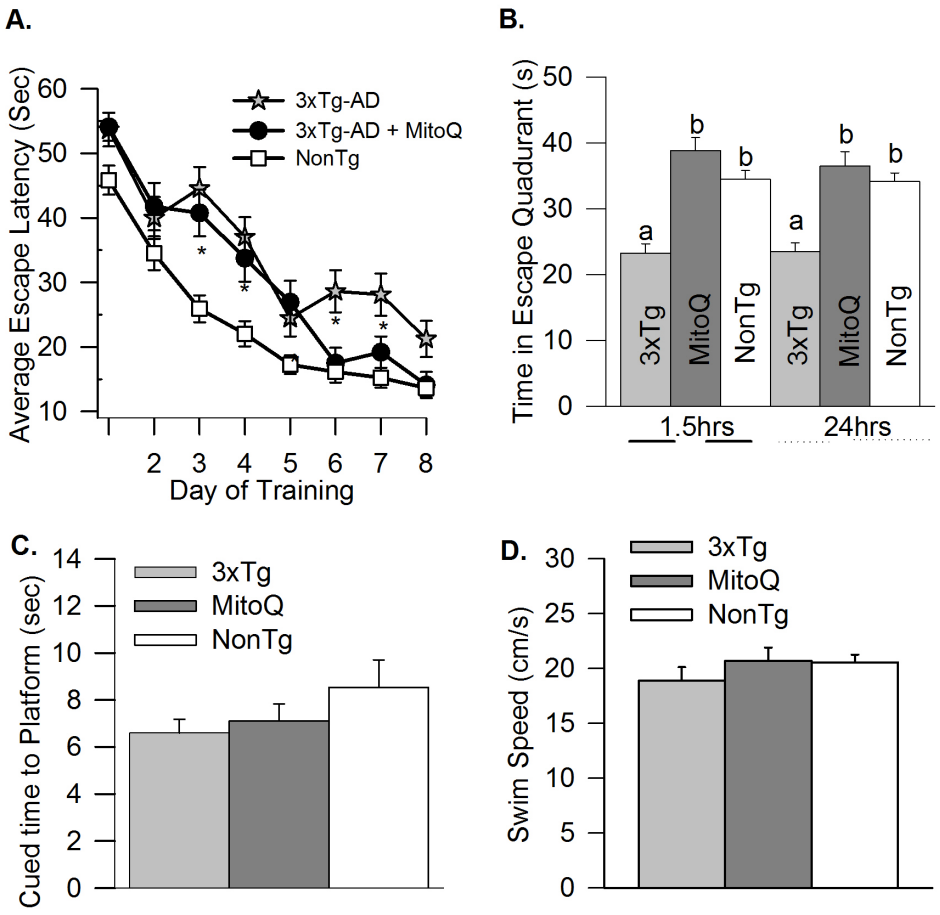


Figure 3.1. MitoQ treatment improved spatial memory retention in 18 month-old female 3xTg-AD mice. ***A***, Time courses of MWM spatial learning and memory acquisition in 18 month-old mice. All groups were able to perform the task with similar escape latencies after 8 d of training. However, MitoQ-treated mice reached criterion escape latency (< 20 s) days earlier than 3xTg-AD mice ($^{\#}p < 0.01$ by repeated measures ANOVA). ***B***, MitoQ-treated mice showed significant improvement in both short- and long-term spatial memory retention compared to 3xTg-AD mice in probe trials conducted 1.5 and 24 h after the last training trial ($p < 0.01$ by ANOVA). ***C***, Swim speeds within the 18 month-old treatment groups were not significantly different indicating that all mice had comparable sensorimotor capabilities ($p = 0.664$; n=12-20). ***D***, Escape latency to the

visible platform. All mice, despite treatment group, were capable of reaching the platform in the same amount of time ($p = 0.107$; n = 10-13). Different letters in this and subsequent figures indicate significant difference.

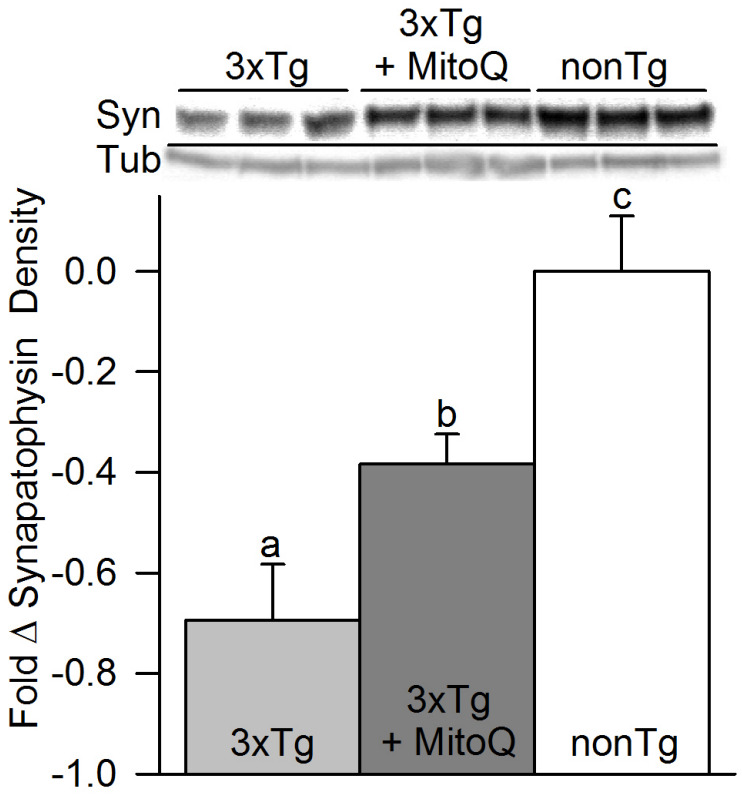


Figure 3.2. MitoQ treatment protected against synapse loss in the brains of aged female 3xTg-AD mice. Synaptophysin (Syn) density was measured in immunoblots from homogenized cortical tissue. MitoQ-treated 3xTg-AD mice showed a significant reduction in the loss of synaptophysin compared to littermate 3xTg-AD controls. However, synaptophysin was also significantly lower in MitoQ-treated 3xTg-AD mice compared to age-matched nonTg mice, suggesting treatment simply slowed or halted further synapse loss. In this and subsequent figures, the data was first normalized to the appropriate tubulin loading control and then to the average value of the similarly normalized nonTg controls ($p < 0.01$; $n = 6$ for each condition).

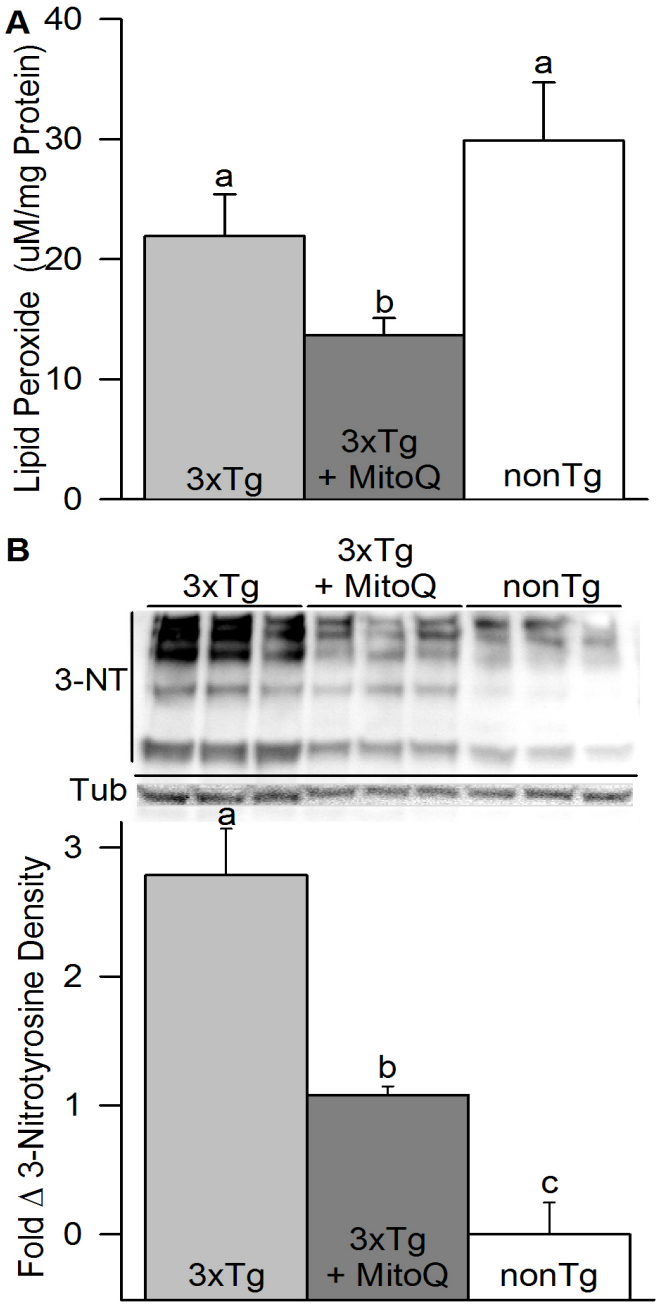


Figure 3.3. Accumulation of oxidative stress markers in the brains of 18 month-old female 3xTg-AD mice that had received 5 m of MitoQ treatment. ***A***, MitoQ treatment reduced lipid peroxidation (MDA levels) in the brains of the 3xTg-AD mice compared to

the same aged nonTg controls ($p < 0.01$). MDA levels were determined using the TBARS assay ($n = 6$-14). ***B***, MitoQ treatment reduced the buildup of nitrated-tyrosine products. Decrease of the nitration marker, 3-nitrotyrosine (3-NT) in the brains of the MitoQ-treated mice indicated that MitoQ inhibited accumulation of nitration produced in them. The bands are individual proteins that have been modified by nitration. Immunoblot densities are shown as fold change relative to average density of nonTg brains ($p < 0.01$; $n = 3$ for each condition).

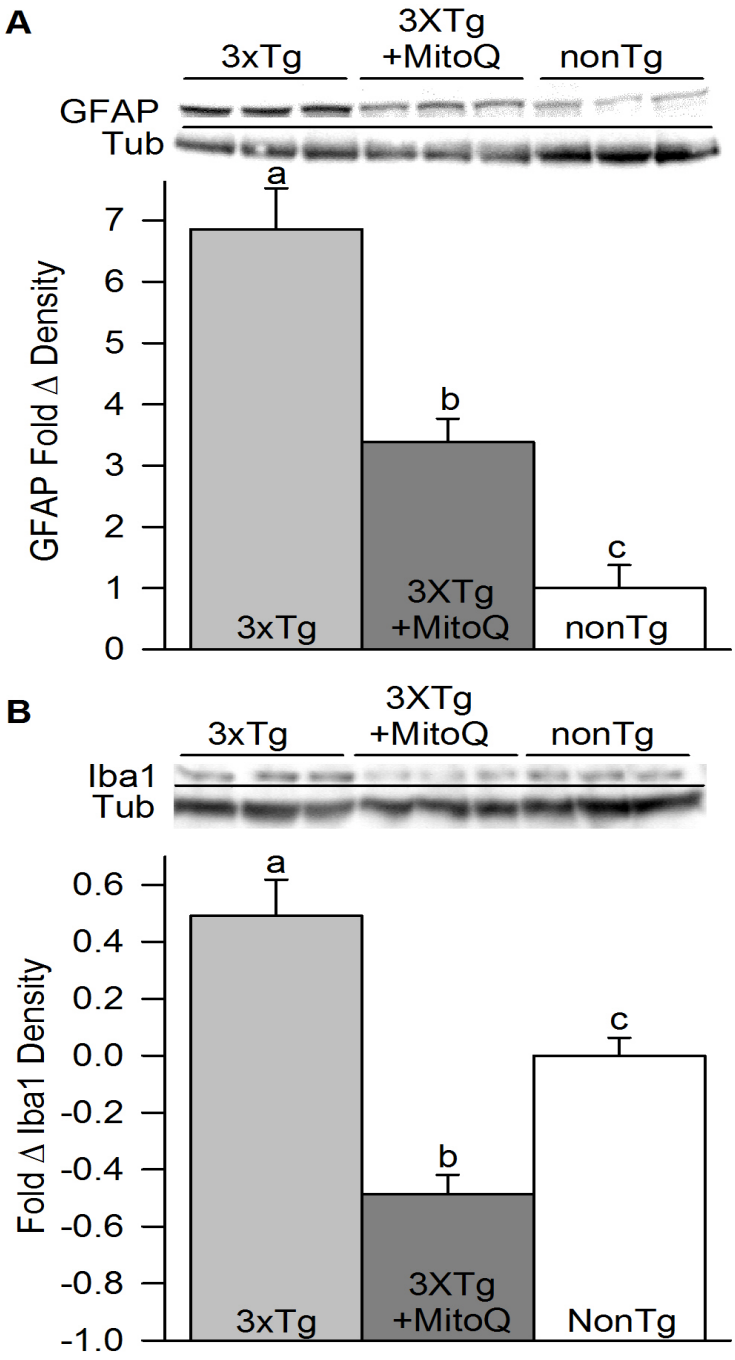


Figure 3.4. MitoQ inhibited astrogliosis and microglial cell proliferation in the brains of aged female 3xTg-AD mice. ***A***, Glial fibrillary acidic protein (GFAP), an astrocyte marker, was elevated in the brains of untreated 3xTg-AD mice compared to the brains of nonTg animals indicating proliferation of astrocytes. MitoQ treatment reduced GFAP

levels to well below those found in nonTG mice indicating that it greatly reduced astrocyte proliferation ($p < 0.01$; $n = 3$ for each condition). ***B***, Iba1, a microglial cell marker, was elevated in the brains of 3xTg-AD mice compared to the brains of nonTg controls. MitoQ treatment reduced brain Iba1 levels to below those of both 3xTg-AD and nonTg animals suggesting significant reduction of microglial cell proliferation ($p < 0.01$; $n = 4$ for each condition).

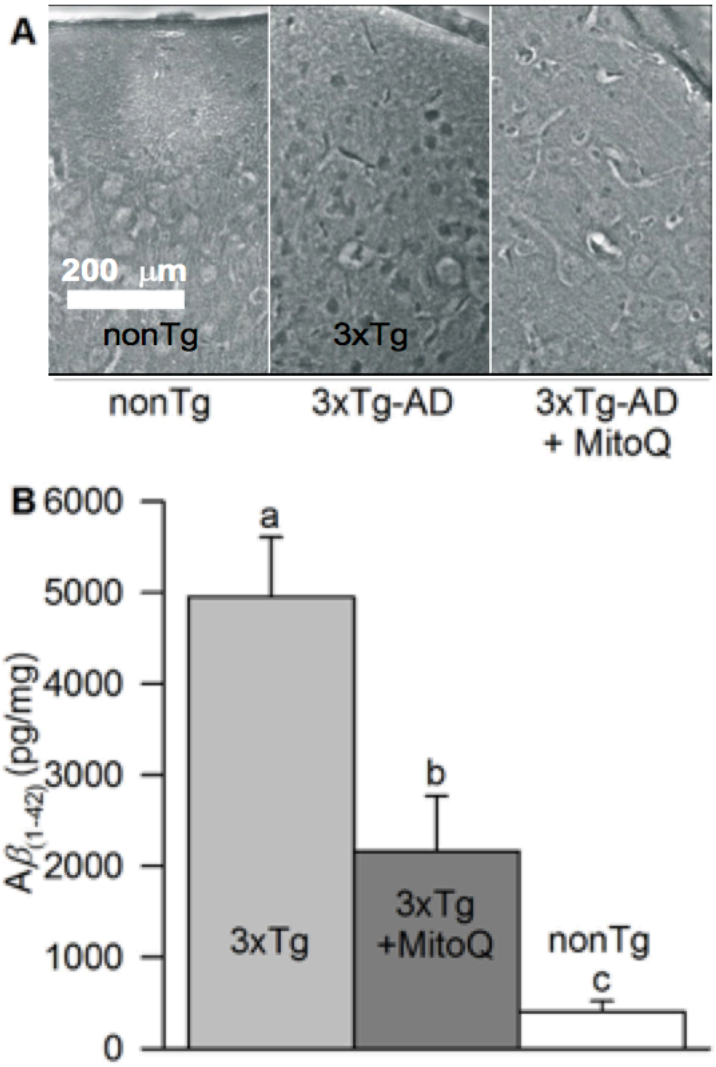


Figure 3.5. MitoQ decreased A$\beta_{(1\text{-}42)}$ burden in the brains of aged female 3xTg-AD mice. ***A***, Representative photomicrographs showing immunostaining for A$\beta_{(1\text{-}42)}$ in the neocortex of 18 month-old female nonTg, 3xTg-AD, and 3xTG-AD mice that had received MitoQ treatment for the preceding 5 m. Intraneuronal and extracellular staining for A$\beta_{(1\text{-}42)}$ was largely absent in nonTg brains and reduced in brains from MitoQ-treated 3xTg-AD mice. ***B***, Quantification of soluble A$\beta_{(1\text{-}42)}$ by ELISA revealed that MitoQ

treatment significantly reduced amyloid burden in the 3xTg-AD mouse brain ($p < 0.01$; n = 6-8 brains for each condition).

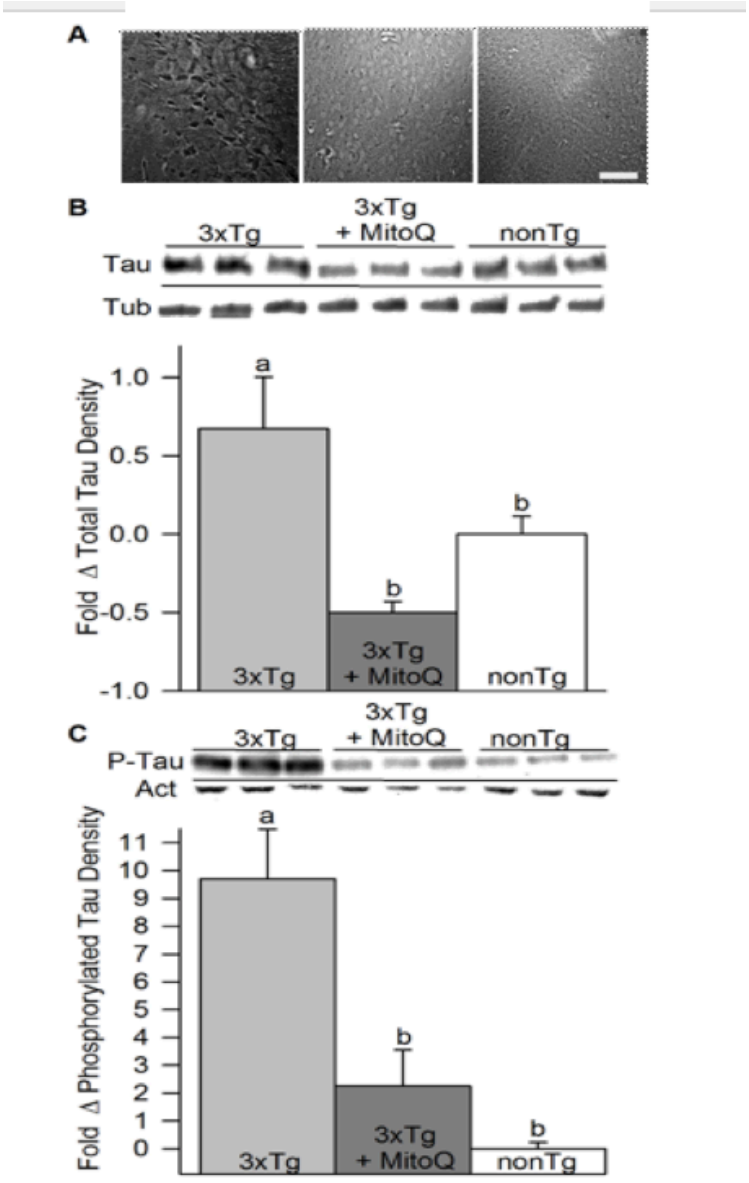
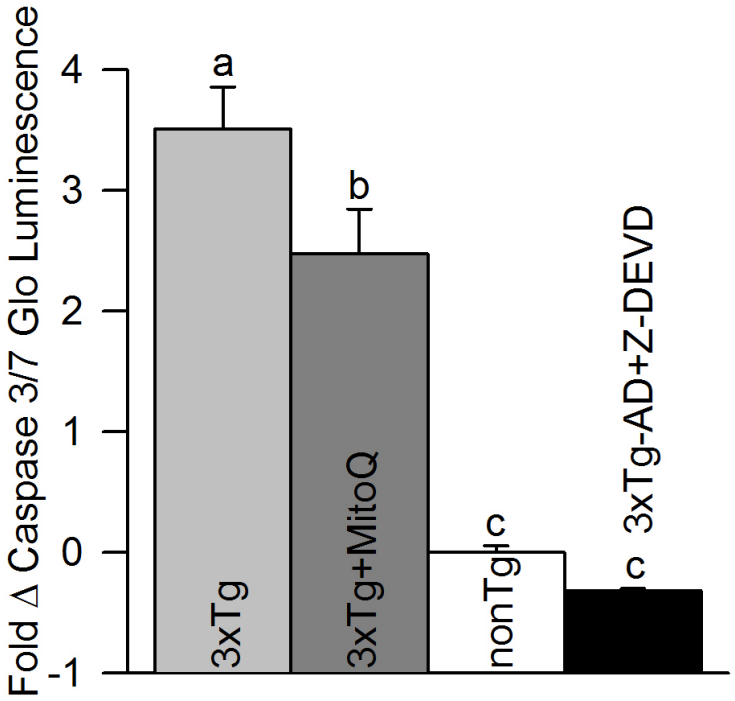


Figure 3.6. MitoQ decreased Tau pathology in the brains of aged female3xTg-AD mice. ***A,*** Representative photomicrographs showing NFT in the neocortex of 18 month-old female nonTg, 3xTg-AD, and 3xTG-AD mice that had received MitoQ treatment for the preceding 5 m. Staining is for total Tau protein. *B*, MitoQ treatment greatly decreased total Tau levels in the brains of these mice as measured by immunoblots ($p < 01$; $n = 3$ for each condition). *C.* MitoQ treatment also greatly inhibited phosphorylated tau levels in the brains of these mice ($p < 0.01$; $n = 3$ for each condition).



. **Figure 3.7** MitoQ treatment reduced Caspase 3/7 activity in the brains of aged female 3xTg-AD mice. Caspase 3/7 activity in brain homogenates was analyzed with the Caspase-Glo 3/7 assay. Relative luminescence values, produced with a caspase 3/7-specific substrate, were measured by a luminometer. Values are normalized to nonTg caspase activity. Z-DEVD (conc), a broad-spectrum caspase inhibitor, reduced elevated caspase activity in 3xTg brain homogenates, confirming caspase activity was measured by the assay ($p < 0.01$; $n = 6$-10 except for Z-DEVD where $n = 2$).

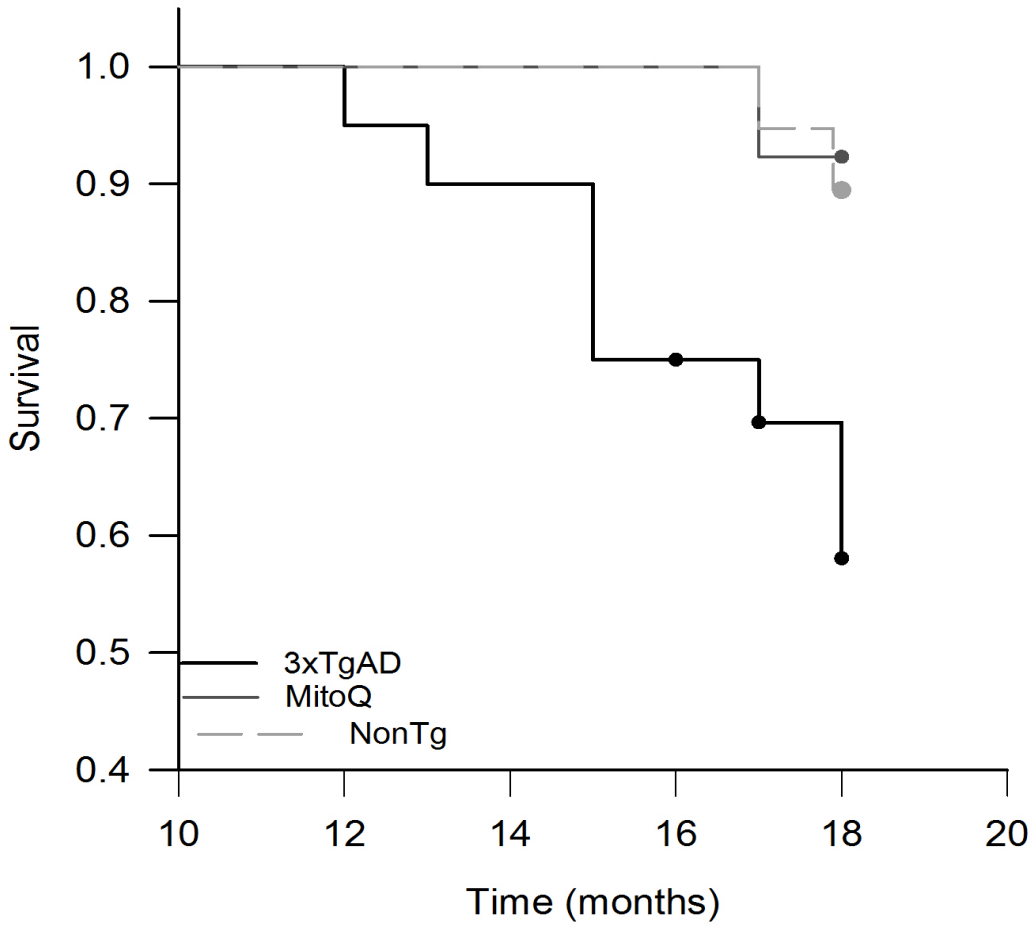


Figure 3.8. MitoQ treatment increased lifespan in aged 3xTg-AD female mice to that of the nonTG controls ($p = 0.07$ comparing MitoQ-treated to untreated 3xTG-AD mice). Survival was determined by Kaplan-Meier log-rank analysis for all mice until an end point of 18 m of age ($n = 15$-20 mice).

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The principal goal of this research was to provide insight into the role of mitochondria-mediated stress in AD-like disease progression. The discoveries herein provide proof of the concept that potent targeted antioxidants are capable of significantly reducing mitochondria-generated reactive species, even in an environment such as the 3xTg-AD mouse model, which is predisposed, genetically altered, and primed for neuronal demise. Additionally, these results further indicate that age-dependent neurodegeneration is facilitated and influenced heavily by mitochondria-generated reactive species. These results further underscore the usefulness of mitochondria-targeted antioxidants as a means to combat excessive reactive species and the destructive damage they facilitate throughout the CNS.

Within our study, we aimed to further elucidate the role that mitochondria-derived oxidative stress plays in AD disease progression. The results we provide indicate not only that mitochondria-generated reactive species are a major source of oxidative stress within the context of AD-like pathology but also that they play a critical role in disease modulation. The suppression of mitochondria- generated reactive species with the targeted antioxidant, MitoQ, not only inhibits the proliferation of markers of oxidative stress but also influences the propagation of several other downstream pathologies. These data provide substantial evidence that mitochondria-derived oxidative stress is not only

involved in increasing the toxicity of prominent pathologies *in vitro*, as we and many other researchers have shown, but also makes mitochondria-derived oxidative stress a viable target for treatment strategies (Szeto, 2006; Moreira et al., 2010; McManus et al., 2011; Mao et al., 2012).

Age-Dependent Neurodegeneration and Mitochondria

Age-dependent neurodegeneration is the results of molecular processes becoming increasingly inefficient with age (Knopman et al., 2016). Consequently, as life expectancy increases, age-dependent neurodegeneration progressively affects a larger proportion of the population. The occurrence of mitochondrial dysfunction, increasingly more present with aging, is a contributor to the overall decline in molecular processes. A series of negative feedback mechanisms, including the decline of several antioxidant defense mechanisms, the accumulation of mitochondrial DNA mutations, and impaired respiration chain function, further increase reactive species production at the mitochondria (Piko et al., 1988; Cortopassi and Arnheim, 1990; Fraga et al., 1990). Though changes in mitochondrial homeostasis occur in the absence of AD, age-related mitochondrial abnormalities are exacerbated or altered to some extent when occurring in conjunction with AD (Hirai et al., 2001), possibly linking mitochondrial aging and neurodegeneration. More recently, it was reported that the imbalance in nuclear and mitochondrial genomic material encoding for OXPHOS transcripts are a potential culprit for compromised OXPHOS efficiency and a trigger for the irregular release of reactive species (Lunnon et al., 2017).

Early supplementation and constitutive genetic manipulations of antioxidant defense mechanisms overcome the detrimental effects of AD-like disease progression in model organisms by specifically reducing mitochondria-related oxidative stress (St-Pierre et al., 2006; Massaad et al., 2009; McManus et al., 2011). However, while the literature clearly identifies mitochondria-generated oxidative stress as a modulator of key pathologies in AD progress, this study demonstrates that age-dependent oxidative stress accumulated throughout the disease state can be affected by and changed after treatment. We treated aged mice with MitoQ well after all known AD pathologies were present, targeting pathologies that developed in our model two months before cognitive deficits, and four and ten months before Aβ and Tau, respectively. The results indicate that not only does mitochondria-mediated oxidative stress mediate the toxic pathologies but also that the process is dynamic, fluid, and reversible enough to ultimately improve cognitive function. The limitation is that our mouse model, 3xTg-AD, lacks neuronal death, which is irreversible; however, our model does display the synapse loss prominently shown in humans (Clark et al., 2015). Furthermore, the timeline of neuronal loss in humans is not well understood and leaves some room for exploration. Because synapse loss is a major contributor to memory deficits that are associated with AD, further inhibition of loss and an overall improvement in memory with treatment using MitoQ positively reinforces the potential of targeted antioxidants to reduce downstream symptoms/phenotypes of AD mechanisms. Additionally, these results further support the literature, which increasingly suggests that the signaling mechanisms influenced by aberrant reactive species production play a mediating role in disease progression (Atzori et al., 2001; Quiroz-Baez et al., 2009; Gandhi and Abramov, 2012; Caccamo et al., 2015).

Mitochondria are essential to the aging of cells. The removal of mitochondria via a method of "artificial mitophagy" from aging cells reverses inflammation and restores DNA levels back to levels comparable to young cells, which suggests that mitochondria biogenesis is a major driver of the aging mechanism (Correia-Melo et al., 2016). While Correia-Melo et al. were the first to definitively prove mitochondria's involvement in aging, the major limitation is that the removal of older mitochondria from cells is not actionable in a clinical setting. However, based on this evidence, if the removal of aging mitochondria can attenuate the aging process, it is reasonable to infer that repairing and improving the bioenergetics of aging mitochondria could perhaps have a similar effect. In the study presented here, MitoQ treatment over time in 3xTg-AD mice appears to be doing just that. MitoQ-treated mice have a life span comparable to nontg-control animals, whereas non-treated 3xTg-AD mice display significant mortality beginning at 12 months of age. It is interesting to note, and perhaps somewhat contrary to our results, that the literature shows that overexpression of non-targeted antioxidant enzymes is not significantly effective in extending lifespan. In addition, dietary supplements also do not improve lifespan. Only one variable distinguishes the antioxidant approach used in the study presented here from other antioxidant approaches currently being assessed—MitoQ. MitoQ directly interacts with the mitochondria and perhaps through some mechanism of improving overall bioenergetics results in the extension of lifespan in our animals.

Mitochondria: Neurological Gate Keepers

Mitochondria are major providers of energy to the cell, and their function is critical for the production of ATP for cellular energy requirements. Tissue and organ functions depend on adequate ATP production, especially when energy demand is high. Mitochondria also play a role in a vast array of important biochemical pathways, including apoptosis, generation and detoxification of reactive oxygen species, intracellular calcium regulation, steroid hormone and heme synthesis, and lipid metabolism. The complexity of mitochondrial structure and function facilitates its diverse roles within the cell but also enhances its vulnerability. For instance, with regard to the production of ATP, dysfunction during production can increase the leakage of reactive species leading to increased oxidative stress, which is a prominent pathology in AD that ultimately results in neuronal death (Devi et al., 2006).

The consequences of Alzheimer's disease are as dependent on dysfunctional mitochondria as on location. The central nervous system, particularly the brain, has a naturally high demand for oxygen, which under normal circumstances can be occasionally converted to the free radical superoxide through interaction with leaked electrons from the ETC. Free radicals are important to signaling and are normally detoxified through a succession of chemical reactions with antioxidant enzymes. However, the brain, an area of the CNS with higher levels of unsaturated fatty acids and relatively low levels of antioxidants, is at a greater risk for oxidative modifications that result in dysfunctional proteins, lipids, and DNA.

Dysfunctional mitochondria, increased reactive species, and a location ripe for oxidative stress all contribute to the overall increase in oxidative stress that is associated

with aging (Sun et al., 2016; Swerdlow et al., 2017). Although aging is the number one risk factor for AD, what further differentiates Alzheimer's from normal aging is the increase in "mitochondria stemming" modulating pathological factors in the disease state (Association, 2017). The literature shows that reactive species affect knowns and well-studied AD pathologies either through direct interaction or indirectly by altering the expression of key enzyme modulators in some way.

Many studies have suggested, with evidence of oxidative stress and mitochondrial impairment occurring early in the disease state, that these factors lie figuratively upstream of other AD-associated pathologies. However, the question remains: If mitochondria-stemming oxidative stress has a major role in AD disease progression, will reducing oxidative stress inhibit the propagation of AD-associated pathologies? Furthermore, will the addition of a targeted antioxidant be enough to reduce the oxidative stress in a late-stage model?

We answered these questions in the study discussed. The most detrimental aspect of AD is memory loss, and our results suggest that targeted antioxidants have the potential to slow that loss.

Clinical Implications of Mitochondria-targeted antioxidants for Neurodegeneration

Alzheimer's disease is vastly complex and multifactorial. Nevertheless, clinical trials have focused on the hallmark pathologies involving misfolded proteins Aβ and tau. However, mitochondrial abnormalities within the disease state are often detectable prior to memory loss and the accumulation of misfolded protein pathologies. These abnormalities include decreased mitochondrial respiration needed for activity and

morphology, which are vital for healthy mitochondrial function. Further, downregulation of oxidative phosphorylation and impairment of import pathways (such as TIM and TOM) are hallmarks of AD noted in patients (Sorrentino, 2017).

Although published data using preclinical models of AD suggest that removal of Aβ or tau pathology is paramount to improving the overall etiology of Alzheimer's disease, the results of human clinical trials have yet to mirror those obtained in animal models (Oddo et al., 2004; Siemers et al., 2006; Doody et al., 2013; Walls et al., 2014; Dai et al., 2017). While animal models do provide valuable insight into mechanistic patterns in disease, they also inherently fail to provide the complete story due to their biological limitations (van der Worp et al., 2010; Webster et al., 2014). Even so, work initially conducted in model organisms has resulted in various discoveries leading to significant progress in human therapy. Thus, one must ask this question: Why are neurodegenerative diseases, including Alzheimer's disease, so difficult to understand? One reason perhaps is the reliance on genetically altered animals modeled after FAD that have been used to understand the development of sporadic AD in humans who do not harbor such mutations. These models have been the best representations we have had thus far, but perhaps still they are not sufficient (mechanistic vs Clinical). Even so, if we take a look at other disease states, genetically modified model organisms have been instrumental in the development of effective treatments. With ever-increasing technological advancements and the occurrence of newer more physiologically relevant models, this concern is being addressed.

Another glaring caveat to the path we have taken to investigate disease progression and treatment strategies perhaps is the focus on hallmark pathologies. A

pathology such as Aβ allows for the distinction of AD from other forms of related dementia that present similar phenotypes clinically. However, once plaques are detectable with current technological advances, they are largely symptomatic of the overall disease state and not causative. Despite the limitations of the model used in this study, I would argue that a shift in focus of pathologies not so dependent on mutations is warranted. Further exploration of upstream pathologies of Aβ and Tau may provide additional unbiased (from familial mutations) insight into the inner workings of the disease and perhaps lead to new and/or simply better ways to target the known mechanisms that lead to disease propagation. First understanding and then targeting factors upstream of Aβ, such as mitochondrial ROS, common in both familial and sporadic AD, are the necessary next steps for progress in developing effective treatment strategies.

It would be irresponsible not to mention that several clinical trials have been conducted with minimal success using dietary supplement antioxidants such as vitamin E, Ginkgo Biloba, and Curcumin (Ringman et al., 2012; Dysken et al., 2014; Yang et al., 2015). However, these dietary supplements lacked the targeting and renewable properties present in MitoQ (Kelso et al., 2001). It is recommended that further research explore targetable antioxidants that concentrate at the area of most concern and therefore have more therapeutic potential.

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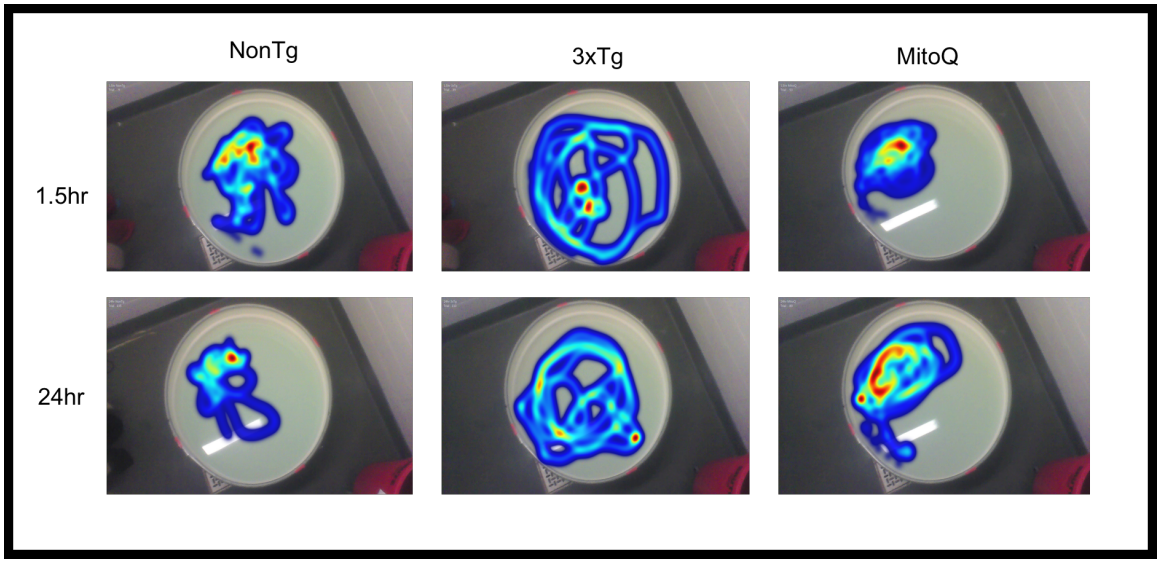
APPENDIX



Appendix figure 5.1 Search strategies. Spatial memory retention was assessed in probe trials conducted 1.5 h and 24 h after the last training acquisition trial. During the probe trials, the platform was removed and mice were allowed a 60 s free swim. Both NonTg and MitoQ treated 3xTg female mice display intentional goal-orientated search strategies further indicating that spatial retention was achieved. 3xTg-AD mice perform exploratory swim patterns indicative of a lack of memory retention. Representative heat maps for the swim paths of the 1.5 h and 24 h probe trials for treatment groups were generated from video analysis by Ethovision tracking software.

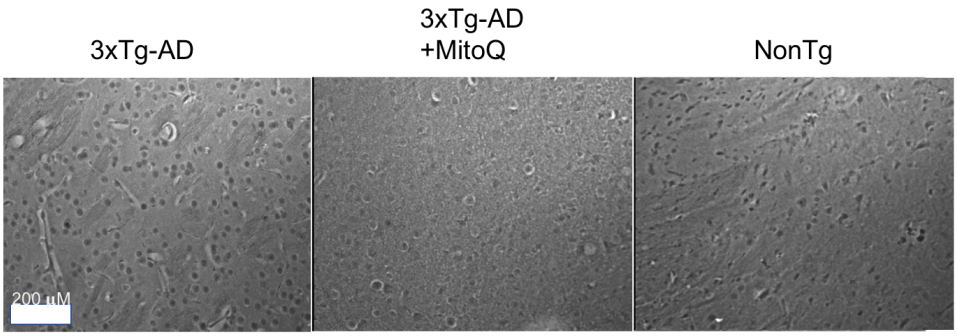


Figure 5.2 Representative photomicrographs showing hyperphosphorylated tau in the neocortex of 18-month-old female nonTg, 3xTg-AD, and 3xTG-AD mice that had received MitoQ treatment for 5 m. Staining is for AT8 specific Tau protein.