THE ROLE OF MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS IN AGE-DEPENDENT NEURODEGENERATION: IMPLICATIONS FOR THE USE OF MITOCHONDRIA-TARGETED THERAPEUTICS

by

MEAGAN J. MCMANUS

(Under the Direction of James L. Franklin)

ABSTRACT

The work presented herein illustrates the multifaceted role of mitochondria in neurodegeneration and provides evidence supporting the use of mitochondria-targeted therapeutics in diseases involving oxidative stress and metabolic failure, namely Alzheimer's disease (AD). The results implicate mitochondria-derived reactive species (RS) as a central mediator of neuronal apoptosis. The data suggest that while JNK activation is involved in many forms of apoptosis, it is not necessary for mitochondrial RS generation, which may explain its limited role in dictating apoptogenic cytochrome *c* redistrubution in trophic factor-deprived, sympathetic neurons isolated from mice. In order to provide a greater understanding of the relationship between mitochondrial oxidative stress and neuronal demise, mitochondria-targeted antioxidants were tested on *in vitro* models of neurodegeneration. MitoQ effectively attenuated all critical, mitochondria-dependent events associated with neuronal apoptosis, including superoxide production from the mitochondrial electron transport chain, cytochrome *c* redistribution, caspase activation, loss of mitochondrial membrane potential, and the ultimate death of the cell. MitoQ also prolonged the commitment to caspase-independent death by

protecting the inner mitochondrial membrane (IMM). Conversely, $MitoE_2$ had no effect on caspase-dependent and caspase-independent events leading to neuronal death, which may be due to inadequate accumulation within the IMM and the inability to sufficiently suppress mitochondrial RS. To determine whether the apparent mitochondrial protection conferred by MitoQ was sufficient to prevent the emergence of AD-associated neuropathology *in vivo*, young, triple-transgenic AD mice were treated with MitoQ for 5 months and the effect on AD progression was analyzed. The results indicate that MitoQ prevented the onset of cognitive decline, oxidative stress, Aβ-accumulation, and activation of apoptotic machinery in female 3xTg-AD mice. These findings provide solid evidence of a role for mitochondrial RS in age-dependent neurodegeneration and suggest mitochondria-targeted therapies hold great promise for the treatment of Alzheimer's disease.

INDEX WORDS: mitochondria; oxidative stress; neuronal apoptosis; mitochondrial therapeutics; MitoQ; neurodegenerative disease; Alzheimer's disease; 3xTg-AD mice; learning and memory

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DEDICATION

I dedicate this work to my family, whose love has made this possible:

To my dad, who looked at a dreamer and saw a scientist: your great heart has been to mine an inspiration beyond words. Long ago, you told me that the "impossible" dreams were the only ones worth chasing, and that has made all the difference. As a child, I always loved "riding on your shoulders" - I guess not much has changed; thank you for enabling me to reach higher.

To my mom, the most talented, creative genius I have ever known: you always said that nothing was worth doing unless you do it with all your mind, body and soul - but you forgot to mention how exhausting that is! Thank you for *showing* me that anything is possible if you work hard enough. You have given me "more" than I could ever ask for in love and support, and I can only hope to repay you day by day with the same.

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To my beautiful grandmother, Sara Louise, who has shown me that the secret to the fountain of youth is in retaining the ability to grow spiritually. Your endless prayers, love, and wisdom have carried me through life's hugest hurtles.

And lastly, to my grandparents, Hazel and Chet Krieger, who exemplified the diverse and devastating effects Alzheimer's disease can have on the most precious, loving people of all. You continue to inspire me and will forever be found in my heart.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Neurodegenerative diseases are those in which a particular population of neurons degenerate and die. The location and the type of neuron within the population affected determine how the pathology manifests symptomatically. For example, in Parkinson's disease, neurons of the substantia nigra degenerate, causing movement disorder (initiation and tremor), whereas in Alzheimer's disease, neurons within the medial temporal lobe that are primarily responsible for learning and memory are most affected. The most prevalent neurodegenerative diseases are Alzheimer's disease (AD) and Parkinson's disease (PD), followed by Amyotrophic Lateral Sclerosis (ALS), Freidrich's ataxia (FA), and Huntington's disease (HD). These diseases are age-dependent, and those that follow a Mendelian form of inheritance typically occur at an earlier age (FA and HD) than those that are largely sporadic (AD, PD, and ALS) (Table 1). Despite the late age of clinical diagnosis associated with the sporadic forms, increasing evidence suggests that critical damage to the nervous system may be occurring years before symptomatic manifestation of the disease. It follows that therapeutics which correct or attenuate the mechanisms responsible for this early damage may prove the most effective strategy for preserving neurological health.

The most consistent data describe increased oxidative stress and impaired bioenergetics in the earliest stages of all of these diseases, suggesting that mitochondria dysfunction may be an important, recurrent theme (Halliwell and Gutteridge, 2007). Since nuclear DNA (nDNA) encodes many of the proteins necessary for proper mitochondrial function, it undoubtedly plays

a part in impaired energetics that lead to these diseases. However, in cytoplasmic hybrid cells(cybrids) expressing mitochondrial DNA (mtDNA) from subjects with the sporadic forms of AD, PD, or ALS, and nDNA from cell lines or control subjects, the cells exhibit metabolic impairments indicative of the disease months later, suggesting mitochondrial dysfunction arises intrinsically, from an unfavorable combination of inherited and age-acquired defects in mtDNA (Swerdlow *et al.,* 1998; Wallace *et al.,* 2010). These findings are quite amazing, considering that almost 80% of mitochondrial metabolic machinery is encoded by nDNA, yet mtDNA alterations alone are sufficient to shift the cellular phenotype.

mtDNA and select nDNA together determine an individual's baseline mitochondrial energetic capacity, and may also predispose people to age-dependent neurodegeneration (Wallace, 2008b). Unlike nDNA, mtDNA is inherited entirely from the female in most species, and is present in thousands of copies per cell, such that both normal and various mutant copies coexist (heteroplasmy). The mutation rate for mtDNA is estimated to be more than 100-fold higher than that of nDNA, perhaps due to the closer proximity of mtDNA to mutagenic mitochondrial reactive oxygen species (ROS) (Nachman *et al.,* 1996; Schriner *et al.,* 2000; Wallace and Fan, 2009). As we age, somatic mtDNA mutations appear to be clonally amplified within post-mitotic cells, which in time, may drive phenotypic expression of impaired mitochondrial function (Reeve, *et al.,* 2008).

Recent animal studies support an old suspicion that the rise in mtDNA mutations and corresponding decline in mitochondrial function are important determinants of mammalian aging (Larsson, 2010). Proofreading deficits induced by a mitochondrial polymerase-γ (POLYG) mutation cause proliferation of somatic mtDNA deletions and mutations, and these young mt- mutator mice exhibit several phenotypes reminiscent of human aging, including

weight loss, alopecia, osteoporosis, cardiomyopathy, anemia and sarcopaenia (Kujoth *et al.,* 2005b). Although overt neuropathology was not seen in the mt-mutator mice, POLYG mutations in humans are associated with neurological dysfunction, such as parkinsonism and ophthalmoplegia, (Luoma *et al.,* 2004; Mancuso *et al.,* 2004).

Interestingly, when mt-mutator mice also express a mitochondria-specific catalase, the mtDNA mutation load is reduced by over 50%, the progeric phenotype is attenuated, and the mice live longer, suggesting that mitochondrial ROS also play a causal role in this process (Dai *et al*., 2009). mtDNA encodes critical subunits of the proton-pumping respiratory complexes (I, III, IV, and V) and the structural RNAs necessary for their expression. The delicate balance within this ~~"c~~hain" of proteins determines the coupling efficiency of oxidative phosphorylation (OXPHOS) (Wallace, 2010c). Therefore, alterations in mtDNA may disrupt this critical balance, which would likely result in less ATP and more ROS, thereby causing damage to mtDNA, proteins, and lipids, and further perpetuating mitochondrial dysfunction (Indo *et al.,* 2007). Oxidative damage to mitochondrial components may also contribute to the development of age- associated diseases by inducing apoptosis and subsequent loss of irreplaceable cells, such as neurons (Kujoth *et al.,* 2005b; Wallace, 2008a). In order to understand the implications of these propagating pathways in age-dependent neurodegenerative disease, we must consider the basic biology and functional significance of the mitochondria to the nervous system.

Table 1.1. Characteristics of the most prevalent neurodegenerative diseases.
Abbreviations: amyloid precursor protein (APP); Leucine-rich repeat kinase 2 (LRRK2); PTEN-induced kinase 1 (PINK1); copper zinc superoxide dismutase (CuZnSOD); 8-hydroxydeoxyguanosine (8OHdG)4-hydroxy-2-*trans*-nonenal (HNE); 3- nitrotryosine (3-NT); advanced glycation end products (AGEs); F_2-Isoprostanes (F_2-IPs); cerebral metabolic rate for glucose (CMR_{GLU}); αketoglutarate dehydrogenase (αKDGH); pyruvate dehydrogenase (PDH); c-Jun N-terminal kinase (JNK). *autosomal dominant mutation, others are recessive. ** (Swerdlow *et al.,* 1998; Trimmer *et al.,* 2000).

Characteristics	Alzheimer's Disease	Parkinson's Disease	Amyotrophic Lateral Sclerosis	Huntington's Disease	Feidreich's ataxia
Anatomical	Medial temporal lobe Cortex	Substantia nigra Locus Coeruleus	Motor Cortex Brainstem Spinal cord	Caudate nucleus Putamen	Cerebellum Spinal Cord
Mendelian Genetic Association	APP*, Presenilin 1 & 2* <1%	LRRK2*, α-synuclein*, Parkin, PINK1 <10%	CuZn SOD* <10%	Huntington*	Frataxin
Age of Onset	>65	>55	>60	>30	>15
Oxidative Stress	3-NT, 8OHdG, AGEs, F_2-IPs, HNE, and more	3-NT, 8OHdG, AGEs, F_2-IPs, HNE, and more	3-NT, 8OHdG AGEs	3-NT , 8OHdG, HNE	Ur-8OHdG
Metabolic Impairment	CMR_{GLU}, Complex IV and V, αKGDH, PDH	Complex I, αKGDH	Complex I, Complex IV	Complex II, III αKGDH Aconitase	Complex I, II, and III Aconitase
Sporadic Forms: mtDNA Cybrids **	• Reduced complex IV activity • Oxidative stress • Reduced ATP • Mitochondrial depolarization • Increased caspase 3activity • Activated stress pathways • Increased intra- & extracellular Aβ	• Reduced complex I activity • Oxidative stress • Reduced ATP • Elevated Bcl2s • Mitochondrial depolarization • Decreased mitochondrial Ca^{2+} • Activated JNK •Altered mitochondrial ultrastructure • Synuclein aggregations	• Reduced complex I activity • Oxidative stress •Reduced mitochondrial calcium •Altered mitochondrial ultrastructure		

Mitochondria: The Source of Neurological Chi

The flow of energy through the human body generates and sustains the biological complexity set by the codes in nucleic acids (Wallace, 2008). For this purpose, there are approximately 10^{17} energy-generating mitochondria within the human body. These intricate, molecular

capacitors possess a highly specialized inner membrane, which compartmentalizes their metabolic functions. In contrast to the permeable outer membrane, the inner mitochondrial membrane (IMM) is protein-rich and extremely selective, allowing only specific molecules entry via discriminatory transporters. The brain relies almost exclusively on glucose as a substrate for energy production, which is broken down via glycolysis in the cytosol to produce a small amount of ATP, NADH, and pyruvate. Pyruvate is then transported through the IMM to the mitochondrial matrix and enters the tricarboxylic acid (TCA) cycle by pyruvate dehydrogenase (PDH). A major function of the TCA cycle is to strip hydrogen off the hydrocarbon chains to provide reducing equivalents to the respiratory chain, located within the IMM. The TCA cycle reduces NAD^+ and FAD^+ to NADH and $FADH_2$, which then donate the electrons from hydrogen to the chain via complex I and II, respectively. Coenzyme Q_{10} is reduced either by complex I or II, producing ubiquinol (QH_2). QH_2 transfers electrons to complex III, which then reduces cytochrome *c*. Cytochrome *c* shuttles electrons to the terminal oxidase (complex IV), which retains all partially reduced intermediates until the full, four-electron reduction of O_2 to H_2O is achieved. The energy produced from this series of favorable redox reactions is harnessed to pump protons from the matrix to the intermembrane space, thus establishing a proton gradient over the IMM and creating the mitochondrial membrane potential ($\Delta\psi_m$). This potential energy is then converted to chemical energy for life, in the form of ATP by the inner membrane H^+- ATPase (Wallace *et al.,* 2010).

The human brain utilizes approximately $4x10^{28}$ ATP molecules per minute, the majority of which is used to operate the electrogenic activity of neurons (Ames III, 2000; Halliwell and Gutteridge, 2007). Adequate energy supply by mitochondria is essential to

maintain axonal transport, ionic homeostasis, neuronal excitability, and survival. In response to synaptic activation, mitochondria accumulate in dendritic spines and are required to generate and maintain their morphological structure (Kroemer *et al.,* 2007). Mitochondria are also abundant in axons and boutons within synaptic terminals, where they serve a vital role in Ca^{2+} regulation and ATP generation required for proper neurotransmission. Mitochondrial distribution within the neuron is dictated in part by mitochondria fission and fusion, which allow these dynamic organelles to meet instantaneous energy demands and exchange components to maintain efficient respiration (Xiang *et al.,* 1996). Mutation or oxidative modification of fission and fusion proteins results in mitochondria mislocalization to the cell body, energetic failure, synaptic loss, and neurodegeneration (Kowaltowski *et al.,* 2001; Cho *et al.,* 2009).

Reactive Species: Production and Peril

The transport of high energy electrons through the mitochondrial respiratory chain provides not only a source ATP, but also of ROS, by the monovalent reduction of molecular oxygen to form the superoxide radical anion ($O_2^{\cdot-}$) (Murphy, 2009). ROS is a collective term that includes both oxygen-centered radicals (unpaired electron), as well as non-radicals that are oxidizing agents or are easily converted into radicals (Turrens, 2003). Due to their transient nature, it is currently difficult to directly measure ROS *in vivo*, and therefore analysis of ROS levels is largely dependent on the molecular footprints left in their wake of destruction (Halliwell and Gutteridge, 2007). When concentrations of ROS exceed the cellular ability to remove ROS via endogenous antioxidants, oxidative stress arises and damage ensues. Such stress may cause damage by the oxidation of critical cellular components, including nucleic acids, proteins, and lipids.

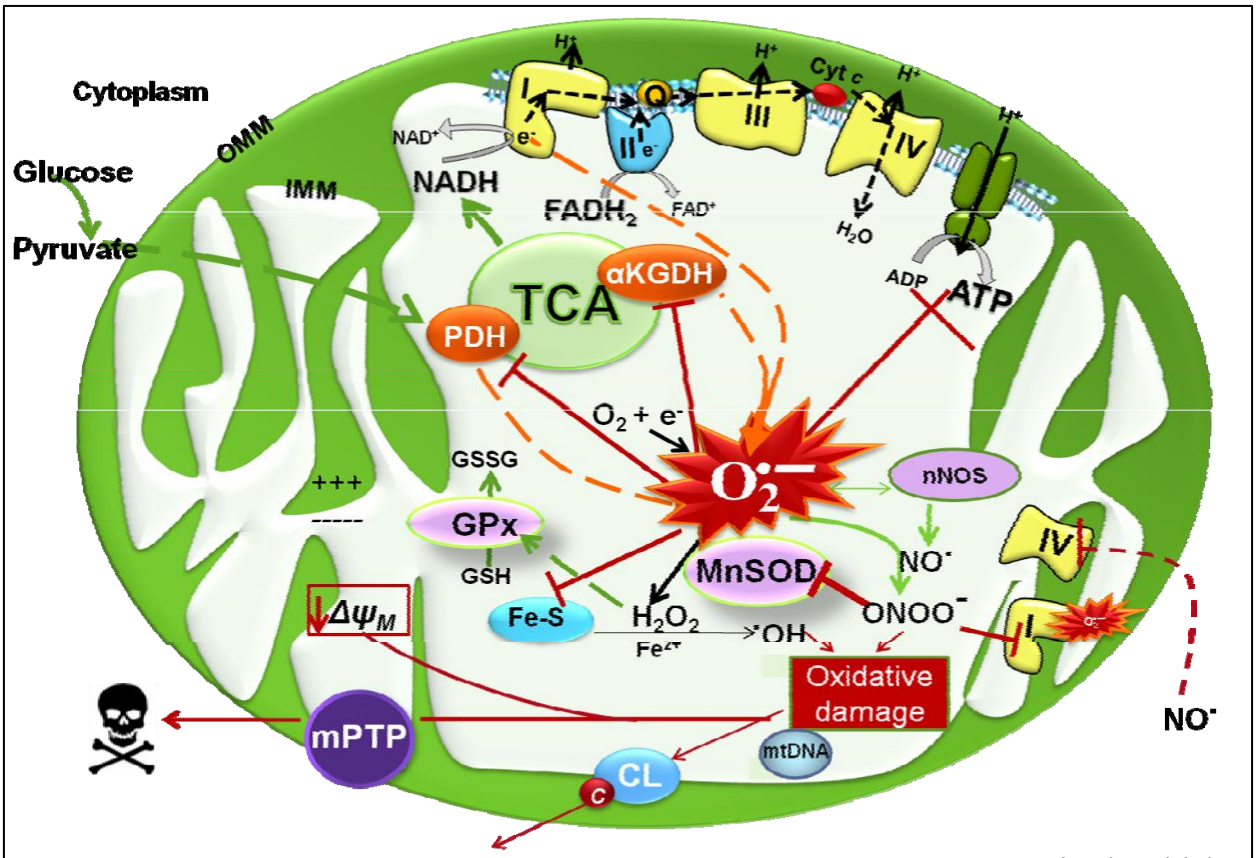

Figure 1.1. Mitochondrial bioenergetics, reactive species, and apoptosis. Mitochondrial generation of RS and regeneration of key antioxidants (i.e., glutathione, GSH) are both intimately linked to the energetic status of the mitochondria. ATP generated by oxidative phosphorylation is dependent on efficient electron transfer and maintenance of $\Delta\psi_m$. $O_2^{\bullet-}$ may be formed toward the matrix via the one-electron reduction of molecular oxygen by respiratory chain components (I and III), as well as theTCA cycle enzymes, pyruvate dehydrogenase (PDH) and αketoglutarate dehydrogenase (αKGDH). Basal $O_2^{\bullet-}$ generation rates are enhanced in many neuropathological situations. $O_2^{\bullet-}$ is mainly detoxified by MnSOD. $O_2^{\bullet-}$ also reacts by a diffusion-controlled rate with $NO^{\bullet}$, forming $ONOO^-$, which inhibits MnSOD, allowing $O_2^{\bullet-}$ levels to rise. $ONOO^-$ and $O_2^{\bullet-}$ oxidatively inhibit bioenergetic enzymes: inhibition of respiratory complexes may decrease ATP; inhibition of TCA enzymes may alter the balance of pyrimidine nucleotides and lead to antioxidant depletion. $ONOO^-$ and $O_2^{\bullet-}$ may also cause cardiolipin (CL) peroxidation, as well as nitration and oxidation of cytochrome *c*, leading to its apoptotic effects. $O_2^{\bullet-}$oxidizes the Fe-S center of metabolic proteins, releasing free Fe^{2+} and, consequently, may generate indiscriminant $^{\bullet}OH$-mediated damage to lipids, protein and DNA. Compromise of the IMM perturbs mitochondrial Ca^{2+} regulation, activates the mitochondrial permeability transition pore (mPTP), abolishes $\Delta\psi_m$ and ATP production, and activates the caspase family of apoptotic executioners, resulting in cell death.

Oxidative stress has been implicated in the pathogenesis of a number of diseases, including cancer, ischemia, and neurodegenerative disorders (Wallace, 2005). Among all organs of the body, the brain is perhaps most vulnerable to oxidative damage due to its high utilization of oxygen, increased levels of polyunsaturated fatty acids and redox transition metals, and relatively low levels of antioxidants (Halliwell and Gutteridge, 2007). These characteristics may explain the prevalence of oxidative damage in the most common neurodegenerative diseases. As seen in Table 1, impairment of metabolic enzymes within the mitochondria is also common to these diseases. Inhibition of complexes within the respiratory chain may cause electrons flowing through the system to slow down, and leak to molecular oxygen, forming the most proximal ROS, $O_2^{\cdot -}$ (Murphy, 2009).

Most of the $O_2^{\cdot -}$ generated by the mitochondria is dismutated by manganese superoxide dismutase (MnSOD; SOD2) to hydrogen peroxide (H_2O_2), and from H_2O_2 to H_2O by oxidation of the tripeptide, glutathione (GSH). However, if $O_2^{\cdot -}$ levels exceed the protection offered by the intramitochondrial antioxidant defense system, $O_2^{\cdot -}$ may damage important mito-enzymes by oxidizing iron-sulfur (Fe-S) centers of non-heme proteins, such as those of the TCA cycle and respiratory chain. This reaction also releases free ferrous iron (Fe^{2+}), which will generate the highly reactive hydroxyl radical ($^{\cdot}OH$) via Fenton chemistry, thus initiating a chain of lipid peroxidation and damage to proteins and DNA (Halliwell and Gutteridge, 2007). Therefore, in addition to causing direct damage, $O_2^{\cdot -}$ can be cytotoxic by generating other reactive oxygen and nitrogen species, collectively termed, reactive species (RS; Table 2).

In the aging brain, $O_2^{\cdot -}$ may be generated primarily within the mitochondrial matrix and inner membrane (Halliwell and Gutteridge, 2007; Murphy, 2009). In order for $O_2^{\cdot -}$ to cause damage, the target must out-compete the favorable reaction of $O_2^{\cdot -}$ with micromolar concentrations of MnSOD in the matrix. One biological molecule that meets these stringent

Table 1.2. Biologically Important Reactive Oxygen and Nitrogen Species

Reactive Species (RS)	Radicals	Non-radicals
Reactive Oxygen Species (ROS)	Superoxide $O_2^{\cdot -}$ Hydroxyl, $OH^{\cdot}$ Hydroperoxyl, $HO_2^{\cdot}$ Carbonate, $CO_3^{\cdot -}$ Peroxyl, $RO_2^{\cdot}$ Alkoxyl, $RO^{\cdot}$	Hydrogen Peroxide, H_2O_2 Organic Peroxides, ROOH Peroxynitrite, $ONOO^-$ Peroxynitrate, O_2NOO^- Peroxynitrous acid, ONOOH
Reactive Nitrogen Species (RNS)	Nitric oxide, $NO^{\cdot}$ Nitrogen Dioxide, $NO_2^{\cdot}$ Nitrate $NO_3^{\cdot}$	Nitrous acid, HNO_2 Nitrosyl cation, NO^+ Nitroxyl anion, NO^- Dinitrogen tetroxide, N_2O_4 Dinitrogen trioxide, N_2O_3 Peroxynitrite, $ONOO^-$ Peroxynitrate, O_2NOO^-

requirements is $NO^{\cdot}$. The reaction between $NO^{\cdot}$ and $O_2^{\cdot -}$ occurs at a faster rate than that of $O_2^{\cdot -}$ and MnSOD ($6.7x10^9 M^{-1}s^{-1}$ and $1x10^9 M^{-1}s^{-1}$, respectively) and $NO^{\cdot}$ is present in high concentrations in neuropathological conditions (Malinski *et al.,* 1993; Beckman and Koppenol,

1996). Because the diffusion rate of $O_2^{\cdot -}$ through hydrophobic compartments is constrained by its electric charge, $O_2^{\cdot -}$ is most likely to cause damage at the site of production. In contrast, $NO^{\cdot}$ is uncharged, barely polar, and possesses a relatively long half-life for a RS, which allows $NO^{\cdot}$ to readily diffuse into the mitochondria from cytoplasmic sources (Denicola *et al.,* 1996; Ferrer- Sueta and Radi, 2009). $NO^{\bullet}$ may also be produced locally by a post-translationally modified version of neuronal nitric oxide synthase (nNOS). While further characterization of mitochondrial nNOS is warranted, several studies suggest the presence of nNOS in association with the IMM, which would produce $NO^{\bullet}$ in close proximity to the principal sites of mitochondrial $O2^{\cdot -}$ generation (Tatoyan and Giulivi, 1998; Elfering *et al.,* 2002; Dedkova *et al.,* 2004). Whether increased mitochondrial $NO^{\bullet}$ arises by diffusion from extra-mitochondrialcompartments or a local nNOS, the proximity of $NO^{\bullet}$ to $O2^{\cdot -}$ may prove perilous.

Even high physiological levels of NO• are considered principally neuroprotective; most damaging nitroxidative modifications often attributed to NO• itself only occur after oxidation to peroxynitrite ($ONOO^-$) or its subsequent decomposition (Fig. 2) (Beckman and Koppenol, 1996; Malinski, 2007).

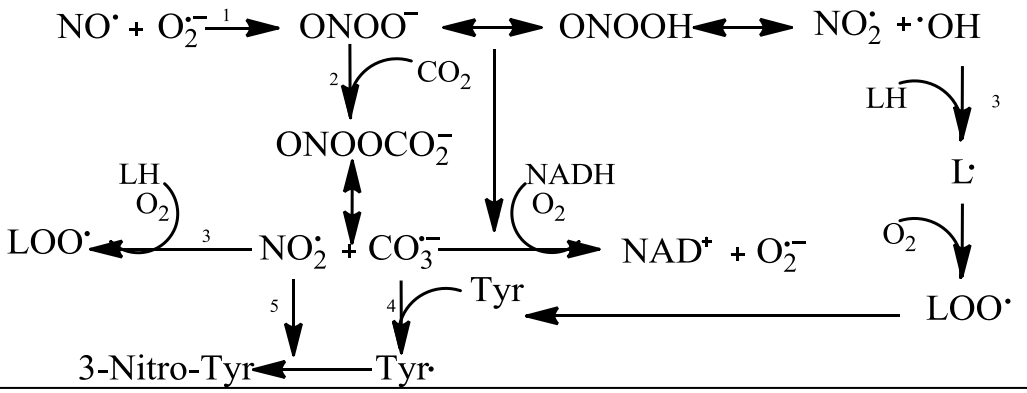

Figure 1.2. Peroxynitrite formation and decomposition: Radical-mediated damage. NO• rapidly reacts with other free radicals, namely $O_2^{\bullet-}$. The product of this reaction (Rxn 1) is peroxynitrite (ONOO-). Mitochondria possess a relatively high concentration of CO_2, which reacts with $ONOO^-$ (Rxn 2; $4.6x10^4$) to produce carbonate radicals ($CO_3^{\bullet-}$) and higher oxides of nitrogen ($NO_2^{\bullet}$) by homolysis of $ONOOCO_2^-$. $CO_3^{\bullet-}$, $NO_2^{\bullet}$, and the hydroxyl radical ($^{\bullet}OH$) propagate the formation of lipid radicals ($LOO^{\bullet}$) following hydrogen abstraction of LH (Rxn 3;~$1x10^{10}$). Oxidation of tyrosine (Tyr) by $CO_3^{\bullet-}$ produces tyrosyl radicals ($Tyr^{\bullet}$) (Rxn 4), which may result in their dimerization ($2.25x10^6$) and protein crosslinking, or nitration (Rxn 5; $1x10^9$) to 3-nitro-tyrosine (3-Nitro-Tyr; 3-NT). $LOO^{\bullet}$ may also mediate tyrosine oxidation processes in hydrophobic compartments (Rxn 6; $4.8x10^3$).

As a major source of $O_2^{\bullet-}$, mitochondria are also a principle target of downstream RS-damage (Murphy, 2009). RS not only damage critical proteins in the IMM, but may also initiate the propagation of lipid-derived radicals within the membrane bilayer. Cardiolipin, the major phospholipid of the IMM, is particularly susceptible to oxidation due to its high degree of unsaturation, which may cause it to dissociate from, and subsequently release, cytochrome *c*

into the cytosol (Shidoji *et al.,* 1999; Iverson and Orrenius, 2004). Nitration and oxidation of cytochrome *c* are also associated with its apoptotic-activation (Hortelano *et al.,* 1999; Cassina *et al.,* 2000; Vaughn and Deshmukh, 2008). In these circumstances, cytochrome *c* acts as the key intrinsic apoptotic trigger, thus providing a plausible link between mitochondrial ROS and apoptosis (Deshmukh and Johnson, 1998; Kirkland *et al.,* 2002c).

Neuronal Apoptosis

Apoptosis is a term describing the morphological characteristics of a programmed process of cell suicide (Green and Kroemer, 2004). These characteristics include cellular atrophy, plasma membrane blebbing, and condensed chromatin. Apoptotic cells signal phagocytes to engulf their shrunken cell bodies, thus preventing damage due to induction of inflammatory responses to cell lysis, as seen in necrotic death. Apoptosis serves many physiological functions which benefit mitotic cells by allowing the removal of those that are compromised, ensuring appropriate turnover, and preventing cancerous growth (Renehan *et al.,* 2001). Apoptosis is also physiologically important in post-mitotic cells, most notably during development of the nervous system. In this process, over half of the neurons originally produced –fall away" when they fail to forge essential connections with the target tissue, and consequently do not acquire adequate levels of trophic factor to survive (Oppenheim, 1991; Putcha and Johnson, 2003).

Apoptosis may proceed via the intrinsic or extrinsic pathway, the key differences of which involve the apoptotic stimulus and the extent of mitochondria involvement. As the name implies, the extrinsic pathway is activated by the binding of an *external* ligand to a death receptor on the cell surface, such as those of the tumor necrosis factor (TNF) superfamily. Conversely, the intrinsic pathway is initiated by intracellular signals and proceeds in a manner ultimately dictated by the mitochondria. These pathways are not entirely exclusive, as

components of the extrinsic pathway target the mitochondria and signaling kinases from the intrinsic pathway induce expression of extrinsic death ligands, and both forms of apoptosis typically depend on activation of effector caspases to execute death (Fig. 3) (Cory and Adams, 2002; Cowling and Downward, 2002; Kondo *et al.*, 2010).

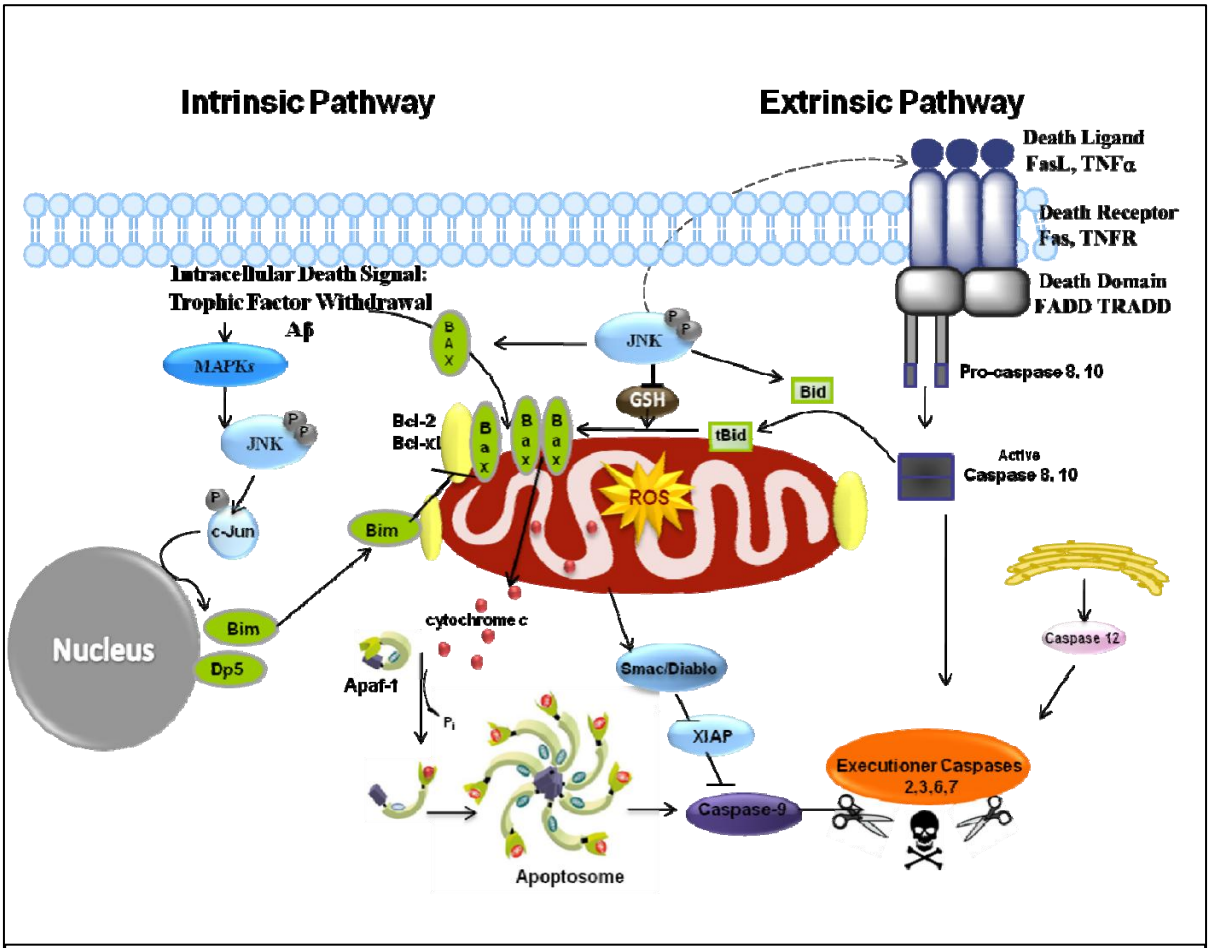

Figure 1.3. Molecular Pathways of Apoptosis: Intrinsic vs. Extrinsic All pathways to apoptosis converge on the activation of caspases, which are cysteinyl aspartate proteases that coordinate the efficient dismantling and engulfment of suicidal cells. In addition, the two pathways are interconnected via JNK and the BH3-only protein Bid. JNK is activated by intrinsic and extrinsic stimuli, and induces the extrinsic death ligand Fas-L. Within the extrinsic pathway, JNK activation also results in caspase-8-independent cleavage of Bid, resulting in jBid. jBid and caspase-8-dependent, truncated Bid (tBid) translocate to the mitochondria and trigger the activation of the pro-apoptotic BCL-2 family members Bax and Bak, leading to OMM permeabilization. This permeabilization may serve as an amplification loop for the activation of effector caspases. See main text for a detailed description of intrinsic pathway events.

While both pathways serve important physiological function, the research discussed herein focuses largely on the intrinsic, mitochondria-mediated form which may be initiated by DNA damage, neurotrophin deprivation, or a variety of toxins. Activation of the intrinsic pathway is primarily responsible for cell death during neural development and also contributes to degeneration of the aging brain (Deshmukh and Johnson, 1997). Therefore, a greater understanding of the orchestration and significance of events within neuronal apoptosis may provide insight into how these events contribute to the pathogenesis of neurodegeneration and may suggest rational targets for new therapeutics.

The key signaling pathways involved in neuronal apoptosis were first delineated by Horvitz and colleagues via genetic manipulation of the developing nematode, *Caenorhabditis elegans* (Ellis and Horvitz, 1986). Subsequent studies identified striking similarities in the molecular machinery involved in neuronal apoptosis within worms, flies, mice, and humans, suggesting this process is highly conserved among different species (Aravind *et al.,* 2001). Some of the most useful models of neuronal apoptosis include primary cultures of sympathetic neurons deprived of their nerve growth factor (NGF), repolarized/serum-starved cerebellar granule neurons, and toxin-treated cortical neurons (Miller and Johnson Jr, 1996; Xiang *et al.,* 1998; Newcombe *et al.,* 2004). Following an intrinsic signal, the characteristic sequence of events typically involves activation of mitogen-activated protein kinases (MAPKs) which phosphorylate c-Jun N-terminal kinase (JNK). JNK phosphorylates c-Jun, which leads to the formation of the activator protein-1 (AP-1) and increased expression of pro-apoptotic genes, such as BH3-only proteins of the B-cell lymphoma-2 (Bcl-2) family. BH3-only proteins interact with and regulate the core Bcl-2 family proteins to promote apoptosis. In mammals, there are at least 12 core BCL-2 family proteins, including BCL-2

itself, and others that share structural similarity. These proteins display a range of bioactivities, from prevention to promotion of apoptosis (Youle and Strasser, 2008).

The net balance of pro-apoptotic (Bax, Bak, Bid, Bim, DP5, Puma) and anti-apoptotic (Bcl2 and Bcl_xL) Bcl-2 proteins at the OMM is a major determinant of cell fate (Putcha *et al.,* 2001; Zou *et al.,* 2002). Induction of BH3-only family members would allow them to dominate the binding site of anti-apoptotic Bcl-2 proteins, removing their protective effects by rendering them unable to sequester other damaging, pro-apoptotic family members, such as Bax (Cory and Adams, 2002; Putcha *et al.,* 2003). Alternatively, BH3-only proteins may directly activate Bak and Bax. During apoptosis, Bax and Bak translocate from the cytosol to the mitochondria, where they may activate OMM channels, or if free to oligomerize, may form pore-like structures that permeabilize the OMM and contribute to mitochondrial ROS generation (Antonsson *et al.,* 1997; Kirkland *et al.,* 2002b; Danial and Korsmeyer, 2004; Annis *et al.,* 2005). Excess mitochondrial ROS modifies critical IMM components, such as cardiolypin and cytochrome *c,* which may be necessary for release of cytochrome *c* into the cytosol (Kirkland *et al.,* 2002a; Jiang *et al.,* 2008; Vaughn and Deshmukh, 2008). Activated cytochrome *c* will then bind apoptosis protease activating factor (APAF-1), allowing its compact structure to unwind, hydrolyze ATP, and polymerize into a seven-spoked, wheel-like structure with a pro-caspase-9 recruitment domain within the central hub (Zou *et al.,* 1999; Acehan *et al.,* 2002). The subsequent recruitment, binding, and activation of seven caspase-9 molecules yields the active apoptosome. Caspase-9 is considered the main initiator caspase in intrinsic apoptosis. Accordingly, caspase-9 activates a host of executioner caspases by cleaving the linker domain within the zymogen dimers. Executioner caspases, such as 3 and 7, then degrade critical cellular components beyond the point of repair, including components of the respiratory chain, which

may further augment ROS production, leading to loss of the mitochondrial membrane potential ($\Delta\psi_m$), and activation of the mystifying mitochondrial permeability transition pore (mPTP) (Ricci *et al.,* 2004; Juhaszova *et al.,* 2008). While the exact structure of mPTP remains elusive, it is thought to span both the inner and outer mitochondrial membranes and to be comprised of cyclophilin D, adenine nucleotide translocase (ANT), and the voltage-dependent anion channel (VDAC; Rasola *et al.,* 2010). In many cell types, opening of the mPTP signifies the decisive loss of mitochondrial integrity and the closing stages of apoptosis. Most, if not all, of these events are highly conserved within various types of neurons undergoing mitochondria-mediated apoptosis due to a variety of insults.

As highlighted above, mitochondria integrate energy metabolism, ROS production, and apoptosis, such that a pathological imbalance in one process will cause a modification in the others (Circu and Aw 2010). Propagation of this vicious cycle is particularly devastating for the adult nervous system as it will ultimately compromise mitochondria membrane integrity and culminate in the loss of precious, post-mitotic neurons.

Alzheimer's Disease

Alzheimer's disease (AD) is the leading cause of dementia among older people and is the most prevalent neurodegenerative disease worldwide. Currently affecting more than 5 million Americans, AD is one of the most serious health problems in the U.S. (Association., 2010). In order to move beyond the use of palliative treatments for the disease to those that effectively forestall its progression, we need a better understanding of its pathogenesis.

AD is characterized by increscent dementia in adulthood accompanied by structural and biochemical changes in the brain, including oxidative stress, decreased glucose utilization,

neuronal and synaptic loss, intracellular aggregates of tau in neurofibrillary tangles (NFTs), and extracellular deposits of beta-amyloid (Aβ) in senile plaques. This insidious and progressive neurodegenerative disease affects up to 15% of individuals over age 65 and doubles every five years, reaching over half of the population 80 and older (Association, 2010). As our life expectancy extends, the incidence of age-associated disease amplifies; cases of dementia and AD are expected to increase by 10 million every quarter century due to the aging population (Association., 2010). The cost will be economically crippling for the American economy, as the current $100 billion spent on research and clinical care per year skyrockets (de la Torre, 2010a). The impact of this dramatic increase would be diminished by the discovery of treatments to effectively delay the onset of AD, placing great need on elucidating the most proximal etiological factors.

For patients with familial Alzheimer's disease (fAD), Aβ was established as the etiological factor with the identification of autosomal dominate mutations in amyloid precursor protein (APP) or the proteins involved in its cleavage (presenilin-1 and -2), all of which lead to excessive Aβ accumulation (Scheuner *et al.,* 1996; Roberson and Mucke, 2006). However, for the remaining 99% of AD patients, there is no obvious Mendelian pattern of inheritance or environmental toxin currently identified. The single greatest risk factor for these sporadic AD patients is advancing age, such that studies suggest most people have the capacity to develop it if they simply live long enough (Hogan, 2008; Association., 2010).

Although the term "sporadic" implies the absence of genetic influence, epidemiological data indicate that maternal family history of longevity and sporadic AD predicts the longevity, cognitive aging, and risk of AD in offspring more accurately than the paternal history, which suggests a maternally inherited factor may be involved (Mosconi *et*

al., 2007). However, X-chromosome inheritance seems unlikely as it would preferentially predispose males to the disease, and this does not coincide with the increased incidence of sporadic AD in women (Viña and Lloret, 2010; Association., 2010). A more plausible explanation may be found in an unfavorable combination of inherited and/or accumulated mtDNA abnormalities, which would explain both the maternal influence and the age-dependence of sporadic AD (Kujoth *et al.,* 2005a; Swerdlow and Khan, 2009a; Wallace, 2010).

Another genetic association in *sporadic* AD is found within two adjacent genes on chromosome 19 (Roses *et al.*, 2010). These genes encode apolipoprotien E (ApoE), which is involved in lipid metabolism, and the translocator of the outer mitochondrial membrane 40 (TOM40), which is necessary for the import of nDNA-encoded mitochondrial proteins (Bu, 2009; Endo and Yamano, 2010). Polymorphisms in APOE and TOMM40 were recently discovered to influence AD onset, but the pathological basis of both the relationship between these genes and their individual roles in AD are uncertain (Pericak-Vance *et al.,* 1991; Roses *et al.,* 2009). Despite the etiological heterogeneity, both forms of AD develop the same prevailing pattern of pathological progression; they both exhibit region-specific, cerebral hypometabolism decades before symptoms emerge and require the same criteria for clinical diagnosis and post- mortem confirmation (Mosconi *et al.,* 2010).

While definitive diagnosis still depends on autopsy analysis of the plaques and tangles first described by Alois Alzheimer, provisional diagnosis of AD is currently based on clinical assessment of cognitive testing. According to the Clinical Dementia Rating (CDR), AD begins with the failure to retain new information and personality changes in mild stage 1, followed by persistent memory loss and deterioration of most cognitive function in moderate stage 2, and

total loss of recognition and ability to care for oneself in the most severe stage 3. This succession correlates with the progressive degeneration of neurons within the memory system of the medial temporal lobe (MTL), which includes the hippocampus, entorhinal cortex, and subiculum. Over half of the neurons within entorhinal cortex layers I and IV are lost in the mildest stages of early AD (CDR=0.05). By stage 3, degeneration in this region reaches 90% and extends into the frontal, parietal, and temporal cortices (de la Torre, 2010).

The prevalence of neurodegeneration in the early stages suggests that cell death pathways are activated prior to the onset of clinical symptoms, yet these symptoms usually emerge long before the amyloid plaques and tau tangles that are still considered pathognomonic of the disease (Castellani *et al.,* 2006). This chronological disassociation suggests another etiological factor may be responsible for the early neurodegeneration.

Mitochondria and Oxidative Stress

Recent advances in noninvasive neuroimaging have provided the unprecedented opportunity to identify and monitor *in vivo* changes in the hypothesized continuum from normal aging to mild cognitive impairment (MCI) and onward throughout AD. MCI is clinically characterized by a memory deficit without loss of general cognitive functions and is widely accepted as a prodrome to AD (Petersen *et al.,* 1999; Association., 2010). Studies in these ~~"tr~~ansitional" patients have yielded great insight into the early pathological changes involved in AD.

Postmortem analysis first identified the selective vulnerability of large pyramidal neurons with long projections anatomically connecting regions of the MTL and the cortex. These neurons have a high dependence on continuous, efficient utilization of glucose and oxygen for the energy required for axonal transport and synaptic transmission (Li *et al.*, 2008).

These characteristic properties led early investigators to suspect impairments in cerebral oxidative metabolism may be responsible for the selective synaptic loss and death (Hoyer and Betz, 1988; Hoyer, 1992). Over the past decade, fluoro-deoxyglucose positron emission tomography (FDG-PET) studies have established a specific pattern of decreased cerebral metabolic rate (CMR) in genetically susceptible, presymptomatic patients and those with MCI (Hoyer *et al.,* 1991; Berti *et al.,* 2010). Longitudinal neuroimaging studies reveal the rate of metabolic decline within the MTL predicts the progression of cognitive deterioration from normal aging to AD with over 80% accuracy and more closely correlates with the level of oxidative damage than other biomarkers tested in preclinical patients (Mosconi et al., 2008a, 2008b). With the exception of cytochrome oxidase (COX; complex IV), metabolic enzymes within the mitochondria that correlate best with early cognitive decline are known both to generate $O2^{\cdot-}$ and to be highly susceptible to oxidative inhibition, which may provide a mechanism underlying this correlation (Gibson et al., 1998; Tretter and Adam-Vizi, 2000; Hinerfeld et al., 2004; Ke and Gibson, 2004; Bubber et al., 2005; Murphy, 2009; Sultana and Butterfield, 2009). While COX may be less susceptible to post- translational modification by RS, the DNA encoding COX may be more susceptible to RS- damage as COX has the highest ratio of mtDNA/nDNA-encoded subunits of all the respiratory complexes.

An overwhelming body of evidence provides clues that link oxidative stress to AD development (Nunomura *et al.,* 2001a; Lin and Beal, 2006a; Gibson, 2010). Virtually every established biomarker of oxidative damage has been reported in MCI and early AD; these include, but are not limited to the following: increased levels of protein carbonyls, 4-hydroxy-2-*trans*-nonenal (HNE), S-nitrosylation, 3-nitrotryosine, advanced glycation end products (AGEs), lipid peroxidation, F_2-Isoprostanes (F_2-IPs), nucleic acid oxidation in postmortem

brain tissue and cerebrospinal fluid, and decreased levels of plasma antioxidant and total antioxidant capacity (Pratico *et al.,* 2002; Rinaldi *et al.,* 2003a; Guidi *et al.,* 2006; Markesbery and Carney, 2006; Halliwell and Gutteridge, 2007). Furthermore, most of the known genetic, environmental, and lifestyle-related risk factors for AD are associated with oxidative stress (Nunomura *et al.,* 2006). Such studies and others suggest that oxidative damage not only occurs earlier in AD, but is also more prevalent in anatomically relevant regions than plaques and tangles (Li *et al*., 2008; Hirai *et al.,* 2001). However, the latter features remain the defining characteristics of AD, it is important to determine whether mitochondrial dysfunction and oxidative stress are epiphenomena, or if they play a causative role in the development of these histological hallmarks.

Mitochondria, oxidative stress, and amyloid

While many AD-hypotheses have been proposed over the years, none have enjoyed the popularity afforded Aβ. According to the "amyloid cascade hypothesis", all biochemical, histological, and clinical changes associated with AD are due to accumulation of Aβ peptide fragments (Hardy and Selkoe, 2002). Proteolytic cleavage of the type-1 transmembrane glycoprotein, APP, by β-secretase (β-site amyloid precursor protein cleaving enzyme; BACE) triggers the amyloidogenic process and is considered the rate-limiting step in Aβ-formation. BACE cleaves APP at the N-terminus, releasing the soluble N-terminal fragment, sAPPβ, while the C-terminal fragment (C99) remains membrane bound. C99 is subsequently processed by presenilin-dependent γ-secretase to generate Aβ. In addition to this major proteolytic pathway, some suggest Aβ is generated by several caspases (2, 3, 6, 8, 9, and 12), either by their direct cleavage of APP (caspase 3) or in a process involving γ-secretase (caspase 2 and 8) (Hortelano

et al., 1999; Chae *et al.*, 2010). Once generated, Aβ peptide fragments readily aggregate into small oligomers, which continue to coalesce into insoluble fibrils and ultimately deposit in extracellular plaques within the AD brain.

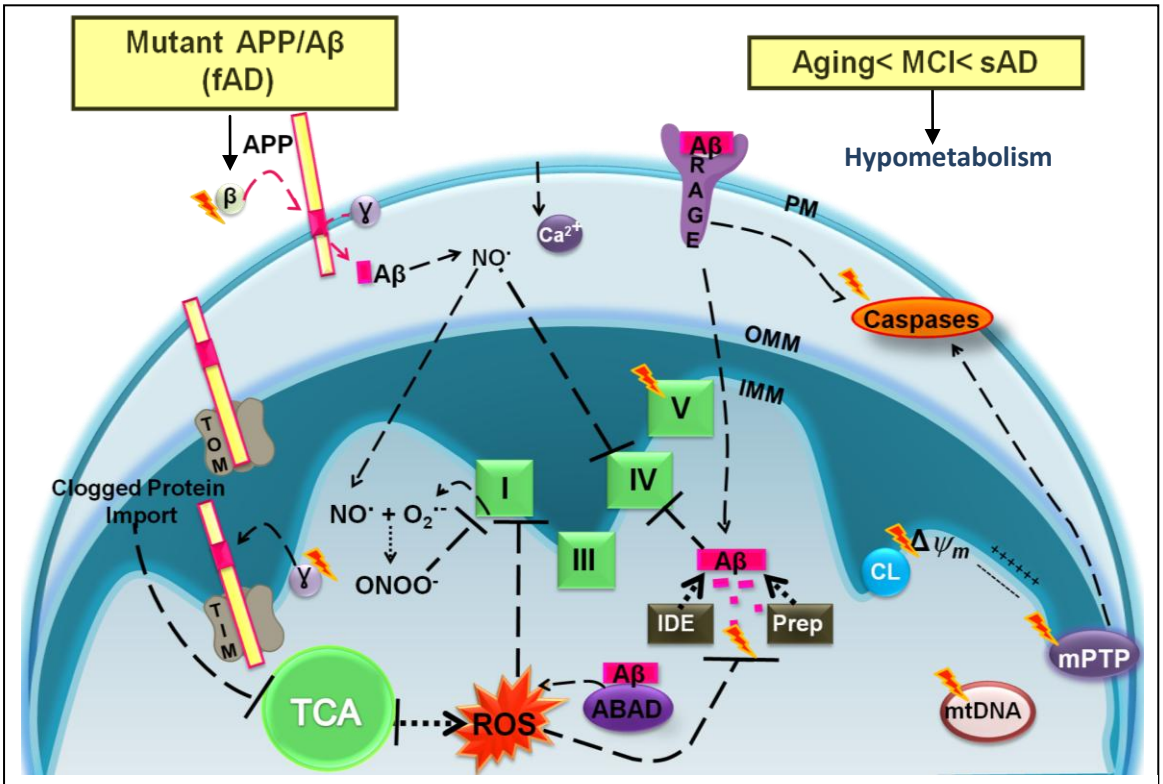

Figure 4. Dynamic interplay between mitochondria dysfunction and amyloid. In early-onset AD, APP or presenilin mutations increase APP and Aβ levels. Intracellular accumulation of Aβ and subsequent mitochondrial damage is dependent on the receptor for advanced glycation end products (RAGE). Both APP and Aβ localize to synaptic mitochondria. APP is imported by TOM, where it is arrested due to its acidic domain. This "clog" results in impaired importation of nDNA-encoded mt-proteins essential for metabolism, and may be responsible for the decreased cerebral glucose utilization. γ-secretase is also found in the mitochondria and is activated by ROS. Mitochondrial Aβ reacts with ABAD and produces ROS, and also inhibits complex IV. Aβ-degrading enzymes IDE and Prep are found in the mitochondrial matrix, where they may be inhibited by ROS.

Cerebral hypometabolism and oxidative damage amplify in aging, MCI, and sAD. These factors activate β- and γ-secretase, thereby facilitating the cleavage of Aβ. The cleaved Aβ may then enter mitochondria, induce additional RS, and magnify the metabolic deficit by the pathways pictured here and discussed in the main text. This interplay between Aβ, metabolism, and RS may be responsible for the synaptic loss and cognitive decline in AD patients. "Lightning bolts" represent alteration by RS.

All of the amyloidogenic proteases aforementioned are influenced by fluctuations in mitochondria function and oxidative stress (Figure 4). Metabolic impairment induced by thiamine deficiency or respiratory chain inhibition augments BACE expression and activity (Zhang et al.; Saravanan *et al.,* 2009). Oxidative stress markers correlate with BACE activation in sporadic AD patients (Borghi *et al.,* 2007). In the early stages of AD, oxidative damage to lipid membranes generates HNE, a neurotoxic aldehyde that covalently modifies proteins. HNE and RS lead to increased expression and activity of BACE, and consequently, Aβ generation, all of which are preventable by antioxidant treatment *in vitro* (Tamagno *et al.,* 2002, 2005; Tong *et al.,* 2005). Oxidative stress- induced BACE1 expression is regulated in part by γ-secretase in a mechanism involving JNK activation (Tamagno *et al.,* 2008; Dong-Gyu *et al.,* 2010). γ-secretase is present in the mitochondria and may also be induced by RS (Minopoli *et al.,* 2007). Oxidative stress is the only factor known to augment APP-cleavage by increasing expression of the γ-secretase catalytic subunit, presenilin-1 (Guglielmotto et al.; Oda *et al.,* 2010). Mitochondrial RS also activate caspases within the intrinsic pathway and may stimulate those of the extrinsic pathway through an ER-mediated mechanism (Eilers *et al.,* 1998; Schweizer *et al.,* 2003). In addition to inducing amyloidogenic processing, oxidative stress inactivates Aβ-degrading enzymes, such as neprilysin and insulin degrading enzyme (IDE), and therefore may be a major determinant of Aβ-accumulation (Green and Kroemer, 2004). While mitochondrial perturbations and oxidative stress clearly alter Aβ production and clearance in cell and animal models (Fig. 4), the etiological relevance of this relationship is confounded by the ability of Aβ to also cause oxidative stress (Hensley *et al.,* 1994).

Oxidative stress and tau

The second histological hallmark that characterizes AD is the accumulation of intracellular neurofibrillary tangles (NFT). NFTs are composed of paired helical filaments (PHF), which contain a highly phosphorylated form of the microtubule-associated protein tau (Alonso *et al.,* 1996). Tau is an important component of the neuronal cytoskeleton and is found primarily in axons. Normal tau promotes tubulin polymerization and stabilizes microtubule structures, allowing efficient cellular trafficking. The phosphorylation state of tau dictates its affinity for microtubule binding. When tau is phosphorylated at certain sites, it detaches from microtubules, destabilizing the cytoskeleton. This destabilization disrupts cellular trafficking, such as the axonal transport of proteins and organelles that are critical for synaptic function (Stamer *et al.,* 2002; Spires-Jones *et al.,* 2009).

Recent studies have begun to clarify the sequence of tau modifications that lead to neurodegeneration (Figure 5). This process may be initiated by activation of the executioner caspases-3 and -7, which cleave tau at Asp42, producing Δtau (Rissman *et al.,* 2004). Production of Δtau may contribute to neuronal dysfunction and cognitive decline by facilitating nucleation- dependent filament formation. ΔTau readily adopts a conformational change recognized by the early pathological tau marker MC-1 (Uboga and Price, 2000, Jicha *et al.,* 1997). Tau may then be phosphorylated by glycogen synthase kinase-β (GSK-3ß), a modification that can be recognized by the NFT antibody PHF-1 (Wagner *et al.,* 1996). Confocal microscopy experiments with fluorescent-labeled Δtau, MC-1, and PHF-1 reveal triple labeling of both neurons and dystrophic neurites in the AD hippocampus and cortex, strengthening the assertion that Δtau is a critical component of tangle formation. In addition, Δtau colocalizes with intracellular Aβ42 accumulation within hippocampal neurons and

dystrophic neurites of MCI and AD patients, as well as middle-aged 3xTg-AD mice, supporting the hypothesis that caspase cleavage of tau is an early event. Furthermore, Δtau inversely correlates with cognitive function in AD, suggesting that caspase cleavage of tau may contribute to the transition from normal aging to MCI and AD (Rissman *et al.,* 2004). Studies *in vitro* and *in vivo* suggest that oxidative stress chronologically precedes NFT formation in humans and transgenic mice (Nunonomura *et al.,* 2001b; Nakashima *et al.,* 2004; Smith *et al.,* 2005). Increased oxidative stress may cause tau nitration and microtubule dissociation (Zhang *et al.,* 2005b). Both oxidative stress and Aβ induce caspase activation and lead to Δtau immunoreactivity *in vitro*. Treatment of primary cortical neurons with the lipid peroxidation product, 4-HNE, results in a 10-fold increase in Δtau immunoreactivity over vehicle-treated cells (Cotman *et al.,* 2005). Oxidative stress also alters the activity of key kinases implicated in the pathological phosphorylation of tau (Lu *et al.,* 1999; Zhang *et al.,* 2005a). Conversely, oxidative stress inhibits the isomerase Pin1, which is crucial for tau dephosphorylation and microtubule binding (Lu *et al.,* 1999). Oxidative inhibition of Pin1 may be particularly important in the clinical presentation of tau pathology, as it is increased in presymptomatic individuals and inversely correlates with NFT levels and neurodegeneration in the AD brain. Furthermore, in transgenic mice, Pin1 deficiency leads to increased NFT formation and cognitive impairments, which are reversed when active Pin1 is reintroduced (Deane *et al.,* 2003). Oxidative alteration of these tau-modulating proteins is prominent in the hippocampus of MCI and AD patients, suggesting that oxidative stress plays a causal role in aberrant tau phosphorylation (Butterfield *et al.*, 2006).

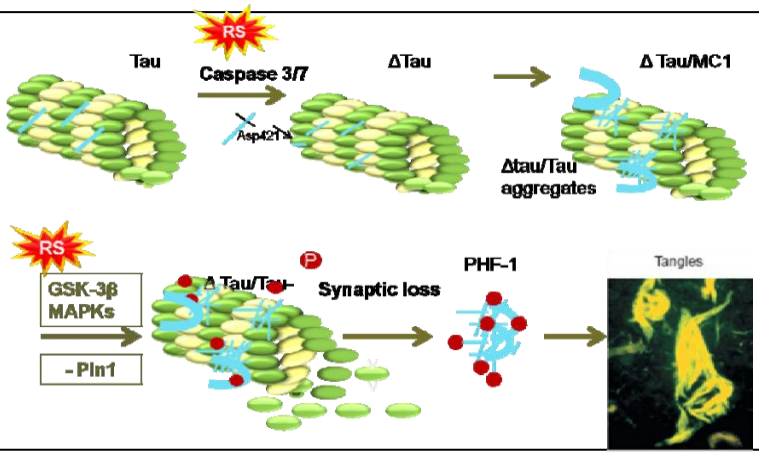

Figure 1.5. Potential role of oxidative Stress in the progression of tau pathology. Tau normally binds and stabilizes microtubules. Increased neuronal RS may activate Caspase 3 and-7 causing increased cleavage of tau at Asp421, resulting in Δtau. Δtau more readily forms the conformational change (MC1) associated with tangle pathology and acts a ~~–~~seed" by stimulating accumulation of tau aggregates. RS also activate glycogen synthase kinase (GSK-3β) and MAPKs implicated in the hyperphosphorylation of tau. RS oxidatively inhibit the isomerase Pin1, which is required for tau de-phosphorylation and restoration of tau function. Hyperphosphorylated tau dissociates from microtubules, leading to their disassembly and impaired axonal transport, which is critical for proper synaptic function. The dissociated, hyperphosphorylated tau aggregates form paired helical fragments (PHF-1) and extracellular tangles.

Summary

Despite tireless efforts, the development of preventative therapeutics for age-dependent neurodegenerative diseases has progressed at a glacial pace for the past decade. The vast majority of disappointing results have come from AD clinical trials. Because of the attractiveness of the amyloid cascade hypothesis, most of these drug candidates have solely targeted Aβ. In my opinion, the data emphasize the need to engage additional hypotheses and investigate therapeutics which more specifically address the underlying metabolic failure and neuronal loss. The following research aims to achieve this goal by providing a greater understanding of the relationship between mitochondrial oxidative stress and neuronal demise.

CHAPTER 2

ELEVATED LEVELS OF REACTIVE OXYGEN SPECIES OCCUR INDEPENDENTLY OF JNK ACTIVATION DURING THE APOPTOTIC DEATH OF SYMPATHETIC NEURONS[1]

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Abstract

Withdrawal of nerve growth factor (NGF) from sympathetic neurons causes apoptotic death. Activation of c-Jun NH_2-terminal kinase (JNK) may contribute to this death by the induction and phosphorylation of pro-apoptotic Bcl-2 proteins, such as Bax, that are involved in reactive oxygen species (ROS) production and cytochrome *c* release from mitochondria. Induction of either JNK or ROS may stimulate the other, and both may regulate release of apoptogenic factors from the mitochondria. In order to discern the relationship between JNK and ROS in apoptosis, we treated NGF-deprived, mouse sympathetic neurons with the selective JNK inhibitor, SP600125, and examined the effect on several important apoptotic events. SP600125 prevented induction of c-Jun expression and resulted in a dose-dependent, yet surprisingly modest, increase in cell survival after 48 hours of NGF deprivation. SP600125 treatment was not sufficient to prevent the elevation in ROS or the release of cytochrome *c* from the mitochondria in NGF- deprived sympathetic neurons. Our results suggest that while JNK activation plays an important role in many forms of apoptosis, it may not be a crucial regulator of Bax-dependent events involved in the apoptotic death of mouse sympathetic neurons deprived of NGF.

Introduction

c-Jun N-terminal kinases (JNKs) belong to the superfamily of mitogen-activated protein kinases (MAPKs) that are involved in the regulation of cell proliferation, differentiation, and apoptosis. Whether JNK activation leads to enhanced survival or apoptosis depends on the stimuli and the cell type involved (Dhanasekaran and Reddy, 2008). It appears that sustained activation of JNK is associated with apoptosis, whereas acute and transient activation may be more important for

signaling cell proliferation, neurite outgrowth and survival (Sanchez-Perez *et al.,* 1998; Yu *et al.,* 2000; Harris *et al.,* 2002 Kamata *et al.*, 2005). JNK plays a role in both extrinsic and intrinsic apoptotic pathways, providing a link between activation of death-receptors and the mitochondria. In response to various ROS-inducing stimuli, JNK is activated by phosphorylation by MAPKs (Kadowaki *et al*., 2005; Matsuzawa and Ichijo, 2008; Katagirl *et al.,* 2010; Circu and Aw 2010). JNK amplifies apoptotic signaling via up-regulation of pro-apoptotic genes through the dual phosphorylation of c–Jun at Ser63/Ser73 residues. In combination with c-Fos, c-Jun forms the transcription factor activator protein 1 (AP-1), which increases expression of immediate early genes (Putcha and Johnson, 2003). JNK also directly modulates the activities of mitochondrial pro- and antiapoptotic proteins through distinct phosphorylation events.

The most important influence of JNK in apoptosis may be the regulation of Bcl-2 proteins, which are critical mediators of mitochondria-dependent, apoptotic events. JNK promotes apoptosis by inhibiting the anti-apoptotic Bcl2-family members Bcl2 and Bcl-xL (Yamamoto *et al.,* 1999). JNK also induces the expression and activation of pro-apoptotic Bcl-2 family members, including Bax and the BH3-only proteins Bim and Bid (Harris and Johnson,

2001; Esposti, 2002; Oleinik *et al.,* 2007). These proteins influence the induction of mitochondrial ROS and the subsequent release of apoptotic proteins such as cytochrome *c* and Smac/Diablo from the mitochondria (Potts *et al.,* 2003). Together these events may determine a neuron's ~~–~~competence to die," allowing activation of executioner caspases, which cleave critical cellular substrates, leading to the morphological characteristics of apoptosis and death of the cell (Deshmukh, 2000).

Many of these events have been extensively characterized in sympathetic neurons deprived of nerve growth factor (NGF). Previous studies in our lab and others have shown that the increase in mitochondrial ROS and subsequent death of these cells is Bax-dependent (Deckwerth *et al.,* 1998; Kirkland *et al.,* 2002b). During apoptosis, Bax localizes to the outer mitochondrial membrane (OMM), where it may oligomerize into pores that allow cytochrome *c* to be released into the cytoplasm. Bax may also allow cytochrome *c* release via activation of other OMM channels, possibly via interaction with the voltage-dependent anion channel (VDAC) (Schwarz *et al.,* 2007). In addition to OMM permeabilition, Bax may contribute to cytochrome *c* release by increasing mitochondrial ROS. Several studies suggest that the mitochondrial redox status may determine the inner membrane association of cardiolipin with cytochrome *c,* as well as the ability of cytochrome *c* to initialize apoptosome formation (Eilers *et al.,* 1998; Kirkland *et al.,* 2002a; Borutaite and Brown, 2007; Vaughn and Deshmukh, 2008; Xu *et al.,* 2008). Considering that JNK activation occurs early in NGF deprivation-induced apoptosis and regulates Bcl-2 proteins that interact with Bax, as well as Bax itself, and that Bax appears to be the primary dictator of mitochondrial ROS, we hypothesized that JNK activation may lie upstream of Bax and thereby play a causal role in mitochondrial ROS production in this paradigm. We sought to determine the effect of JNK on these Bax-dependent events by treating

mouse sympathetic neurons with the selective JNK inhibitor SP600125 and investigating the outcome on cell survival, ROS, and cytochrome *c*.

Materials and Methods

Reagents CM-H_2DCFDA and MitoSOX were purchased from Molecular Probes. Nerve growth factor 2.5S was purchased from Cedarlane (Hornby, Ontario, Canada). All other reagents were purchased from Sigma unless otherwise indicated.

Mouse breeding and genotyping. DNA for genotyping was extracted from the tail of each pup using a Wizard Prep kit (Promega, Madison, WI) or a Quick Extraction kit (Epicentre Biotechnologies, Madison, WI). Mice deficient in both bax alleles ($bax^{-/-}$) are poor breeders (Knudson *et al.,* 1995). Therefore, mice hemizygous for the bax allele ($bax^{+/-}$; C57Bl/6 genetic background) were mated to generate $bax^{-/-}$ offspring. Founding breeders were obtained from The Jackson Laboratory (Bar Harbor, ME). Both the mutant and the wild-type alleles were amplified by a single PCR. Primers and PCR protocol are as described previously (Kirkland *et al.,* 2002c).

Cell Culture. Primary sympathetic neuronal cultures were prepared from the superior cervical ganglia of neonatal C57Bl/6 mouse pups. Following extraction, the ganglia were digested by sequential incubation in collagenase and trypsin, and the neurons further dissociated by trituation in growth media as described (Johnson and Argiro, 1983). The cells were plated on collagen- coated, #1 glass coverslips and maintained in 35 mm culture dishes. Cultures were incubated in medium containing Eagle's minimum essential medium with Earle's salts (Invitrogen/GIBCO Life Technologies, Inc., Carlsbad, CA). This medium was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, 20 mM

fluorodeoxyuridine, 20 mM uridine, 1.4 mM L-glutamine, and 50 ng/ml 2.5S NGF. All cultures were maintained at 35º C in an incubator having an atmosphere of 95% air and 5% CO_2. In order to deprive neurons of NGF, the cultures were washed twice in standard culture medium devoid of NGF, and incubated in media containing a NGF-neutralizing antibody (Cedarlane). All experiments were performed at 6-9 days *in vitro* (DIV).

Cell Survival. To assess the frequency of neuronal apoptosis, cultures were deprived of NGF as described above, both in the absence and presence of various concentrations of the JNK inhibitor, SP-600125. After 48 h, the treatment medium was washed out and the neurons were ~~"r~~escued" by incubation in NGF-supplemented medium for 5 days. The cultures were then fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.2, for 30 min at 4°C, washed, and stained with crystal violet. The distinguished cellular outlines of plump, crystal violet-positive neurons highlight viable neurons over the dead cells, which received little or no staining. Percent survival was calculated by dividing the number of viable neurons within each deprivation treatment by the total number of neurons in NGF-maintained, sister cultures, $n \geq 4$.

Confocal and Fluorescent Microscopy. A Nikon C1 laser-scanning confocal microscope mounted on a Nikon Eclipse TE 300 inverted microscope was used for all confocal microscopy. The microscope was controlled by EZC1 software running on a Dell computer. Neurons, observed with a 60X-plan oil immersion lens (N.A. 1.4), were chosen at random and scanned by the confocal microscope at 512 X 512 pixel resolution. Images were quantified by

measuring the raw pixel intensity of neuronal somas with MetaMorph software (Universal Imaging Co.). The quantified area covered a 60 mm^2 area of the soma. The fluorescence intensity of each neuron was normalized to that of NGF- maintained neurons receiving the same concentration of dye for the same time as the experimental cells. All microscopy was done at room temperature.

Immunocytochemistry. Cultures from $bax^{+/+}$ and $bax^{-/-}$ were maintained in NGF or deprived for 12h, fixed as described above, washed with Tris-buffered saline [TBS: 0.1 M Tris-HCl (pH 7.6), 0.9% NaCl], and incubated in blocking solution (5% normal goat serum in TBS, containing 0.3% Triton X-100) for 1 h at room temperature. The cultures were then incubated with anti-phospho- c-Jun (Ser63 II) (1:1000, Cell Signaling) in 1% normal goat serum in TBS, containing 0.3% Triton X-100, overnight at 4 °C. The next day, the cultures were washed three times with TBS and incubated in antibody solution containing Alexa Fluor 610 (1:2,000, Molecular Probes). After three washes with TBS, the cultures were mounted for fluorescence microscopy.

Reactive Oxygen Species (ROS) Measurement. The redox-sensitive dye 5-(and-6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate (CM-H_2 DCFDA) were used to determine ROS levels. CM-H_2 DCFDA is readily membrane-permeant and is trapped in cells by binding of the chloromethyl group to cellular thiols. It becomes intensely fluorescent when oxidized by hydrogen peroxide and downstream free-radical products of hydrogen peroxide (Royall and Ischiropoulos, 1993). Our lab previously characterized the use of this dye in rat and mouse sympathetic neurons (Kirkland *et al.,* 2001; 2002). Cultures were incubated for 20 min at 35^{o}C in the appropriate experimental medium containing CM-H_2DCFDA (10 mM). They were then

washed 2X with Leibovitz's L-15 medium and left in the last wash for confocal microscopy. CM-H_2DCFDA was excited with the 488-nm line of the confocal laser. The FITC photomultiplier of the confocal microscope was used for image acquisition. As a measure of the mitochondria redox status, relative mitochondrial $O_2^{\cdot-}$ levels were determined by MitoSOX Red (Zhao *et al.,* 2005). To selectively detect the $O^{\cdot-}$-MitoSOX product, we used the 408 nm line of the confocal laser, and the red photomultiplier channel of the confocal microscope was used for image acquisition (Robinson *et al.,* 2006; Johnson and Cadwell, 2007). Cultures were incubated for 10 min at 35°C in the appropriate experimental medium containing MitoSOX (2 μM). After incubation in MitoSOX, cultures were washed twice with L-15 and kept in the second wash for microscopy.

Immunoblotting. At the conclusion of treatment, cultures were washed once with cold PBS, scraped, and transferred in 1.0 ml PBS into microfuge tubes. The samples, consisting of ~2.5 ganglia each, were pelleted at 5000 rcf for 3 min at 4°C. The supernatants were removed and 30 μl of lysis buffer consisting of 25 % glycerol, 60 mM Tris (pH 6.8), 100 mM dithiothreitol, 1 mM EDTA, 0.1 % bromophenol blue, 0.1 % SDS, and a 1:100 dilution of a protease inhibitor cocktail (Sigma) was added. The pellets were then homogenized for 15 s with a disposable pellet pestle (Kontes, Vineland NJ). After homogenization, samples were boiled for 7 min, allowed to return to room temperature, and then spun briefly at 13 rpm in an Eppendorf Mini-spin table-top centrifuge to pellet debris. The lysates and a prestained molecular weight marker (7 ml; Bio-Rad) were loaded into the wells of 12% Tris-HCL pre-cast gels (Bio-Rad), and run for one hour at 150 V. Proteins were transferred from the gels onto an equilibrated Immobilon-P polyvinylidene difluoride membrane for one hour at 100 V (Millipore). The

membrane was briefly immersed in MeOH, air dried, and blocked for at least 1 h at room temperature with TBST (10 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween-20) containing 2% ECL blocking agent (Bio-Rad). The membrane was then incubated overnight at 4°C with one of the following primary antibodies: c-Jun (1:1000, Cell Signal); monoclonal p-JNK/SAPK (1:1000, Cell Signal); β-tubulin (1:1000, Sigma). The membrane was then washed several times in TBST, followed by incubation for 60 min at room temperature in anti-mouse or rabbit, HRP-linked secondary antibody (1:2000, Amersham). The membrane was then washed in TBST, and incubated in chemiluminescent substrate for 3-5 min.

Statistics. Statistical analysis and graph preparation were done with Sigmaplot 11.1 (Systat Software, Inc., San Jose, CA). Appropriate statistical measures were determined from analysis of data distribution. Unless otherwise indicated, statistical comparisons were made by Kruskal- Wallis one-way ANOVA on ranks followed by the Dunn's multiple comparisons p*ost-hoc* test. Error bars are ± SEM.

Results

Effect of SP600125 on JNK activation and Apoptosis

Mouse sympathetic neurons were treated with a range of SP600125 concentrations at the time of NGF deprivation, and the effect on JNK-phosphorylation (p-JNK) was assessed with immunoblots. Previous reports describe an increase in JNK activity, approximately two-fold, beginning soon after the initial burst of ROS and leading to enhanced levels of phosphorylated c- Jun in sympathetic neurons (Eilers *et al.,* 1998; Harris *et al.,* 2002). However, we did not see a large increase in p-JNK after NGF deprivation, possibly due to the relatively high level of p-

JNK in NGF-maintained neurons, which supports a role for JNK activity in healthy cells (Newbern *et al.,* 2007; Barnat *et al.,* 2010). p-JNK levels were slightly increased from 6-24 h after NGF- withdrawal, and these levels were inversely proportional to the concentration of SP600125, suggesting inhibition was achieved (Figure 1). However, the concentration (50 μM) necessary to attain significant p-JNK inhibition was much higher than that of similar studies (Mesner *et al.,* 1992; Bruckner *et al.,* 2001).

p-JNK translocates to the nucleus and induces c-Jun expression, or activates c-Jun in the cytoplasm, which then translocates to the nucleus, where it may autoregulate its own expression (Eilers *et al.,* 1998; Ip and Davis, 1998). An increase in c-Jun protein levels were evident after 24 h of NGF withdrawal, and were diminished by SP600125 treatment. These experiments further suggest high concentrations of SP600125 (50 μM) significantly prevented JNK activation in NGF-deprived sympathetic neurons (Figure 1).

In order to determine the effect of blocking JNK-activation, we deprived sympathetic neurons of NGF for 48 h and compared the rate of survival to sister cultures treated with SP600125 from the time of deprivation. Surprisingly, SP600125 treatment only produced a modest increase in survival at concentrations that significantly prevented evidence of JNK- activation (Figure 2).

Relationship between JNK/c-Jun activation, Bax, and ROS

In some systems it has been reported that JNK/c-Jun activation is a consequence of Bcl-2 activation and oxidative stress (Frisch *et al.,* 1996; Hsuuw *et al.,* 2005; Wang *et al.,* 2008a). NGF deprivation induces c-Jun phosphorylation on residues (Ser63/73) recognized by JNK. In order to determine whether Bax was required for this process, we examined NGF-deprived

neurons with an antibody that only recognizes c-Jun phosphorylation at Ser63 and does not recognize unphosphorylated c-Jun (Watson *et al.,* 1998). Immunocytochemistry of NGF-deprived wild type and *bax*-deficient neurons revealed that 12 h of NGF deprivation induced c-Jun phosphorylation and translocation to the nucleus, consistent with the activation of JNK early in this paradigm (Figure 3). Phosphorylated c-Jun protein accumulated in the nuclei of NGF- deprived sympathetic neurons irrespective of Bax, indicating that Bax is not required for the activation of JNK/c-Jun. These findings support those previously established by Deckwerth, *et al.* (1998).

Because of the importance of Bax-dependent, mitochondrial ROS production in the apoptotic death of these cells and the known influence of JNK on pro-apoptotic proteins that dictate ROS levels, we sought to determine the effect of JNK-inhibition on ROS levels in sympathetic neurons deprived of NGF for 24 h. Previous studies in our lab have shown that the ROS produced in these neurons derive from the production and dismutation of $O_2^{\cdot -}$ produced by electrons leaking from the mitochondrial electron transport chain (Eilers *et al.,* 1998; Kirkland *et al.,* 2002c). Using the redox sensitive dye, CM_2-DCFDA, in conjunction with confocal microscopy, we found that SP600125 treatment had no effect on H_2O_2-associated ROS in this paradigm (Figure 3). Taken together, these results suggest that JNK/c-Jun activation is not dependent on Bax, but JNK/c-Jun also does not determine the effects of Bax activation.

Effect of SP600125 on Mitochodrial Cytochrome c Stores

Mitochondria begin dumping cytochrome *c* approximately 24 h after NGF-withdrawal in mouse sympathetic neurons (Eilers *et al.,* 1998). This process is dependent on the balance of Bcl-2 proteins, and therefore may be influenced by JNK activation (Kirkland *et al.,* 2002b;

Putcha *et al.,* 2003). To determine whether SP600125-inhibition of JNK was sufficient to block this effect, we quantified the retention of cytochrome *c* in sympathetic neurons deprived of NGF for 48 h and maintained in the pan-caspase inhibitor Boc-aspartyl fluoromethyl ketone (BAF). Since SP600125 only modestly prevented cell death, BAF was included from the time of deprivation in all NGF-deprived cultures in order to prevent any cell death due to caspase activation during this time. Caspase activation occurs downstream of cytochrome *c* release, and consequently, does not affect cytochrome *c* retention in this paradigm. Immunocytochemistry revealed depletion of mitochondrial cytochrome *c* stores after 48 h of NGF deprivation of sympathetic neurons ($p<0.001$ for NGF-deprived vs. control). SP600125 co-treatment did not appear to affect the release of mitochondrial cytochrome *c* during NGF deprivation ($p \geq 0.1$ for –NGF and all concentrations of SP600125) (Figure 5).

Discussion

Depriving neonatal sympathetic neurons of NGF *in vitro* constitutes a well-defined model of neuronal apoptosis which recapitulates the physiologically appropriate cell death necessary for nervous system development. New protein synthesis is necessary for this death to occur, particularly that of c-Jun (Martin *et al.,* 1988; Deckwerth *et al.,* 1998; Harris *et al.,* 2002). Many of the AP-1 responsive genes induced by c-Jun encode proteins of the Bcl-2 family. JNK also translocates to the mitochondria during apoptosis and directly activates these pro-apoptotic proteins via phosphorylation (Kharbanda *et al.,* 2000). In order to determine if JNK activation was necessary for the downstream mitochondrial events and death in sympathetic neurons, we employed the JNK inhibitor SP600125.

Surprisingly, we did not see an impressive effect on survival by SP600125

treatment. This JNK inhibitor has shown enhanced efficacy in many other apoptotic models, including NGF-differentiated PC-12 cells (Bruckner *et al.,* 2001; Chauhan *et al.,* 2003; Schwabe *et al.,* 2004). These results may also suggest that the same type of neuron derived from different sources, such as seemingly closely related species (i.e., rat and mouse) or differentiated cell lines, can exhibit disparate dependence on JNK signaling during apoptosis induced by the same stimuli.

Other studies examined the effect of JNK in sympathetic neurons isolated from rats by treating the cells with CEP-1347, a mixed lineage kinase (MLK) inhibitor that inhibits the JNK pathway by preventing upstream activation of SEK1/MKK4 and MKK7. CEP-1347-treatment demonstrated remarkable trophic effects, including preservation of metabolism, neurite outgrowth, and anti-apoptotic effects including retention of cytochrome *c* (Harris *et al.,* 2002). The beneficial effects of CEP-1347 may involve other events downstream or independent of MKK4/7 (Wang *et al.,* 2005), since selective JNK inhibition in our study was less effective at preventing apoptosis and certainly did not appear to lend trophic benefit.

JNK-dependent phospho-c-Jun and Bax-dependent mitochondrial ROS are both increased early in this apoptotic paradigm. However, our results and others reveal that Bax does not affect phospho-c-Jun levels, suggesting either Bax is downstream of JNK/c-Jun activation or their pro- apoptotic effects occur in parallel pathways (Deckwerth *et al.,* 1998). Our results also show that JNK inhibition by SP600125 did not affect Bax-dependent ROS, lending more support for the latter hypothesis. This divergence in JNK/c-Jun signaling and Bax-dependent ROS was also evident in previous studies in SP600125-treated PC-12 cells and sympathetic neurons from JNK3-deficent mice in which the level of ROS was unaffected during apoptosis induced by NGF deprivation (Bruckner *et al.,* 2001). The low rate of survival

conferred by SP600125 combined with the apparent lack of effect on ROS also affirm a pivotal role for mitochondrial ROS in this apoptotic paradigm (Kirkland *et al.*, 2002b; Vaughn and Deshmukh, 2008).

The results reported herein support a role of JNK/c-Jun activation in apoptosis, but suggest that it is not primarily responsible for Bax-dependent events, such as mitochondrial ROS production and cytochrome *c* release, in apoptotic sympathetic neurons isolated from mice. Our results also suggest that therapeutic intervention by selective JNK inhibition may not be ideal for pathological conditions in which oxidative stress and neuronal death are heavily implicated, such as age-dependent neurodegenerative disease.

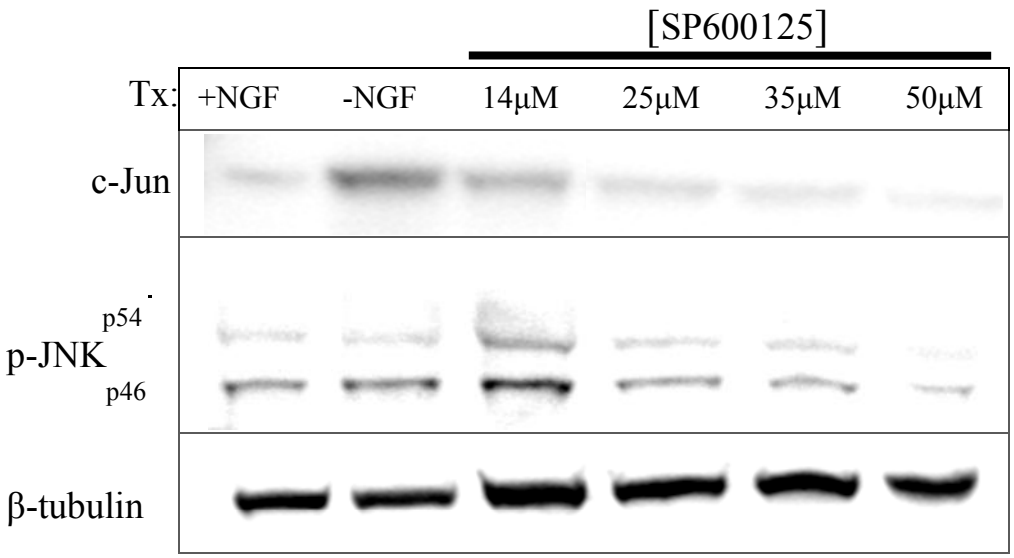

Figure 2.1. SP600125 prevented p-JNK/c-Jun pathway activation after NGF deprivation in sympathetic neurons. Immunoblot analysis revealed the effect of SP600125 on phospho-JNK (p54 and p46 residues) and c-Jun levels in cultures deprived of NGF with a neutralizing antibody. There was a dose-dependent effect of SP600125 treatment in the induction of c-Jun and activation of JNK after 24 h of NGF deprivation. Beta-tubulin III provided a loading control.

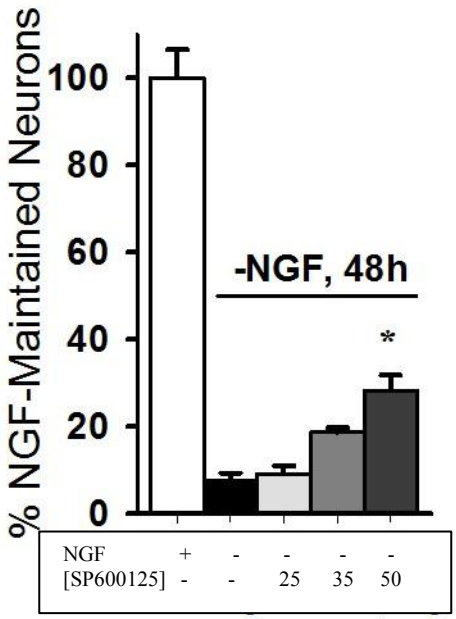

Figure 2.2. Inhibition of JNK activation by SP600125 increased survival of NGF deprived sympathetic neurons. Sympathetic neurons were grown in the presence of NGF for 6 days and then deprived of NGF alone, or in the presence of the JNK-inhibitor SP600125 at concentrations of 25 μM, 35 μM, or 50 μM for 48 h. Neurons were subsequently rescued with NGF, allowed to establish for 5 days, then fixed and stained with crystal violet to assess viability. Viability expressed as percent of sister cultures maintained in NGF throughout the treatment period (*$p<0.05$ for –NGF+SP50μM verses –NGF alone). Results represent findings from four independent experiments; error bars are ± SEM..

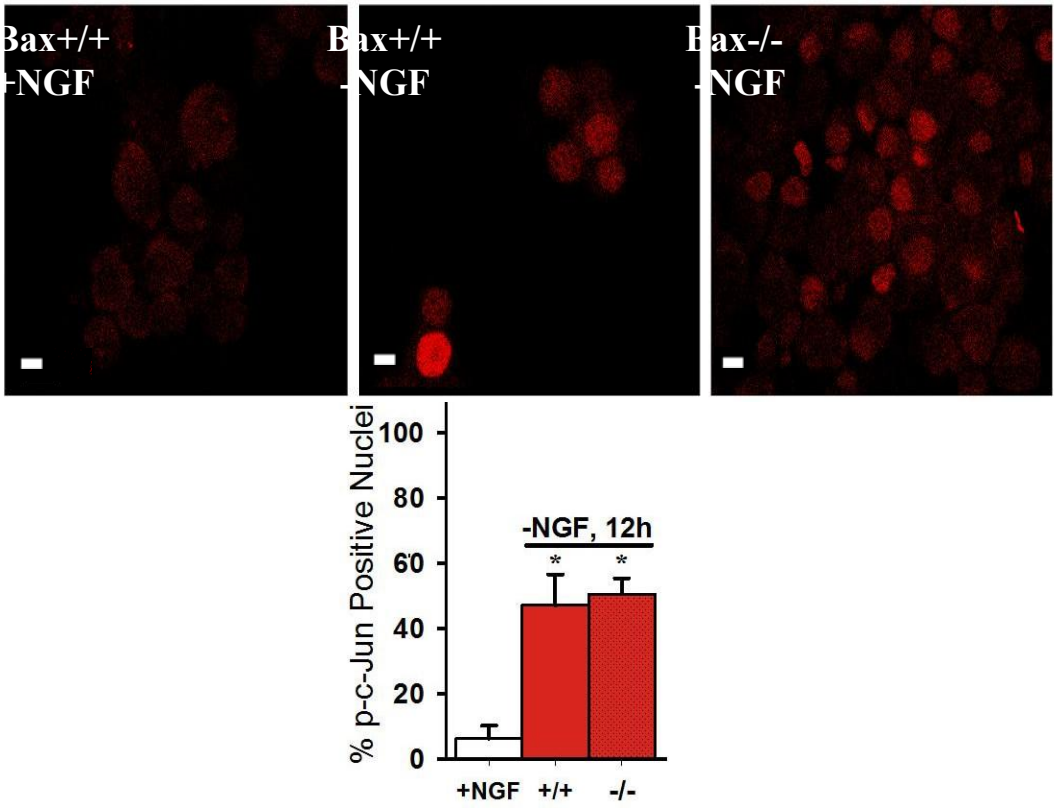

Figure 2.3. Apoptotic nuclei accumulated phospho-c-Jun (Ser 63) immunoreactivity irrespective of Bax. Cultures of *bax* $^{+/+}$ and *bax* $^{-/-}$ sympathetic neurons were maintained in NGF for 6 days. The cells were then allowed to remain in NGF (+NGF) or deprived of NGF (-NGF) for 12 h, fixed and stained with the phospho-c-Jun antibody. In the presence of NGF, faint staining of the cell body was observed with the phospho-c-Jun antibody. After NGF withdrawal, nuclear staining of phospho-c-Jun was increased. Similar induction of p-c-Jun occurred both in wild type (+/+) and Bax-deficient (-/-) neurons during apoptosis due to NGF-withdrawal ($p < 0.05$ for all –NGF vs. +NGF). Error bars represent ± SEM.

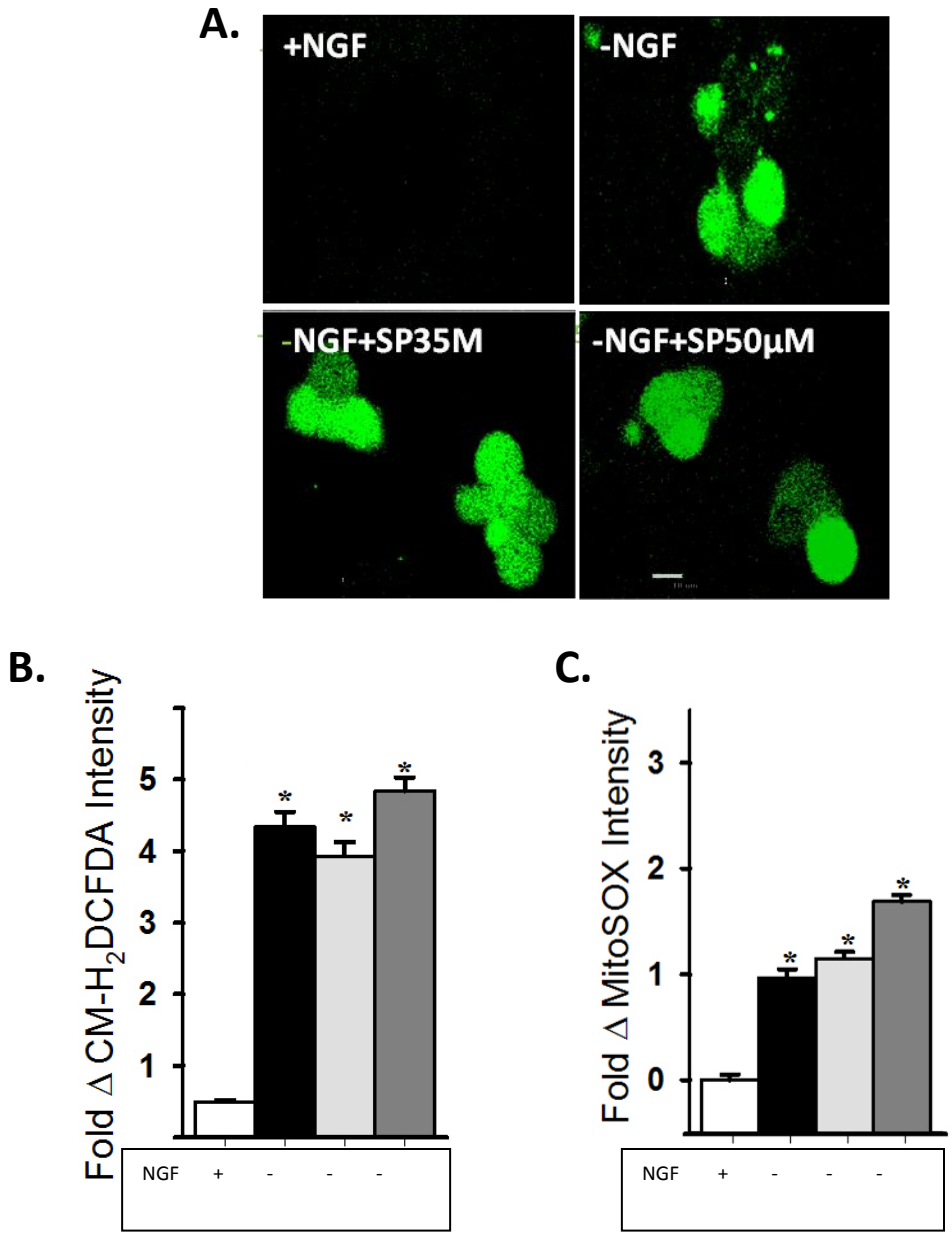

Figure 2.4. Mitochondrial ROS production persisted despite p-JNK/c-Jun inhibition by SP600125. A. Confocal micrographs show a dramatic increase in ROS in sympathetic neurons deprived of NGF for 24 h, as evident by the increased intensity of the redox sensitive dye, CM-H_2DCFDA over NGF-maintained, control neurons. Inhibition of p- JNK/c-Jun activation by 35 µM and 50 µM SP600125 produced no effect on these ROS. Quantification of the average intensity of CM-H_2DCFDA staining (B.) and MitoSOX (C.) over NGF-maintained sister

cultures revealed that the increase in ROS at 24 h of NGF deprivation was not effected by JNK-inhibition (*$p < 0.05$ for -NGF, SP35, SPSO versus +NGF). Error bars respresent ± SEM.

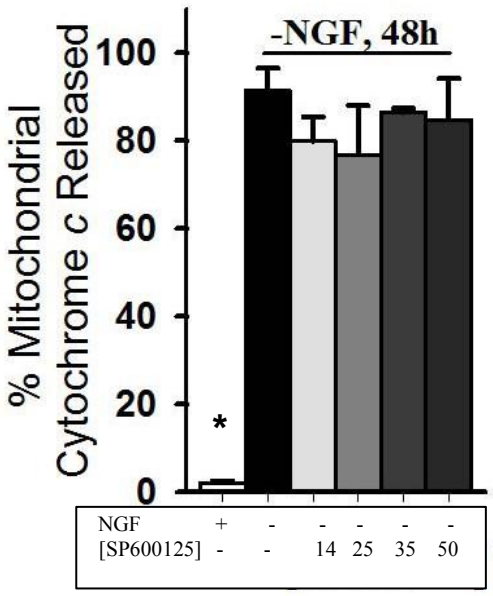

Figure 2.5. SP600125 treatment did not prevent the loss of cytochrome *c* during apoptosis of NGF-deprived sympathetic neurons. After 6-DIV, sympathetic neurons were deprived of NGF for 48 h in the presence of BAF and various concentrations of SP600125 (14 μM, 25 μM, 35 μM, and 50 μM). The release of cytochrome *c* as detected by immunofluorescence was not affected by SP600125 treatment ($p < 0.05$ for all NGF-maintained vs. -NGF deprived cultures, regardless of SP600125 treatment).

CHAPTER 3

MITOCHONDRIAL REACTIVE OXYGEN SPECIES MEDIATE THE PROGRESSION OF CASPASE-DEPENDENT AND CASPASE-INDEPENDENT NEURONAL DEATH[2]

[2]McManus, M.J., M.P. Murphy, and J.L. Franklin
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Abstract

Mitochondrial dysfunction and oxidative stress are implicated in many neurodegenerative diseases. Mitochondria-targeted drugs that effectively decrease oxidative stress, protect mitochondrial energetics, and prevent neuronal loss may therefore lend therapeutic benefit to these currently incurable diseases. The results provided herein indicate that the mitochondria-targeted antioxidant, $MitoQ_{10}$, effectively suppresses mitochondria-derived reactive oxygen species (ROS) and prevents cytochrome *c* release, caspase activation, and mitochondrial damage implicated in the death of trophic factor-deprived sympathetic neurons in culture, while $MitoE_2$ was largely ineffective. In this paradigm, the most proximal point of divergence was found in the enhanced ability of $MitoQ_{10}$ to scavenge mitochondrial superoxide ($O_2^{\cdot}$), as detected by redox-sensitive dyes. The mitochondrial protection provided by $MitoQ_{10}$ was sufficient to prevent both caspase-dependent and caspase-independent events that determine the commitment to neuronal death, suggesting the generation of mitochondria-derived ROS is an important regulator of both pathways leading to neuronal demise. The data highlight the use of mitochondria-targeted antioxidants as tools to delineate the role of mitochondria-derived ROS in pathological pathways involving mitochondrial dysfunction and suggest $MitoQ_{10}$ may provide therapeutic value for neurodegenerative conditions involving multiple modes of death.

Introduction

Although the genetic and environmental factors that trigger neuronal apoptosis differ amongst physiological and pathological settings, most of the subsequent signaling events are highly conserved. In the early stages of life, neurons compete for a limited supply of growth factors. Those that fail to acquire adequate levels will die off by inducing both genetic and biochemical events that converge on the mitochondria and end in cell suicide. This attrition highlights the physiologically appropriate role of apoptosis, as pruning of excess neurons is necessary for proper sculpting of the nervous system during development (Oppenheim, 1991). Perhaps the most extensively characterized model of this process is that of sympathetic neurons deprived of nerve growth factor (NGF) (Davies, 1996; Putcha and Johnson, 2003). Withdrawal of NGF from sympathetic neurons in culture causes a cessation of somatic growth and neurite outgrowth, as well as a sharp decline in glucose uptake (Chang *et al.,* 2003). Shortly after deprivation, Bax permeabilizes the outer mitochondrial membrane (OMM) and enhances superoxide ($O_2^{\cdot -}$) production by the mitochondrial electron transport chain (Kirkland *et al.,* 2002b, 2007, 2010). These events orchestrate mitochondrial release of apoptogenic cytochrome *c* into the cytosol where it binds apoptotic protease activating factor-1 (Apaf1) and induces formation of the apoptosome (Kirkland *et al.,* 2002b; Vaughn and Deshmukh, 2008). Apoptosome formation amplifies activation of the apical caspase 9 and executioner caspase 3, which cleave critical cellular components and cause death (Wright *et al.,* 2006). If these caspases are inhibited, death proceeds in a caspase-independent manner and the point-of-no-return is extended to the loss of mitochondrial membrane potential ($\Delta\psi_m$) (Deshmukh *et al.,* 2000; Chang *et al.,* 2002). NGF-deprived sympathetic neurons provide an ideal experimental paradigm to investigate the effect of potential therapeutics on caspase-dependent and -

independent events that dictate neuronal death, as cultured sympathetic neurons undergo the processes described above in a most synchronous fashion (Deshmukh and Johnson, 1997).

As postmitotic cells, neurons are irreplaceable. The fate of a neuron is largely determined by the balance of death-promoting and life-preserving signals that both congregate at and emanate from its mitochondrial membranes (Kroemer *et al.,* 2007). If the flow of electrons through the inner mitochondrial membrane (IMM) is disturbed, cellular energetics may decline and free radicals will form as electrons leak out, reducing molecular oxygen to $O_2^{\cdot-}$ (Murphy, 2009). As the primary source of $O_2^{\cdot-}$ in mammalian cells, mitochondria are also the principle targets of damage due to $O_2^{\cdot-}$ or the downstream radicals and non-radicals that are formed, collectively termed reactive oxygen species (ROS) (Turrens, 2003). To protect against this damage, mitochondria possess an extensive antioxidant defense system, comprised of both enzymatic and non-enzymatic components, including manganese superoxide dismutase (MnSOD), glutathione (GSH), ubiquinone, and α-tocopherol (Murphy, 2009). Many mitochondrial antioxidants require reducing equivalents derived from glucose metabolism for their transport to and regeneration within the mitochondria (Kaplowitz *et al.,* 1985; Kowaltowski *et al.,* 2001b; Sheeran *et al*., 2010). A significant imbalance in interdependent bioenergetic and redox signaling systems will eventually lead to mitochondrial dysfunction and neuronal death (Circu and Aw, 2010; Wallace, 2010).

Mitochondrial dysfunction is prominent in both acute and progressive neurodegenerative conditions (Green and Kroemer, 2004; Lin and Beal, 2006); therefore, therapeutics that protect these organelles may prevent the neuronal loss that follows (Stefanis, 2005). In this regard, mitochondria-targeted antioxidants may prove useful (Bruckner *et al.,* 2001). The high membrane potential across the IMM allows antioxidants, such as ubiquinone or vitamin E, to be targeted to the negatively charged mitochondrial matrix by covalent

attachment to the lipophilic cation, triphenylphosphonium (TPP^+; Figure 1) (Murphy, 2001). These compounds easily traverse the plasma membrane and the OMM, and selectively accumulate within the IMM of treated cells (James *et al.,* 2007; Murphy and Smith, 2007). In the current study, we examined the effects of two such compounds, $MitoQ_{10}$ and $MitoE_2$, on the critical events that dictate the commitment to neuronal death (Chang *et al.,* 2002). The findings suggest that the mitochondria-targeted antioxidant, $MitoQ_{10}$, prevented the progression of both caspase-dependent and -independent pathways to death in cultured sympathetic neurons by protecting the organelle from excessive mitochondrial $O_2^{\cdot -}$ production and the subsequent damage that leads to irrevocable neurodegeneration.

Materials and Methods

Chemicals. [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cycloheexadienl-yl)decyl] triphenylphosphonium methanesulfonate (MitoQ10), [2-(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl) ethyl] triphenylphosphonium chloride (MitoE2), and decyltriphenylphosphonium bromide (dTPP) were synthesized as described previously (Kelso, 2001; Smith 1999). MitoSOX Red and 5-(and -6)-chloromethyl-2`, 7`-dicholorodihydrofluorescein diacetate (CM-H2DCFDA) were purchased from Invitrogen (Eugene, OR). Nerve growth factor (NGF) 2.5S was purchased from Harlan Bioproducts

(Indianapolis, IN). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise noted.

Neuronal Culture and Treatment. Primary cultures of sympathetic neurons from the superior cervical ganglia (SCG) were prepared from neonatal C57B1/6 mice based on techniques described (Johnson and Argiro, 1983). Briefly, the SCG were enzymatically dissociated by successive 30-min treatments in 1 mg/ml collagenase and 2.5 mg/ml trypsin at 35° C, washed with Leibovitz-15 (L-15), then mechanically dissociated by trituration into growth media, plated onto collagen-coated 24-well Costar plates (Corning, Inc., Corning, NY) for survival assays or #1 glass coverslips for microscopy experiments, and maintained at 35°C in a humidified incubator containing 5% CO_2/95% air. The growth medium (Eagle's minimum essential medium with Earle's salts; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, 20 μM uridine, 1.4mM L-glutamine, and 50 ng/ml 2.5S NGF) was replaced after 3-4 days *in vitro* (DIV).

After 6 DIV, NGF deprivation was achieved by rinsing cultures twice with medium lacking NGF, followed by addition of medium containing goat anti-mouse 2.5S NGF neutralizing antiserum (anti-NGF; Cedarlane Labs, Burlington, Ontario). Mitochondria-targeted compounds were included in media at the time of deprivation, unless otherwise stated. The concentrations (0.25-1 μM) of these compounds were chosen based on results from initial toxicity screens, which revealed that long-term exposure (7 days) to concentrations above this range were toxic to NGF-maintained neurons.

Survival Assays. The ―commitment to die" was quantified as the portion of neurons that could be rescued from death by readdition of NGF to the deprived cultures at the indicated time points, as described (Deshmukh *et al.,* 2000). Briefly, sympathetic neurons were equally distributed among 24-well plates and allowed to establish for 6- DIV. The cultures were deprived of NGF for various periods ranging from 24 to 96 h, after which time they were rinsed to remove any residual anti-NGF, and incubated in NGF-containing media for at least 5 days. In this rescue paradigm, neurons that are not committed to die regain phenotypic characteristics of NGF-maintained neurons in the days following NGF-readdition, while those neurons that have made the decision to die continue to atrophy and detach from the collagen substrate. After rescue, the cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.2) for more than 1 h, washed with PBS, and stained by a brief rinse with 0.1% crystal violet. Survival within each experimental group is presented as a percent of the total number of neurons in sibling cultures that were maintained in NGF from the time of plating. The cultures were counted in a blinded manner. The treatments were run in triplicate or quadruplicate within each of at least three independent experiments.

Microscopy. All confocal microscopy was performed using a Nikon (Melville, NY) C1 laser scanning confocal microscope mounted on a Nikon Eclipse TE 300 inverted microscope. The confocal microscope was controlled by EZC1 software running on a Dell computer. Neurons were observed with a 60x plan oil immersion lens, chosen at random and scanned by the confocal microscope. Laser power, pinhole size, and photo- multiplier gain were maintained at constant levels during each experiment. Experiments were replicated at least three times. Fluorescence microscopy was conducted using a 20x objective. Light was provided by a Xenon lamp and images collected by a cooled CCD camera (Micro-Max; Princeton

Instruments, Trenton, NJ). Filters were operated by a Lambda 10–2 optical filter changer (Sutter Instruments, Novato, CA).

All images were quantified by measuring the raw pixel intensities in the cytoplasm of neuronal somas with the region tool of MetaMorph software (Universal Imaging, West Chester, PA). The area quantified covered a 60 μm^2 area of cytoplasm in confocal microscopy images. The intensity of each neuron was normalized to that of control neurons receiving the same concentration of dye for the same time as the experimental cells. Normalized data are shown as fold change from the intensity of the dye measured in sister cultures of sympathetic neurons maintained in NGF-containing medium from the time of plating. All microscopy was conducted at room temperature.

ROS measurement. As a measure of the mitochondria redox status, relative mitochondrial $O_2^{\cdot-}$ levels were determined by MitoSOX Red. MitoSOX is a redox-sensitive dye composed of hydroethidine linked by a hexyl carbon chain to a TPP^+ group that targets the dye to the mitochondria. Most of the fluorescent intensity of MitoSOX presumably originates from oxidation of the hydroethidine moiety by $O_2^{\cdot-}$ to the fluorescent product, 2- hydroxyethidium, that is subsequently retained in the mitochondria due to the high affinity of ethidium for DNA (Zhao *et al.,* 2005). To selectively detect the $O_2^{\cdot-}$-MitoSOX product, we used the 408 nm line of the confocal laser, and the red photomultiplier channel of the confocal microscope was used for image acquisition (Robinson *et al.,* 2006; Johnson and Cadwell, 2007). Our lab has extensively characterized the use of this dye to detect $O_2^{\cdot-}$ in apoptotic sympathetic neurons; selectivity of MitoSOX for mitochondria-derived $O_2^{\cdot-}$ in these neurons is supported by its co-localization with Mito-Tracker Green, increased intensity upon treatment with the superoxide-generator menadione, a lack of fluorescent induction in response to H_2O_2, and more (Kirkland *et al.*, 2007; 2010). Cultures were incubated for 10 min at 35°C in the appropriate experimental

medium containing MitoSOX (2 μM). After incubation in MitoSOX, cultures were washed twice with L-15 and kept in the second wash for microscopy. ROS were also detected using the redox-sensitive dye CM-H_2DCFDA. This dye is membrane permeant and is trapped in cells by binding of the chloromethyl group to cellular thiols. The reduced form of CM-H_2DCFDA becomes intensely fluorescent after oxidation by multiple reactive species, thus providing an general indicator for the intracellular redox state (Royall and Ischiropoulos, 1993). We extensively characterized the use of this dye in rat and mouse sympathetic neurons (Kirkland *et al.,* 2001; 2002). This characterization shows that when CM-H_2DCFDA is trapped in these cells, it is insensitive to pH changes within the physiological range and is not photo-oxidized at the laser power used in our experiments. Cultures were incubated in the appropriate experimental medium containing CM-H_2DCFDA (10 μM) for 20 min at 35°C. They were then washed twice with L-15 medium and left in the last wash for confocal microscopy. CM-H_2DCFDA was excited with the 488 nm line of the confocal laser. The green photomultiplier channel was used for image acquisition.

Mitochondrial membrane potential ($\Delta\psi_m$) measurement. The cationic fluorescent probe, tetramethylrhodamine methyl ester ($TMRM^+$), accumulates in cellular compartments in response to local potential differences. Neurons were incubated with low concentrations (10 nM) of $TMRM^+$ for 30 min, rinsed once with L-15, and quantified bathed in the appropriate amount of $TMRM^+$ in L-15. At equilibrium, these low concentrations of $TMRM^+$ provide a quantitative estimate of $\Delta\psi_m$ in ~~"n~~on-quenched" mode, such that the fluorescence produced (ex543/em573) is a direct function of $\Delta\psi_m$, and complications due to plasma membrane redistribution are diminished (Nicholls and Ward, 2000; Duchen *et al.,* 2003). Using this technique, we measured $TMRM^+$ fluorescence within individual neurons and normalized these values to that of control cultures maintained in NGF. Specificity of $TMRM^+$ fluorescence for

$\Delta\psi_m$ was confirmed by the complete loss ($p < 0.001$) of staining upon treatment with the uncoupling agent, carbonyl cyanide p-trifluromethoxy-phenylhydrazone (FCCP), which dissipates $\Delta\psi_m$ without altering plasma membrane potential (Duchen, 1999).

Immunocytochemistry. Neurons were immunostained for cytochrome *c* based on methods previously described (Eilers *et al.,* 1998). Cultures were fixed for at least 60 min at 4°C with 4% paraformaldehyde in PBS. They were then washed with Tris-buffered saline (TBS; pH 7.2) and incubated for at least 60 min at room temperature in blocking buffer (TBS containing 0.3% Triton X-100 and 5% normal goat serum), rinsed, and incubated overnight at 4°C in blocking buffer containing the anti-cytochrome *c* primary antibody (200 ng/ml; PharMingen, San Diego, CA). The following day, cultures were washed 3X with cold TBS and incubated for 2-4 h at RT in blocking buffer containing a FITC-conjugated anti-mouse secondary antibody (1.7 µg/ml; PharMingen), washed, and then viewed by fluorescence microscopy. Cytochrome *c* appears to be rapidly degraded after redistribution to the cytosol in mouse sympathetic neurons in culture (Putcha *et al.,* 2000). As such, neurons with a bright, mitochondrial pattern of staining were counted positive for cytochrome *c* retention, whereas those with faint or no staining were counted negative (Neame *et al.,* 1998).

Caspase 3/7 Activity Assay To assess caspase 3 activity, the Caspase-Glo™ Assay (Promega, Madison, WI) was used according to the manufacturer's protocol. Briefly, sympathetic neurons were grown in a 96-well plate for 6-DIV, and then deprived of NGF for 24 h in the presence or absence of $MitoQ_{10}$ or $MitoE_2$ (500 nM). Additional NGF- deprived cultures were treated with the pan-caspase inhibitor, Boc-aspartyl fluoromethyl ketone (BAF; 50 µM), for comparison. After the treatment period, an equal amount of Caspase-Glo 3/7 Reagent was added to each

well, resulting in cell lysis, followed by caspase cleavage of the pro-luminescent caspase 3/7 DEVD-aminoluciferin. The magnitude of the luminescence generated by consumption of free amino-luciferin by luciferase represents both caspase 3 and 7 activity; however, only caspase 3 is active in these cells (Wright *et al.,* 2006). Luminescence intensity was recorded with a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Results represent the average of four independent experiments in which the treatments were run in duplicate or triplicate, and are shown as fold change from the respective NGF-maintained sister cultures.

Statistical Analysis. Statistical comparisons were made by Kruskal-Wallis one-way ANOVA, followed by Dunn's *post hoc* test for individual differences, unless otherwise stated. Data analysis and statistical comparisons were performed using SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA). All error bars represent the mean ± standard error (SEM).

Results

$MitoQ_{10}$ Prevents Apoptosis in NGF-deprived Sympathetic Neurons

NGF deprivation induces apoptosis in mouse sympathetic neurons that becomes irreversible within 24-48 h. After 24 h of deprivation, approximately half of the neurons are unable to be rescued by NGF readdition and are thus "committed to die"; practically all sympathetic neurons in culture reach this point after 4 h of deprivation (Deshmukh and Johnson, 1997). To assess the ability of $MitoQ_{10}$ and $MitoE_2$ to prevent neuronal apoptosis, sympathetic neurons were deprived of NGF for 48 h alone or in presence of $MitoQ_{10}$ or $MitoE_2$ (250 nM-1 μM), or a combination of both (250 nM each). Approximately 70% of sympathetic neurons treated with $MitoQ_{10}$ (250 nM-1 μM) from the time of deprivation were capable of rescue by

NGF-readdition 48 h later. Concentrations of $MitoQ_{10}$ lower than 250 nM produced no protective effect (data not shown), and those above 1 μM were cytotoxic, as evident by the sharp decline in survival with 5 μM $MitoQ_{10}$ (Figure 2*A*). dTPP consists of the mitochondria-targeting moiety (TPP^+) attached to a ten-carbon chain, and was used to control for nonspecific effects of the lipophilic cation. dTPP produced no detectable changes in the survival rate of NGF- deprived neurons, thus the ubiquinone moiety of $MitoQ_{10}$ was responsible for its anti- apoptotic effect. $MitoE_2$ was ineffective in preventing neuronal death at all concentrations studied.

Ubiquinol enhances the efficacy of vitamin E in membranes and cells by reducing the tocopheroxyl radical back to the active antioxidant after its oxidation by a reactive species (Burton and Traber, 1990; Kagan and Tyurina, 1998; James *et al.,* 2004). We considered that the mitochondria-targeted analogs may perform similarly. To investigate the ability of $MitoQ_{10}$ to enhance the efficacy of $MitoE_2$, cultures were treated with a combination of $MitoE_2$ and $MitoQ_{10}$ (250 nM each) from the time of deprivation, and the effect on the decision to die was determined as described. Even though the total concentration of mito-antioxidant was equal to that of the most effective dose of $MitoQ_{10}$ (500 nM), there was no significant increase in survival over that of NGF-deprived neurons and no benefit over the same concentration of $MitoQ_{10}$ alone ($p > 0.05$, $MitoQ_{10}$ 250 nM versus $MitoE_2$+$MitoQ_{10}$) (Figure 2*A*). The most effective concentration of each mitochondria-targeted compound was 500 nM, and this concentration was used for all subsequent experiments shown.

To determine the effect of $MitoQ_{10}$ and $MitoE_2$ on caspase-independent death, neuronal cultures were deprived for 72 h in the presence of the caspase inhibitor, BAF, alone, or with $MitoQ_{10}$ or $MitoE_2$. Using the same rescue paradigm, previous studies have shown that BAF-

treated neurons can be rescued by NGF readdition up to 60 h after NGF-withdrawal, but by 72 h death prevails by a caspase-independent mechanism (Deshmukh *et al.,* 2000). Accordingly, BAF-treatment alone was not sufficient to prevent death after this degree of deprivation. The results show that $MitoQ_{10}$-treated neurons escaped impending death and recovered upon NGF readdition after 72 h of deprivation. Although $MitoQ_{10}$ treatment did not prevent the commitment to die at later time points (96 h; data not shown), these results indicate that $MitoQ_{10}$ significantly delayed the commitment to die beyond that of caspase inhibition by BAF.

Commitment 1: $MitoQ_{10}$ prevented cytochrome c redistribution and caspase activation

During NGF deprivation, the two most critical check-points that dictate a sympathetic neuron's commitment to die involve the depletion of mitochondrial cytochrome *c* stores and the terminal loss of $\Delta\psi_m$ (Neame *et al.,* 1998; Deshmukh *et al.,* 2000; Chang *et al.,* 2002). Since cytochrome *c* release begins around 18 h after NGF deprivation, approximately half of the deprived sympathetic neurons become committed to die and substantially degraded due to caspase activation by 24 h of NGF withdrawal, which confounds quantification of the effect additives may have on further cytochrome *c* release and the subsequent, terminal loss of $\Delta\psi_m$ (Deshmukh and Johnson, 1997; Putcha *et al.,* 1999). Numerous reports have shown that the ultimate loss of both cytochrome *c* and $\Delta\psi_m$ is not dependent on caspase activity in this paradigm (Deshmukh and Johnson, 1998; Desagher *et al.,* 1999; Putcha *et al.,* 1999; Deshmukh *et al.,* 2000; Kirkland and Franklin, 2001; Chang *et al.,* 2002). Therefore, in order to more accurately assess the ability of mito-antioxidants to modulate these endpoints, substantial degeneration was prevented by inhibiting caspase activity with BAF. Sympathetic

neurons were deprived of NGF in the presence of BAF, $MitoQ_{10}$, or BAF plus dTPP, $MitoQ_{10}$, or $MitoE_2$ for 48 h, and the effect on cytochrome *c* redistribution was quantified by immunocytochemistry. Only $MitoQ_{10}$-treated, NGF- deprived neurons maintained mitochondrial cytochrome *c* staining throughout NGF deprivation (Figure 3*A*). Since $MitoQ_{10}$ prevented death up to 72 h of NGF deprivation, this treatment did not require BAF addition. The results indicate no difference between $MitoQ_{10}$ alone and Mito+BAF treatment in this paradigm. To determine the time course by which $MitoQ_{10}$ prevented loss of mitochondrial cytochrome *c* after NGF deprivation, sympathetic neurons were deprived of NGF in the presence of BAF, and $MitoQ_{10}$ or dTPP was added to parallel cultures 18 h and 24 h from the initial time of deprivation. The effect on cytochrome *c* redistribution was assessed 24 h later. $MitoQ_{10}$ addition significantly blocked any further release of cytochrome *c* from mitochondria of NGF- deprived neurons (Figure 3*B*). In contrast, dTPP failed to abort mitochondrial cytochrome *c* release, as neurons lost a significant amount of cytochrome *c* after the time of dTTP addition. The results indicate that $MitoQ_{10}$ acutely prevented mitochondrial cytochrome *c* depletion by a mechanism dependent on the antioxidant moiety.

The release of cytochrome *c* from the intermembrane space (IMS) causes imminent death of the cell following apoptosome formation and the activation of executioner caspases (Commitment 1) (Chang *et al.,* 2002; Putcha *et al.,* 2002; Wright *et al.,* 2006). To investigate the apoptogenic activity of cytochrome *c* that had been released from the mitochondria of NGF-deprived neurons treated with $MitoQ_{10}$ or $MitoE_2$, sister cultures of equal density were treated accordingly and induction of executioner caspase 3 was determined 24 h later. The results confirm that the increase in caspase 3 activity in NGF-deprived cultures is completely blocked by treatment with the pan-caspase inhibitor BAF (Figure 3*C*). NGF-deprived,

$MitoQ_{10}$-treated neurons had similar levels of caspase activity as cultures that had been maintained in NGF since plating ($p > 0.05$), suggesting that $MitoQ_{10}$ effectively prevented apoptotic induction ofcaspase 3, while $MitoE_2$ treatment produced no detectable effect on caspase induction during NGF deprivation.

ROS Scavenging Divergence between $MitoQ_{10}$ *and* $MitoE_2$

Considering the antioxidant properties of ubiquinol and vitamin E (Burton and Traber, 1990; Mordente *et al.,* 1998; James *et al.,* 2004), we anticipated that any benefit seen from the mitochondria-targeted compounds would likely correspond to their effects on mitochondrial ROS. To explore this relationship, the effect of dTPP, $MitoQ_{10}$, and $MitoE_2$ on relative mitochondrial $O_2^{\cdot-}$ levels in NGF-deprived sympathetic neurons was assessed with the mitochondria-specific, redox-sensitive dye, MitoSOX. Withdrawal of NGF from mouse sympathetic neurons increases mitochondria-derived ROS within 6 h of deprivation that rise to peak levels after 18-24 h of NGF deprivation (Kirkland *et al.,* 2002, 2010). NGF deprivation induced a 1.4 ± 0.2-fold increase in MitoSOX fluorescence emission over that of NGF-maintained neurons that was significantly prevented by $MitoQ_{10}$ (Fig. 4*B*). To establish the efficiency of mitochondrial $O_2^{\cdot-}$ suppression by $MitoQ_{10}$, $MitoQ_{10}$ was added after various time points of NGF deprivation up to 24 h, at which time MitoSOX intensity was again quantified and normalized to NGF-maintained controls. Consistent with its rapid uptake into cells and accumulation in the mitochondria (Smith *et al.,* 2003; Ross *et al.,* 2008), the results show that $MitoQ_{10}$ acutely (<1 h) attenuated $O_2^{\cdot-}$-sensitive MitoSOX levels in NGF-deprived neurons. dTTP had no effect on mitochondrial $O_2^{\cdot-}$ levels compared to NGF-deprived alone ($p > 0.05$; Fig 4*B*), excluding the possibility that nonspecific effects of lipophilic cation accumulation could

account for the decreased MitoSOX intensity. These results are supported by previous studies describing $O_2^{\cdot-}$ suppression by $MitoQ_{10}$ in isolated mitochondria, neurons, and astrocytes (Pehar *et al.,* 2007; Cassina *et al.,* 2008; Maroz *et al.,* 2009). Interestingly, $MitoE_2$ had no effect on mitochondrial ROS detected by MitoSOX during NGF deprivation.

To investigate the effects of $MitoQ_{10}$ and $MitoE_2$ on the intracellular redox state, CM-H_2DCFDA levels were determined in sympathetic neurons deprived of NGF alone, and in the presence of $MitoQ_{10}$ or $MitoE_2$, for 24 h. Previous studies with sympathetic neurons in culture have determined that CM-H_2DCFDA is relatively insensitive to $O_2^{\cdot-}$ oxidation, and the fluorescence it produces in response to NGF deprivation is primarily due to H_2O_2-associated species (Kirkland *et al.,* 2001; 2002; 2007). NGF deprivation resulted in a 5.6 ± 0.4 fold increase in CM-H_2DCFDA intensity over that of NGF-maintained controls (Fig. 4*C*). Both $MitoQ_{10}$ and $MitoE_2$ significantly attenuated the increased intracellular ROS detected by CM-H_2DCFDA, but only $MitoQ_{10}$ decreased these ROS to the level of NGF-maintained neurons ($p > 0.05$ for –NGF+$MitoQ_{10}$ and +NGF). $MitoQ_{10}$ also suppressed CM-H_2DCFDA-sensitive ROS within an hour of treatment. Taken together, the data indicate that while $MitoE_2$ has antioxidant activity in these neurons, it is not sufficient to modulate the mitochondrial redox state during NGF deprivation.

Commitment 2: $MitoQ_{10}$ p*revented loss of* $\Delta\psi_m$ *independently of cas*pase *activation*

NGF-deprived neurons are able to maintain $\Delta\psi_m$ after cytochrome *c* release, and in some cases, for a surprisingly extensive period of time (Neame *et al.,* 1998; Deshmukh *et al.,* 2000; Chang *et al.,* 2002; Chang and Johnson, 2002). Although transient decreases in $\Delta\psi_m$ occur in healthy and degenerating neurons, permanent loss of $\Delta\psi_m$ is intimately associated with cell death (Kroemer *et al.,* 2007). The majority of mitochondrial functions that are important for neuronal vitality, such

as ATP generation, cytosolic Ca^{2+} buffering, protein import, and antioxidant regeneration, are dependent on $\Delta\psi_m$ (von Ahsen *et al.,* 2000; Duchen, 2004; Wallace, 2005). Sustained ATP production is necessary to prevent caspase-independent death; however, sympathetic neurons rely principally on glycolysis for ATP during NGF deprivation (Chang *et al.,* 2003; Vaughn and Deshmukh, 2008). Therefore, maintenance of mitochondrial inner membrane integrity is required for prolonged neuronal survival aside from its role in ATP production, as less than 15% of NGF-deprived, BAF-maintained sympathetic neurons that have lost $\Delta\psi_m$ are capable of rescue from death (Deshmukh *et al.,* 2000).

Apart from the most obvious, $MitoQ_{10}$ and $MitoE_2$ have structural differences that may allow $MitoQ_{10}$ to accumulate within the IMM to a greater degree. Previous studies have shown that the length of the alkyl chain connecting these antioxidants to the TPP^+ moiety is directly correlated with the extent of IMM accumulation (Asin-Cayuela *et al.,* 2004; James *et al.,* 2004; James *et al.,* 2007). Since $MitoQ_{10}$ has a much longer hydrophobic linking chain than $MitoE_2$ (Fig. 1), we sought to determine if improved protection of the IMM by $MitoQ_{10}$ would correlate with its enhanced efficacy over $MitoE_2$. The ability of $MitoQ_{10}$ and $MitoE_2$ to preserve the functional integrity of the IMM was estimated by measuring $\Delta\psi_m$ with the potential-dependent fluorescent dye, $TMRM^+$. In accordance with the time-line previously established (Deshmukh *et al*., 2000), our results reveal that the majority of BAF-treated sympathetic neurons had lost $\Delta\psi_m$ after 48 h of NGF deprivation, signifying the involvement of caspase-independent mechanisms (Fig. 5). Sister cultures were treated with BAF plus dTPP, $MitoQ_{10}$, or $MitoE_2$, or $MitoQ_{10}$ alone, from the time of deprivation. As expected from the increased survival evident at 72 h, $MitoQ_{10}$-treated, NGF-deprived neurons retained $TMRM^+$ staining to the level of NGF-maintained neurons, while neither $MitoE_2$ nor dTPP produced any detectable protection.

When caspase activation is not prevented by pharmacological or genetic means, $\Delta\psi_m$ decreases following cytochrome *c* release in a portion of the NGF-deprived neurons in culture (Kirkland *et al*., 2010; Deshmukh *et al.,* 2000), and to a significant degree in other cell types, possibly due to respiratory chain compromise by activated caspases (Ricci *et al.,* 2004; Stranahan *et al.,* 2009). Therefore, caspase activation may contribute to the $\Delta\psi_m$ loss in NGF-deprived cultures lacking BAF (Kirkland *et al.*, 2010). In this regard, the ability of NGF-deprived neurons treated with $MitoQ_{10}$ alone to retain $TMRM^+$ intensity indistinguishable from that of cultures treated with $MitoQ_{10}$ plus BAF ($p > 0.05$) after 48 h of deprivation indicate that $MitoQ_{10}$ prevented both caspase-dependent and -independent influences on IMM integrity.

Discussion

This study evaluated the ability of mitochondria-targeted antioxidants, $MitoQ_{10}$ and $MitoE_2$, to prevent the progression of caspase-dependent and -independent neuronal death. In trophic factor-deprived sympathetic neurons in culture, $MitoQ_{10}$ prevented all critical checkpoints that dictate the neuron's commitment to die, while $MitoE_2$ had no effect (Fig. 6) (Deshmukh *et al.,* 2000; Chang *et al.,* 2002). In the classical apoptotic paradigm, $MitoQ_{10}$ treatment restored mitochondrial redox balance, prevented apoptogenic cytochrome *c* release, activation of caspase 3, and delayed the commitment to die for 72 h. The mitochondrial protection afforded by $MitoQ_{10}$ also prevented the progression of death in BAF-treated cultures, suggesting that mitochondria-derived ROS are actively involved in the mechanisms responsible for caspase-independent death as well.

Generation of $O_2^{\bullet-}$ by the mitochondrial respiratory chain is one of the earliest apoptotic events that occurs following NGF withdrawal from sympathetic neurons in culture (Greenlund *et al.,* 1995; Dugan *et al.,* 1997; Kirkland *et al.,* 2007). Accordingly, it was the most proximal point of divergence between $MitoQ_{10}$ and $MitoE_2$ in this study. $MitoQ_{10}$ acutely attenuated increased mitochondrial $O_2^{\bullet-}$, while $MitoE_2$ only affected cellular ROS detected by CM-H_2DCFDA, suggesting that the antioxidant activity of $MitoE_2$ does not involve direct modification of mitochondrial $O_2^{\bullet-}$ production during NGF deprivation. These results suggest that efficient suppression of mitochondrial ROS was of paramount importance in dictating the cascade of apoptotic events that follow in this paradigm. These results also suggest that the mitochondrial redox state may be a more influential mediator of apoptotic progression than the cellular redox state (Martensson *et al.,* 1990; Kim, 2007; Circu *et al.,* 2008), in part by influencing cytochrome *c* release and caspase activation. Within a short period, $MitoQ_{10}$ addition appeared sufficient to restore both the mitochondrial and cytosolic redox states and prevent cytochrome *c* redistribution in NGF-deprived sympathetic neurons. Previous studies have clearly shown that $MitoQ_{10}$ directly detoxifies $O_2^{\bullet-}$ /$HOO^{\bullet}$ and lipid-derived radicals, and effectively prevents peroxidation of the IMM within isolated mitochondria and cells (Kelso *et al.,* 2001; James *et al.,* 2005, 2007; Maroz *et al.,* 2009). Our results suggest that $MitoQ_{10}$ may act similarly in sympathetic neurons, as oxidation of the major IMM phospholipid, cardiolipin, dissolves its association with cytochrome *c,* and may be necessary to release mitochondrial cytochrome *c* into the cytosol of NGF-deprived sympathetic neurons (Kirkland *et al.,* 2002a; Iverson and Orrenius, 2004). A combination of increased ROS and cytochrome *c* release is essential for apoptosome formation (Vaughn and Deshmukh, 2008), which may explain the ability of $MitoQ_{10}$ to block downstream induction of caspase 3. Since caspase inhibition delays

death, partial loss of $\Delta\psi_m$, and mitochondrial $O_2^{\cdot-}$ production in this paradigm (Deshmukh *et al.,* 2000; Werth *et al.,* 2000; Kirkland *et al.,* 2007, 2010), $MitoQ_{10}$ may have indirectly prevented mitochondrial dysfunction by blocking the consequences of active caspase 3 feeding back on mitochondria substrates, such as respiratory complex 1 (Ricci *et al.,* 2004).

The divergent effects of $MitoQ_{10}$ and $MitoE_2$ on mitochondrial ROS and neuronal death induced by NGF deprivation offer other important implications. First, selective accumulation within the IMM and effective recycling of radical scavengers appear critical determinants of long-term antioxidant activity and anti-apoptotic efficacy in compromised biological systems. Both $MitoQ_{10}$ and $MitoE_2$ are lipophilic, radical scavengers that are targeted to the mitochondria, however the active antioxidant moiety of $MitoQ_{10}$ is recycled after it detoxifies reactive species, whereas there is no available evidence of this for $MitoE_2$ (Kelso *et al.,* 2001; James *et al.,* 2007). Thus, the ability of $MitoQ_{10}$ to be continuously regenerated by respiratory complex II may have sustained the protective effect of $MitoQ_{10}$ in both pathways to neuronal death. Although oxidative phosphorylation does not produce significant ATP in the deprived neurons, electron transport does maintain $\Delta\psi_m$ throughout NGF deprivation (Chang *et al.,* 2003), and therefore would be available to regenerate $MitoQ_{10}$ until the point of terminal $\Delta\psi_m$ loss. The divergence in efficacy between these compounds also reflects structural differences that allow improved accumulation and penetration of $MitoQ_{10}$ in the IMM (Kelso *et al.,* 2001; Asin-Cayuela *et al.,* 2004; James *et al.,* 2007). Increasing the length of the alkyl chain that connects these antioxidant moieties to their targeting sequence (TPP^+) significantly enhances uptake into the mitochondria, membrane binding, and the extent to which the antioxidant penetrates the IMM (Ono *et al.,* 1994; Asin-Cayuela *et al.,* 2004; James *et al.,* 2007; Ross *et al.,* 2008). $MitoQ_{10}$ has a much longer alkyl chain than $MitoE_2$ (Fig. 1), allowing $MitoQ_{10}$ to accumulate to a greater degree

within the IMM. Second, previous studies have shown that anti-apoptotic agents, such as GSH and NGF, restore the cytosolic redox balance (CM-H_2DCFDA levels) and not that of the mitochondria (MitoSOX levels) when added to NGF-deprived sympathetic neurons (Kirkland *et al.,* 2007); however, the similar antioxidant character produced by MitoE_2 in this study was not sufficient to prevent the same apoptotic events. Therefore, our results may signify the importance of metabolic pathways that are regulated by GSH and NGF (Hall, 1999; Vaughn and Deshmukh, 2008; Franco and Cidlowski, 2009; Wallace *et al.,* 2010), but not by vitamin E, that may be necessary to prevent death during stressful events, such as loss of trophic support (Yan *et al.,* 1995). Conversely, MitoQ_{10} acts solely as an antioxidant (James *et al.,* 2005), yet it was sufficient to restore cytosolic redox balance in NGF-deprived neurons to the level of those metabolically supported with media containing NGF. Taken together, these results suggest that the mitochondrial redox balance is an important determinant of the cytosolic redox state (Franco and Cidlowski, 2009), but the reverse may not be true when the bioenergetic balance is challenged by apoptotic stimuli.

Sympathetic neurons normally maintain a highly reduced intracellular environment by NGF-stimulation of the pentose phosphate pathway, which generates the reducing potential (NADPH) necessary for effective recycling of GSH (Ho *et al.,* 2007). In these neurons, this environment controls cytochrome *c*-mediated apoptosis by ensuring that the release of cytochrome *c* alone will not lead to death (Wright *et al.,* 2006; Vaughn and Deshmukh, 2008). Evidence to support the redox control over the apoptogenic activity of cytochrome *c* is found in the selective ability of oxidized cytochrome *c* to induce apoptosome formation, caspase 3 activation and death in cultures of NGF-deprived sympathetic neurons (Vaughn and Deshmukh, 2008). Our results support a functional role of the mitochondrial redox state in regulating this

check-point (Lluis *et al.,* 2007), as $MitoQ_{10}$ addition to NGF-deprived cultures promptly decreased mitochondrial free radicals and derailed this check-point (Commitment 1; Fig. 6), while $MitoE_2$ treatment appeared to only affect general, cytosolic ROS levels and did not prevent progression of this commitment to death (Chang *et al.,* 2002; Kirkland *et al.,* 2002).

As highlighted in Fig. 6, the role of mitochondrial free radicals in the commitment to caspase-dependent death of NGF-deprived sympathetic neurons is becoming more apparent; however, insights into the mechanisms leading to the collapse of $\Delta\psi_m$ that dictates caspase-independent death (Commitment 2) have been largely unrealized (Deshmukh *et al.,* 2000). The available evidence suggests that mitochondrial events that occur downstream of Bax and upstream of caspase activation are required for caspase-independent death (Chang *et al.,* 2003). Perhaps one of the most influential actions of Bax in cultured sympathetic neurons is the induction of mitochondrial $O_2^{\cdot -}$ (Kirkland *et al.,* 2002, 2007, 2010; Vaughn and Deshmukh, 2008). The ability of $MitoQ_{10}$ to prevent Bax-dependent events and extend Commitment 2 in these neurons may indicate that mitochondrial $O_2^{\cdot -}$ mediates Bax-dependent events in caspase-independent death as well. In many cell types, the ultimate loss of $\Delta\psi_m$ occurs by activation of the mitochondrial permeability transition pore (mPTP) due to increased levels of mitochondrial ROS or calcium (Ca^{2+}) and other unknown events (Kroemer *et al.,* 2007; Papa *et al.,* 2007; Juhaszova *et al.,* 2008), but the effect of mPTP inhibition on caspase-independent $\Delta\psi_m$ loss in mouse sympathetic neurons appears inconsequential (Chang and Johnson, 2002). Interestingly, in another model of caspase-independent neuronal death in which mPTP appears minimally involved, antioxidants (including vitamin E) that did not affect $O_2^{\cdot -}$ -associated dihydroethidium (DHE) intensity, also did not prevent loss of $\Delta\psi_m$, or death of cultured cortical neurons, whereas more effective free radical scavengers capable of decreasing DHE oxidation also decreased

caspase-independent death (Lang-Rollin *et al.*, 2003). While such results and ours may suggest the importance of the particular radicals scavenged by $MitoQ_{10}$ and not $MitoE_2$, at present this difference is not possible to definitively decipher with fluorescent probes (Murphy, 2009; Zielonka and Kalyanaraman, 2010). Taken together, our results at least establish a role for mitochondrial ROS generation in orchestrating the ultimate loss of $\Delta\psi_m$, independently of the known effects on caspase activation and mPTP formation (Juhaszova *et al.*, 2008). Mitochondrial ROS may lead to loss of $\Delta\psi_m$ by directly damaging the respiratory complexes (I, III, and IV) that are responsible for generating $\Delta\psi_m$, or by initiating lipid peroxidation events that ultimately dismantle the IMM and drive the cell to death (Ricci *et al.*, 2004; Kroemer *et al.*, 2007; Kondo *et al.*, 2010).

In summary, our results indicate that the ability of $MitoQ_{10}$ to suppress free radicals within the mitochondria was both necessary and sufficient to protect the functional integrity of the IMM in caspase-dependent and caspase-independent pathways to death in a model of age-dependent neurodegeneration. This finding suggests that mitochondria-ROS production may orchestrate the execution of cell death apart from the influence on caspase activity. As such, mitochondria-targeted therapeutics may enhance treatment options for neurodegenerative conditions that involve multiple modes of death (Friedlander, 2003; Stefanis, 2005; Spires-Jones *et al.*, 2009).

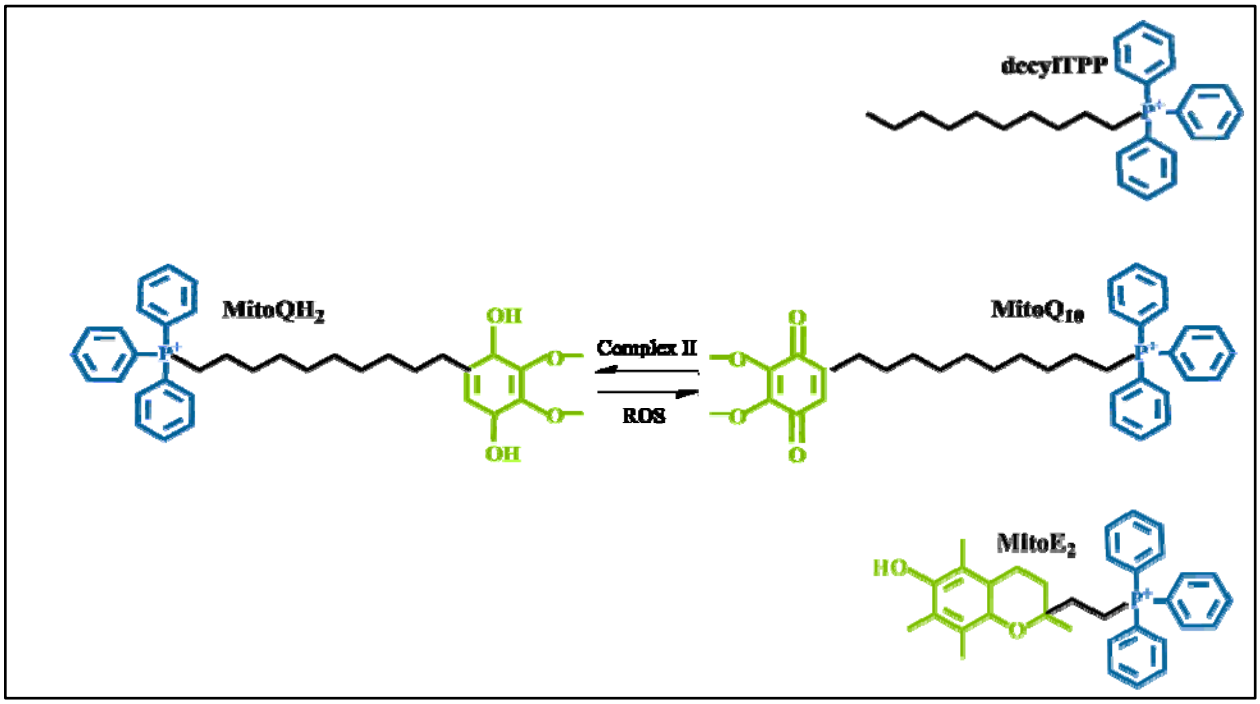


Figure 3.1. Mitochondria-targeted compounds. These compounds pass directly through lipid membranes to accumulate within the mitochondria driven by the attraction of the lipophilic triphenylphosphonium cation (TPP^+) to the negatively charged matrix. DecylTPP (dTPP) is similar to $MitoQ_{10}$ (Asin-Cayuela *et al.*, 2004), but lacks the antioxidant moiety; ubiquinone is attached to the ten-carbon chain to produce $MitoQ_{10}$ (James *et al.*, 2007), while a two-carbon bridge connects the α-tocopherol moiety of vitamin E to TPP^+ in $MitoE_2$ (Smith *et al.*, 1999). In these compounds, the length of the hydrophobic alkyl bridge determines the extent of accumulation and penetration in the mitochondrial inner membrane (Asin-Cayuela *et al.*, 2004; Porteous *et al.*, 2010), and therefore influences their potential to suppress ROS derived from the mitochondrial electron transport chain. Within the mitochondria, $MitoQ_{10}$ is continuously reduced to the ubiquinol form ($MitoQH_2$) by respiratory complex II after detoxifying a ROS.

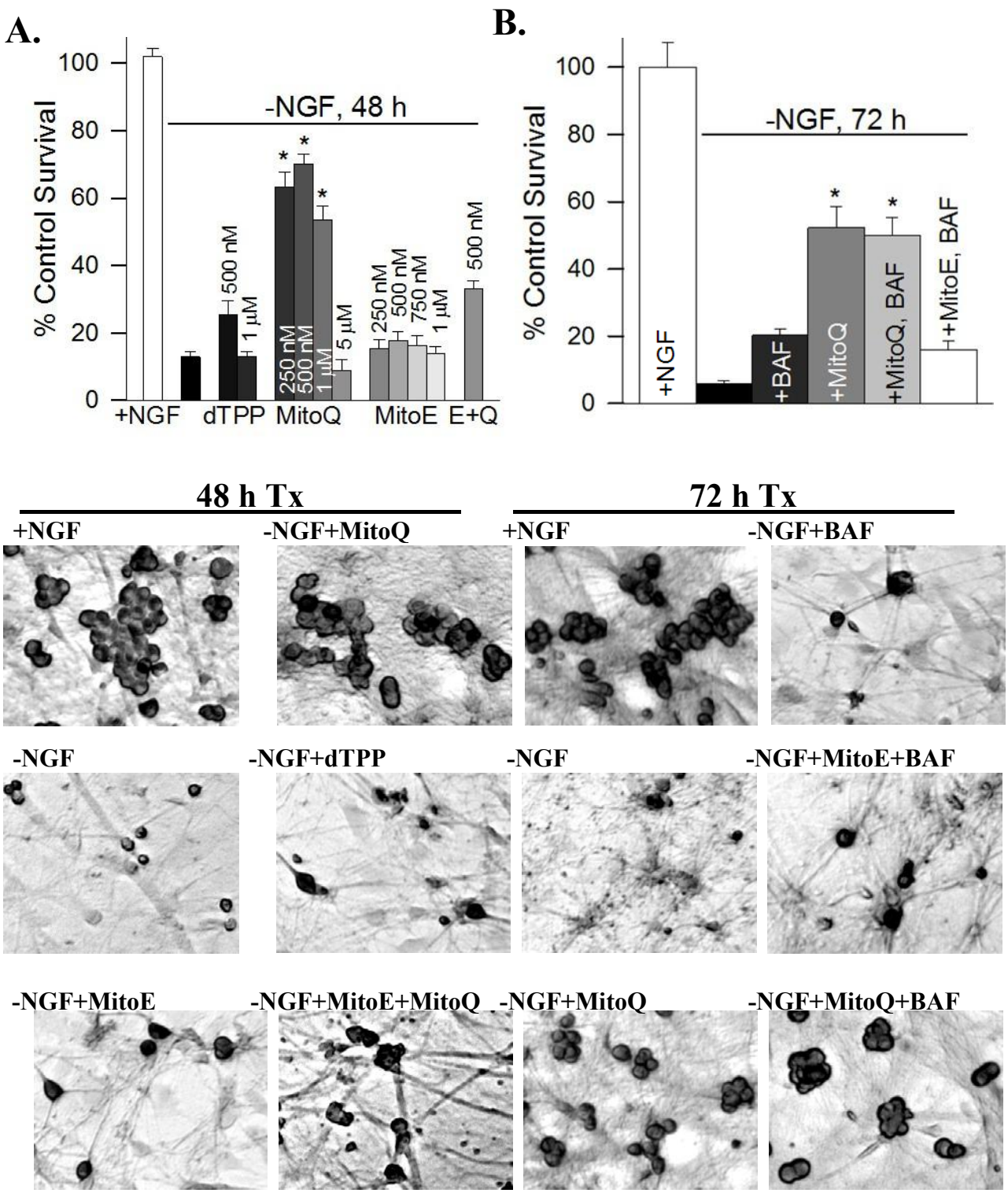
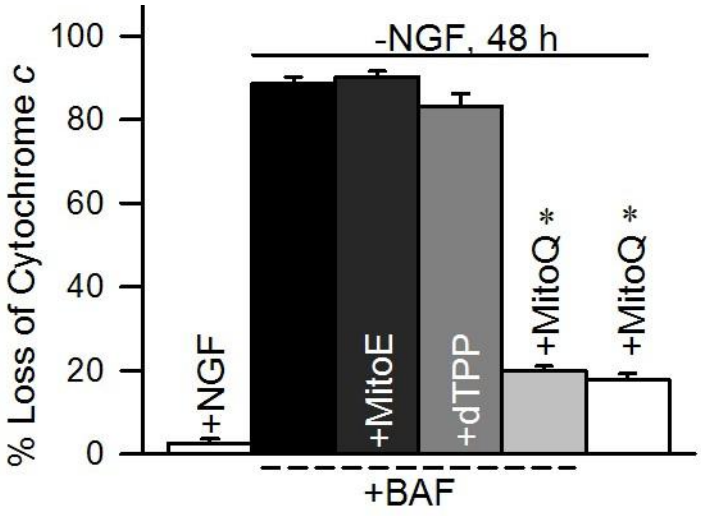


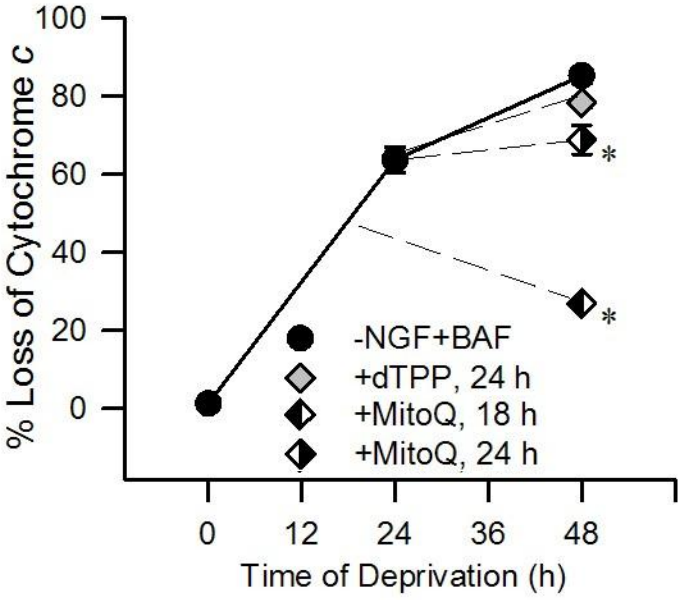
Figure 3.2. MitoQ$_{10}$ prevented caspase-dependent and independent death induced by NGF deprivation of sympathetic neurons in culture. ***A,*** To determine the effects of the mitochondria-targeted agents on apoptosis in sympathetic neurons, cultures were

deprived of NGF, alone or in the presence of the indicated concentrations (ranging from 0.25-5μM) of dTPP, $MitoQ_{10}$, $MitoE_2$, or $MitoE_2$+$MitoQ_{10}$ (E+Q; 250 nM of each). Neurons that were not committed to die by caspase-dependent death (Commitment 1) were rescued by NGF replacement at 48 h; five days later the cells were fixed, counted, and quantified as the percent of viable neurons in sister cultures that were maintained in NGF from the time of plating. Only $MitoQ_{10}$ treatment (0.25-1 μM) prevented caspase- dependent death of NGF-deprived sympathetic neurons (*$p < 0.001$). ***B,*** To determine the effect of $MitoQ_{10}$ and $MitoE_2$ (500 nM) in caspase-independent death, cultures were deprived of NGF alone, in the presence of the pan-caspase inhibitor BAF (50 μM), or with $MitoQ_{10}$ or $MitoE_2$ plus BAF from the time of deprivation. These cultures were rescued with NGF 72 h later, and quantified as described above. Cultures treated with BAF continued to die after 72 h, despite NGF readdition, indicating that the neurons were committed to die by caspase-independent death (Commitment 2). Cultures receiving $MitoQ_{10}$ alone and $MitoQ_{10}$ plus BAF treatment escaped impending death to a similar degree ($p = 0.76$), indicating that $MitoQ_{10}$ treatment was neuroprotective against both caspase-dependent and -independent death after 72 h of deprivation (*$p < 0.001$ for $MitoQ_{10}$ and $MitoQ_{10}$+BAF versus –NGF+BAF). $MitoE_2$ had no effect on caspase- independent death. Results are presented as ±SEM from at least four independent experiments (n = 12-34 cultures). Statistical analysis was performed by ANOVA, followed by the Holm-Sidak p*ost hoc* test for multiple comparisons between groups. ***C,*** Representative micrographs of cultures treated as indicated, rescued with NGF after 48 or 72 h and fixed. Easily distinguishable cellular outlines of plump, crystal violet-stained neurons highlight viable neurons over the dead cells that show little or no staining.


-NGF, 48 h
% Loss of Cytochrome c
100
80
60
40
20
0
+NGF
+MitoE
+dTPP
+MitoQ *
+MitoQ *
+BAF

A.

B.


% Loss of Cytochrome c
100
80
60
40
20
0
-NGF+BAF
+dTPP, 24 h
+MitoQ, 18 h
+MitoQ, 24 h
*
*
0
12
24
36
48
Time of Deprivation (h)

C.

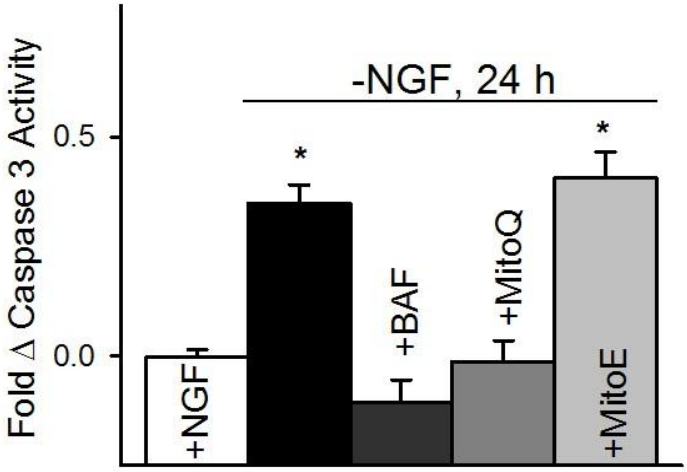

-NGF, 24 h
Fold Δ Caspase 3 Activity
0.5
0.0
*
*
+NGF
+BAF
+MitoQ
+MitoE

Figure 3.3. MitoQ$_{10}$ prevented release of cytochrome *c* from mitochondria and caspase 3 activation during apoptosis induced by NGF deprivation. ***A,*** Sympathetic neurons were deprived of NGF in the presence of MitoQ$_{10}$, or the pan-caspase inhibitor BAF (50 μM) alone, or with MitoE$_2$, dTPP, or MitoQ$_{10}$. The concentration of all mito-compounds was 500 nM in each of the experiments below. The cultures were fixed after 48 h of treatment and processed for cytochrome *c* immunocytochemistry. Neurons that lost mitochondrial cytochrome *c* staining were counted and are shown as the percent of total neurons per treatment. Only MitoQ$_{10}$ treatment prevented cytochrome *c* redistribution (*$p < 0.001$, for –NGF+MitoQ$_{10}$+BAF and -NGF+MitoQ$_{10}$ versus -NGF+BAF). ***B,*** To determine if MitoQ$_{10}$ could prevent release of mitochondrial cytochrome *c* when added at later time points, sympathetic neurons were deprived of NGF and maintained in BAF; dTPP or MitoQ$_{10}$ was added to parallel cultures 18 and 24 h from the time of deprivation, fixed and quantified after 48 h total deprivation. -NGF+BAF neurons were also fixed at 24 h after deprivation to quantify the effect of dTPP and MitoQ$_{10}$ addition. dTPP failed to attenuate cytochrome *c* release ($p > 0.05$ for +dTPP and –NGF+BAF at 24 h). MitoQ$_{10}$ prevented further release of cytochrome *c* when added at 18 h or 24 h (*$p < 0.001$ for cytochrome *c* retention in -NGF+BAF 24 h and –NGF+MitoQ$_{10}$ cultures at 48 h) ($n \tilde{} 500$ neurons). ***C,*** Caspase 3 activity was measured with the Caspase Glo assay. Sympathetic neurons were deprived of NGF for 24 h alone, or with BAF (50 μM), MitoQ$_{10}$, or MitoE$_2$. Caspase activity was normalized to NGF-maintained, sister cultures of equal density. NGF deprivation alone significantly increased caspase 3 activity. MitoQ$_{10}$ and BAF prevented this increase, while MitoE$_2$ had no effect (*$p < 0.001$ for –NGF and MitoE$_2$ versus +NGF). Data are shown as ± S.E.M of four independent experiments (n = 12-20 cultures).

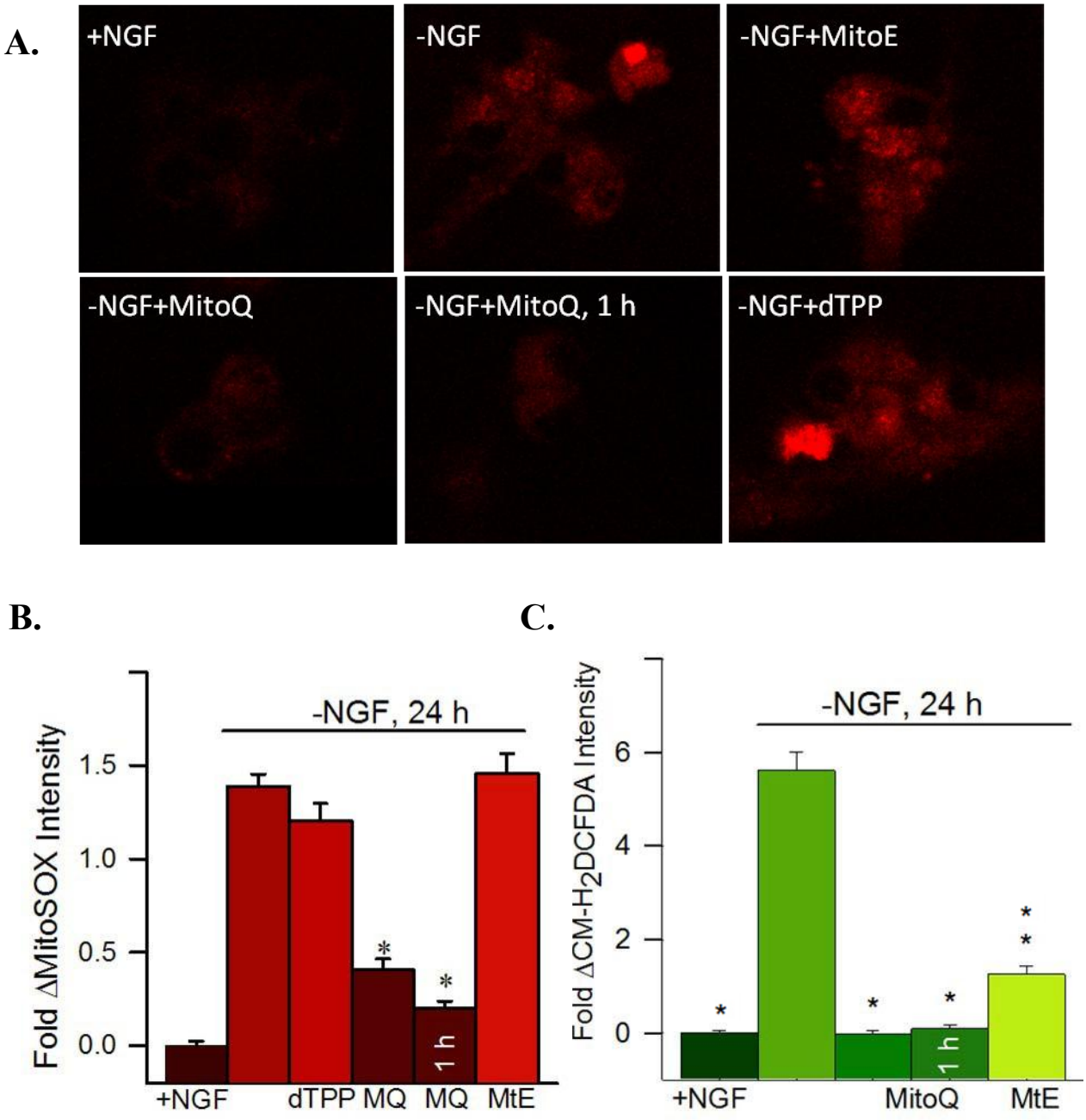


Figure 3.4. Effect of mito-antioxidants on ROS levels in NGF-deprived sympathetic neurons. ***A,*** Representative micrographs comparing MitoSOX levels in NGF-maintained sympathetic neurons to those deprived of NGF for 24 h alone or with the indicated treatments. The cultures were exposed to MitoSOX (2 μM) for 10 min, washed, and

immediately analyzed by confocal microscopy. MitoSOX staining is cytoplasmic and punctate, consistent with its demonstrated localization to the mitochondria (Kirkland *et al.*,2007; 2010) ***B,*** Mitochondrial $O^{\cdot -}_2$ levels were estimated by the fluorescent intensity of MitoSOX in neurons treated with dTPP, $MitoQ_{10}$, or $MitoE_2$ from the time of NGF-withdrawal (24 h), or with $MitoQ_{10}$ for less than 1 h prior to analysis. MitoSOX dye intensities are normalized to the intensity average of sibling cultures maintained in NGF from the time of plating. Both chronic (24 h) and acute (<1 h) $MitoQ_{10}$ treatments prevented the increase in $O_2^{\cdot -}$ sensitive MitoSOX intensity ($p < 0.001$ versus -NGF). Conversely, neither dTPP nor $MitoE_2$ attenuated mitochondrial $O^{\cdot -}_2$ detected by MitoSOX (n = 91-331). ***C,*** The effect of $MitoQ_{10}$ and $MitoE_2$ on cellular ROS levels was measured by the dye intensity of CM-H_2DCFDA (10 μM for 20 min) and quantified similarly to the method described above**.** Both $MitoQ_{10}$ (24 h and <1 h) and $MitoE_2$ significantly prevented the increased CM-H_2DCFDA intensity in NGF-deprived neurons ($p < 0.001$ for MQ and MtE versus -NGF), but only $MitoQ_{10}$ decreased CM-H_2DCFDA to the level of NGF-maintained neurons ($p > 0.05$ for –NGF+$MitoQ_{10}$ versus +NGF) (n = 166-254). Stars indicate statistical difference from neurons deprived of NGF for 24 h ($p < 0.001$).

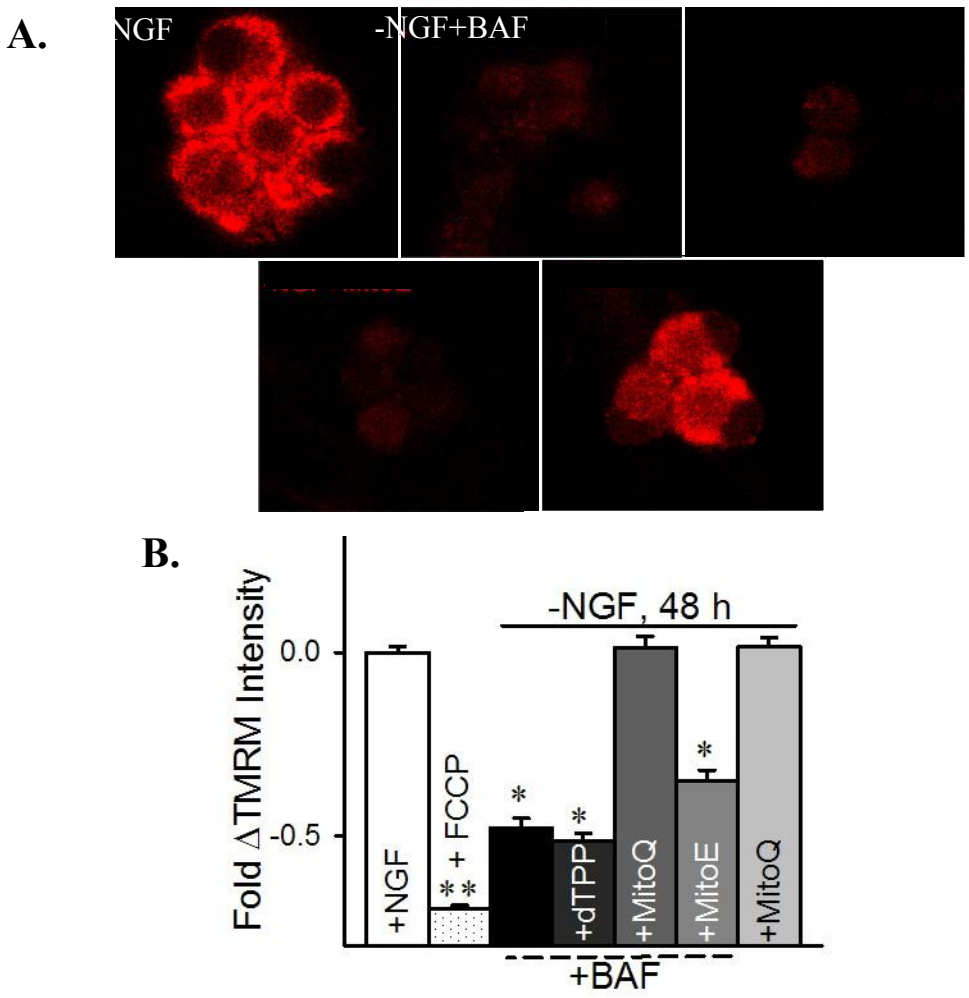


Figure 3.5. MitoQ$_{10}$ prevented loss of $\Delta\psi_m$ in NGF-deprived sympathetic neurons. Sympathetic neurons were deprived of NGF in the presence of BAF alone, or with 500 nM of MitoE$_2$, MitoQ$_{10}$, or dTPP. The effects of mitochondria-targeted compounds on $\Delta\psi_m$ were measured by the fluorescent intensity produced by the membrane potential– dependent dye TMRM$^+$. The average TMRM$^+$ intensity of NGF-deprived, BAF-maintained neurons within each treatment group was normalized to that of sister cultures maintained in NGF from the time of plating. TMRM$^+$ fluorescence was $\Delta\psi_m$-specific, as 1 h treatment of NGF-maintained neurons with the uncoupling agent FCCP (1.6 μM) completely abolished TMRM$^+$ staining (**$p < 0.001$, for FCCP versus +NGF and -NGF). All NGF-deprived, BAF-treated cultures

exhibited significant loss of $\Delta\psi_m$ by 48 h ($p < 0.001$ versus +NGF), except those also treated with $MitoQ_{10}$ ($p > 0.05$ for $MitoQ_{10}$+BAF versus +NGF). $MitoQ_{10}$ treatment prevented $\Delta\psi_m$ in both caspase-dependent (without BAF) and caspase-independent conditions ($p < 0.001$ for $MitoQ_{10}$ and $MitoQ_{10}$+BAF versus -NGF+BAF) (n = 42-212).

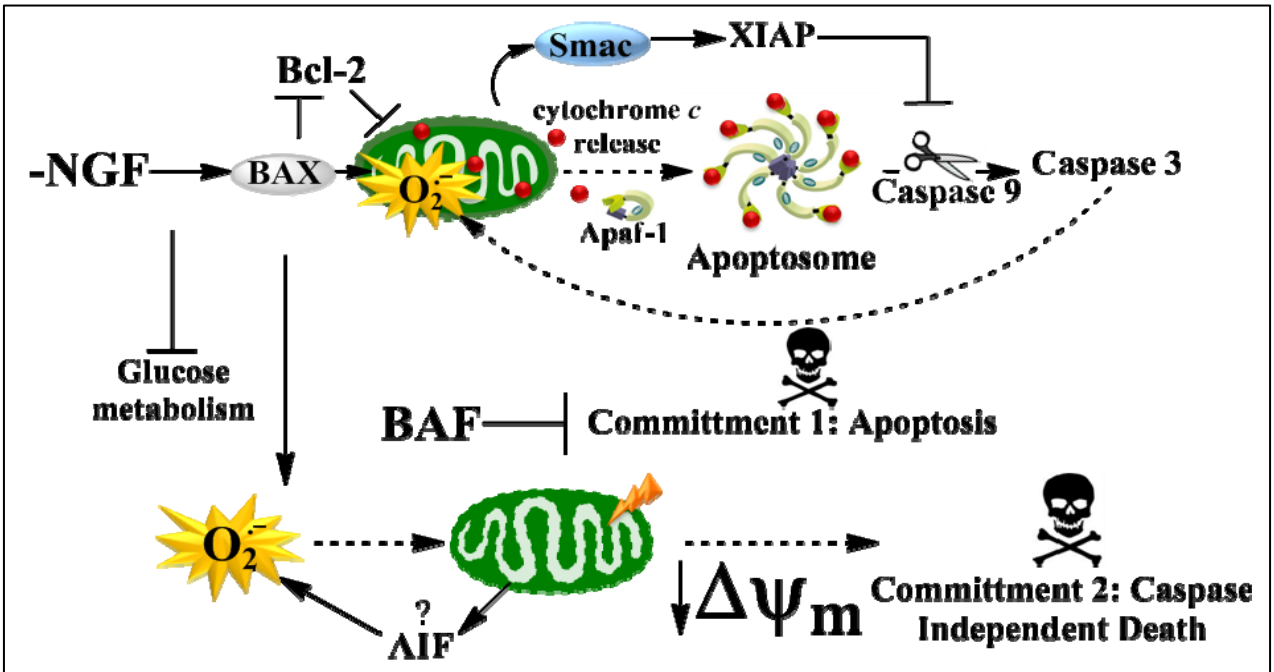


Figure 3.6. Role of Mitochondrial Reactive Oxygen Species in the Commitment to Neuronal Death: Implications from mitochondria-targeted antioxidants. In the absence of NGF, the neurons shift to a pro-oxidant state as metabolism slows and mitochondria produce more ROS, thus compromising endogenous antioxidant defenses. Pro-apoptotic Bcl-2 proteins, such as Bax or Bid, may directly permeabilize the OMM, mobilizing proteins from the intermembrane space, such as the sequestered second mitochondria-derived activator of caspases (Smac) and cytochrome *c*. Released Smac may bind the X-linked inhibitor of apoptosis proteins (XIAPs), removing the block and allowing caspase activation. A potentially moderate level of caspase-3 activation may then provoke mitochondrial $O_2^{\cdot-}$ production as caspases now have access to the electron transport chain due to OMM compromise. The induction of mitochondrial ROS supports further cytochrome *c* release by oxidizing cardiolipin. Mitochondrial ROS convert respiratory cytochrome *c* into a molecular villain via formation of the apoptosome and the inevitable onset of apoptotic death (Commitment 1). Neurodegenerative stimuli also evoke caspase-independent neuronal death, which can be followed in this system by inhibiting caspases with BAF. In BAF-treated neurons, Bax-dependent events lead to mitochondrial $O_2^{\cdot-}$ accumulation by currently unknown mechanisms that require further investigation, but likely involve metabolic decline and possibly apoptosis inducing factor (AIF)(Kondo *et al.*, 2010). The mitochondrial ROS that escape detoxification may then damage metabolic enzymes and lipids of the IMM, both of which become deleterious for sympathetic neuron vitality as mitochondrial dysfunction ensues with the collapse of $\Delta\psi_m$ (Commitment 2). Dashed arrows represent pathways that were blocked by MitoQ treatment in NGF-deprived sympathetic neurons.

CHAPTER 4

THE MITOCHONDRIA-TARGETED ANTIOXIDANT, MITOQ, PREVENTS LOSS OF SPATIAL MEMORY RETENTION AND EARLY NEUROPATHOLOGY IN A MOUSE MODEL OF ALZHEIMER'S DISEASE[3]

[3]McManus, M.J., M.P. Murphy, and J.L. Franklin.
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Abstract

Considerable evidence suggests that mitochondrial dysfunction and oxidative stress contribute to the progression of Alzheimer's disease (AD). We examined the ability of the novel mitochondria-targeted antioxidant, MitoQ to prevent AD-associated pathology in cultures of cortical neurons and a triple transgenic Alzheimer's mouse model (3xTg- AD). MitoQ attenuated beta-amyloid (Aβ)-induced neurotoxicity in these neurons and also prevented increased production of reactive species (RS) and loss of mitochondrial membrane potential ($\Delta\psi_m$). To determine whether the mitochondrial protection conferred by MitoQ was sufficient to prevent the emergence of AD-associated neuropathology *in vivo*, we treated young female 3xTg-AD mice with MitoQ for 5 months and analyzed the effect on the progression of AD-like pathologies. Our results show that MitoQ prevented the onset of cognitive decline, oxidative stress, Aβ-accumulation, and activation of apoptotic machinery in these mice. The work presented herein suggests a central role for mitochondria in neurodegeneration and provides evidence supporting the use of mitochondria-targeted therapeutics in diseases involving oxidative stress and metabolic failure, namely Alzheimer's disease.

Introduction

Alzheimer's disease is the most prevalent of the neurodegenerative diseases and the leading cause of dementia among the elderly. Current therapies are palliative and do not delay the progression of this devastating illness. Extensive evidence indicates that alterations in glucose metabolism and mitochondrial energetics occur along with the accumulation of oxidative damage prior to the cardinal signs of AD pathogenesis in the brains of transgenic animal models and patients (Gibson *et al.,* 1998; Hirai *et al.,* 2001; Rinaldi *et al.,* 2003b; Lin and Beal, 2006b; Sultana *et al.,* 2006; Yao *et al.,* 2009). Such findings have inspired clinical trials of various antioxidant compounds for treating AD. To date, none of the tested compounds have proven to be effective (Sano and Growdon, 1997; Petersen *et al.,* 2005; DeKosky *et al.,* 2008). One possible explanation for these failures is that the compounds tested were not efficient at detoxifying relevant RS, perhaps because they did not achieve sufficient concentrations at the site of most RS production, the mitochondria. This potential problem may be overcome by antioxidants that traverse the blood brain barrier (BBB) and selectively concentrate in mitochondria (Shigenaga *et al.,* 1994). The recently developed, mitochondria-targeted antioxidant, MitoQ possesses these qualities (Bruckner *et al.,* 2001; Rodriguez-Cuenca *et al.,* 2010).

MitoQ is produced by covalently binding the active moiety of ubiquinone, an endogenous antioxidant and component of the mitochondrial electron transport chain, to a triphenylphosphonium cation (TPP^+) by a ten-carbon linking group. MitoQ crosses the BBB and neuronal membranes to concentrate several hundred-fold in mitochondria driven by the high membrane potential across the inner mitochondrial membrane (IMM; (Bruckner *et al.,* 2001; Rodriguez-Cuenca *et al.,* 2010). The ubiquinone moiety is delivered to the matrix side of

the IMM, placing it at the site of most cellular RS generation (Murphy, 2009). The TPP^+ moiety adsorbs to the matrix side of the IMM and the ubiquinone penetrates into the membrane where it is reduced to the active antioxidant ubiquinol by respiratory complex II. The ubiquinol acts as an antioxidant by preventing lipid peroxidation or reducing other RS, and in doing so is oxidized to ubiquinone. Complex II then reduces ubiquinone back to ubiquinol. MitoQ is a poor substrate for complex I and is negligibly oxidized by complex III. Therefore, it cannot substitute for endogenous ubiquinone in the electron transport chain, but primarily acts as an antioxidant capable of continuous regeneration by complex II (James *et al.,* 2005; James *et al.,* 2007; Rodriguez-Cuenca *et al.,* 2010).

To evaluate the therapeutic potential of MitoQ for treating AD and to examine a role for mitochondria-generated RS in the progression of AD, we investigated the effect of MitoQ on Aβ toxicity in primary cortical neurons and on cognitive deficits and neuropathology in a young triple transgenic (3xTg-AD) mouse model of AD. We found that MitoQ treatment prevented Aβ-induced oxidative stress and apoptosis in cortical neurons in culture and the early cognitive decline, oxidative stress, Aβ accumulation, and caspase activation in the brains of 3xTg-AD mice.

Materials and Methods

Reagents. Decyltriphenylphosphonium bromide (dTPP) and MitoQ as MitoQuinone mesylate: [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cycloheexadienl-yl) decyl triphenylphosphonium methanesulfonate], complexed with β-cyclodextrin (MS-010) was provided by Antipodean Pharmaceuticals, Inc. (*Auckland, New Zealand CA*). 5-(and –6)-chloromethyl-2`, 7`-dicholorodihydrofluorescein diacetate (CM-H_2DCFDA) and tetramethyl rhodamine methyl ester ($TMRM^+$) were purchased from

Invitrogen/Molecular Probes (Eugene, OR). Aβ (22-35 and 1-40) peptides were purchased from Bachem (Torrance, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Mice. The 3xTg-AD mouse model used in this study expresses three mutant human genes (amyloid precursor protein, APP_{swe}; presenilin-1, $PS1_{M146V}$; and four-repeat tau, tau_{P301L}; Oddo *et al.,* 2003). The first two are associated with early onset forms of human AD and the latter with human frontotemporal dementia. These mice and nontransgenic (NonTg) mice from the same 129/C57BL6 hybrid background strain were kindly provided by Dr. Frank LaFerla (University of California, Irvine). The mice were group-housed and kept on a 12 h light:12 h dark schedule. All mice were given *ad libitum* access to food and water. Starting at 2 months of age, female 3xTg-AD or NonTg mice were given MitoQ (100 μM) or the control for MitoQ, decyl triphenylphosphonium bromide (dTPP; 100 μM) continuously in their drinking water for 5 months. Other age-matched 3xTg-AD and NonTg mice received water without MitoQ or dTPP.

Cognitive impairment in 3xTg-AD mice emerges as a long-term retention deficit at 4-6 months of age and further develops in an age-dependent manner. There is a transient sex-divergence in 3xTg-AD pathology, such that young- to middle-aged (6-12 month) female 3xTg-AD mice exhibit enhanced cognitive deficits compared to age- matched male 3xTg-AD (Clinton *et al.,* 2007). Therefore, only female mice were used in this study. Each mouse was handled every day for at least a week prior to behavioral testing and given a general health evaluation. A total of 117 mice were used in these experiments. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture. Cortical neurons were isolated from the cerebral cortex of neonatal C57B1/6 mice. After removal of the meninges, the cortices were digested in 2 mg/ml trypsin in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS) for 20 min at 35° C. The solution was diluted with an equal volume of cold HBSS containing 50 μg/ml DNAse and centrifuged at 200*g* for 5 min at 4° C. The supernatant was removed and the tissue washed 2X with cold HBSS. The cortical tissue was then dissociated by trituration in growth media (Neurobasal-A medium containing 2% B27 supplement, 1% Penicillin/Streptomycin, 0.1% L-glutamine) and distributed to poly-L lysine-coated #1 glass coverslips or 24-well plastic Costar tissue culture plates (Corning, Corning, NY). The cells were seeded in 100 μl of growth medium for a minimum of 2 h in a 5% CO_2 atmosphere (35° C) in a humidified cell culture incubator. The medium was then aspirated and replaced. Coverslips were maintained in growth medium in 35 mM plastic cell culture dishes (Costar). Plating density was 3.5 X 10^5 cells/ml of medium. Medium was replaced at 3-4 days *in vitro* and experimental treatments began on day 7. Cortical neuron survival was determined by blinded counting of phase-bright/morphologically intact cells in 10-randomly selected fields of view per treatment and was normalized to the average percent survival of non-treated, sibling cultures.

Microscopy. Microscopy experiments were conducted with a laser scanning confocalmicroscope (Nikon C1; Southern Micro Instruments, Marietta, GA) attached to a Nikon Eclipse TE300 inverted microscope (Melville, NY). Cells were observed with a 60X- plan oil-immersion objective (N.A. 1.4) and scanned at 512 X 512 or 2048 X 2048 pixel resolution. The confocal microscope was controlled by a Dell computer running EZC1 software (Nikon). Confocal pinhole and gain were maintained at constant levels during each experiment. Laser power was

10% of maximum. Phase contrast images were taken by a cooled Princeton Micro-Max CCD camera (Princeton Instruments, Trenton, NJ) mounted on the Nikon TE-300 microscope and controlled by MetaMorph software (Molecular Devices, Sunnyvale, CA).

RS were detected using the redox-sensitive dye CM-H_2DCFDA. This dye is membrane permeant and is trapped in cells by binding of the chloromethyl group to cellular thiols. The reduced form of CM-H_2DCFDA becomes intensely fluorescent upon oxidation by multiple RS, thus providing an assay of generalized RS production(Royall and Ischiropoulos, 1993). We extensively characterized the use of this dye in rat and mouse neurons (Kirkland and Franklin, 2001; Kirkland *et al*., 2002). This characterization shows that CM-H_2DCFDA trapped in neurons is insensitive to pH changes within the physiological range and is not photo-oxidized at the laser power used in these experiments. Cultures were incubated in the appropriate experimental medium containing CM-H_2DCFDA (10 μM) for 10 min at 35°C. They were then washed twice with Leibovitz's L-15 medium and left in the last wash for confocal microscopy. CM- H_2DCFDA was excited with the 488 nm line of the confocal laser. The green photomultiplier channel of the microscope was used for image acquisition. The cationic fluorescent probe, $TMRM^+$, accumulates in mitochondria because of the high membrane potential across the IMM and, therefore, provides a semi-quantitative estimate of $\Delta\psi_m$. Neurons were incubated with low concentrations (20 nM) of $TMRM^+$ in experimental medium for 30 min, rinsed once with L-15, and viewed in L-15 containing 20 nM $TMRM^+$. At equilibrium, the fluorescence produced by this low concentration of $TMRM^+$ (excitation 543/emission red photomultiplier channel) is a direct function of $\Delta\psi_m$, and complications due to self-quenching of the dye are eliminated (Nicholls and Budd, 2000; Nicholls and Ward, 2000; Nicholls and Ferguson, 2002).

Dye intensities in images were quantified by measuring the raw pixel intensities in neuronal somas with the region tool of MetaMorph software. The area quantified covered 60 μm^2 of the somas. The dye intensity measured in each neuron was normalized to the average dye intensity of control NonTg neurons receiving the same concentration of dye for the same period as the experimental cells. Normalized data are shown as fold change from the average intensity of the dye measured in control cultures (sibling cultures of cortical neurons without treatment).

Spatial learning and memory retention. After 4.5 months of experimental treatments, spatial learning and memory retention were determined by the Morris Water Maze (MWM; Morris, 1984) behavioral test. The apparatus used for the MWM tasks consisted of a circular aluminum tank (1.22 m) painted white and filled with water maintained at 24
± 1° C. Non-toxic, white tempura paint was used to conceal the slightly submerged, circular plexiglass platform (14 cm diameter). MWM tests were conducted according to Billings *et al*. (2005) with minor modifications. Mice were placed on the platform for 10s prior to the first training trial to reduce stress and associate them with the presence of the escape platform. During the acquisition trails, the mice were placed in the tank at one of four designated start points and allowed 60 s to find and escape onto the platform using at least three distinct, extra-maze cues on the curtains surrounding the tank. If a mouse failed to find the platform within the allotted time, she was manually guided there and the escape latency was recorded as 60 s. In either case, the mouse remained on the platform for 30 s in order to consolidate the spatial cues (Arendash *et al.,* 2006). After this time, the mouse was allowed to rest in a holding cage fitted with a warm towel for 30 s until the start of the next trial. Each mouse was given 4

consecutive trials per day for 7 days (Vorhees and Williams, 2006). Short- and long-term retention of the spatial training was assessed in probe trials (platform removed) conducted 1.5 h and 24 h, respectively, after the last acquisition trial. Spatial bias for the platform location was determined by the number of crosses over the previous platform location during the 60 sec free swim and average distance from the platform.

Cued acquisition trials were conducted after the last probe trial to determine whether differences in learning and memory could be attributed to impaired eyesight, swimming ability, or motivation to escape (Morris, 1984). In the cued trials, extra-maze cues were removed and the platform was made visible by a mounted flag. Multiple parameters in all MWM tasks were calculated and analyzed by Ethovision XT tracking software (Noldus Information Technology, Leesburg, VA).

Tissue Acquisition. At 7 months of age mice were sacrificed by cervical dislocation and intercardially perfused with phosphate buffered saline (PBS, pH 7.4). Brains were rapidly removed and sagitally split. One hemi-brain was fixed in 4% paraformaldehyde for immunohistochemistry. After removal of the brainstem and cerebellum, the other hemi-brain was snap-frozen in liquid nitrogen, and stored at -80°C for use in biochemical analysis.

Immunoblotting. Previously frozen hemi-brains were homogenized in 2% SDS lysis buffer (50 mM Tris, 2 mM EDTA, 150 mM NaCl) supplemented with 100x protease inhibitor cocktail (Sigma) and centrifuged at 4° C for 1 h at 100,000x *g* in a Beckman Coulter Optima TLX-120 ultracentrifuge. Protein concentration of the supernatant was determined by the Bradford assay (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of protein from each sample were

separated by SDS-PAGE and transferred to equilibrated PVDF membranes (Millipore, Bedford, MA). Membranes were blocked for at least 1 h at room temperature with TBST (10 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk, followed by incubation in primary anti-Δtau (1 μg/ml; Invitrogen) or anti-3-nitrotyrosine (1 μg/ml; Cayman Chemical Co, Ann Arbor, MI) diluted in 1% nonfat dry milk overnight at 4°C. The membranes were then washed for 20 min in TBST and then incubated for 1h at room temperature in anti-mouse HRP- linked secondary antibody (1:2000, Bethyl Labortories, Inc., Montgomery, TX) followed by another 20 min wash. The membranes were stripped with Restore Stripping Buffer (Pierce), washed in TBST and processed as described above for *β*-actin or *β*-tubulin (Santa Cruz Biotechnologies, Inc., Santa Cruz, Ca) as a loading control. Proteins were detected using the chemiluminescent SuperSignal substrate (Pierce).

Lipid Peroxidation. Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS). Brain homogenates (0.02 g/ml in 50 mM Tris-HCl) were diluted in 2 volumes of 15% trichloroacetic acid and centrifuged at 1000 X *g* for 10 min at 4°C. The supernate was added to an equal volume of 0.375% TBA in 0.25 M HCl and heated at 100°C for 15 min. To prevent further peroxidation of lipids during the assay, 0.015% 2,6-di-*tert*-butyl-4-methylphenol was added to the mixture before the acid- heating stage. After cooling and centrifugation at 100 X *g* for 5 min, the formation of TBARS was determined by the absorbance of the colorimetric product at 532 nm by a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). The amount of TBARS in the samples was calculated from a standard curve produced by hydrolysis of tetraethoxypropane. Results were normalized to protein concentration of each sample.

Glutathione Assay. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was determined by a luminescent assay (GSH-Glo; Promega, Madison, WI). Brains were Dounce-homogenized (10 mg/ml) in PBS containing 2 mM EDTA, briefly centrifuged, and the supernatant collected. The GSH standards and samples were diluted accordingly in lysis buffer containing luciferin substrate. N-ethylmaleimide (NEM) was included in aliquots of each sample to block GSH and allow quantification of GSSG. The samples were then incubated in buffer containing glutathione S-transferase for 30 min at room temperature. The reaction was stopped with addition of the detection reagent, incubated for 15 min, and the luminescence quantified by the SpetraMax M2 microplate reader.

Immunohistochemistry. Hemi-brains were fixed for 48h in 4% paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and mounted on glass slides. Sections were deparaffinized, rehydrated, and heated in 10 mM sodium citrate, pH 6.0, to ~95°C to assist in antigen retrieval. The sections were incubated in blocking buffer (150 mM NaCl + 100 mM Tris pH 7.4 + 0.1% Triton X-100 + 2% BSA) for 1 h at room temp, followed by anti-Aβ(1-42) antibody (1:500; BioSource Int., Camarillo, CA) overnight at 4° C (Oddo *et al.*, 2003). Aβ (1-42) was visualized by using an ABC immunoperoxidase kit from Vector Laboratories (Burlingame, CA) and diaminobenzidine substrate. Images were taken by a CCD camera mounted on our Nikon TE-300 microscope. Image acquisition was controlled by MetaMorph software.

Amyloid (1-42) Elisa. Soluble A*β* (1-42) was extracted as described by Oddo *et al*. (2003), with modifications necessary for the ELISA kit (Covance, Inc., Princeton, NJ). Previously

frozen hemi-brains were Dounce-homogenized in the appropriate amount (150 mg/ml wet weight) of ice-cold 0.6% SDS lysis buffer (50 mM Tris, 2 mM EDTA, 150 mM NaCl) supplemented with 100X protease inhibitor cocktail (Sigma). The resulting homogenate was sonicated and centrifuged at 4°C for 1 h at 100,000 X *g* in the ultracentrifuge. The supernatant was collected and further analyzed according to the ELISA kit instructions. Briefly, 100 µl of the samples or Aβ-42 peptide standards were added to each well in duplicate or triplicate and incubated overnight at 4° C. The following day, the 96-well plate was thoroughly washed, incubated in tetramethylbenzidine substrate for 40 min at room temp, and the absorbance at 620 nm was recorded by the SpectraMax M2 microplate reader. Quantification of soluble Aβ (1-42) levels in the samples was achieved by normalizing the Aβ (1-42) concentration detected to the total protein in each sample, as determined by the Bradford protein assay (Pierce).

Caspase 3/7 activity assay. Caspase 3/7 activity was measured using the Caspase-Glo 3/7 kit (Promega) according to the manufacturer's instructions with modifications for tissue homogenates (Werth *et al.,* 2000). Briefly, brain extracts were prepared by Dounce homogenization in ice-cold hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA), and centrifuged for 15 min at 13,000 rpm. Protein concentrations of the supernate were determined using the Bradford assay (Pierce) to ensure equal loading. Samples were diluted accordingly in PBS and loaded in duplicate or triplicate into a white-walled, 96-well plate for 1 h incubation in an equal volume of Caspase-Glo reagent at room temperature. The luminescence of each sample, a measure of caspase 3/7 activity, was measured by a SpectraMax M2 microplate reader and normalized to the respective NonTg control. This assay

does not distinguish between the activities of caspases 3 and 7.

Statistics. Statistical analysis and graph preparation were done with Sigmaplot 11.1 (Systat Software, Inc., San Jose, CA). Appropriate statistical measures were determined from analysis of data distribution. Unless otherwise indicated, statistical comparisons were made by Kruskal-Wallis one-way ANOVA on ranks followed by the Dunn's multiple comparisons *post-hoc* test. Error bars are ± SEM.

Results

MitoQ *attenuated Aβ-toxicity in cortical neurons in cell culture*

The amyloid hypothesis posits that A*β* peptides lie upstream of all the pathological events in AD including formation of neurofibrillary tangles (NFT), synaptic dysfunction, and neuronal death (Nicholls and Budd, 2000; Oddo *et al.,* 2003). A*β* peptides are cleaved from a protein known as amyloid precursor protein (APP), a ubiquitously expressed protein of uncertain function (Nicholls and Ferguson, 2002). Cortical neurons in culture can be killed by exposure to these peptides. Considerable evidence suggests that toxicity induced by both endogenous A*β* and extracellularly-applied A*β* is mediated, at least in part, via the intrinsic, mitochondria-dependent apoptotic pathway (Harada and Sugimoto, 1999; Cardoso *et al.,* 2001; Casley *et al.,* 2002b; Caspersen *et al.,* 2005; Hansson Petersen *et al.,* 2008; Cho *et al.,* 2009; Takuma *et al.,* 2009). Manczak *et al*. (2010) recently demonstrated that MitoQ protects mitochondria from A*β*-induced toxicity in N2a cells. They also demonstrated that MitoQ enhances neurite outgrowth in cultured hippocampal neurons from A*β*PP expressing mice but did not present direct evidence of a protective effect of MitoQ on the mitochondria in these cells. In order to determine whether MitoQ can prevent A*β*-toxicity in primary neurons and to explore its effect on mitochondria, we investigated its effects on mouse cortical neurons treated with Aβ (22-35) in cell culture. This amyloid fragment contains the residues essential for formation of A*β* aggregates and exhibits a toxicity profile that closely parallels that of full length A*β* 1-40 and 1-42 in hippocampal and cortical neurons (Walencewicz-Wasserman *et al.,* 1995; Casley *et al.,* 2002b). Aβ (22-35) treatment caused a progressive reduction in cortical neuron survival that declined by 48 h to ~35% that of untreated controls (Figure 1*A*, *B*). Low concentrations of MitoQ (1-100 nM) greatly inhibited this toxicity. dTPP, a compound that is structurally similar

to MitoQ but lacks the ubiquinone moiety was used to control for nonspecific effects of the lipophilic cation. dTPP did not increase survival of Aβ (22-35)-treated neurons. Figure 1*C* shows that 1 nM MitoQ also prevented death of cortical neurons treated with A*β* 1-40.

MitoQ attenuated Aβ-induced RS and mitochondrial damage in cortical neurons in cell culture

A*β*, and its precursor APP, localize, in part, to mitochondria where they can cause mitochondrial dysfunction as evidenced by increased production of RS, enzyme inhibition, loss of cytochrome *c*, and mitochondrial depolarization as indicated by loss of $\Delta\psi_m$ (Casley *et al.,* 2002a; Kim *et al.,* 2002; Anandatheerthavarada *et al.,* 2003; Jang and Surh, 2003; Lustbader *et al.,* 2004; Liang *et al.,* 2009; Takuma *et al.,* 2009). To evaluate the effect of MitoQ on A*β*-induced RS and mitochondrial damage, cortical neurons in cell culture were treated with A*β* (22-35) for 24 h in the presence or absence of MitoQ. Confocal microscopy allowed simultaneous assessment in single cells of changes in RS by the redox-sensitive dye, CM-H_2DCFDA, and $\Delta\psi_m$ by the potential-dependent fluorescent dye, $TMRM^+$ (Figure 2*A*). A*β* (22-35) treatment for 24 h induced a 3.4 ± 0.2- fold average increase in CM-H_2DCFDA intensity indicating elevated neuronal RS production. This treatment also significantly depolarized $\Delta\psi_m$ ($p < 0.05$) suggesting damage to the IMM. MitoQ (1 nM and 5 nM) prevented the increased RS caused by A*β* (22-35) treatment and maintained $\Delta\psi_m$ at levels indistinguishable from controls (Figure 2*B, C*). A*β* (22-35) + dTPP-treated neurons had RS and $\Delta\psi_m$ levels that were not significantly different from those of cells treated with A*β* (22-35) alone, indicating the antioxidant moiety of MitoQ was responsible for its protective effects.

A*β* induces production of superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), and multiple RS

downstream of these radicals in many cells including cortical neurons (Keller *et al.,* 1998; Longo *et al.,* 2000; Keil *et al.,* 2004; Malinski, 2007; Stepanichev *et al.,* 2008; Díaz *et al.,* 2010). The Aβ (22-35)-induced increases in $O_2^{\cdot-}$ and $NO^{\cdot}$ leads to formation of peroxynitrite ($ONOO^-$) because of the diffusion-controlled reaction between $O_2^{\cdot-}$ and $NO^{\cdot}$ (Murphy, 2001). Given the sensitivity of CM-H_2DCFDA to oxidation by $ONOO^-$, in combination with the propensity of $ONOO^-$ formation following Aβ-induced stress, we reasoned that the primary RS detected in our paradigm was $ONOO^-$ (Setsukinai *et al.,* 2003; Malinski, 2007). Co-treatment of cultures with Aβ (22-35) and the nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NNA) decreased CM-H_2DCFDA fluorescence intensity to that found in control cultures, confirming that the RS responsible for CM-H_2DCFDA oxidation were primarily nitrogen-associated species ($p < 0.001$ for Aβ vs. Aβ + L-NNA; Figure 2B). The generation of RS by electron leakage from the mitochondrial electron transport chain is highly dependent on maintenance of the proton gradient across the IMM (Turrens, 1997; Murphy, 2009). We used the protonophore, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) to break down this gradient. By decreasing the proton gradient across the IMM, FCCP increases rate of electron transport through the respiratory chain, thereby, inhibiting electron leakage and consequently mitochondrial $O_2^{\cdot-}$ production (Turrens, 1997). Brief incubation with FCCP (1.5 μM) caused the loss of most CM-H_2DCFDA fluorescence in Aβ (22-35)-treated cortical neurons (data not shown) suggesting that the RS detected by this dye was of mitochondrial origin. Taken together, these results suggest that MitoQ decreased CM-H_2DCFDA oxidation in Aβ -treated cortical neurons by suppressing RS produced by mitochondria. $\Delta\psi_m$ not only influences RS generation but other critical mitochondrial functions, such as electron transport, ATP synthesis, and Ca^+ sequestration, and is therefore considered the primary dictator of mitochondrial energetics, as well as an indicator of the

overall health of mitochondria in neurons (Nicholls and Ward, 2000). The ability of MitoQ treatment to maintain $\Delta\psi_m$ at control levels suggests that MitoQ preserves the functional integrity of neuronal mitochondria by preventing Aβ-induced $NO^{\cdot}$/ $O_2^{\cdot-}$/$ONOO^-$ damage to critical components of the IMM.

MitoQ improved cognitive performance in young 3xTg-AD mice

To evaluate the therapeutic potential of MitoQ for treating AD and examine a possible role for oxidative stress in AD progression, we investigated the effect of MitoQ on cognitive performance in the 3xTg-AD mouse model of AD. This mouse strain develops cognitive dysfunction as well as both plaque and neurofibrillary tangle (NFT) pathology in AD-relevant brain regions in an age-dependent fashion that closely resembles AD progression in humans (Oddo *et al.,* 2003). Notably, the earliest pathological changes in 3xTg-AD mice involve mitochondrial impairment and increased oxidative stress (Resende *et al.,* 2008; Yao *et al.,* 2009). Figure 3 shows the ages at which the various neuropathologies appear in these mice (Oddo *et al.,* 2003; Rissman *et al.,* 2004; Billings *et al.,* 2005; Rohn *et al.,* 2008; Yao *et al.,* 2009)

Female 3xTg-AD mice were either left untreated or received a continuous supply of 100 μM MitoQ or 100 μM dTPP in their drinking water from 2-7 months of age. Age-matched NonTg female mice either received no treatment or 100 μM MitoQ in their drinking water from 2-7 months of age. After 4.5 months of treatment, the cognitive performance of these 6 groups was determined by performance in the MWM (Morris, 1984). All mice were able to learn the MWM task, as the average escape latency for each group gradually decreased to reach a pre-determined criterion (< 25 s average latency) during 7-day, hidden-platform training trials. Untreated NonTg, NonTg receiving MitoQ treatment, and 3xTg-AD mice receiving MitoQ

treatment performed at a similar rate, reaching criterion on day 4. The untreated 3xTg-AD and 3x-dTPP-treated mice required additional training to learn the task (Figure 4*A*). MitoQ treatment significantly enhanced 3xTg-AD performance on acquisition trials to a level indistinguishable from NonTg and NonTg receiving MitoQ treatment ($p < 0.01$). Conversely, treatment with the control, dTPP, did not affect the performance of 3xTg-AD mice ($p > 0.05$ for 3xTg-AD + dTPP vs. 3xTg-AD on all training days). To determine the effect of MitoQ treatment on short and long-term spatial memory retention, the platform was removed and spatial bias for the previous platform location in the MWM was analyzed in probe trials conducted 1.5 h and 24 h after the last training trial. MitoQ prevented the long-term retention deficit in 3xTg-AD mice ($p < 0.05$ for 3xTg-AD + MitoQ vs. 3xTg-AD; Figure 4*B*). These mice performed at the same level as NonTg controls ($p > 0.05$ for 3xTg-AD + MitoQ vs. NonTg controls), as highlighted by the similar, focused search pattern in these two groups as compared to the random searching in other 3xTg-AD groups (Figure 8). An analogous trend was evident in the short-term probe.

The effect of MitoQ on the rate of learning in 3xTg-AD mice could not be explained by a stimulatory effect of MitoQ or by an effect of MitoQ on vision. Cued acquisition trials in which the escape platform was made visible to the mice were conducted after the last probe trial (Figure 4*C*). There were no differences in the average times in which mice in each group reached the platform, indicating no visual problems. The average swim speed was ~ 21 cm/sec for each group ($p = 0.84$) indicating that motor performance was the same for all and that the MitoQ did not have a stimulatory effect. Rather, MitoQ enhanced learning and spatial memory retention. Therefore, the 3xTg mice receiving MitoQ treatment performed as well as NonTg mice on all tasks indicating that MitoQ therapy effectively prevented the onset of AD-associated cognitive decline in them. The control, dTPP, produced no effect on acquisition or probe trials indicating

that the cognitive enhancement in MitoQ-treated mice was due to the antioxidant moiety.

MitoQ decreased oxidative stress in the cortex of 3xTg-AD mice.

Resende *et al*. (2008) reported that several markers for oxidative stress were higher in the brains of 3-5 month old 3xTg-AD mice than in NonTg mice of the same age. Among these markers were decreased levels of GSH, increased levels of GSSG, and increased levels of malondialdehyde (MDA), a marker for lipid peroxidation. Figure 5*A* shows that 5 months of MitoQ treatment prevented the decrease in the ratio of GSH/GSSG within 3xTg-AD brains. This treatment had a similar effect on lipid peroxidation, as Figure 5*C* shows MitoQ decreased MDA levels in the brains of these mice.

Lipid-derived radicals contribute to oxidative modifications in proteins (Kaplowitz *et al.,* 1985; Lakatos *et al.,* 2010). Tyrosine nitration provides a footprint for free radical-mediated damage involving oxidation of $NO^{\cdot}$ to $ONOO^{-}$, or products of its subsequent decomposition, and the formation of a tyrosyl radical intermediate (Radi *et al.,* 2002) . Nitration of tyrosine residues alters the structure of proteins, and often their biological activity, which could play an important role in AD development (Putcha *et al.,* 2002; Malinski, 2007; Wei *et al.,* 2009). Because of the apparent decrease in $ONOO^{-}$-associated RS levels in our *in vitro* analysis of MitoQ- treated neurons, we investigated a related effect of MitoQ treatment in 3xTg-AD mice by measuring cerebral nitrotyrosine levels. Figure 5*B* shows that MitoQ prevented the increase in 3-nitrotyrosine in young 3xTg-AD brains, suggesting MitoQ is also effective at detoxifying these RS *in vivo*. Taken together the data indicate that the brains of young 3xTg-AD mice are under oxidative stress and that MitoQ treatment prevented this stress from occurring.

MitoQ *decreased Aβ accumulation in the brains of 3xTg-AD mice*

The amyloid hypothesis states that A*β* peptides lie upstream of all the pathological events occurring in AD, including NFT formation, synaptic dysfunction, and neuronal death (Nicholls and Budd, 2000). This hypothesis is supported by the early A*β* accumulation in the rare, autosomal dominant forms of AD and transgenic mice harboring these human mutations (Hardy and Selkoe, 2002). Intraneuronal A*β* immunoreactivity is one of the earliest histopathological events in the 3xTg-AD brain (Billings *et al.,* 2005). Evidence suggests that A*β* increases cellular RS, and there is the possibility that these RS may in turn increase production of A*β* in a destructive autocatalytic cycle (Tamagno *et al.,* 2002; Tamagno *et al.,* 2005; Lin and Beal, 2006; Quiroz-Baez *et al.,* 2009). Therefore, we sought to determine the effect of MitoQ treatment on A*β* accumulation (Cardoso *et al.,* 2001; Hansson *et al.,* 2004; Caspersen *et al.,* 2005; Manczak *et al.,* 2006; Anandatheerthavarada and Devi, 2007). Figure 6*A* shows that 7–month-old 3xTg-AD mice that had received MitoQ treatment for 5 months prior to death had reduced staining for intraneuronal A*β*1-42 in their hippocampus and neocortex than did untreated 3xTg-AD mice. The ability of MitoQ to decrease A*β*1-42 burden was confirmed by ELISA (Figure 6*B*). These findings are consistent with the hypothesis that an autocatalytic feedback cycle exists between A*β* deposition and oxidative stress.

*MitoQ decreased cas*pase *activation in the cortex of 3x-Tg-AD mice*

Recent evidence suggests that executioner caspases 3 and 7 initiate tau aggregation and phosphorylation, which may lead to NFT formation and cognitive decline in AD (Cotman *et al.,* 2005; Rohn *et al.,* 2008; de Calignon *et al.,* 2010). Because the intrinsic apoptotic pathway may

involve mitochondrial RS generation (Eilers *et al.,* 1998), we hypothesized that the decrease in oxidative stress caused by MitoQ treatment may prevent aberrant caspase activity in the 3xTg-AD model (Rohn *et al.,* 2008). Using a proluminescent caspase 3/7 substrate, we measured the effect of MitoQ treatment on caspase 3/7 activity in the brains of young female 3xTg-AD mice. There was a 3.7 ± 0.7-fold increase in cerebral caspase 3/7 activity in 7-month-old untreated 3xTg-AD mice compared to NonTg controls. This increase was completely blocked by treatment with MitoQ during the preceding 5 months (Figure 7). Unfortunately, we were unable to determine any downstream effect of this decrease on tau pathology, as levels of caspase-cleaved tau (Δtau) were below the level detectable in immunoblots of our 7-month-old, untreated 3xTg-AD mouse brain homogenates (Rissman *et al.,* 2004).

Discussion

The recent development of antioxidants that selectively concentrate in mitochondria provides an unprecedented opportunity to decipher the impact of mitochondria-generated RS on the pathogenesis and progression of both sporadic and inherited forms of AD (Smith *et al.,* 2003; Lin and Beal, 2006b; Reddy, 2008; Swerdlow and Khan, 2009b; Moreira *et al.,* 2010). Perhaps the most extensively studied compound of this class is the mitochondria-targeted ubiquinone derivative, MitoQ (Smith *et al.,* 2003; Rodriguez- Cuenca *et al.,* 2010). The antioxidant moiety of MitoQ detoxifies RS in isolated mitochondria and cells and is effectively recycled by complex II (James *et al.,* 2005; James *et al.,* 2007; Cassina *et al.,* 2008; Maroz *et al.,* 2009; Rodriguez-Cuenca *et al.,*
2010).

We first investigated the ability of MitoQ to prevent the death of cortical neurons in cell culture caused by exposure to A*β* peptides. We found that low nanomolar concentrations

of MitoQ prevented Aβ-induced death in these cells. These concentrations also blocked an Aβ-induced increase in NO$^{\cdot}$-associated RS and an Aβ- induced depolarization of $\Delta\psi_m$. These data indicate that death was associated with mitochondrial dysfunction and suggest that it was caused by mitochondria-derived RS. Bolstered by these findings, we tested the ability of MitoQ to inhibit the decline in spatial memory retention that occurs in the 3xTg-AD mouse model from 2-7 months after birth (Oddo *et al.,* 2003). MitoQ prevented the onset of cognitive decline in these mice, which was previously attributed to intraneuronal Aβ-accumulation in 3xTg-AD mice of this approximate age (Billings *et al.,* 2005). More recent studies suggest that the Aβ deposition and early impairment are preceded by, and correlate with, mitochondrial dysfunction and increased oxidative stress in 3xTg-AD brains (Resende *et al.,* 2008; Yao *et al.,* 2009). MitoQ blocked the elevated oxidative stress and also prevented Aβ accumulation and elevated caspase 3/7 activity in 3xTg-AD brains. Our results suggest that mitochondria lie at the hub of a putative autocatalytic cycle in which Aβ peptides induce RS that in turn perpetuate Aβ production, activate apoptotic machinery, and culminate in the neuronal atrophy that may underlie cognitive decline in the AD brain (Gibson and Shi; Lin and Beal, 2006).

Aβ induces increased mitochondrial RS production, at least in part, via interaction with Aβ alchohol dehydrogenase and by direct inhibition of cytochrome oxidase (respiratory complex IV; Canevari *et al.,* 1999; Lustbader *et al.,* 2004; Rhein *et al.,* 2009). Aβ indirectly inhibits other respiratory complexes through NOS-dependent generation of reactive nitrogen species, an event that may increase leakage of electrons from the mitochondrial electron transport chain, further augmenting RS production (Radi *et al.,* 1994; Radi *et al.,* 2002; Keil *et al.,* 2004). In addition to our mouse data, increasing evidence suggests that Aβ-induced RS may have significant functional consequences in the pathogenesis of AD in humans as well. Nitration of mitochondrial proteins is associated with pathological changes in mitochondrial

redox state, morphology, and bioenergetics in preclinical and early AD patients (Anantharaman *et al.,* 2006; Butterfield *et al.,* 2006; Butterfield *et al.,* 2007a; Yao *et al.,* 2009). Evidence also supports a causative role for these modifications in mediating the early synaptic degeneration and cognitive decline in AD (Wang *et al.,* 2008b; Dumont *et al.,* 2009; Massaad *et al.,* 2009; Yao *et al.,* 2009). Overexpression of mitochondrial superoxide dismutase, MnSOD, decreases Aβ-processing, preserves synaptic integrity, and eliminates the earliest cognitive deficits in transgenic AD mice (Massaad *et al.,* 2009). MitoQ may prevent damage downstream of $O_2^{\cdot -}$ by the ubiquinol version directly detoxifying lipid-derived radicals and reactive nitrogen species, or indirectly, through effective recycling of α-tocopherol (James *et al.,* 2005). In addition, the ubiquinone moiety of MitoQ reacts rapidly with $O_2^{\cdot -}$ and, thus, may also enhance mitochondrial antioxidant defense by complimenting $O_2^{\cdot -}$ degradation by MnSOD (Maroz *et al.,* 2009).

MitoQ selectively accumulates in the mitochondrial matrix and is preferentially adsorbed to the IMM, and is therefore optimally positioned to prevent RS damage to, and subsequent inactivation of, mitochondrial enzymes that may be involved in AD pathogenesis. Post-translational modification of alpha-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and critical components of the respiratory chain by RS may contribute to impaired glucose metabolism found in preclinical AD subjects (Sultana and Butterfield, 2009a; Yao *et al.,* 2009). Longitudinal neuroimaging studies reveal the rate of metabolic decline within the medial temporal lobe predicts the progression of cognitive deterioration from normal aging to AD with over 80% accuracy and more closely correlates with the level of oxidative damage than other biomarkers tested in preclinical patients (2008b; Mosconi *et al.,* 2008a). This correlation may prove fundamental, as mitochondrial dysfunction and oxidative stress encourage the development of the plaques and NFTs that have diagnostically defined AD for the past century

(Gibson and Shi, 2010; Nicholls and Budd, 2000). Studies *in vitro* show that mitochondrial perturbations and oxidative stress alter the activity of the proteolytic enzymes associated with Aβ production and clearance. The predominant pathway of Aβ production occurs by sequential cleavage of APP by β- and γ-secretases. Metabolic impairment induced by thiamine deficiency or respiratory chain inhibition augments β- secretase expression and activity (Nachman *et al.,* 1996; Schriner *et al.,* 2000). Oxidative stress also induces β-secretase upregulation, and consequently, Aβ generation, both of which are preventable by antioxidant treatment *in vitro* (Tamagno *et al.,* 2002; Tong *et al.,* 2005). The final step in Aβ production is achieved by γ-secretase, which is present in mitochondria and may also be up-regulated by RS-induced expression of the catalytic subunit, presenilin-1 (Guglielmotto et al.; Minopoli *et al.,* 2007; Oda *et al.,* 2010). Mitochondrial RS also activates caspases, which may prove amyloidogenic (Hortelano *et al.,* 1999; Cassina *et al.,* 2000). Aβ-accumulation may also result from oxidative inactivation of Aβ-degrading enzymes, such as neprilysin and insulin-degrading enzyme (Green and Kroemer, 2004). Intracellular Aβ may then enter the mitochondria where it induces additional RS and magnifies the metabolic deficit (Lustbader *et al.,* 2004; Hansson Petersen *et al.,* 2008). Both amyloid and mitochondrial RS activate apoptotic machinery (caspase 3 and 7) which may culminate in NFT formation (Cotman *et al.,*2005). The ability of MitoQ to block each of these early events in 3xTg-AD mice suggests that it may effectively prevent analogous events that influence clinical changes in AD.

The early hippocampal-based, spatial retention deficits in the MWM behavioral test emerge in 3xTg-AD mice at 4-6 months of age and are reminiscent of spatial learning deficits and delayed recall seen in patients with mild cognitive impairment, which is the proposed prodrome of AD (Grundman *et al.,* 2004; Mosconi *et al.,* 2008a).

Consequently, young 3xTg-AD mice provide a valuable model for the evaluation of prophylactic therapeutics (Billings *et al.,* 2005). The ability of MitoQ to suppress all AD-associated neuropathology investigated in this model suggests a possible influence of mitochondria-induced oxidative stress in the progression of AD. Failure to address this underlying mitochondrial dysfunction may explain the disappointing results of Aβ-centered therapy in humans. Recent clinical trials attempting to replicate the benefits of Aβ-immunotherapy seen in mouse models have been largely unsuccessful in preventing early cognitive decline in AD patients despite significantly decreasing the amyloid burden (Holmes *et al.,* 2008a; Holmes *et al.,* 2008b; Sabbagh, 2009). Others describe a lack of clinical correlation between Aβ levels and AD progression, and propose brain atrophy and cerebral metabolic rate provide the best predictive parameters for clinical changes in AD (Giannakopoulos *et al.,* 2003; Li *et al.,* 2008; Walhovd *et al.,* 2010). These findings suggest that, while Aβ is undoubtedly involved, it may not exclusively dictate AD pathogenesis, and auxiliary approaches may be required to optimize therapeutic benefit and prevention of AD.

MitoQ has been developed as a pharmaceutical for use in humans and has undergone two phase II trials (Green *et al.,* 2007). MitoQ was protective in humans against liver damage (Manczak *et al.,* 2010), however it was not protective in a PD study (Green *et al.,* 2007). The reason for the lack of protection in PD is unclear, but may be because by the time a patient shows symptoms of PD any protection afforded by MitoQ may be too late to reverse the neuronal death that underlies PD. Consequently, while there are similarities in pathology between PD and AD, the negative result in PD does not preclude testing of MitoQ and related compounds in AD, but suggests that any mitochondria-protective interventions in AD should be

applied as early as possible. Advancements in neuroimaging may soon provide the opportunity to administer preventative therapeutics to individuals at risk for AD years before irrevocable neurodegeneration ensues (Berti *et al.,* 2010), and could determine whether mitochondria-targeted antioxidants are sufficient to prevent metabolic failure in early stages of AD.

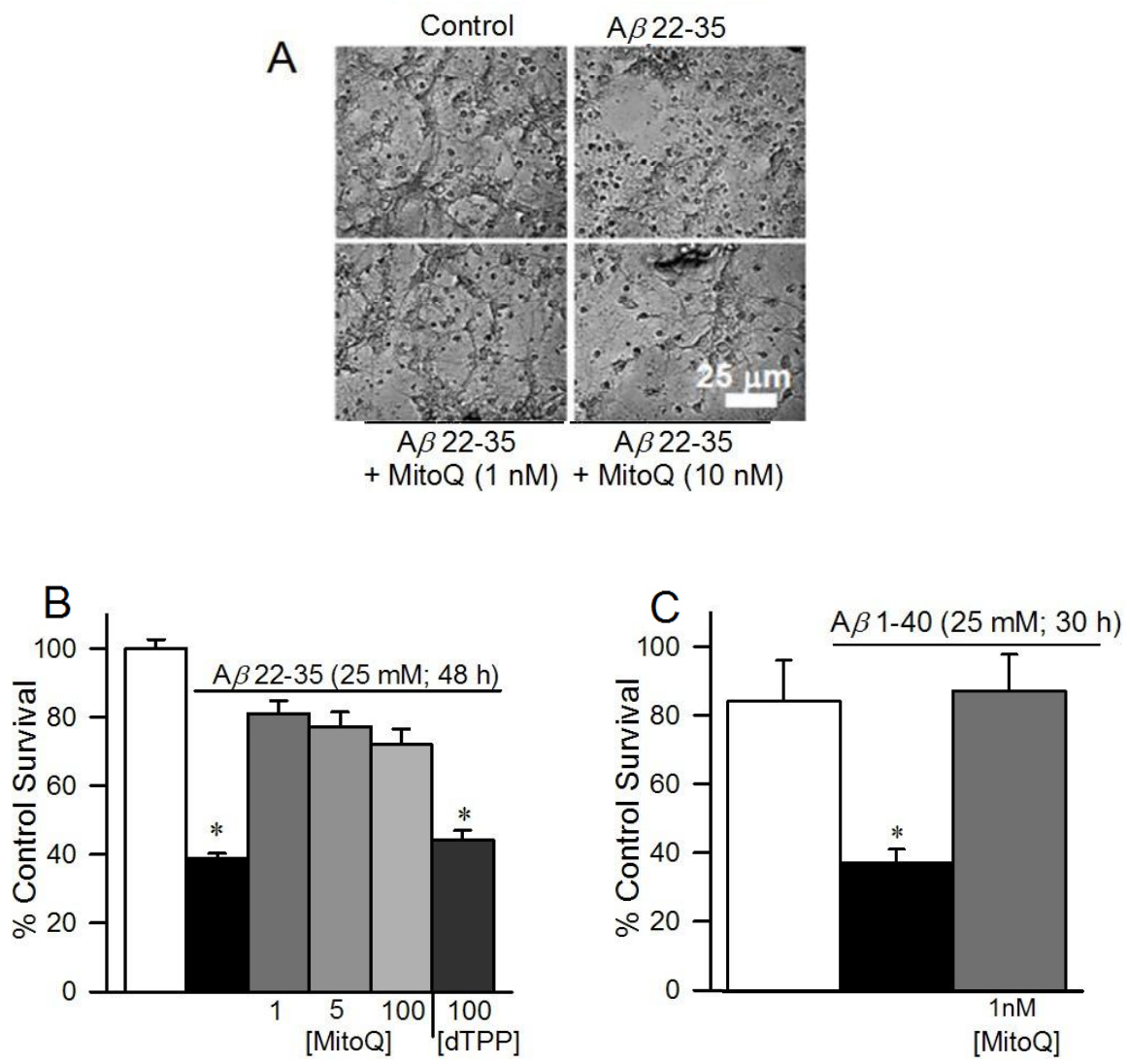


Figure 4.1. **MitoQ inhibited A*β*-induced death of mouse cortical neurons in cell culture**. ***A***. Photomicrographs of untreated cortical neurons and cortical neurons exposed to A*β* (22-35; 25 µM) alone or with MitoQ for 48h. ***B***. MitoQ prevented most cell death caused by A*β* (22-35). (*n* = 30 cultures per condition except for 100 µM MitoQ or dTPP where *n* = 9 cultures per condition). ***C***. MitoQ also prevented A*β* (1-40)-induced death (*n* = 9 cultures per condition). After treatments, neurons were fixed, counted, and quantified as percentage of the average

number of healthy (phase-bright) neurons in control, sibling cultures. Stars indicate a significant difference relative to control ($p < 0.001$).

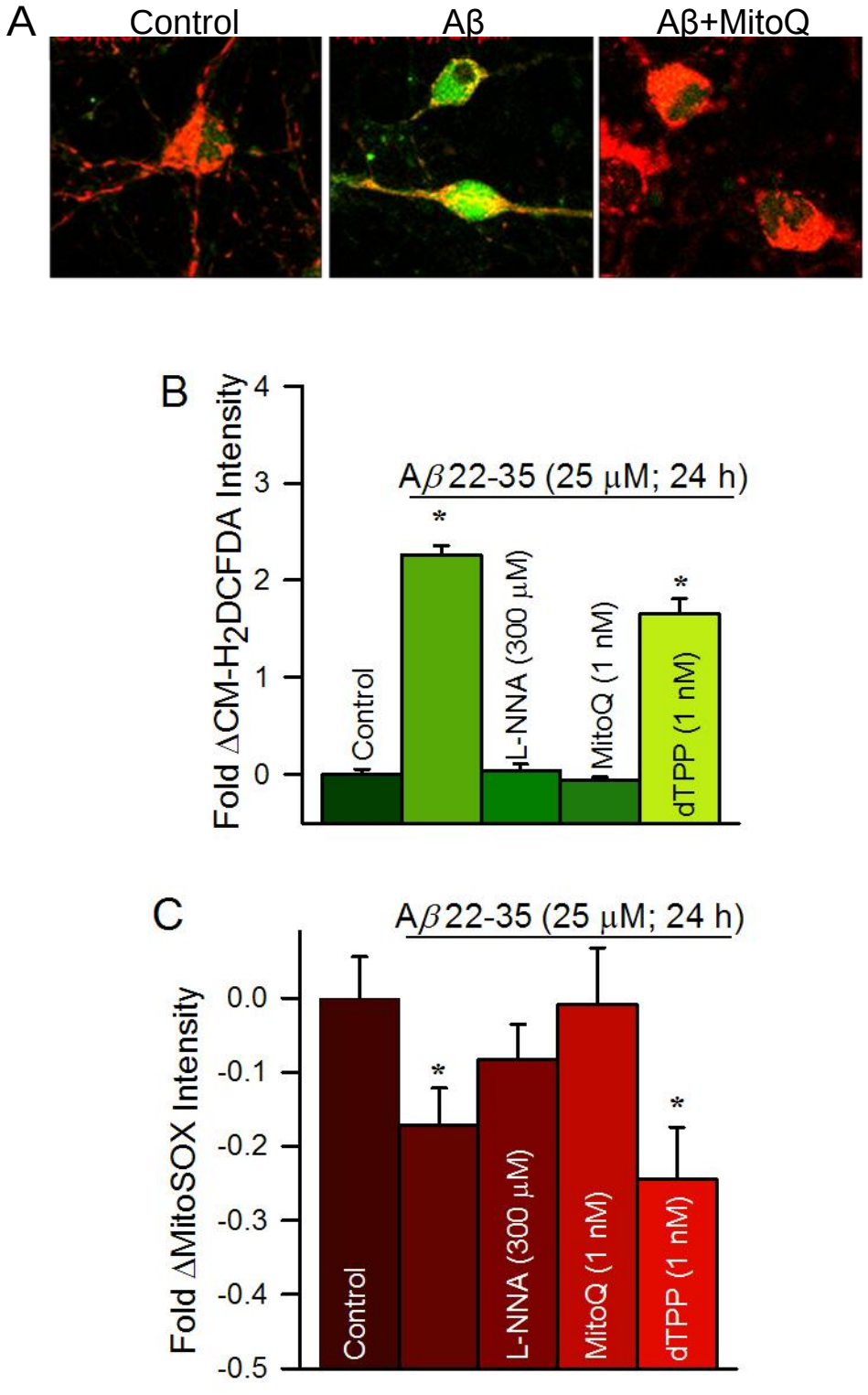

A
Control
Aβ
Aβ+MitoQ
B
Fold ΔCM-H2DCFDA Intensity
4
3
2
1
0
Aβ22-35 (25 μM; 24 h)
*
*
Control
L-NNA (300 μM)
MitoQ (1 nM)
dTPP (1 nM)
C
Aβ22-35 (25 μM; 24 h)
Fold ΔMitoSOX Intensity
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
*
*
Control
L-NNA (300 μM)
MitoQ (1 nM)
dTPP (1 nM)

Figure 4.2. **MitoQ prevented increased Aβ-induced RS production and Aβ-induced $\Delta\psi_m$ depolarization in mouse cortical neurons in cell culture.** Confocal microscopy allowed simultaneous assessment of RS by the redox-sensitive dye, CM-H_2DCFDA and $\Delta\psi_m$ by the potential-dependent fluorescent dye, $TMRM^+$. ***A.*** Confocal micrographs of mouse cortical neurons costained with CM-H_2DCFDA (green) and $TMRM^+$ (red). RS levels, indicated by increased CM-H_2DCFDA intensity, were much higher in cells treated for 24 h with Aβ (22-35; 25 μM) peptide. MitoQ (1 nM) blocked the increased RS caused by exposure of neurons to Aβ (22-35). ***B***. MitoQ and the NOS inhibitor L-NNA suppressed increased RS caused by Aβ (22-35) exposure. dTPP, which mimics the mitochondria targeting moiety of MitoQ but contains no ubiquinone, had no significant effect. ***C.*** MitoQ and L-NNA also blocked depolarization of $\Delta\psi_m$ (decreased $TMRM^+$ intensity) caused by Aβ (22-35) peptide while dTPP had no effect. Dye intensity was determined in confocal micrographs with MetaMorph software and normalized to the average dye intensity of control cultures plated at the same time. Dye intensities are shown as fold change from these values. The specificity of $TMRM^+$ fluorescence for $\Delta\psi_m$ was confirmed by treating control cultures with the protonophore, FCCP; 0.5 μM; (Duchen, 1999). This treatment caused greatly decreased $TMRM^+$ staining (not shown). Stars indicate significantly different from controls ($p < 0.005$). n = 43-143 neurons

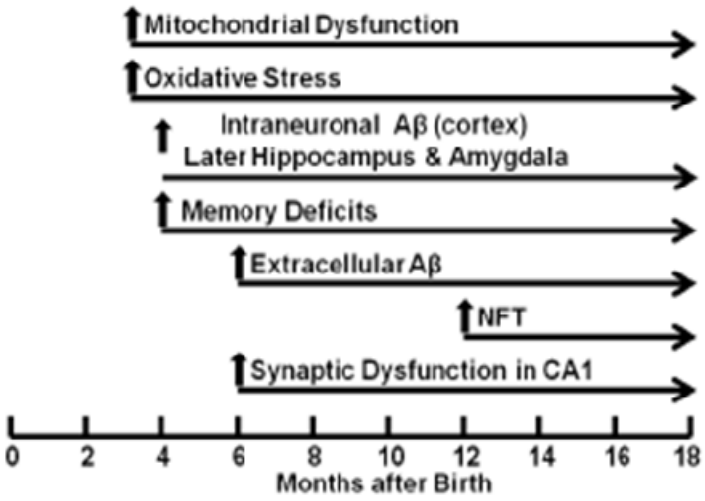
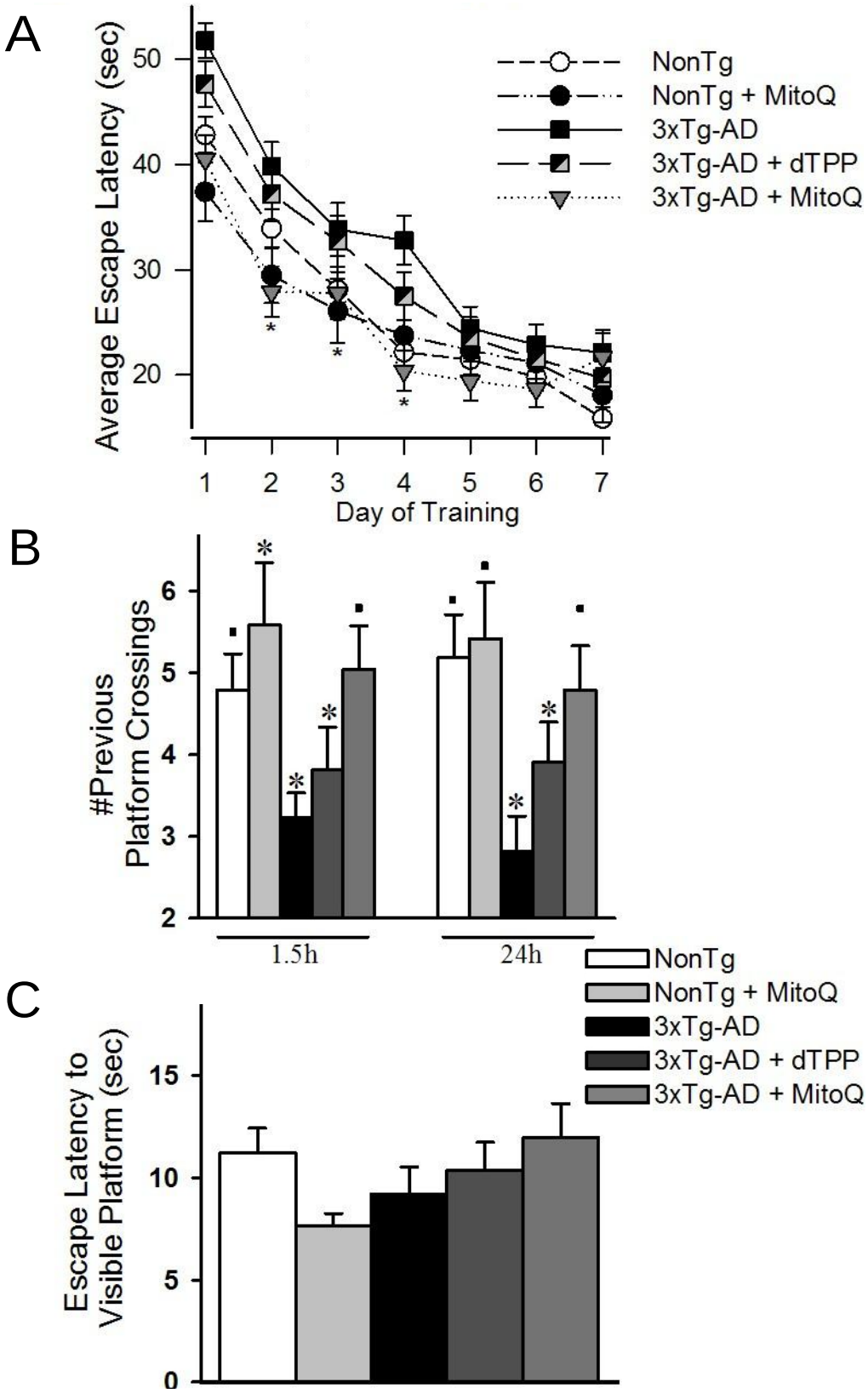


Figure 4.3. Mitochondrial dysfunction occurs in young female 3xTg-AD mice.

Summary of the ages at which known cognitive and neuropathological changes occur in 3xTg-AD mice (Oddo *et al.,* 2003; Rissman *et al.,* 2004; Resende *et al.,* 2008; Rohn *et al.,* 2008; Yao *et al.,* 2009).


A
Average Escape Latency (sec)
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40
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1
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6
7
Day of Training
NonTg
NonTg + MitoQ
3xTg-AD
3xTg-AD + dTPP
3xTg-AD + MitoQ
B
#Previous Platform Crossings
6
5
4
3
2
1.5h
24h
C
Escape Latency to Visible Platform (sec)
15
10
5
0

Figure 4.4. **MitoQ treatment prevented the onset of cognitive deficits in youngfemale3xTg-AD mice**. ***A***. Time-courses of spatial learning and memory acquisition in the MWM. At 6.5 months of age, 6 groups of female mice having the specified genotypes and that had received the indicated treatments for 4.5 months were trained in the MWM (see Materials and Methods for details). Each mouse performed 4 training trials per day for 7 consecutive days, and the latencies in seconds for each animal to reach a platform hidden under opaque water per day were averaged. All trials were filmed by digital camera and the data analyzed by EthoVision software. All groups were able to perform the task with equal latencies after 7 days of training. However, the NonTg, NonTg + MitoQ, and 3xTg-AD + MitoQ mice learned the task more quickly than did untreated or dTPP-treated 3xTg-AD mice ($p < 0.01$ by ANOVA). Treated mice received either dTPP (100 μM) or MitoQ (100 μM) in their drinking water from 2-7 months after birth. ***B***. MitoQ treatment prevented loss of short- and long-term spatial memory retention in the 3xTg-AD mice. Memory retention was assessed in MWM probe trials conducted 24 h after the last training trials. In the probe trials, the platform was removed from the water tank and mice were allowed a 60 s free swim. Spatial bias is shown as the number of previous platform location crosses as determined by video analysis using Ethovision tracking software. MitoQ significantly prevented the long-term retention deficit in 3xTg-AD mice ($p < 0.05$ compared to NonTg control). Conversely, dTPP treatment did not affect learning or memory in 3xTg-AD mice ($p > 0.05$). Short-term probe trials (1.5 h after last training period) showed a similar trend. ***C***. Cued acquisitions trials in which the platform was not hidden revealed that all mice were capable of swimming to the visible platform in the same amount of time ($p = 0.3$ by ANOVA). Stars indicate significantly different from untreated NonTg controls (1.5 or 24 h as appropriate); squares indicate significantly different from untreated 3xTg-AD animals (*n* = 21-38 mice except for the MitoQ-exposed NonTg where 12

animals were treated).

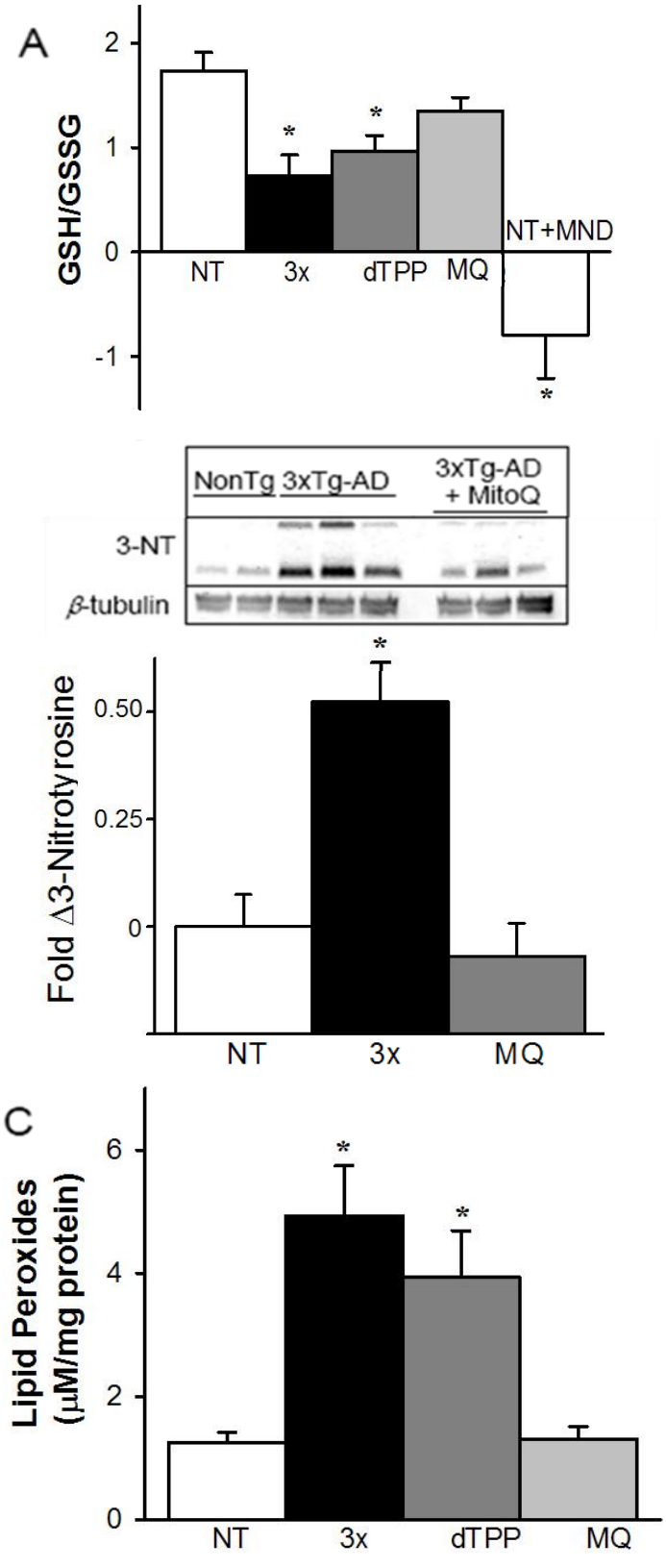
A
GSH/GSSG
2
1
0
-1
NT
3x
dTPP
MQ
NT+MND
*
NonTg
3xTg-AD
3xTg-AD
+ MitoQ
3-NT
β-tubulin
Fold Δ3-Nitrotyrosine
0.50
0.25
0
NT
3x
MQ
C
Lipid Peroxides
(μM/mg protein)
6
4
2
0
NT
3x
dTPP
MQ

Figure 4.5. MitoQ prevented oxidative stress in the brains of young female 3xTg- AD mice. ***A***. MitoQ treatment inhibited increased GSH/GSSG ratios in the brains of 3xTg-AD mice ($p < 0.01$). As a positive control, NonTg samples were incubated with 40 μM menadione to deplete GSH and increase GSSG for 45-60 min before the assay (Di Monte, 1984). The results are expressed as the average molar ratio of GSH/GSSH for each group (*n* = 4 brains for each condition). ***B***. MitoQ treatment suppressed the development of elevated levels of 3-nitrotyrosine (3-NT) in the brains of 3xTg-AD mice. Top, representative immunoblots showing that 3-NT levels were higher in two proteins, which ran at approximately 26 and 77 kDa, in the brains of 3xTg-AD mice than in NonTg mice. 3-NT levels in these proteins were lower in the brains of MitoQ-treated 3xTg-AD animals than in untreated ones. Bottom, quantitative analysis of 3-NT band density indicated nitrated proteins were significantly increased in untreated 3xTg-AD mice at 7 months of age and that this increase was prevented by MitoQ treatment. Density was determined for entire lanes and is shown as fold change from the average density in NonTg brains (*n* = 5-6 brains). ***C***. MitoQ treatment prevented increased lipid peroxidation as determined by the TBARS assay in the brains of 3xTg-AD mice ($p < 0.01$) (*n* = 6 brains for each except dTPP where *n* = 3 brains). The brains used in this and subsequent assays were obtained from the 7 month-old female mice (Figure 4) that had been treated as indicated for 5 months. Brains were removed two weeks after the end of MWM probe trial

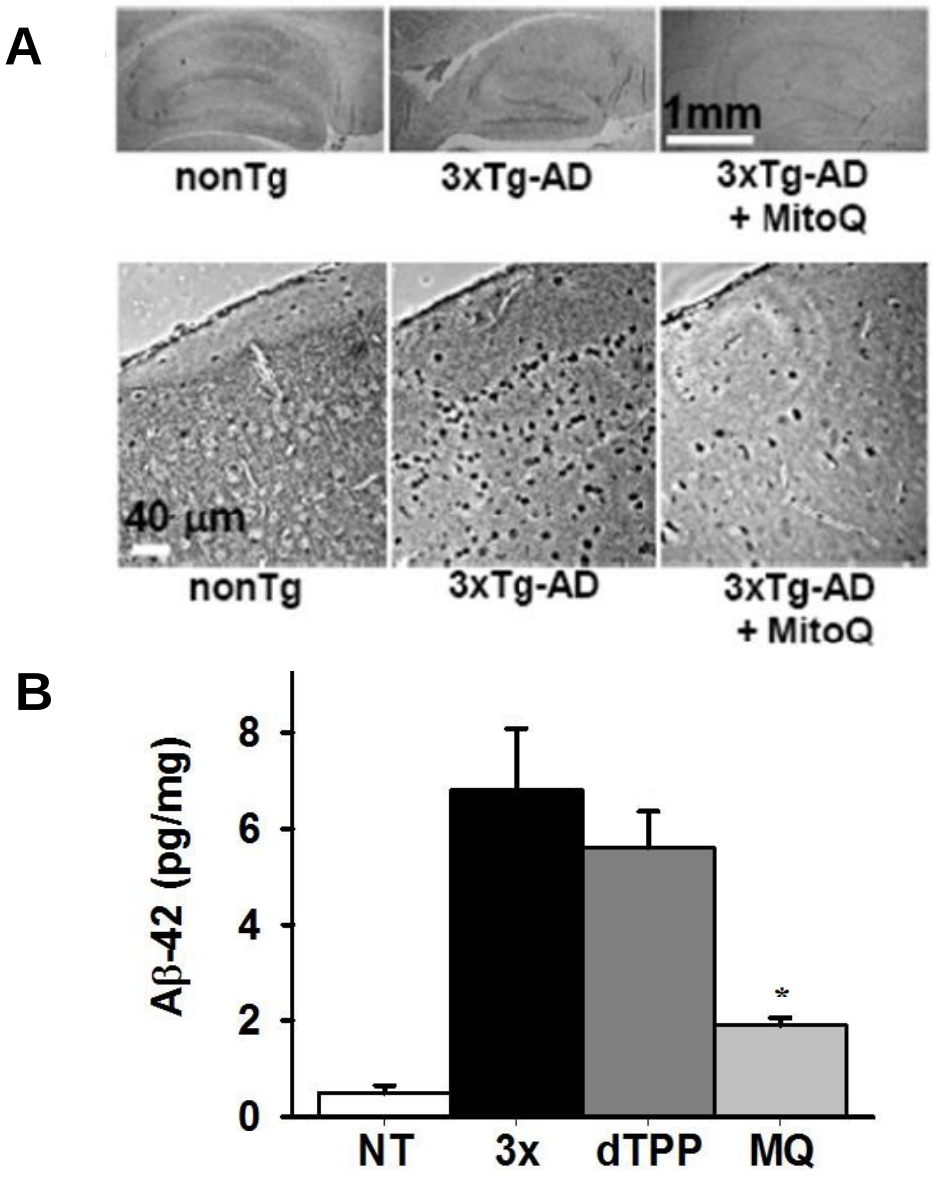

Figure 4.6. **MitoQ reduced intraneuronal Aβ1-42 burden in brains of young 3xTg- AD mice.** ***A***. Representative photomicrographs showing immunostaining for Aβ1-42 within the hippocampus (top) and neocortex (bottom) of NonTg, untreated 3xTg-AD mice, and 3xTg-AD mice that had received MitoQ treatment for 5 months. Many cells in 3xTg-AD mice receiving

no treatment exhibited cytosolic staining for Aβ1-42 while 3xTg-AD mice that had received MitoQ-treatment exhibited much less staining for Aβ1-42. Sections are representative of 4 brains per condition. ***B***. Quantification of Aβ1-42 by ELISA revealed that MitoQ treatment prevented increased Aβ1-42 burden in the 3xTg-AD mouse brain (n = 4 for each condition).

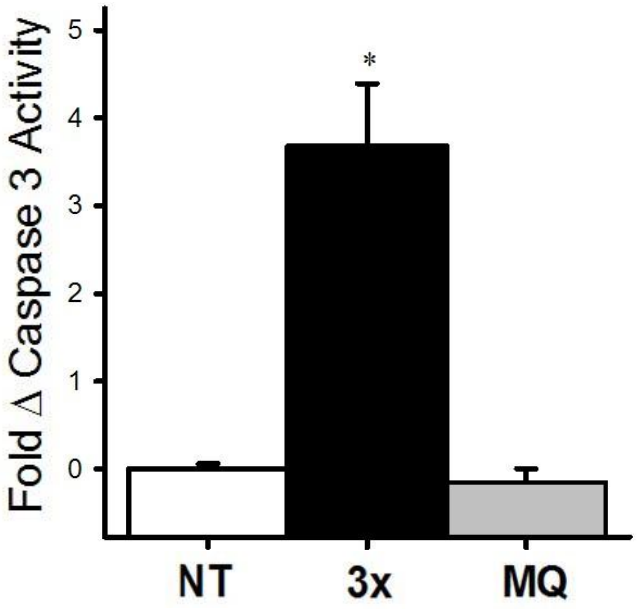

Figure 4.7. MitoQ treatment blocked increased caspase 3/7 activity in young female 3xTg-AD mouse brains. Caspase 3/7 activity was assessed by the Caspase Glo 3/7 assay. Brain homogenate was incubated with a proluminescent caspase 3/7-specific substrate and luminescence quantified by a luminometer. More intense luminescence indicates higher caspase 3/7 activity. Values are normalized to the average luminescence of the NonTg controls (n = 8-10 brains).

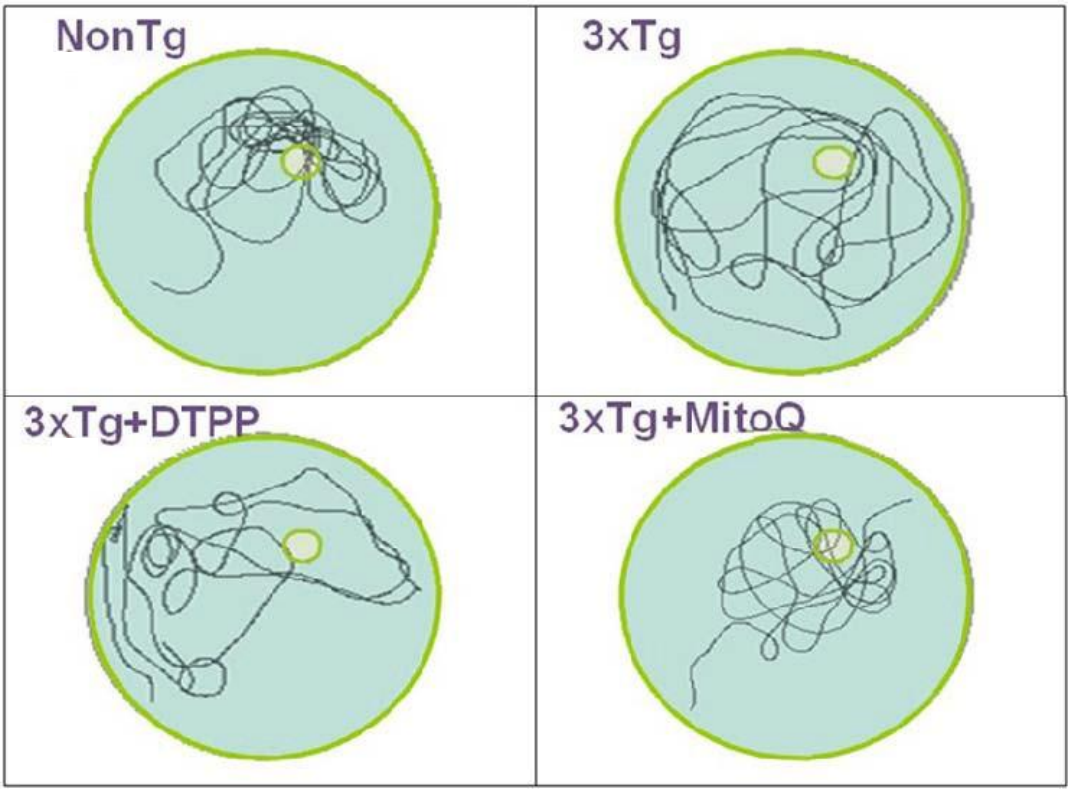

Figure 4.8. Search strategies in the long-term probe trials. Short- and long-term retention of spatial learning was assessed in probe trials conducted 90 min and 24 h after the last training trial, respectively. In the probe trials, the platform was removed and mice were allowed a 60s ~~–f~~ree" swim. Representative swim paths of the long-term (24 h) probe trials for the indicated treatment groups were generated from video analysis by Ethovision tracking software. NonTg and 3xTg+MitoQ mice retained spatial bias as evident by the goal-oriented swim pattern. In contrast, the untreated 3xTg and 3xTg+dTPP mice failed to show focused searching.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Discussion

The principal goal of this research was to provide mechanistic insights into the consequences of mitochondrial oxidative stress in neurodegenerative conditions. The discoveries herein indicate that mitochondria-generated reactive species (RS) promote a permissive environment that may be necessary for the activation of signaling pathways responsible for neuronal demise during age-dependent neurodegeneration. The suppression of mitochondrial RS with the targeted antioxidant, MitoQ, prevented critical apoptotic events and neuronal death in multiple *in vitro* models, as well as phenotypic expression of the most common age-dependent neurodegenerative disease. The beneficial effect of MitoQ in the paradigms tested could be due to a multitude of signaling cascades influenced by mitochondrial RS.

Bioenergetics and Redox Balance

Virtually every aspect of neuronal growth, communication, and death is controlled by the flux of energy through the nervous system. As an individual's energy demands fluctuate throughout life, reversible alterations in bioenergetic physiology are required that cannot be achieved by relatively static genetic changes. While epigenomic changes (histone actetylation, methylation, and phosphorylation) provide moderately stable adaptations, they are not sufficient for short-term, reversible alterations required within the dynamic environment of the nervous system. These fluctuations are accomplished by signal transduction pathways that are largely controlled by intermediates produced by mitochondrial regulation of the following: ATP for

phosphorylation, RS for driving oxidative modifications, acetyl-CoA for acetlyation, NAD^+ for sirtuin-mediated deacetylation, S-adenosylmethionine (SAM) for methylation, and oxidation-reduction (redox) couples for thiol-regulation (Wallace, 2010).

Redox reactions drive ATP production and coordinate a plethora of signal transduction pathways that enable the mitochondria to control cellular fate (Shigenaga *et al.,* 1994; Zhivotovsky, 2003; Kujoth *et al.,* 2005b; Lin and Beal, 2006a; Kim, 2007; Jarrett *et al.,* 2008; Pop *et al.,* 2010). Redox control networks within the mitochondria and cytosol are somewhat connected via shuttling systems in the inner mitochondrial membrane (IMM); thus, while they may influence each other, the distinct redox pools will differ from one another based on the concentration of reducing equivalents and RS within each respective compartment (Contestabile, 2009; Devore *et al.,* 2010). These thiol-based redox couples are maintained by the flow of reducing equivalents into the mitochondria via the respiratory chain and into the cytosol through the mitochondria, glycolysis, and the pentose phosphate shunt, and are therefore intimately connected to metabolism (Bolanos *et al.*, 2008; Kim *et al.*, 2007; Wallace 2010). In mitochondrial and degenerative diseases, an imbalance in this interdependent network exploits an innate threshold and pushes the cell into a path of self-destruction (Zhivotovsky, 2003; Circu and Awe 2010).

While the signaling pathways involved in this network are incredibly complex, the imbalance that leads to death can be recapitulated in cultures of sympathetic neurons deprived of nerve growth factor (NGF). Following NGF withdrawal, metabolism slows as mitochondria generate more RS, collectively overwhelming endogenous antioxidant defenses and shifting critical redox couples to an oxidized state that activates apoptotic machinery and ends in neuronal death (Bough, 2008; Vaughn and Deshmukh, 2008). Of the compounds tested in this

study, the only one capable of aborting this process was also the only compound that effectively suppressed the pathological induction of mitochondrial RS, suggesting that mitochondrial RS production is a major determinant of neuronal fate.

Each of the investigated compounds exhibited an unique effect on the cytosolic and mitochondrial redox states as determined by redox-sensitive probes: the c-Jun N-terminal kinase (JNK) inhibitor effected neither; mitochondria-targeted vitamin E ($MitoE_2$) decreased cytosolic RS, but had no effect on the mitochondria redox state; mitochondria-targeted ubiquinone (MitoQ) restored both mitochondrial and cytosolic redox environments to the level of neurons in cultures that were metabolically supported in medium containing NGF. Using the same methods, previous studies in our lab have demonstrated NGF-readdition to deprived cultures returns cytosolic RS to basal levels in a mechanism involving restoration of the glutathione redox state (GSH/GSSG), but this is not sufficient to alter mitochondrial RS for at least 6 h, possibly longer (Kirkland *et al.,* 2007). Conversely, this study indicates that effective RS-scavenging in the mitochondria may be sufficient to restore redox balance in the cytosol within 1 h. Taken together, these results suggest that the mitochondrial redox balance is an important determinant of the cytosolic redox state (Bolanos *et al.*, 2008; Barnat *et al.,* 2010; Devore *et al.,* 2010), but the reverse may not be true when the bioenergetic network is challenged by apoptotic stimuli.

Mitochondrial protection may overcome caveats of caspase-targeted therapy

Due to the range of neurological disorders associated with caspase activation, caspases have been suggested as a therapeutic target. There are two major caveats to this strategy. First, caspases have several important physiological roles. Caspases are required for embryonic development, adult stem cell differentiation, and the prevention of cancer (Zhivotovsky, 2003;

McLaughlin, 2004; Kroemer and Martin, 2005). Moreover, caspase activation may be necessary for neurological function, as low levels of caspase 3 contribute to axon guidance, synaptic plasticity, and may even promote neuroprotection by inducing heat-shock protein 70 (Hsp70) and degrading pro-apoptotic proteins such as Bax (Choi *et al.,* 2001; Gilman and Mattson, 2002; McLaughlin *et al.,* 2003). In stressful conditions, caspase activation occurs rapidly, as multiple caspases are simultaneously activated via the apoptosome, and downstream executioner caspases (caspase 3) can also auto-activate (Lang-Rollin *et al.,* 2003; Riedl and Salvesen, 2007; Peterson *et al.,* 2009). If low levels of caspase activity can occur without causing large scale proteolysis of cellular substrates, there must be a powerful intrinsic signaling mechanism in place to limit the extent of caspase activation (Schwerk and Schulze-Osthoff, 2003; McLaughlin, 2004). Previous studies in sympathetic neurons have eloquently shown that the sequestration of apoptotic proteins within the mitochondria prevents the induction of caspase activation that leads to death (Lang-Rollin *et al.,* 2003; Chen and Yan, 2007; Vaughn and Deshmukh, 2008; Xu *et al.,* 2008). Recent studies suggest the cellular redox environment may determine the "competence to die" (Deshmukh *et al.,* 1998), and the current study solidifies an important role for mitochondrial RS in dictating the cellular redox state and the amplification of caspase activation in response to apoptotic stimuli. Therefore, therapeutics that protect the integrity of mitochondrial membranes may provide a more rational approach to inhibiting caspase-dependent death, as basal levels of caspases are not likely to be affected. Evidence to support this proposal is found in the ability of $MitoQ_{10}$ to protect mitochondria from RS-induced damage and prevent the pathological induction of caspase 3 *in vitro* and *in vivo,* without significantly decreasing caspase activity from that in healthy controls.

The second potential limitation to caspase-targeted therapy is that caspase inhibitors do not prevent mitochondrial dysfunction and the progression of caspase-independent death in neurons exposed to apoptotic stimuli (Lang-Rollin *et al.,* 2003; Cregan *et al.,* 2004; Nylen *et al.,* 2009; Ashe and Zahs, 2010). While some refer to all caspase-independent death as entirely non- apoptotic or necrotic, these terms are vague, if not misleading, in that caspase-independent death often encompasses a myriad of morphological characteristics (Kroemer and Martin, 2005; Kroemer *et al.,* 2008). Regardless, it appears that some caspase-independent death is regulated by the mitochondria, and may occur in a programmed, self-destructive process (1, 307, 308).

In our model system and others, caspase inhibition merely shifts the morphological manifestation of cell death, while caspase-independent self-destruction proceeds in a manner ultimately determined by the integrity of the mitochondrial inner membrane (21, 22, 180, 304). When the IMM is compromised, mitochondrial membrane potential ($\Delta\psi_m$) collapses, and the neuron can no longer survive. Therapeutic manipulation of caspase-independent death is lacking in part because the events between Bax induction and $\Delta\psi_m$ loss are still largely undefined (21, 168, 304). The results of this study suggest that mitochondrial RS may dictate the collapse of $\Delta\psi_m$ by directly damaging critical components of the IMM (23, 206), which can be prevented by effective antioxidants that selectively accumulate within and protect this membrane. The mitochondrial protection provided by MitoQ in NGF-deprived neurons extended survival beyond the point of caspase-inhibition, suggesting that effective, mitochondria-targeted therapeutics may supplement the shortfalls of strategies that solely modify caspase activity.

Mitochondrial RS: "the good, the bad, and the ugly"

The results from this study and others suggest that MitoQ suppresses mitochondrial $O_2^{\bullet-}$ (~~"t~~he bad"; Beckman and Koppenol, 1996), which may be responsible for its ability to restore redox balance in cell culture and in transgenic AD mice. MitoQ decreased several markers of oxidative stress, including GSH/GSSG. Previous studies have shown that the level of reduced glutathione (GSH) plays a critical role in maintaining cellular vitality (Eilers *et al.,* 1998; Schafer and Buettner, 2001; Kirkland *et al.,* 2002b; D'Alessio *et al.,* 2005; Vaughn and Deshmukh, 2008; Bolanos *et al.,* 2008). The suppression of mitochondrial $O_2^{\bullet-}$ by MitoQ may have prevented neuronal loss by indirectly preserving GSH levels, as $O_2^{\bullet-}$ depletes GSH stores by oxidizing it to GSSG (Dikalov *et al.,* 1996; Wink *et al.,* 1997; Jones, 2006). However, the direct reaction between mitochondrial GSH and $O_2^{\bullet-}$ may only become significant when manganese superoxide dismutase (MnSOD) is depleted or inhibited by other RS, as MnSOD has a greater affinity for $O_2^{\bullet-}$ and is present in high concentrations within the mitochondria (Kaplowitz *et al.,* 1985; Beckman and Koppenol, 1996). Interestingly, this may be the case in AD, as patients and transgenic animals have increased levels of nitrated MnSOD, which results in a loss of function (MacMillan-Crow *et al.,* 1996; Aoyama *et al.,* 2000; Anantharaman *et al.,* 2006; Dumont *et al.,* 2009).

Protein nitration appears to be a permanent modification (Radi *et al.,* 1994; Beckman and Koppenol, 1996; Radi *et al.,* 2002); therefore the AD brain will undoubtedly suffer from an accumulation of $O_2^{\bullet-}$ and downstream RS if the loss of MnSOD activity is not compensated. Evidence of mitochondrial $O_2^{\bullet-}$ accumulation in AD is found in the inhibition of metabolic enzymes with iron-sulfur centers, which are major endogenous targets of mitochondrial $O_2^{\bullet-}$ damage (Hoyer and Betz, 1988; Hoyer, 1992; Murphy, 2009). In this study, 3xTg-AD mice

had consistently higher levels of nitrated tyrosine residues in at least two cerebral proteins of approximately 26 and 77 kDa. While analysis of the individual proteins was not performed, it is at least conceivable that the lower band may represent nitrated MnSOD (~24 kDa). MitoQ-treated 3xTg-AD mice had similar levels of nitrated proteins as NonTg mice, suggesting MitoQ blocked $ONOO^-$ formation.

While others have shown the mitochondrial energetic failure and oxidative stress in this AD model (Resende *et al.,* 2008; Yao *et al.,* 2009), we provide the first evidence of nitro-oxidative damage. These protein modifications by RS may be more methodical than typically envisioned (Sultana *et al.,* 2006; Butterfield *et al.,* 2007b; Sultana and Butterfield, 2009a). In relevance to age-dependent neurodegeneration, accumulating mtDNA aberrations during aging and the decline in GSH may initially generate levels of RS that selectively oxidize or nitrate proteins, such as TCA cycle enzymes (Halliwell and Gutteridge, 2007; Gibson and Shi, 2010), in order to engage an adaptive response to the accruing oxidative stress by signaling to slow the supply of electrons to the respiratory chain, thereby reducing their consequent leak to O_2. Mitochondrial RS may further shift metabolism away from OXPHOS by increasing glycolytic enzymes to provide an alternate source of ATP while simultaneously boosting antioxidant defenses by NADPH production (Singh *et al.,* 2007, 2008). In AD, RS may also attempt to rectify mtDNA issues by signaling amyloid precursor protein (APP) and Aβ translocation to the mitochondria, for an as yet unknown, protective function. Within the mitochondria of AD patients, neuronal APP accumulates exclusively in protein import channels (TOM and TIM), but it is relatively absent in age-matched, control mitochondria. If mitochondrial APP or Aβ localization is a protective strategy, the attempt ultimately fails in the AD brain because levels of TOM/TIM-arrested APP directly correlate with mitochondrial dysfunction (Montiel *et al.,* 2006).

Accumulation of mitochondrial Aβ augments RS via binding ABAD or inhibiting complex IV (Canevari *et al.,* 1999; Lustbader *et al.,* 2004; Rhein *et al.,* 2009). The ensuing excess of mitochondrial RS may then damage respiratory complexes and lipids in the IMM (Radi *et al.,* 1994, 2005), release and activate apoptotic machinery, and result in mitochondrial demise, local synaptic degradation, or even neuronal suicide, all of which occur in the early stages of AD (Mosconi *et al.,* 2006, 2008a; Gibson and Shi, 2010).

While MitoQ prevented neuronal death in trophic factor-deprived sympathetic neurons and Aβ-treated cortical neurons in culture, the *in vivo* effect of MitoQ on synaptic or neuronal loss was precluded because these mice, like most other Aβ-generating genetic lines, do not recapitulate the extensive brain atrophy characteristic of human AD (Oddo *et al.,* 2003; Green *et al.,* 2007; Ashe and Zahs, 2010). Another curiosity that warrants direct investigation is whether localization of APP and Aβ to the mitochondria occurs as a cause, consequence, or epiphenomenon in relation to oxidative stress within the AD brain. Since MitoQ treatment abolished all evidence of oxidative stress, MitoQ-treated 3xTg-AD mice could have, ostensibly, provided a means to elucidate this influence. However, analyzing the effect of MitoQ on mitochondrial-Aβ load in the age-group of 3xTg-AD mice in this study was not likely to be a productive endeavor, as appreciable mitochondrial Aβ-accumulation may not occur until at least 9 months of age in 3xTg-AD mice (Yao *et al.,* 2009), and sufficient mitochondrial isolation from mouse brain tissue typically requires substantial material.

Agents that effectively suppress mitochondrial $O_2^{\bullet-}$, such as MitoQ, may not only relieve hypometabolism by preventing further inhibition of metabolic enzymes by $O_2^{\bullet-}$/$ONOO^-$ damage, but may also act to increase cerebral blood flow by preventing the catastrophic conversion of the vasodilator $NO^\bullet$ (~~–t~~he good") to $ONOO^-$ (~~–t~~he ugly") (Beckman and Koppenol, 1996; Aliev *et*

al., 2004; de la Torre, 2010b). An important implication from the fast reaction between $O_2^{\bullet-}$ and $NO^{\bullet}$, is that each antagonizes the biological actions of the other. Hence, therapeutics that increase the level of $NO^{\bullet}$ likely prevent damage due to $O_2^{\bullet-}$, while increased $O_2^{\bullet-}$ will prevent the cardiovascular benefits of $NO^{\bullet}$ (Moncada and Higgs, 1995; Koga *et al.,* 2002). Increased mitochondrial $O_2^{\bullet-}$ would decrease $NO^{\bullet}$-induced vasodilation, thereby decreasing oxygen tension. In such conditions, complex IV uses nitrite instead of oxygen as the terminal electron acceptor, generating more $NO^{\bullet}$ in order to increase oxygen supply to the brain (Halliwell and Gutteridge, 2007). However, in the AD brain, this intrinsic control may be lost, which could be explained by mitochondrial DNA defects or Aβ inhibiting complex IV activity, possibly preventing this shift while generating $O_2^{\bullet-}$. The results provided here suggest that MitoQ could prevent the resulting hypertension by suppressing $O_2^{\bullet-}$ and restoring the level of bioavailable $NO^{\bullet}$. An informative investigation into the ability of MitoQ to prevent the hypometabolism and/or hypofusion that is responsible for decreased cerebral metabolic rate for glucose (CMR_{GLC}) in presymptomatic and early AD stages would also provide great insight into a plausible mechanistic link between failing metabolism and increased oxidative stress in AD progression (Gabuzda *et al.,* 1994; Gibson *et al.,* 1998; Ames III, 2000; Mosconi *et al.,* 2006, 2007, 2008, 2010; Butterfield *et al.,* 2007b; de la Torre, 2008; Li *et al.,* 2008; Liang *et al.,* 2008; Reddy, 2008; Berti *et al.,* 2010; Gibson and Shi, 2010). With continued improvement in neuroimaging resolution and specificity, this study may be optimized in small animals, such as transgenic mice. However, the greatest possible benefit to be gained from such studies awaits the development of a valid animal model that effectively recapitulates the pathogenesis and progression of sporadic AD in aged humans (Trifunovic *et al.,* 2004; Fukui *et al.,* 2007; Ashe and Zahs, 2010).

Bioenergetic Therapy for the Brain

The central nervous system has proved to be a challenging area for drug discovery, which undoubtedly reflects the complexity of both the architecture of the nervous system and the bioenergetic network on which its function relies. The majority of clinical candidates may fail because the etiological hypotheses remain incomplete despite years of investigation prior to testing in patients. AD clinical trials epitomize this exasperating process. Alzheimer's is a devastating disorder that ravages the brain, progressively robbing its victim of their memory, intellect, personality, and eventually, life itself. Although millions of people are diagnosed with AD per year, not a single patient has ever been cured (Association, 2010). When an exorbitant amount of research funding, talent, and time is focused on a problem that appears well-defined, and no solution is made, it is necessary to reexamine the hypothesis. As such, the repetitive failure of the most effective amyloid-reducing therapeutics to prevent AD debilitation suggests that the amyloid hypothesis is insufficient and that there is at least another critical, age-related process pulling the pathological trigger.

The data provided by this study add credence to the genomic, proteomic, and neuroimaging studies that place the mitochondria at the nexus of AD progression (Mosconi et al. 2008; Sultana and Butterfield, 2009b; Swerdlow and Khan, 2009b; Gibson and Shi, 2010; Wallace, 2010). During conditions of neurological stress the level of mitochondrial RS rises, spinning the bioenergetic network into self-destructive disarray. Autocatalytic cycles increase oxidative stress and decrease energy which may damage the mitochondria and propel the cell to death. These perpetuating events become even more complex when considered in the context of the aging Alzheimer brain. The characteristic features of AD include extracellular plaques and intracellular tangles which are generated by aberrant processing and aggregation of amyloid and

tau, respectively. Both of these proteins compromise mitochondrial function and increase RS generation (Casley *et al.,* 2002a; Lustbader *et al.,* 2004; David *et al.,* 2005; Rhein *et al.,* 2009; Yao *et al.,* 2009), while mitochondrial dysfunction and oxidative stress, in turn, regulate the expression and activity of multiple enzymes implicated in their pathological processing (Rissman *et al.,* 2004; Szabados *et al.,* 2004; Petersen *et al.,* 2005; Velliquette *et al.,* 2005; Sultana *et al.,* 2006; Butterfield *et al.,* 2007b; Quiroz-Baez *et al.,* 2009; Sultana and Butterfield, 2009a).

For sporadic AD patients, this cycle may begin with an unfavorable combination of inherited and age-accumulated mtDNA abnormalities that eventually surpass an innate threshold and begin to foster a degenerative phenotype (Wallace, 2005; Swerdlow and Khan, 2009a). mtDNA variability could potentially explain the early metabolic impairment, the predominant maternal influence, and the age-dependence of sporadic AD (Denicola *et al.,* 1996; Schwerk and Schulze-Osthoff, 2003; Kujoth *et al.,* 2005a; Hayashi *et al.,* 2008; Swerdlow and Khan, 2009a; Dai *et al*., 2009; Wei *et al.,* 2009). The most recent data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) study has associated several mtDNA haplotypes with the propensity for AD; however, distinct, etiological mutations or deletions remain to be discovered (Schwerk and Schulze-Osthoff, 2003). We are just beginning to understand the complexity of mtDNA, and effective mitochondrial gene therapy may not be available for some time (Wallace, 2010), placing great need on the development of pharmacological agents that restore bioenergetic and redox balance.

Administration of individual antioxidants and metabolic agents has shown potential as a neuroprotectant strategy in numerous studies, but have frustratingly failed to deliver on that promise in clinical trials for mitochondrial diseases and neurodegenerative conditions (Wallace and Fan, 2009; Wallace *et al.,* 2010). An important point that distinguishes such AD-trial

failures from those of amyloid-therapy, is that the antioxidants and metabolic agents investigated to date do not alleviate mitochondrial dysfunction and oxidative stress in the brain, whereas, amyloid therapeutics effectively remove the amyloid burden, yet still do not alleviate the symptoms. There are several explanations for the lack of efficacy in the former, but these all reduce to one major problem: the agents were simply not sufficient to restore the deviations in the bioenergetic-redox network. Because typical antioxidants and metabolites are constrained by the blood-brain barrier, and those that do enter the brain may never actually reach mitochondria (Petersen *et al.,* 2005; Pham and Plakogiannis, 2005; Halliwell and Gutteridge, 2007; DeKosky *et al.,* 2008), they cannot be expected to elucidate the role of mitochondrial RS in development of this age-dependent neurodegenerative disease.

In order to effectively decipher the sequence and significance of mitochondrial RS in the bioenergetic disruptions and cell death that characterize age-dependent neurodegenerative diseases, this study utilized a recyclable antioxidant that selectively and sufficiently accumulates within the mitochondrial inner membrane (Kelso *et al.,* 2001; Murphy, 2001; James *et al.,* 2005; Halliwell and Gutteridge, 2007; James *et al.,* 2007; Gane *et al.,* 2010). The results define MitoQ as an ideal candidate to decrease the harmful effects of mitochondrial RS in neurodegenerative conditions. While agents that completely eliminate mitochondrial RS may produce unexpected and undesirable results due to the complex interplay within the bioenergetic network (Wallace *et al.,* 2010), this is not likely to be a concern for MitoQ. The antioxidant moiety of MitoQ detoxifies RS through the formation of an ubisemiquinone radical intermediate (Schöpfer *et al.,* 2000; James *et al.,* 2005; James *et al.,* 2007; Maroz *et al.,* 2009). This intermediate is capable of acting as a pro-oxidant as well, and therefore may generate low levels of mitochondrial RS. As evident in this study and others, RS generated by MitoQ do not appear to reach appreciable

levels to cause damage (James *et al.,* 2005; Cassina *et al.,* 2008; Rodriguez-Cuenca *et al.,* 2010) but instead may act to induce protective genes in pathogenic conditions (Manczak *et al.,* 2010).

Current research efforts are providing hope for early detection of individuals at risk for AD. If identified early enough, the current study suggests effective, mitochondria-targeted antioxidants may provide a promising preventative approach to slow the disease process before definitive mitochondrial damage ensues. Based on the pharmacokinetic properties of MitoQ and other similar compounds (Green *et al.,* 2007; Manczak *et al.,* 2010; Rodriguez-Cuenca *et al.,* 2010; Smith and Murphy, 2010), mitochondria-targeted antioxidants may be available for use in most patients. Until then, the best preventative strategies may involve lifestyle changes that enhance mitochondrial and neuronal health, such as physical and mental exercise, and dietary changes (Willis *et al.,* 2006; Circu *et al.,* 2008; Franco and Cidlowski, 2009; Pop *et al.,* 2010).

Physical exercise is useful in the treatment of mitochondrial diseases (Wallace, 2010a), and therefore, may also accommodate mitochondrial perturbations in age-dependent neurodegenerative diseases. Aerobic endurance training increases oxygen utilization (Jeppesen *et al.,* 2006; Taivassalo *et al.,* 2006), while resistance exercise beneficially alters the balance of wild-type versus mutant mtDNA and increases OXPHOS capacity (Taivassalo *et al.,* 1999; Murphy *et al.,* 2008). mtDNA may only be affected in muscle tissue, but the benefits will extend to other organ systems, most notably, the cardiovasculature. As a result, physical exercise may slow AD progression, as some propose AD is not a disease of the brain, but rather a vascular disorder with neurodegenerative consequences (Aleiv *et al.,* 2004; de la Torre, 2010b).

Several cross-sectional, prospective studies of age-dependent dementia and AD reveal that high dietary intake of antioxidants and the use of antioxidant supplements may synergistically reduce the prevalence and incidence of AD (Martensson *et al.,* 1990; Carelli *et*

al., 2004). Unfortunately, the benefit of dietary supplementation pales in comparison with those of caloric restriction. While the mechanisms underlying its ability to extend healthy aging and AD prevention may not be fully realized, caloric restriction appears to increase cellular resilience via modulation of mitochondrial biogenesis, energetics, and RS (Fernando *et al.,* 2002; Kang *et al.,* 2004; Kroemer and Martin, 2005; Haigis and Guarente, 2006). Notably, many of these effects are dependent on an increase in $NO^{\cdot}$ that is stimulated by the anti-aging sirtuin family of proteins (Guarente, 2008). Slightly less painful than starvation, the ketone diet (KD) may provide similar effects on metabolism, longevity, and memory by mitochondria-centered mechanisms (Wallace, 2010). In the hippocampus of KD-fed rats, mitochondrial density increases primarily within synaptic projections, over 93% of mitochondrial proteins increase, mtDNA is more resistant to oxidative damage, and mitochondrial GSH stores double (Bough, 2008; Jarrett *et al.,* 2008; Nylen *et al.,* 2009). If these effects extrapolate to humans, a synthetic form of ketone bodies that avoids the vascular problems associated with the high-fat KD would possess incredible potential as an integrative approach to restore bioenergetics and forestall the onset of sporadic AD in at-risk individuals.

Summary

The signaling pathways described here conjure appreciation of mitochondria as the hub of both genetic and environmental influences that ultimately dictate our vitality. The results from this study strengthen the ever-growing interconnection of mitochondrial dysfunction and oxidative stress in aging and every major pathological event implicated in AD. Therapeutic strategies that prevent declining neuronal mitochondria may be necessary to sustain cognitive function in

advanced stages of life. The research presented here suggests that mitochondrial RS provide an important signal for the orchestration of neurodegeneration, which can be attenuated with effective antioxidants that selectively accumulate in the inner membrane of neuronal mitochondria. While recent studies have shown significant attenuation of the AD phenotype by genetic manipulation of mitochondrial antioxidant status and metabolic function in AD models, to our knowledge, this study is the first to validate mitochondria-targeted pharmaceuticals as an effective and feasible approach to preserve cognitive function and neuropathological progression in a transgenic model of Alzheimer's disease.

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