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Strategies for Preventing Age and Neurodegenerative Disease-associated Mitochondrial Dysfunction

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Strategies for Preventing Age and Neurodegenerative Disease-associated Mitochondrial Dysfunction

by

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DEDICATION

I dedicate this work to my siblings Hana and Haris Delic who continue to be the source of inspiration for every experiment I do. I also dedicate this work to U.S. Army Staff Sergeant (retired) Charles Claybaker for his unwavering support for neurodegenerative research and for his service to this country.

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ABBREVIATIONS

Aß: Amyloid beta
AD: Alzheimer's disease
ADP: Adenosine diphosphate
AMPK: AMP-activated protein kinase
AICAR: Aminoimidazole-4-carboxamide ribonucleotide
ALS: Amyotrophic lateral sclerosis
ARE: Antioxidant response element
α-syn: Alpha-synuclein
ATP: Adenosine triphosphate
βHB: beta-hydroxybutyrate
CBP-1: CREB binding protein-1
Complex I: NADH dehydrogenase
Complex II: Succinate dehydrogenase
Complex III: Cytochrome bc_1 complex or CoQH2-cytochrome c reductase
Complex IV: Cytochrome C oxidase
Complex V: F_oF_1 ATPase
COX: Cytochrome C oxidase
CR: Calorie restriction
CytC: Cytochcrome C
DCF: 2',7'-dichlorodihydrofluorescein diacetate
ETC: Electron transport chain
FCCP: Protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
HEK: Human embryonic kidney cells
HGM: High glucose media
HGPM: High glucose pyruvate media
HGPUM: High glucose pyruvate uridine media
IDH2: Isocitrate dehydrogenase 2
JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolycarbocyanine iodide
MEF: Mouse embryonic fibroblasts
Mfn1: Mitofusin
MMP: Mitochondrial membrane potential
MT1 and MT2: Melatonin receptors
mtDNA: Mitochondrial DNA
NAD: Nicotinamide adenine dinucleotide
NVU: Neurovascular unit
Nrf2: Nuclear factor erythroid 2–related factor 2
NRF-1 and NRF-2: Nuclear respiratory factor 1 and 2
Ntg: Non transgenic

OXPHOS: Oxidative phosphorylation
PD: Parkinson's disease
PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1α
POLG: Polymerase gamma
Q: Ubiquinone
QH2: Ubiquinone
ROS: Reactive oxygen species
SILAC: stable isotope labeling by/with amino acids in cell culture
SOD2: Superoxide dismutase 2
TCA: Tricarboxylic acid cycle
Tg: Transgenic

ABSTRACT

Mitochondrial dysfunction plays a pivotal role in the development of aging phenotypes and aging-associated neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS). Strategies that restore mitochondrial dysfunction may rescue the deficits of central metabolism in these disorders and improve cell survival. For example, we found that modulating the mTOR signaling pathway in a tissue culture model of aging-induced mitochondrial DNA mutation enhanced mitochondrial function as evidenced by increased oxygen consumption. Our previous melatonin studies also led us to hypothesize that caloric restriction and the hormone melatonin would reverse brain mitochondrial dysfunction in animal models of AD. Although caloric restriction did not improve mitochondrial function in a transgenic P301L tau model of AD, novel insight into the regulation of F_0-F_1 ATP synthase activity under CR was gained that may help explain the protective effects of CR in other disease models. In addition, we determined the effects of melatonin treatment on brain mitochondrial cytochrome c oxidase (COX) activity using the transgenic APP_{SWE} mouse model of AD bred to double melatonin receptor (MT1 and MT2) knockout mice. COX activity declined with aging in control mice, but increased with aging in AD mice, most likely as a response to mitochondrial reactive oxygen species (ROS) induced by amyloid-beta generated through APP proteolysis. Both effects were blunted by melatonin treatment. The effects of melatonin were partially dependent on the G-protein coupled melatonin receptorsWe also used PD models to identify therapies that restore mitochondrial dysfunction. We showed that

overexpression of wild-type alpha synuclein (α-syn) in human neuroblastoma M17 cells resulted in mitochondrial oxygen consumption deficits; similar to the levels observed when PD mutant forms (A30P α-syn, E46K α-syn, and, A53T α-syn) were overexpressed. Mitochondria from cells overexpressing α-syn were more sensitive to a high iron environment, mimicking the physiological conditions in which dopaminergic neurons are found. Diethyl oxaloacetate, succinate, and several amino acids were protective, suggesting the possibility for effective dietary interventions for PD. Lastly, we delineated the level of mitochondrial complex IV activity between gray and white matter in human cervical and lumbar spinal cord, as well as mitochondrial aggregation in the entire neurovascular units (NVU) as a consequence of ALS. At the conclusion of these projects a better understanding of the molecular mechanisms leading to mitochondrial dysfunction in AD, PD, ALS, and aging was gained and promising strategies to delay or reverse these dysfunctions were developed.

CHAPTER ONE: INTRODUCTION

Mitochondria are maternally inherited organelles with a bacterial origin. Mammals contain their own circular ~ 16,500 bp mitochondrial DNA genome, which replicates independently. Mitochondria undergo dynamic processes of fusion and fission. The vast majority of genes necessary for mitochondrial function have been purged from the mitochondrial genome. These genes were either transferred to the nuclear genome or were lost as the endo-symbiotic relationship between proto eukaryotic and proto mitochondrial cells developed. Mitochondria are found in almost all eukaryotes, from single celled eukaryotic organisms, to plants and animals, where they serve as the primary source of cellular energy production. The primary function of mitochondria is the production of intracellular adenosine triphosphate (ATP), the energy currency of the cell. Although some ATP is produced via glycolysis and substrate level phosphorylation, the bulk of ATP is produced through oxidative phosphorylation (OXPHOS). Other important functions performed by mitochondria include iron-sulfur cluster biogenesis, activation of stress responses, regulation of cell death, and intracellular signaling. In this chapter we provide an overview of mitochondrial function. We also provide a review of the role of mitochondrial dysfunction in the progress of aging, amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease.

Mitochondrial electron transport chain and oxidative phosphorylation

Following the breakdown of glucose in glycolysis, the resulting 2 molecules of pyruvate are decarboxylated, oxidized, and bound to coenzyme A. This is accomplished by the pyruvate dehydrogenase enzyme and results in the formation of acetyl-CoA. Similarly, during beta-oxidation of fatty acids, acetyl groups are bound to a coenzyme A yielding acetyl-CoA. A condensation reaction catalyzed by citrate synthase breaks the high energy thioester bond between the acetyl group and coenzyme A, and condenses the acetyl group with a molecule of oxaloacetate to form citrate. This is the first step in the tricarboxylic acid cycle (TCA) (Krebs and Johnson 1980). The subsequent series of oxidations of the intermediates by nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), and decarboxylations, yield metabolic intermediates and high energy carrier molecules NADH and $FADH_2$. The metabolic intermediates feed into important anabolic reactions such as nucleotide, fatty acid, and amino acid biosynthesis. NADH shuttles high energy electrons and hydrogens to the electron transport chain (ETC). The TCA cycle reactions are summarized in Figure 1.

The ETC is made up of enzymatic complexes I, II, III, and IV (Hederstedt and Rutberg 1981; Weiss, Friedrich et al. 1991; Benit, Lebon et al. 2009; Srinivasan and Avadhani 2012), that are involved in redox reactions. NADH molecules are oxidized by complex I (NADH:ubiquinone oxidoreductase), shuttling hydrogen ions (H^+) out of the mitochondrial matrix and into the intermembrane space. In addition to proton translocation from the matrix to the intermembrane space, electrons are also passed onto the ubiquinone molecule. Of the 37 mitochondrial genes only 13 code for peptides of ETC complex I, III, IV, and V, the rest code for tRNA or rRNA molecules used for mitochondrial translation.

To date 45 proteins have been identified that make up the ETC complex I (Carroll, Fearnley et al. 2006). Of the 45 proteins, 14 are conserved catalytic subunits, essential for oxidation/reduction reactions (Hirst 2013). The 14 conserved subunits form a hydrophobic domain in the inner mitochondrial membrane and a hydrophilic domain. The hydrophilic domain extends into the mitochondrial matrix and contains subunits that interact with NADH, while the 7 hydrophobic subunits are essential for ubiquinone binding and transfer of electrons from NADH (Nakamaru-Ogiso, Han et al. 2010). The soluble conserved subunits are encoded in the nuclear DNA while the hydrophobic subunits are encoded in the mitochondrial DNA (Yagi and Matsuno-Yagi 2003; Brandt 2006). The other 31supernumerary proteins are also encoded in the nuclear DNA (Hirst 2013). These supernumerary proteins vary by species, and likely play an essential role in the stability of complex I, since mutations in their genes can cause diseases (Valsecchi, Koopman et al. 2010). The most common complex I disorders are however caused by mutations in the conserved regions. These disorders include Leber's hereditary optic neuropathy (LHON), Leigh syndrome (LS), and mitochondrial encephalomyopathy with stroke-like episodes (MELAS) (La Morgia, Caporali et al. 2014). At 45 subunits and 1,000 kDa in size, complex I is the single largest ETC complex (Hirst 2013). Further study is ongoing and warranted to determine the involvement of complex I in mitochondrial dysfunction of hereditary and non-hereditary neurodegenerative disorders.

Complex II (succinate dehydrogenase) oxidizes succinate into fumarate, in a flavin adenine dinucleotide (FAD)-dependent manner. Unlike complex I, no protons are translocated across the inner membrane by complex II. Protons harvested at complex II are used to reduce ubiquinone. Complex II is composed of 4 nuclear-encoded subunits, which work in concert to oxidize succinate using FAD and shuttle protons and electrons via iron-sulfur clusters to

ubiquinone. Mutations in complex II subunits contribute to LS, mitochondrial encephalomyopathy, optic neuropathy, and tumor formation. Complex II has been described as a tumor suppressor (Selak, Armour et al. 2005)

The electrons are transferred to lipid soluble ubiquinone molecules, which carry the electrons (e-) to complex III (cytochrome bc_1 complex) where they are transferred to cytochrome c. Complex III is a multi-subunit protein complex with both mitochondrial DNA and nuclear DNA encoded proteins. Complex III is composed of 11 subunits, of which cytochrome b is the only subunit encoded by mitochondrial DNA (Iwata, Lee et al. 1998). Complex III oxidizes ubiquinol back to ubiquinone, and in the process translocates 4 protons from the matrix to the intermembrane space. In the process complex III reduces cytochrome c.

Mutations in the genes coding for Complex III subunits can result in a deficits ranging from exercise intolerance to encephalomyopathy.

Cytochrome c, a small soluble heme-containing protein in the intermembrane space, shuttles electrons from complex III to complex IV, where they are transferred onto a molecule of O_2. By addition of e- to molecular oxygen, two more hydrogens are consumed from the matrix, and two molecules of water are generated. Complex IV (cytochrome c oxidase) is the last complex in the mitochondrial ETC. It is composed of 14 subunits, 11 of which are encoded in the nuclear DNA and 3 of which are encoded by the mitochondrial DNA (Balsa, Marco et al. 2012). Complex IV oxidizes cytochrome c by sequentially transferring electrons onto oxygen, reducing it to water, consuming hydrogens in the process. It also translocates 4 additional hydrogens from the matrix into the intermembrane space. Mutations associated with nuclear encoded subunits may cause LS and cardiomyopathy. Genetic studies in yeast show that most complex IV deficits are due to mutations in nuclear-encoded genes (Zee and Glerum 2006).

Movement of H^+ out of the matrix creates both an electrical and a chemical gradient across the inner mitochondrial membrane. The electrical and chemical contribution to energy available for work is summarized with the proton motive force (Nernst) equation $\Delta G = -2.303\ RT\ \Delta pH + nF\Delta\psi_m$. Where ΔG is Gibbs free energy, $\Delta\psi_m$ is the electrical membrane potential, F is the Faraday constant, R is the gas constant, and T is the temperature. ΔpH indicates the difference in the concentration of protons inside the mitochondrial matrix compared to the intermembrane space. This energy is utilized to convert adenosine diphosphate (ADP) into ATP by the F_0-F_1-ATP synthase or complex V.

Complex V is a large 16 subunit complex responsible for the bulk of ATP synthesis in eukaryotic cells. At 600 kDa it is second only to complex I in size and complexity (Walker, Lutter et al. 1991). It is an F type ATPase, with two main components, the matrix facing F_1 catalytic component and the proton pump F_o component. F_o consists of a rotating cylinder made up of multiple c protein subunits, which bind hydrogen. Two other subunits of F_o are the a and b subunits, which help secure the F_o to the catalytic F_1. As the F_o turns due to hydrogen binding and proton motive force, it turns the gamma stock, projecting from its center (which itself is part of F_1). This conformational change then induces one beta subunit at a time to bind ADP and inorganic phosphate and synthesize ATP. The F_1 multi-subunit complex is a trimer of alpha-beta dimers whose beta subunits cycle through open and closed conformation and catalyze ATP synthesis. In order to generate one molecule of ATP, approximately four hydrogens need to be translocated into the matrix through the ATP synthase (Turina, Samoray et al. 2003). The ratio of H^+/ATP likely varies under different cellular requirements. Except for 2 mitochondrial DNA encoded subunits (A6 and A8), the rest of the complex V subunits are encoded by the nuclear

DNA (Schon, DiMauro et al. 2012) Mutations in either the mitochondrial genes or nuclear genes coding for complex V can cause neonatal death and encephalopathy.

For a graphical summary of the ETC please see Figure 2. The nascent ATP molecule is either consumed in the matrix or is transported out of the mitochondria for use in the cytosol via the adenine nucleotide transporter, which transports 1 molecule of ATP across the inner mitochondrial membrane to the inner membrane space for every 1 molecule of ADP it brings in to the matrix (Liu and Chen 2013). Damage to TCA cycle enzymes or ETC complexes can result in profound energy and anabolic intermediate deficits. This makes highly dynamic and energy demanding cells like neurons more sensitive to mitochondrial damage.

Mitochondrial fusion and fission

Mitochondria replicate by fission and combine to exchange membrane, metabolites, and proteins by fusion. Fission is mediated by a conserved cytosolic GTPase Drp1. This protein is conserved in flies, worms and mammals. Drp1 attaches to the outer membrane of the mitochondria and constricts the mitochondria, severing both the inner and outer membrane. Mitochondrial fusion is facilitated by 3 enzymes: Mitofusin-1 (Mfn1), Mitofusin-2 (Mfn2), and Opa1. Mfn1 and Mfn2 facilitate fusion of the outer membrane between two mitochondria, while Opa1 governs the fusion of inner membranes. The processes are regulated by post-translational modifications and proteolysis (Hoppins, Lackner et al. 2007). Mitochondrial fission and fusion are necessary for dividing and non-dividing cells, i.e. neurons. Defects in mitochondrial fusion result in neurological problems like dominant optic atrophy and Charcot Marie Tooth disease type 2A (Youle and van der Bliek 2012). Single mitochondria contain multiple copies of their

genome, and at any time a mitochondrion can contain both normal copies and copies that have damage or mutations. This presence of both types of mitochondrial DNA in one cell is called heteroplasmy. In order to compensate for DNA damage, mitochondria fuse and exchange proteins, membrane, and RNA, but not DNA, a process called transcomplementation (Schon and Gilkerson 2010). Failure of proper mitochondrial fusion and fission can lead to aggregation of mitochondria, formation of mega-mitochondria, and eventual cytochrome c release and cell death signaling. The formation of mega-mitochondria is a structural change that is different from simple mitochondrial swelling. It is a last attempt of mitochondria to decrease ROS production at the organelle level before the cell undergoes apoptosis. The mega-mitochondria produce significantly less ATP and consume less oxygen in an attempt to lower ROS production (Wakabayashi 2002).

Cell signaling, activation of a stress response, and iron metabolism

Mitochondrial function extends beyond its roles as the powerhouse of the cell. It is essential in intracellular signaling, activation of a stress responses, and heme metabolism.

Mitochondrial cell signaling

Proper signaling from the nucleus to the mitochondria (anterograde signaling) and from the mitochondria to the nucleus (retrograde signaling) is essential for proper mitochondrial and overall cellular function. Proteins involved in the TCA cycle, fatty acid beta oxidation, mitochondrial protein trafficking, mitochondrial transcription, and DNA replications are encoded in the nucleus (Dimmer, Fritz et al. 2002; Bolender, Sickmann et al. 2008). Transcription of mitochondrial genes coded in the nucleus is under tight control. Specifically, by the nuclear

respiratory factors 1 and 2 (NRF-1, NRF-2) that are involved in the regulation of mitochondrial biogenesis, and quality control (Kelly and Scarpulla 2004). NRF-1 also controls the transcription of mitochondrial transcription factor A, TFAM, which is essential for both mitochondrial DNA replication and transcription (Kelly and Scarpulla 2004) . This suggests that nuclear NRF-1 has a high degree of control over mitochondrial DNA replication and transcription.

NRF-1 and NRF-2 are activated by a transcriptional co-activator that is responsive to nutrient availability and exercise. This transcriptional co-activator is the peroxisome proliferator-activated receptor gamma (PPAR-γ) coactivator-1 alpha (PGC-1α) (Puigserver, Wu et al. 1998; Wu, Puigserver et al. 1999). The gene coding for PGC-1 alpha, has a cyclic AMP response element, CRE, in its promoter region, which is under the transcriptional control of CRE binding protein, CREB. Transcription is also controlled by a coactivator CREB binding protein CBP. Cyclic AMP is generated by the adenylyl cyclase enzyme from ATP, after adenylyl cyclase is activated by a G protein coupled receptor following receptor stimulation by nutrients, hormones, or neurotransmitters.

It is important to distinguish between NRF-1, NRF-2, and nuclear factor related factor 2 (Nrf2). Nrf2 is often incorrectly spelled as NRF2, while NRF-1 and NRF-2 are frequently incorrectly spelled without the dash further adding to the confusion. While NRF-1 and NRF-2 are transcription factors increasing the transcription of complexes of the ETC in mitochondria (Scarpulla 1997), Nrf2 is a transcriptional activator of nuclear encoded genes containing the antioxidant response element (ARE), and code for proteins involved in ROS stress response and xenobiotic metabolism (Nguyen, Nioi et al. 2009). This distinction is essential, considering that both activation of NRF-1, NRF-2, and Nrf2 can occur under the same conditions (Lerner, Bitto et al. 2013). For example, Lemer *et al*. showed that inhibition of the molecular target of rapamycin

(mTOR) resulted in increased expression of mitochondrial ETC complexes as well as increased expression of antioxidant response element containing proteins. Rapamycin was discovered in the 1970s in a soil sample from Easter Island, also called Rapa Nui, from which rapamycin was named. Rapamycin is produced by *Streptomyces hygroscopicus* and has been found to have antifungal properties (Vezina, Kudelski et al. 1975). Subsequent studies have shown that rapamycin resistance is conferred by mutation in any of the conserved regions of the TOR1 and TOR2 genes in yeast, which are necessary for the drug to effectively bind (Heitman, Movva et al. 1991). Inhibition of mTOR results in G1 cell cycle arrest in yeast and in mammalian T-lymphocytes (Magnuson, Ekim et al. 2012). The reason for cell cycle arrest are due to the pleotropic downstream effects resulting from the inhibition of the master metabolic sensor mTOR, including stimulating autophagy and inducing a lack of ribosomal activation by phosphorylation of the ribosomal S6K subunit.

Retrograde signaling from mitochondria to the nucleus governs stress response, differentiation, and maintenance of stem cell populations (Xu, Duan et al. 2013). Mitochondrial DNA damage can lead to profound energy and metabolic intermediate deficits. It is essential that genes necessary for a stress response, mitochondrial biogenesis, and repair are activated by proper retrograde and anterograde communication between the nucleus and the mitochondria.

Mitochondrial stress response

If mitochondrial damage is great enough, mitochondria can induce apoptosis by the release of cytochrome c into the cytosol. Cytochrome c (cytC) is released into the cytosol following ROS-mediated oxidation of cardiolipin, an inner mitochondrial membrane phospholipid (Orrenius and Zhivotovsky 2005). Cardiolipin normally anchors cytC to the outer

leaflet of the mitochondrial inner membrane. Cytoplasmic cytC interacts with endoplasmic reticulum inositol trisphosphate receptors, which in turn leads to Ca^{2+} release. Excessive Ca^{2+} levels lead to even higher levels of cytC release from mitochondria. In the cytoplasm cytC stimulates caspase-mediated apoptosis.

To prevent ROS-induced apoptosis, Nrf2 is translocated from the cytoplasm into the nucleus where it initiates transcription of ROS responsive genes. Under normal conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) and sequestered in the cytoplasm (Itoh, Wakabayashi et al. 1999). Possible mechanisms initiating this translocation are the interaction of ROS with cysteine sulfhydryl groups in Keap1 and subsequent Nrf2 phosphorylation by MAPK or PI3K (Kim, Cha et al. 2010). Once in the nucleus Nrf2 forms a heterodimer with a small Maf protein (sMaf) and binds to antioxidant response elements (ARE) 5'-GTGACNNNGCN-3' in the promoter region of genes coding for proteins essential for ROS detoxification (Itoh, Wakabayashi et al. 1999; Kensler, Wakabayashi et al. 2007). Some of the proteins include catalase, glutathione-S-transfereases, peroxiredoxin I, and superoxide dismutase (Lee and Johnson 2004; Zhu, Itoh et al. 2005).

ROS-mediated damage to ETC complexes can lead to alterations in ATP synthesis resulting in decreased ATP production. Ahigh ADP/ATP ratio can activate AMP-activated protein kinase (AMPK), which can in the short term stimulate catabolic pathways and in the long term activate mitochondrial biogenesis through PGC1-α or stimulate autophagy by inhibition of mTOR (Garcia-Roves, Osler et al. 2008; Egan, Shackelford et al. 2011). Like mTOR, AMPK is another sensor of cellular metabolic needs. In addition to AMP, AMPK can be activated by 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR). AICAR is an intermediate in *de novo*

nucleotide biosynthesis. These examples of retrograde signaling are essential for maintaining proper mitochondrial function and cellular bioenergetics.

NAD^+ can directly activate poly (ADP-ribose) polymerase 1 (PARP1), which stimulates nuclear DNA repair and induces transcription of NRF-1 (Hossain, Ji et al. 2009; Thomas and Tulin 2013). Other retrograde signaling pathways (such as those activated by calcium influx, calcineurin, and hypoxia induced factor-1 alpha (HIF-1α)) exist, but were not explored in the following studies.

As mentioned above succinate dehydrogenase has been previously described as a tumor suppressor (Selak, Armour et al. 2005). Under normal conditions, succinate is oxidized to fumarate. However, if damage or mutation to succinate dehydrogenase occurs, excess succinate is released into the cytoplasm, where it can inhibit hypoxia-inducible factor-1α (HIF-1α) prolyl hydroxylase, which results in HIF-1α stabilization and oncogenic signaling. HIF-1α responds to hypoxic environments and activation can result in angiogenesis. Abnormal signaling due to inefficient succinate dehydrogenase activity, therefore, can result in tumor formation.

Iron metabolism

Iron is essential for proper enzymatic activity in the cytoplasm and nearly every organelle of the cell including mitochondria (Sheftel, Stehling et al. 2010). Iron's ability to readily undergo a change in oxidative state is why it is so essential in many redox enzymes. It is also the reason why inappropriately transported and sequestered iron can contribute to many neurodegenerative diseases, including Parkinson's disease, by participating in Fenton chemistry. During the Fenton reaction hydrogen peroxide is converted to the hydroxyl radical, by interacting with Fe^{2+}. To avoid generation of ROS, iron is sequestered by proteins or protein cofactors, as iron-sulfur

clusters or heme functional groups. Proper iron sequestration is essential, since heme synthesis and iron sequestration take place in the mitochondria. Mitochondria are also the largest producer of intracellular hydrogen peroxide from monoamine oxygenase activity and superoxide dismutase activity.

Iron is brought into the cell via transferrin receptor 1 (TfR1)-mediated endocytosis and is directed to mitochondria for further processing (MacKenzie, Iwasaki et al. 2008). The endosome is acidified reducing the pH to 6, which releases iron from Tf, allowing it to bind to divalent metal transporter (DMT1) after the Fe^{3+}, now free from Tf, is reduced by endosomal membrane bound ferric reductase (Steap3) (Ohgami, Campagna et al. 2005). The endosome fuses with mitochondria in a "kiss-and-run" model delivering iron to the intermembrane space for further processing (Sheftel, Zhang et al. 2007). Fe^{2+} is transported into the matrix via mitoferrins, iron transporters (Shaw, Cope et al. 2006). Once in the matrix, Fe^{2+} can either be sequestered (into mitochondrial ferritin oligomers), incorporated into heme groups, or into iron-sulfur clusters (Fe-S clusters). For detailed reading on Fe-S clusters and heme biosynthesis in mammals we direct readers to the following reviews (Ajioka, Phillips et al. 2006; Lill and Muhlenhoff 2008). Dysfunctional endosomal transport of iron to mitochondria can predispose the cell to excessive ROS, as is suggested from much Parkinson's disease research.

Roles of mitochondria in aging and neurodegenerative diseases

Mitochondrial dysfunction is a major contributor to the progress of neurodegenerative diseases, for which no cures or effectives treatments exist. Mitochondrial dysfunction is also a well-established contributor to the normal progress of biological aging. The mechanisms by which mitochondrial dysfunction contributes to the progress of these diseases, and methods by

which this dysfunction can be ameliorated remain incompletely understood. This lack of knowledge is underscored by the ongoing research into mitochondrial dysfunction in diseases such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (ALS). Here we propose mechanisms by which mitochondrial dysfunction occurs in Alzheimer's disease models, Parkinson's disease models, and in a novel *in vitro* model of mitochondrial mutation and age-related mitochondrial dysfunction. We also explored metabolic and nutritional approaches that could potentially improve mitochondrial dysfunction and improve outcomes of these diseases. Additionally, we speculate on causes of mitochondrial dysfunction in the spinal cord of ALS patients. In this chapter we will discuss the role mitochondria play in each of these diseases followed by chapters detailing the research performed, the results, and a short discussion of the relevance of the research. Lastly, translational approaches and clinical applications will be discussed.

Aging and mitochondria

Mitochondrial dysfunction has been shown to contribute to human phenotypes of aging, which include alopecia, neuronal degeneration, kyphosis, and sarcopenia (Marzetti, Calvani et al. 2013), and to age related neurodegenerative diseases (Osiewacz 2010). Recently, these phenotypes were recapitulated in a premature aging mouse model (Kujoth, Hiona et al. 2005; Loeb, Wallace et al. 2005). In this model, the exonuclease activity of the mitochondrial DNA polymerase γ (POLG) was knocked out ($POLG^{D257A/D257A}$), resulting in a premature adult onset presentation of aging phenotypes. We have used immortalized mouse embryonic fibroblasts (MEFs) from these mice as a model to study the efficacy of mTOR inhibition or AMPK activation, on delaying mitochondrial dysfunction in these cells to get a better understanding of

what effect these agents might have on the aging phenotypes of the mice. AICAR and rapamycin are known modulators of AMPK and mTOR respectively. As previously discussed, inhibition of mTOR or activation of AMPK in some cell types can improve mitochondrial function. Inhibition of mTOR has also been shown to extend life in a heterogeneous population of mice (Harrison, Strong et al. 2009). We also determined how these modulators differentially affected mitochondrial function when glucose and pyruvate levels in the culture media were varied.

Alzheimer's and mitochondria

Hyperphosphorylated tau protein and improperly processed amyloid precursor protein are well established contributors to the progress of Alzheimer's disease and mitochondrial dysfunction (Villemagne, Ataka et al. 2009; Cohen, Guo et al. 2011; Huang and Mucke 2012; Garcia-Escudero, Martin-Maestro et al. 2013). Amyloid-beta is a product of sequential processing of amyloid precursor protein (APP) by beta and gamma secretases. Several mutations in either the gene coding for APP, presenilin-1 (*PSEN1*) or presenilin-2 (*PSEN2*) result in increased amyloid-beta formation due to improper processing of APP. Presenilin proteins comprise a subunit of the gamma secretase complex. These findings and others have led to the dominant hypothesis that in the field of Alzheimer's research coined the "amyloid cascade" hypothesis by Hardy and Higgins (Hardy and Higgins 1992). The exact role of APP is unknown, but its detection early in embryogenesis suggests it is important in early neuronal development (Ott and Bullock 2001). APP knockout mice are viable but show mild neurological deficits, suggesting that APP is important in maintaining normal neuronal function (Dawson, Seabrook et al. 1999).

The tau protein functions to stabilize microtubule networks within the cells. It has several serine/threonine and tyrosine sites at which it can be phosphorylated. Hyperphosphorylation creates neurofibrilary tangles that impede intracellular trafficking, including that of mitochondria. Several mutations in APP have been found that result in improper amyloid processing but no mutations in tau exist that cause Alzheimer's disease. Hyperphosphorylated tau is none the less a hallmark of AD pathology. Since the discovery of the APP mutants, and their contribution to early onset AD, much drug discovery research has been focused on amyloid-beta clearance. Familial forms of frontotemporal dementia and Parkinsonism, such as the form caused by a tau P301L missense mutation result in tau hyperphosphorylation, and AD-like pathology (Mirra, Murrell et al. 1999). Mouse models of this FTD have been created and can be used to advance neurofibrillary tangle therapeutic research.

Calorie restriction has been shown to be the only robust method to extend the lifespan and health span in multiple species including rodents and non-human primates (Weindruch, Walford et al. 1986; Colman, Beasley et al. 2014). This includes delaying the age of onset for many neurodegenerative diseases. Whether CR accomplishes this by improving global mitochondrial function in the brain by improving mitochondrial function in a specific subpopulation of cells, or by improving cognitive function in a mitochondria- independent manner remains incompletely understood (Lee, Seroogy et al. 2002; Levenson and Rich 2007) . Therefore, it is of interest to determine if CR can delay hyperphosphorylation of tau or improve mitochondrial dysfunction induced by hyperphosphorylated tau.

Parkinson's disease and mitochondria

Parkinson's disease (PD) is a movement disorder brought on by degeneration and death of dopaminergic neurons in the substantia nigra pars compacta region of the brain. The alpha-synuclein (α-syn) protein aggregates with other proteins to form Lewy bodies, which have been found in post mortem tissue of PD patients and have been hypothesized to be a major contributor to the progression of PD. Several mutations in the SNCA (α-syn) gene have been shown to result in familial form of PD. These include the A30P, A53T, and E46K α-syn mutations and they continue to be studied (Mbefo, Fares et al. 2015). Mutations in other genes can also contribute to the development of PD. These include mutations in leucine-rich repeat kinase 2 (LRRK2), PARK2 (Parkin), PTEN-induced putative kinase 1 (PINK1), DJ-1, and ATP13A2 (Kitada, Asakawa et al. 1998; Davie 2008). PINK1 and LRRK2 are associated with mitochondrial function and mitochondrial quality control. Pesticides, rotenone, paraquat, and exposure to heavy metals have been implicated in the development of sporadic PD (sPD) (de Lau and Breteler 2006; Nistico, Mehdawy et al. 2011; Tanner, Kamel et al. 2011; Moretto and Colosio 2013). These environmental factors could contribute to the onset of PD, without the presence of a single causative mutation, which is the case in the late onset sporadic form of PD. The substantia nigra region of the brain is a rather harsh cellular environment, due to the presence of high levels of iron and frequent calcium transients. Exposure to pesticides and herbicides, which are powerful oxidants, can expose dopaminergic neurons to excessive ROS levels, resulting in their death.

Although no mutations in α-syn have been shown to cause sporadic PD, Lewy bodies are present nonetheless. Recently, a dinucleotide polymorphism found in the promoter region of the α-syn gene (SNCA) has been linked to a sporadic form of PD (Chiba-Falek and Nussbaum 2001; Maraganore, de Andrade et al. 2006). This polymorphism results in higher expression of α-syn

and predisposition to PD. Overexpressed α-syn oligomerizes and may create amyloid pores, which can interfere with endosomal iron transport and mitochondrial iron metabolism as discussed above (Volles and Lansbury 2002; Maries, Dass et al. 2003) and also may alter cellular Ca^{2+} homeostasis. This disruption of cation homeostasis would result in increased ROS production, accelerated by Fenton chemistry. Excessive ROS can damage mitochondrial ETC complexes and enzymes involved in the tricarboxylic acid (TCA) cycle. It would be interesting to determine if mitochondrial susceptibility in the substantia nigra in PD is caused by cellular mitochondrial superoxide dismutase (SOD2) mislocalization and degradation. This has been reported in prion disease, which in many ways parallels the pathophysiology of PD (Sinclair, Lewis et al. 2013). SOD2 mislocalization could contribute both to the decrease of energy production and to the inability of mitochondria to provide metabolic intermediates necessary for anabolic reactions. Essential amino acid supplementation and strategies aimed at restoring these catabolic and anabolic deficits remain unexplored as potential therapeutic approaches for PD. Their use as potential therapeutics is particularly attractive since they are generally regarded as safe (GRAS) by the food and drug administration (FDA). Ester forms of amino acids and TCA cycle intermediates might be even more effective, since they would not require active transport to cross the blood brain barrier. Since amino acids are frequently co-transported with other amino acids, treatments with multiple amino acids could prove to be synergistic. Furthermore, transporters of essential amino acids serve as sensors and increase transcription of other amino acid transporters, which might further increase the effectiveness of this therapeutic approach.

ALS and mitochondria

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease resulting in motor neuron degeneration and eventual death. Mitochondrial dysfunction is a well-established contributor to the progress of ALS (Shi, Gal et al. 2010). Much of the focus of ALS research has been neurocentric, since mitochondrial dysfunction is indeed apparent in the upper and lower motor neurons. In particular, two competing hypothesis have emerged, the die back and die forward hypotheses(Baker 2014). The die back hypothesis, which states that in ALS pathogenesis, death is caused first by events at the neuromuscular junction, and the die forward hypothesis, states that neuronal degeneration originates in the neuronal cell body, located in the spinal cord or motor cortex. Both of these hypotheses can be reconciled with respect to mitochondrial function. In either case mitochondrial dysfunction can occur, contributing to energy deficits and neuronal cell death. However, this narrow view does not consider the interaction of neurons, glia, and blood vessels as a whole. Mitochondrial dysfunction could also contribute to collapse of the entire neurovascular unit. Furthermore, degeneration of the phrenic nerve, which originates in the cervical spine (C3-C6), results in the earliest ALS deaths. Loss of diaphragm innervation is the main cause of ALS related-death. We hypothesized that mitochondrial dysfunction could be more severe in the cervical spinal cord than the lumbar region. Therefore we initiated studies to delineate any differences between mitochondrial dysfunction in the cervical vs. lumbar spine, and focused on the role of mitochondrial dysfunction in the entire neurovascular unit.

In the following studies we determined 1) the effects of calorie restriction (CR) on P301L tau Alzheimer's mice mitochondria, 2) the effects of melatonin on mitochondrial complex IV activity in young, aged, and in an amyloid-beta producing Alzheimer's mouse model. We 3)

determined the effects of altered glucose and pyruvate levels on mitochondrial electron transport chain (ETC) function following AICAR or rapamycin treatment of transformed murine embryonic fibroblasts (MEFs) from wild-type and homozygous mitochondrial DNA mutator ($POLG^{D257A/D257A}$) mice. We also 4) characterized an α–syn overexpressing human neuroblastoma cell line for mitochondrial dysfunction and used it to screen dozens of metabolic intermediates for mitochondrial protection. And most recently, we 5) determined mitochondrial ETC alterations in postmortem spinal cord tissue from ALS patients. From studying mitochondrial function in these diverse disease models, we have gained a better understanding of electron transport chain dysfunction and the most appropriate ways to ameliorate mitochondrial dysfunction to delay the onset and progression of disease.

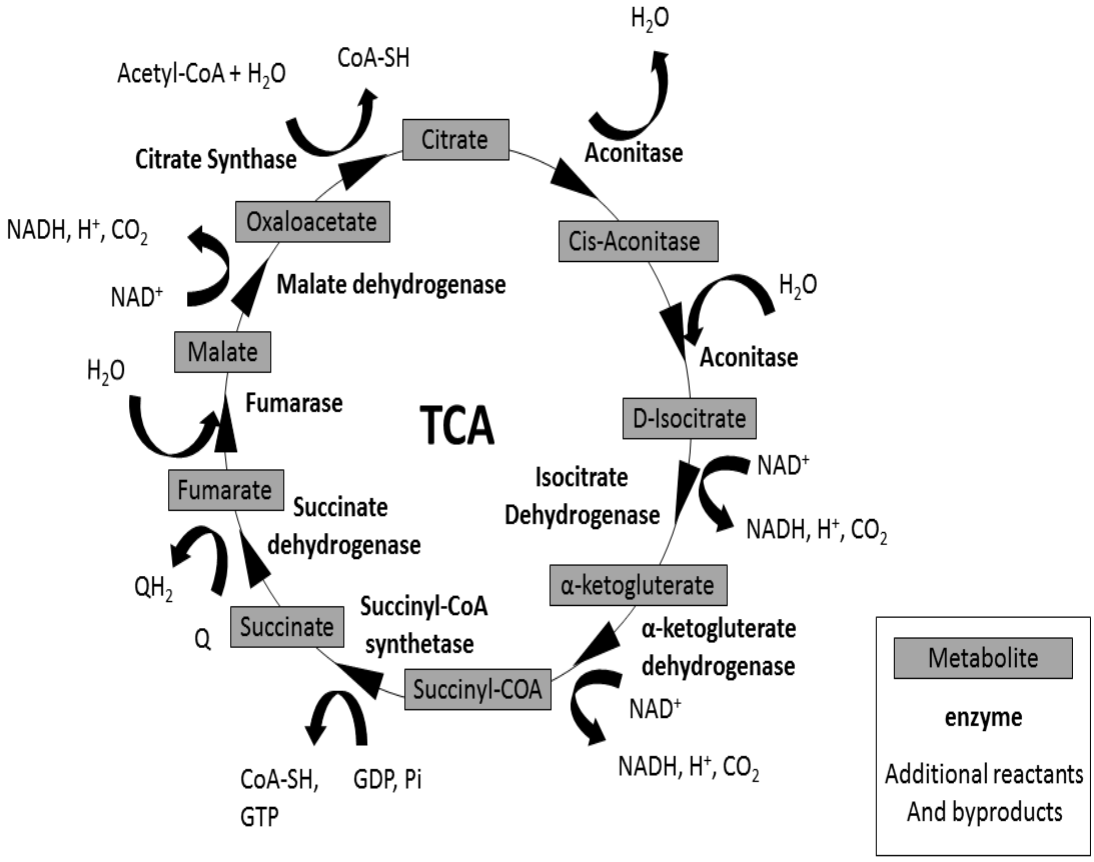

Figure 1 Tricarboxylic acid cycle diagram
Diagram of the tricarboxylic acid (TCA) cycle above. Gray boxes indicate metabolite produced by enzymes in bold. Arrows indicate oxidation of metabolic intermediates and formation of the reduced high energy carriers. The triangles indicates the normal direction of the cycle.

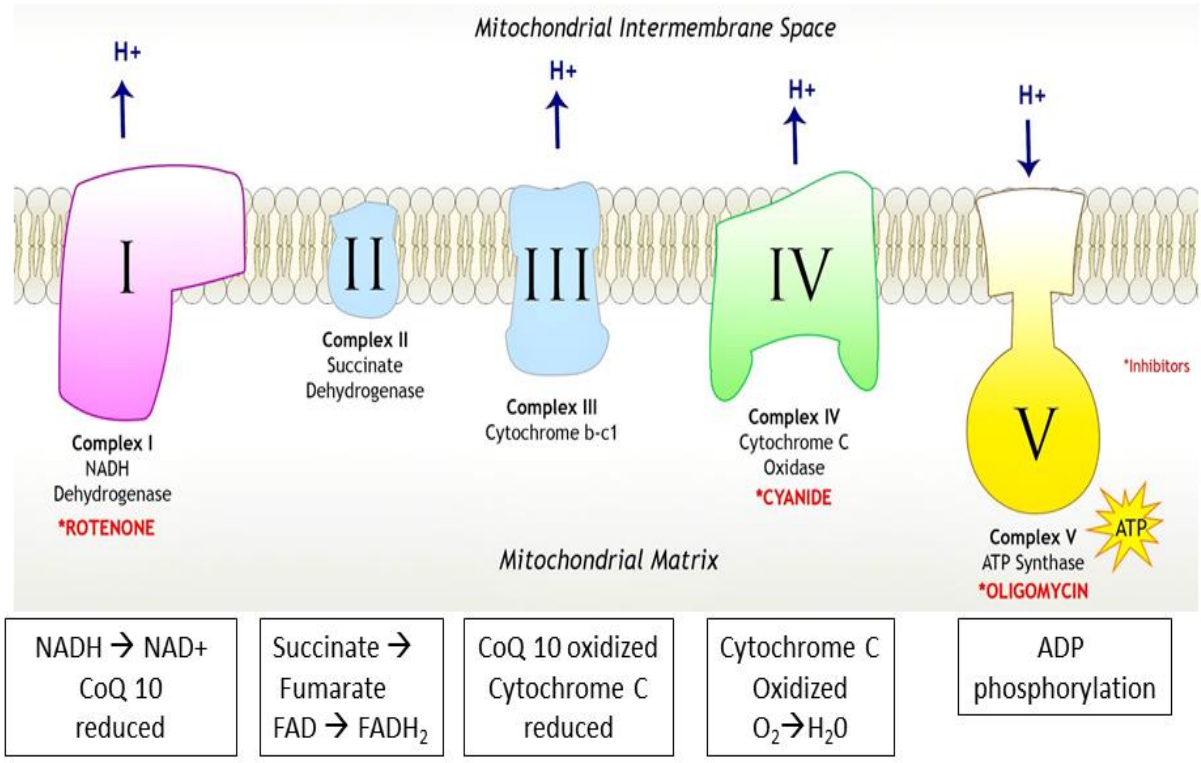

Figure 2 Electron transport chain diagram

The diagram above shows the four multi-protein complexes that make up the electron transport chain, and complex V F_oF_1-ATPase. Complexes I-IV use the flux of high energy electrons obtained from the TCA cycle to drive the vectoral translocation of H^+ from the mitochondrial matrix to the mitochondrial intermembrane space. This process generates the electro-chemical gradient that is then used by complex V to generate ATP.

Descriptive statistical analysis

For the following studies descriptive statistics such as mean, standard deviation, standard error of the mean along with 95% confidence intervals were determined. Means were compared using a two sample student's t-test. A normality test (Shapiro-Wilk) was performed. Equal variance tests were also performed. Analysis of variance using one-way or two-way ANOVA was performed where appropriate. When normality assumptions failed, conservative Bonferroni corrections were performed. All the statistical analyses were performed using Sigmaplot software.

Outcomes of the following studies

In these studies, strategies to prevent or reverse age or neurodegenerative disease-induced mitochondrial dysfunction were explored. These strategies included: calorie restriction, melatonin treatment, supplementation with metabolic intermediates, and modulation of carbon sources along with activation of AMPK or inhibition of mTOR.

In two mouse models of Alzheimer's disease (APP/PS1 and P301L tau) calorie restriction and melatonin treatment were studied. In a cell culture model of sporadic Parkinson's disease, supplementation with metabolic intermediates was studied. In a cell culture model of age-related mitochondrial dysfunction we explored how modulating carbon sources will affect mitochondrial function following pharmacological activation of AMPK or deactivation mTOR. Of these strategies, melatonin treatment and supplementation with metabolic intermediates were found to be the most effective against Alzheimer's disease and Parkinson's disease respectively.

CHAPTER TWO: CALORIE RESTRICTION DOES NOT RESTORE BRAIN MITOCHONDRIAL FUNCTION IN P301L TAU MICE, BUT IT DOES DECREASE MITOCHONDRIAL F_OF_1-ATPASE ACTIVITY

Note to Reader: This chapter was published in Molecular and Cellular Neuroscience. 2015 Jun 3;67:46-54. This chapter has been reproduced with permission from the publisher. The lead author was responsible for designing, executing, and supervising all the mitochondrial assays in this study. The lead author was also responsible for generating figures, statistical analysis, and writing up the manuscript.

Abstract

Calorie restriction (CR) has been shown to increase lifespan and delay aging phenotypes in many diverse eukaryotic species. In mouse models of Alzheimer's disease (AD), CR has been shown to decrease amyloid-beta and hyperphosphorylated tau levels and preserve cognitive function. Overexpression of human mutant tau protein has been shown to induce deficits in mitochondrial electron transport chain complex I activity. Therefore, experiments were performed to determine the effects of 4-month CR on brain mitochondrial function in Tg4510 mice, which express human P301L tau. Expression of mutant tau led to decreased ADP-stimulated respiratory rates, but not uncoupler-stimulated respiratory rates. The membrane potential was also slightly higher in mitochondria from the P301L tau mice. As shown previously, tau expression decreased mitochondrial complex I activity. The decreased complex I activity, decreased ADP-stimulated respiratory rate, and increased mitochondrial membrane potential occurring in mitochondria from Tg4510 mice were not restored by CR. However, the CR diet did result in a genotype independent decrease in mitochondrial F_0F_1-ATPase activity.

This decrease in F_0F_1-ATPase activity was not due to lowered levels of the alpha or beta subunits of F_0F_1-ATPase. The possible mechanisms through which CR reduces the F_0F_1-ATPase activity in brain mitochondria are discussed.

Introduction

Alzheimer's disease (AD) is characterized clinically by memory and functional deficits culminating in progressive cognitive decline. At the tissue level, AD is characterized by increased levels of intracellular and extracellular amyloid beta (Aβ) (LaFerla, Green et al. 2007) and by the presence of intracellular neurofibrillary tangles (NFTs) resulting from the hyperphosphorylation of tau protein (Grundke-Iqbal, Iqbal et al. 1986). Mutations in the human tau gene lead to the progressive formation of NFTs, resulting in early onset frontotemporal dementia with Parkinsonism (Mirra, Murrell et al. 1999) and other tauopathies. When expressed in mice, mutant tau also results in the progressive formation of NFTs (Lewis, McGowan et al. 2000).

Association of mitochondrial dysfunction with AD is well established (Perry, Perry et al. 1980; Sorbi, Bird et al. 1983; Parker 1991). Furthermore, mitochondrial dysfunction occurs with advanced age (Chomyn and Attardi 2003; Romano, Greco et al. 2014) and is hypothesized to play a role in the development of AD as outlined in the mitochondrial cascade hypothesis of AD (Swerdlow, Burns et al. 2014). This hypothesis suggests that a patient's basal mitochondrial function and rate of loss of mitochondrial function with age are determined genetically and contribute to AD susceptibility. Compensatory mechanisms occurring as a consequence of the accrued mitochondrial dysfunction then lead to the AD phenotypes observed (Swerdlow, Burns et al. 2014). Past experiments using transgenic P301L tau mice have shown deficits in

mitochondrial electron transport chain complex I and uncoupled respiration (David, Hauptmann et al. 2005). Proteomic analysis of mitochondria further identified a decline in the level of ATP synthase subunit D in the P301L mice, which was also identified in postmortem brain from human subjects with the P301L tau mutation (David, Hauptmann et al. 2005). Mitochondrial cytochrome c oxidase (complex IV) activity was not altered in the tau transgenic mice (David, Hauptmann et al. 2005), but is commonly found in mouse models of AD overexpressing mutant forms of amyloid precursor protein (APP) (Du, Guo et al. 2010).

CR is the only well-established regimen that extends lifespan and healthspan in a multitude of organisms including mice and non-human primates (Weindruch, Walford et al. 1986; Colman, Anderson et al. 2009). CR, a dietary regimen providing 30-40% less calories than normal *ad libitum*-fed animals, has been shown to be the most robust method of slowing down many of the detrimental effects of aging (Sohal and Weindruch 1996), such as increased oxygen free radical generation by ETC complex I (Sanz, Caro et al. 2005) and the reduced rate of oxygen consumption due in part to declines in ETC complex I and IV activities (Hepple, Baker et al. 2006; Singh, Lakhanpal et al. 2012). However, the degree to which CR increases mitochondrial biogenesis remains contested (Nisoli, Tonello et al. 2005; Hancock, Han et al. 2011; Cerqueira, Cunha et al. 2012). In some tissues, CR has been shown to prevent the aging-related decline of mitochondrial function without inducing mitochondrial biogenesis, while in other tissues, such as adipose tissue (Lambert, Wang et al. 2004), mitochondrial biogenesis is induced by CR. CR and intermittent fasting (IF) have previously been found to be protective for the performance of behavioral tasks, which measure cognitive function in triple transgenic (3xTgAD) mice that show increased levels of amyloid beta peptide and express P301L tau (Oddo, Caccamo et al. 2003; Halagappa, Guo et al. 2007). This protection is likely mediated by

decreased levels of amyloid-beta and phospho-tau in the brains of the CR animals, but the protection occurred without significant changes in the levels of these proteins in the IF mice. In another study using the 3xTgAD mice, dietary protein restriction cycles were found to decrease phospho-tau levels but not Aβ levels in the brain (Parrella, Maxim et al. 2013). However CR has been shown to be successful in reducing Aβ levels in another report (Mouton, Chachich et al. 2009), so the specific mouse model, dietary protocol used, or the duration of time on the restricted diet appears to be important. Our current study sought to determine the effects of 4-month CR on brain mitochondrial function in P301L tau-expressing Tg4510 mice.

Material and methods

Mice and experimental design

Tg4510 mice and parental mutant tau and tetracycline-controlled transactivator protein lines were generated and maintained as described previously (Santacruz, Lewis et al. 2005). The major tau pathology in Tg4510 mice is in the forebrain neurons due to tet activator-driven expression by the CAM kinase II promoter. These animals develop discernible tau deposition by 3 months of age, which progresses into neuronal atrophy and loss by 6 months of age. All animals were 3 months old at the start of the study and nontransgenic (Ntg) littermates were used as positive control groups (FVB/129S background) (n=10 per group). Tg4510 mice are hyperphagic, but hyperactive and leaner than controls (Brownlow, Joly-Amado et al. 2014). Mice were maintained in a specific pathogen-free environment (NIH Guidelines for the care and use of laboratory animals) and kept on a twelve-hour light/dark cycle. Water was provided *ad libitum* (AL) throughout the experiment.

All animals were individually caged for accurate assessments of food intake and body weight as described in (Brownlow, Joly-Amado et al. 2014). Measurements of daily food consumption started when the animals were 3 months old and were carried out for 4 weeks before the start of the CR procedure. The CR group received a diet identical in chemical constituency to the *ad libitum* diet except that it was supplemented with micronutrients to maintain normal vitamin and mineral intake (diet devised by Dr. Robert Engelman) and manufactured at Harlan Teklad (Madison, WI). Thus, the CR diet provided all necessary nutrients in a smaller quantity of food. The non-CR mice were fed *ad libitum*. Due to possible Tg4510 deaths from acute weight loss due to abrupt initiation of the CR diet, the CR groups were slowly transitioned onto the fortified CR diet as described in (Brownlow, Joly-Amado et al. 2014). For example, in the first week of the diet the CR mice were given 10% food reduction with further reduction in food added on each week for 6 weeks. The goal of the CR was to achieve a 35% reduction in body weight, which was achieved following 6 weeks of the diet and then maintained thereafter. Body weight was assessed 3 times a week for careful observation of body weight loss so adjustments could be made to the food offered. A detailed list of macronutrient components of each diet used in this experiment is presented in Table 1.

At seven months of age the mice were submitted to a battery of behavioral testing as recently reported (Brownlow, Joly-Amado et al. 2014). At 8 months of age, after four months on their respective diets, mice were weighed, euthanized with a solution containing pentobarbital (100 mg/kg) and transcardially perfused with 25 ml of 0.9% normal saline solution. Brains were collected immediately following perfusion. One hemisphere was collected for mitochondrial assays and the second hemisphere was frozen for Western blot analysis. The results from the

Western blot analysis assaying tau levels and phosphorylation status are presented in (Brownlow, Joly-Amado et al. 2014).

Mitochondrial isolation

Single mouse cerebral hemispheres were placed in 3 ml of ice cold mitochondrial isolation buffer (MIB) (75 mM sucrose, 0.1% BSA, 1 mM EGTA, 215 mM mannitol, 20 mM K^+ HEPES, pH 7.2) immediately after brain extraction. Cerebral hemispheres were quickly minced using a surgical scalpel, scooped into a spatula, and transferred to a glass dounce homogenizer. After homogenization using 3-4 slow strokes, the homogenate was aliquoted into 4 microcentrifuge tubes and spun at a speed of 1,300 x *g* for 5 minutes at 4^0C. The supernatant was transferred to new microcentrifuge tubes and centrifuged at 13,000 x *g* for 10 minutes at 4^0C pelleting the mitochondria. The pellet was re-suspended in 500 µl of ice cold mitochondrial isolation buffer without EGTA yielding roughly 6 mg protein/ml of mitochondrial suspension. The tube was kept on ice for up to 2 hours for the experiments.

Oxygen consumption analysis

From the mitochondrial suspension, 50 µl was placed into a Strathkelvin Mitocell MT200A respiratory chamber with Clark type electrode, containing 300 µl of respiratory buffer (125 mM KCl, 1 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM pyruvate, 2.5 mM malate, 500 µM EGTA, 20 mM HEPES, pH 7.2) at 37^0C with a stir bar. Respiratory state 2 (mitochondria in buffer with respiratory substrates), state 3 (with the addition of 1 mM ADP), state 4 (with the further addition of 11 µM oligomycin), and state 5 (with the further addition of 457 nM FCCP) were obtained. Each state was recorded for 2 minutes. The middle 1 minute was chosen for slope

analysis. 1 mM ADP, 457 nM FCCP, and 11 µM oligomycin (final concentrations) were added through the injection port using a Hamilton syringe.

Reactive oxygen species level measurements

2',7'-dichlorodihydrofluorescein diacetate was used to detect reactive oxygen species (ROS) levels. Following hydrolysis of the acetate esters by mitochondrial esterases, dichlorodihydrofluorescein is produced. Dichlorodihydrofluorescein, when oxidized by ROS becomes the highly fluorescent compound dichlorofluorescein (DCF). A 2',7'-dichlorodihydrofluorescein diacetate stock solution of 500 µM was made up in 2% DMSO in PBS and the tube was wrapped in foil to prevent exposure to light. An assay concentration of 50 µM was obtained by mixing 10 µl of the stock solution with 80 µl of mitochondrial respiratory buffer plus 10 µl of the mitochondrial suspension per well in a 96-well plate. In some assays, 5 mM pyruvate was omitted and replaced with 5 mM DL-beta-hydroxybutyrate (βHB). As a positive control, 0.5 µl of 100% EtOH was added, as it can greatly stimulate mitochondrial ROS production at complexes I and III (Cunningham and Bailey 2001). The plate was incubated in the dark at 37°C for 30 minutes. The fluorescence was then measured using a Biotek Synergy-2 microplate reader with an excitation filter of 485/20 nm and emission filter of 528/15 nm.

Mitochondrial membrane potential determination

The cationic carbocyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolycarbocyanine iodide) was used to monitor the mitochondrial membrane potential. JC-1 selectively accumulates in mitochondria based on the membrane potential where its emission shifts from green (~530 nm) to red (~600 nm). A 10 µM stock solution of JC-1 was

made in PBS. The mitochondrial sample was incubated with 1 μM JC-1 in mitochondrial respiratory buffer for 10 minutes at room temperature. For the negative control the mitochondrial membrane potential was dissipated by including 457 nM of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) in the suspension. Following the incubation mitochondria were spun down for 10 minutes at 4°C at 13,000 rpm in a microcentrifuge. Mitochondria were washed twice with mitochondrial isolation buffer and then suspended in 450 μl of PBS, and aliquoted into a 96 well microplate in triplicate (100 μl per well). The microplate was read using a Biotek Synergy-2 microplate reader using an excitation filter of 485/20 nm and an emission filter of 528/15 nm for green fluorescence and an excitation filter of 530/20 nm and an emission filter of 590/35 nm for red fluorescence. We report the signal as red fluorescence (polarized) / green fluorescence (depolarized).

ATP synthesis assays in isolated mitochondria

CellTiter-Glo (Promega, Madison, WI) ATP detection reagent was used to determine the levels of medium ATP following a 10 minute 37°C incubation of isolated mitochondria at a concentration of ~ 2 mg/ml in respiratory buffer with 10 mM ADP. CellTiter-Glo reagent was combined with the mitochondrial suspension in a 1:1 ratio in a 96-well microplate and then shaken for 2 minutes. Luminescence was read using a Biotek Synergy 2 microplate reader.

F_0F_1-ATPase activity measurements

A molybdenum-based assay measuring free phosphate was used to determine the F_0F_1-ATPase hydrolytic activity of frozen-thawed isolated mitochondria. The reaction buffer consisted of 80 mM KCl, 3 mM $MgCl_2$, 5 mM sodium azide, 0.2 mM EGTA, 0.2 mM $CaCl_2$,

and 25 mM MOPS. The pH was adjusted to 7.0 using Tris base. 2 µM oligomycin was added to control samples to obtain the F_0F_1-ATPase-independent rate of phosphate release, which was subtracted off. The coloring solution consisted of 0.7 mM ammonium heptamolybdate, 3 mM ascorbic acid, 100 µM potassium antimony tartrate, and 140 mM H_2SO_4 made from stock concentrations of 0.3 M ascorbic acid, 4 mM potassium antimony tartrate and 24 mM ammonium heptamolybdate. 1 mM ATP and 10 µg/ml of mitochondrial protein were added to the reaction buffer at 37°C. After a 10 minute incubation, 270 µL of coloring solution was added to 30 µL of reaction buffer (containing ATP and mitochondrial protein) and the absorbance was read at 850 nm on a Biotek Synergy 2 microplate reader 4 minutes later.

ATP synthesis assays in permeabilized N2a and HEK293 cells

N2a and HEK293T cells stably expressing human P301L tau were grown in T-75 flasks using M10 culture media (DMEM with 10% FBS, 30 µg/ml penicillin, 50 µg/ml streptomycin, and 2 mM glutamine). Following trypsinization, cells were pelleted and re-suspended in buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 0.1% BSA, 10 mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) containing 75 µg/ml digitonin. Cells were incubated for 2 minutes at room temperature while gently tapped in a 1.5 ml microcentrifuge tube. The permeabilized cells were spun down by centrifugation at 1,200 x *g* for 5 min. The permeabilized cells were washed with buffer A (without digitonin), spun down again, and then suspended in 1.4 ml of buffer A with 5 mM malate and 10 mM pyruvate or 5 mM succinate and 5 µM rotenone. 140 µl of a 3 mM di-adenosine pentaphosphate stock solution yielding a final concentration of 267 µM was added to tubes containing the permeabilized cell suspensions. 25 ul of the suspension was placed into each well of a 96-well microplate. 2.6 µl of 1 mM oligomycin (made up in DMSO) yielding a

final concentration of 94 µM was added to control wells. The plate was then incubated for 1 minute at room temperature. 25 µl of 0.2 mM ADP yielding a final concentration of 0.1 mM was added to all wells. 50 µl of CellTiter-Glo solution was added to each well at 1 minute intervals quenching the reaction. The luminescence was read using a Biotek Synergy 2 microplate reader 10 minutes later. Control values, those obtained in the presence of oligomycin, were subtracted off those obtained in the absence of oligomycin. Best fit straight lines through the 0, 1, and 2 minute data points were used to obtain the initial mitochondrial ATP synthesis rates.

Western blots

For the western blots, the mitochondrial fraction was centrifuged 10,000 x *g* for 10 min at 4°C. The supernatant was poured off and the mitochondrial pellet was resuspended in 8.2 µl/10 mg protein of buffer B, consisting of 10 mM HEPES, 420 mM NaCl, 0.5 mM dithiothreitol, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktails I & II (Sigma). 15 µg of protein for each sample was loaded and ran on an SDS-PAGE gel. The protein was transferred to a 0.2 µm pore size nitrocellulose membrane and immuno-blotted using mouse monoclonal antibodies against ATP synthase subunit alpha and ATP synthase subunit beta, which were obtained from Life Technologies and used at 1:1,000 and 1:2,000-fold dilution, respectively. Fluorescently tagged goat anti-mouse secondary antibodies (IRDye 800CW, LI-COR Biosciences) were used at a dilution of 1:10,000. Band intensities were quantified by densitometric analysis and normalized to the band intensity of β-actin (antibody obtained from Sigma and used at a dilution of 1:1000) used as a loading control.

Protein assays

A determination of the protein concentration was performed using the BCA protein assay (Thermo Scientific Pierce). All results were normalized using the protein concentrations present for the assays.

Statistical analysis

Error bars represent the standard error of the mean. Two-way ANOVA tests were performed with genotype and diet as variables, followed by Fisher's LSD post hoc means comparison on all samples. * indicates a p value of < 0.05, ** indicates a p-value < 0.005, and *** indicates a p-value < 0.001. Stat View software version 5.0 (SAS Institute Inc., Cary, NC) was used for statistical analysis. Graphs were generated using GraphPad Prism version 5.01 (La Jolla, CA).

Results

Mitochondria from Tg4510 mice show decreased state 3 oxygen consumption rates and respiratory control ratios

Cerebral mitochondria were isolated from Tg4510 (expressing P301L tau) and nontransgenic (Ntg) control mice fed either an *ad libitum* or CR diet. State 2, state 3, state 4, and state 5 oxygen consumption rates (as outlined in Table 2) were measured using the isolated mitochondria. The results are shown in Fig. 3 A. No genotype or diet differences were found in the state 4 and state 5 respiratory rates. However, we did find that Tg4510 mice had slightly lower state 2 and state 3 (ADP-stimulated) respiratory rates than Ntg controls (ANOVA genotype effect, $p<0.05$), regardless of diet. Fig. 3 B shows the respiratory control ratios (RCRs)

defined as the ratio of state 3 to state 4 respiration, as well as the ratios of state 5 to state 4 respiration. These ratios provide an excellent indication of both electron transport chain activity and the impermeability of the inner mitochondrial membrane to protons as a direct indicator of the ATP synthesizing capability of the organelle. The Tg4510 mice showed a decreased RCR compared to the Ntg mice (ANOVA genotype effect, p=0.02), as shown previously (David, Hauptmann et al. 2005). The CR diet had no appreciable effect on the RCRs. No genotype or diet-induced differences in the state 5 to state 4 respiratory ratios were observed as well.

Mitochondria from Tg4510 mice show increased ROS production in the presence of ethanol stress

We next measured reactive oxygen species (ROS) production from the mitochondria. Either malate plus pyruvate or malate plus beta-hydroxybutyrate (βHB) were used as respiratory substrates. βHB was used because it is a respiratory substrate present at higher levels during CR conditions when pyruvate and glucose levels are low. Under standard conditions, no genotype or diet-induced differences in ROS production were observed when using either malate plus pyruvate or malate plus βHB as respiratory substrates (Fig. 4 A), although there were trends toward increased ROS production when assaying mitochondria from the *ad libitum* fed Tg4510 mice. However, following incubation with 0.5% ethanol, which stimulates ROS production at electron transport chain (ETC) complexes I and III (Bailey, Pietsch et al. 1999), genotype dependent differences were revealed (ANOVA genotype effect, p =0.01, Fig. 4). Under the ethanol stress, the Tg4510 mitochondria produced more ROS than Ntg controls independent of the respiratory substrates oxidized. This is consistent with the results from a previous study

(David, Hauptmann et al. 2005). In addition, the presence of βHB instead of pyruvate in the respiratory buffer consistently led to substantially increased ROS production ($p=0.001$).

Mitochondria from Tg4510 mice show increased membrane potential

A mitochondrial membrane potential assay was performed using the potentiometric fluorescent probe JC-1. Surprisingly, mitochondria from Tg4510 mice showed a trend for higher membrane potential than Ntg controls (ANOVA genotype effect, $p=0.09$) and this effect was independent of the diet (Fig. 5). In addition when comparing only mice on the CR diet, Tg4510 mitochondria had a higher membrane potential than Ntg controls (t-test, $p<0.05$). The uncoupler FCCP was used as a negative control to dissipate the membrane potential.

CR does not restore the reduced mitochondrial ETC complex I activity in Tg4510 mitochondria

We next determined ETC complex I activity from the mitochondrial extracts. As shown in Fig. 6, mitochondria from Tg4510 mice showed a decreased complex I activity (ANOVA genotype effect, $p=0.001$). This finding is similar to the findings in previous reports (Rhein, Song et al. 2009; Lasagna-Reeves, Castillo-Carranza et al. 2011). Consistent with the inability of CR to restore other mitochondrial functional parameters in this study, the CR diet did not prevent the decreased complex I activity in the Tg4510 mice.

No change in the levels of the alpha and beta subunits of ATP synthase in isolated mitochondria from Tg4510 mice

The fact that ADP-stimulated (state 3) respiratory rates were lower in mitochondria from the Tg4510 mice than controls, but uncoupler-stimulated (state 5) respiratory rates were not lower in the Tg4510 mice suggests a possible P301L tau-induced deficiency of mitochondrial ADP transport or ATP synthesis. Therefore, Western blot analysis was performed on the alpha and beta subunits of the F_0F_1-ATP synthase complex to determine if lower levels were present in isolated Tg4510 mitochondria. No difference in the amount of these subunits was detected in any of the mitochondrial samples (Fig. 7 A and 7 B). In addition, no tau band could be detected on the blots from the mitochondrial fractions (data not shown). Therefore, a direct interaction between tau and complex I is not likely responsible for the decreased complex I activity present.

No change in isolated mitochondrial ATP production in Tg4510 mice or in CR mice

To obtain further data regarding the ability of mitochondria from Tg4510 mice to transport and phosphorylate ATP, we performed assays measuring the ATP synthesizing capacity of the isolated mitochondria using malate plus pyruvate (Fig. 8 A) or malate plus βHB (data not shown) as respiratory substrates. For this assay, energized mitochondria were incubated in the presence of ADP and phosphate for 10 minutes and then the ATP in the suspension was measured. Due to experimental constraints, time course experiments were subsequently performed in immortalized cells. No genotype or diet-induced differences in ATP levels were observed between groups. However, mitochondria oxidizing malate plus pyruvate produced significantly more ATP than mitochondria oxidizing malate plus BHB ($p < 0.05$) (data not shown).

A decreased rate of mitochondrial ATP synthesis in digitonin-permeabilized N2a-P301L tau cells

To further determine if mutant tau expression has an effect on mitochondrial ATP synthesis, we used cell culture models and measured the rate of oligomycin-sensitive ATP synthesis in digitonin-permeabilized N2a (Fig. 8 B) or HEK293T (Supplementary Fig. 10) cells with or without stable expression of P301L tau. The permeabilized cells were given the respiratory substrates malate plus pyruvate that primarily generate NADH, which is oxidized through ETC complex I. Strikingly, P301L tau expression decreased the rate of ATP synthesis in the neural N2a cell line, but not in the non-neural HEK293T cell line. To determine if the decreased rate of ATP synthesis in the N2a-P301L tau cells was primarily due to decreased complex I activity, a similar ATP synthesis experiment was performed, but this time administering succinate, an ETC complex II linked respiratory substrate instead of malate plus pyruvate. Rotenone, an ETC complex I inhibitor was also added to prevent oxidation of endogenous complex I-linked respiratory substrates. As shown in Fig. 8 B, the cells in both succinate treated groups generated ATP at a much lower rate than when oxidizing malate and pyruvate. In addition, there was no significant difference in the rate of ATP synthesis when comparing digitonin-permeabilized N2a and N2a-P301L tau cells indicating that complex I dysfunction was the major determinant for the decline in ATP synthesis in these cells when oxidizing NAD-linked respiratory substrates. The data do not suggest that there is a functional deficit in mitochondrial ADP/ATP transport or F_0F_1-ATP synthase activity in the P301L tau-expressing cells.

Mitochondria from CR mice show decreased F_0F_1-ATPase activity

One last assay was performed on isolated brain mitochondria to determine if any alteration of mitochondrial F_0F_1-ATPase activity was induced by P301L tau expression. Mitochondria samples were frozen and thawed several times to disrupt the membrane integrity, ATP was added, and oligomycin-sensitive ATPase activity was measured by monitoring the release of free phosphate. We found no difference in F_0F_1-ATPase activity as a result of P301L tau expression. But surprisingly, there was a significant decrease in oligomycin-sensitive ATPase activity in mitochondrial homogenates from mice on the CR diet regardless of the genotype (ANOVA, diet effect p=0.0003) (Fig. 9).

Discussion

In this study we found that 4 months of CR did not restore cerebral mitochondrial ETC complex I deficits mediated by P301L tau overexpression. In addition there was a strong decrease in cerebral mitochondrial F_0F_1-ATPase activity in mitochondria from mice of all genotypes on the CR diet.

Tau inhibition of mitochondrial ETC complex I

Consistent with published data (David, Hauptmann et al. 2005), we observed P301L tau expression to decrease mitochondrial ETC complex I activity. Somewhat unexpectedly, this reduced activity was not prevented by CR (as shown in Fig. 2.4), as CR has previously been shown to decrease phospho-tau levels (Halagappa, Guo et al. 2007). In our study, since the uncoupled respiration of mitochondria oxidizing the complex I-dependent substrates malate and pyruvate was not affected by tau expression, but tau expression decreased complex I activity,

there must be spare respiratory capacity in complex I so that the activity of complex I does not limit the maximal (uncoupled) respiratory rate.

Tau expression does not decrease F_0F_1-ATPase activity or the levels of two ATP synthase subunits

The mitochondrial levels of the alpha and beta subunits of the F_0F_1-ATPase did not change with mutant tau expression or following a CR diet (Fig. 7 A and 7 B) suggesting that in our model, neurofibrillary tangles (NFTs) did not prevent F_0F_1-ATPase subunit translocation into the mitochondrion, in contrast to a report that found the accumulation of the ATP synthase alpha subunit in the cytoplasm in association with neurofibrillary tangles (Sergeant, Wattez et al. 2003) . In mammals all but two of the F_0F_1-ATPase subunits are encoded by nuclear DNA, synthesized by cytosolic ribosomes, and translocated into the mitochondria. We did not find any evidence of mitochondrial F_0F_1-ATPase deficiency induced by P301L tau expression, while others have found mutant tau expression to decrease ATP levels and the levels of ATP synthase D chain (David, Hauptmann et al. 2005). However, we did not measure the level of this subunit.

Behavior of Tg4510 mice on the CR diet

We have recently published the results of a thorough behavioral characterization of the mice used in this study (Brownlow, Joly-Amado et al. 2014). The Tg4510 mice had decreased body weight even though they consumed more food, likely due to increased locomotor activity. The CR diet was shown to increase short term memory in the Tg4510 mice in the novel object recognition test and there was a trend for improvement in contextual memory with mice undergoing CR. However, there were no improvements in spatial memory deficits as well as no

effect on tau deposition markers in the Tg4510 mice on the CR diet. Since the CR diet did not improve mitochondrial respiratory function of the mice as shown here, some other positive effects of CR are likely responsible for the improved behavior. In this regard mutant tau is known to both inhibit mitochondrial transport and alter mitochondrial fission and fusion dynamics (Eckert, Nisbet et al. 2014), which could potentially be normalized by the CR diet. In addition, CR decreases neuroinflammation (Morgan, Wong et al. 2007) and increases neurogenesis (Levenson and Rich 2007), which may also be responsible for the protective effects of CR on the behavior.

Possible mechanisms for the decreased state 3, but not state 5 respiration rate in mitochondria from Tg4510 mice

Another group studying mitochondrial function in P301 tau transgenic mice found defects in both the state 3 (ADP-stimulated) and the state 5 (uncoupler-stimulated) respiratory rates (David, Hauptmann et al. 2005), where we only found a deficit in the state 3 respiratory rate. This is likely due to the different strains of mice used in the two studies. In the previous study tau expression in the mice was driven by the neuron-specific mThy1.2 promoter, while in our study tau expression is driven by the CAM kinase II promoter. The decreased state 3 but not state 5 respiratory rates in the Tg4510 cerebral mitochondria used here (Fig. 3), suggests that either ADP transport into mitochondria is reduced or that the F_0F_1-ATP synthase activity is decreased in these mice.

We found the F_0F_1-ATPase (ATP-hydrolyzing) activity to be normal in the Tg4510 mice. However, a limitation to our study is that we did not directly measure the reverse reaction, the ATP synthase activity or the rate of ADP/ATP transport into and out of the isolated

mitochondria. In addition, the RCR of the isolated mitochondria was low, even in the Ntg controls, possibly indicating damage during the isolation procedure that could have affected our results. A further limitation is that our measurement of ATP synthesis in isolated brain mitochondria only captured a single time point and did not measure a rate, so small changes in ATP synthesis activity could easily have been missed. So, we cannot rule out the possibility that Tg4510 brain mitochondria possess a slightly decreased F_0F_1-ATP synthase activity with a normal F_0F_1-ATPase activity that could explain the decreased state 3, but normal state 5 respiratory rates in the Tg4510 mitochondria in our study.

Another possible explanation for the decreased ADP-stimulated respiratory rate in Tg4510 mitochondria is hindered transport of ADP and ATP into or out of the mitochondria. The adenine nucleotide translocase (ANT) is responsible for the reversible exchange of ADP/ATP into and out of the mitochondrial matrix, while VDAC (voltage-dependent anion channel) proteins are responsible for the transport of nucleotides and other solutes across the outer mitochondrial membrane. Adenine nucleotide translocase-1 (ANT-1) has been shown to be inhibited by a caspase-cleaved, truncated form of tau (Amadoro, Corsetti et al. 2012). A tau mediated inhibition of ANT function may also be responsible for the increased mitochondrial membrane potential observed in mitochondria from Tg4510 mice. The ANT can mediate both basal and fatty acid-mediated mitochondrial uncoupling (Brustovetsky and Klingenberg 1994; Shabalina, Kramarova et al. 2006), which decreases mitochondrial membrane potential. Another possible explanation for the decreased state 3 respiratory rate is that tau associates with VDAC and inhibits ADP and ATP transport across the outer mitochondrial membrane in neural mitochondria. In this regard, tau has been shown to associate with VDAC1 and high levels of VDAC1 occur in AD brain (Manczak and Reddy 2012).

Possible mechanisms through which CR decreases F_0F_1-ATPase activity

CR has previously been shown to slightly decrease F_0F_1-ATP synthase activity in rat heart (Colom, Oliver et al. 2007). We have shown a much larger decrease in F_0F_1-ATPase activity in cerebral mitochondria as a result of CR in both mice expressing mutant tau and age-matched Ntg counterparts. Since the ADP-stimulated respiratory rate was not altered in Ntg CR mice, the rate of the forward reaction of F_0F_1-ATP synthase is not likely altered by CR, but the reverse F_0F_1-ATPase reaction appears greatly slowed by the CR diet. We predict that the decreased F_0F_1-ATPase activity plays a protective role in neural cells during CR. In times of low glucose in the brain such as in times of CR, the TCA cycle and mitochondrial NADH and $FADH_2$ levels may decline due to respiratory substrate limitation resulting in decreased ETC-mediated proton pumping and a decreased mitochondrial membrane potential (MMP). To compensate for the decreased MMP, the F_0F_1-ATP synthase likely reverse its normal direction and hydrolyzes ATP to pump protons. This reversal could potentially deplete cellular ATP levels resulting in bioenergetic collapse and cell death. Limiting mitochondrial F_0F_1-ATPase activity under these conditions would minimize loss of ATP, maintaining cellular energy reserves, thus protecting from cell death. But the MMP must not be totally dissipated under these conditions as it is required for protein import and other essential mitochondrial functions.

There are several mechanisms through which CR may lead to decreased F_0F_1-ATPase activity. One possibility is through alteration of F_0F_1-ATP synthase post-translational modification such as by protein acetylation. In this regard, the majority of ATP synthase subunits are acetylated, with many of them being acetylated at multiple residues (Foster, Liu et al. 2013). For example ATP synthase subunit alpha was found to have 26 acetylation sites, the

most of any mitochondrial protein. The mitochondrial protein deacetylase SIRT3 is upregulated by CR (Someya, Yu et al. 2010). The deacetylation of the OSCP (oligomycin-sensitivity conferring protein) subunit of ATP synthase was shown to stimulate ATP synthase activity in muscle (Vassilopoulos, Pennington et al. 2014). In liver, even though SIRT3 deacetylase was upregulated by CR, there was an increase in total protein acetylation levels (Hebert, Dittenhafer-Reed et al. 2013; Nakamura, Kawakami et al. 2013). SIRT3-mediated deacetylation of ATP synthase beta subunit was also shown to increase ATP synthase activity (Rahman, Nirala et al. 2014).

Another possible mechanism as to how CR may decrease F_0F_1-ATPase activity is through increasing the levels or activity of its natural inhibitor, mitochondrial ATPase Inhibitor Factor 1 (IF1). IF1 overexpression has been described to promote cell survival through transcriptional activation of the NFκB pathway (Formentini, Sanchez-Arago et al. 2012). CR may induce IF1 expression or increase its activity through post-translational modification. Overexpression of IF1 has been shown to preserve mitochondrial morphology and inner membrane architecture and prevent apoptotic signaling (Faccenda, Tan et al. 2013). IF1 is found to be acetylated under normal physiological conditions in Hartley guinea pig hearts (Foster, Liu et al. 2013), so we speculate that deacetylation of IF1 by SIRT3 could play an active role in its regulation during CR.

Conclusions

Four months of CR did not rescue the decreased cerebral ETC complex I activity in mice expressing P301L tau. Since mutant tau expression caused decreased ADP-stimulated, but not uncoupler-stimulated respiratory rates, hyperphosphorylated tau may induce functional deficits

in mitochondrial ANT, VDAC, or ATP synthase activities in brain. Future studies will focus on determining the effects of P301 tau expression on the transport of ADP and ATP across mitochondrial membranes and the effects of P301 tau expression on the rate of ATP synthase activity in freshly isolated brain mitochondria. In addition, CR significantly reduced F_0F_1-ATPase activity in brain, which is likely an important neuroprotective mechanism under these conditions. Therefore, future studies will determine the CR-induced changes in the post-translational modifications of IF1 and the many subunits of the mitochondrial F_0F_1-ATP synthase and the consequence of these modifications on function.

Acknowledgments

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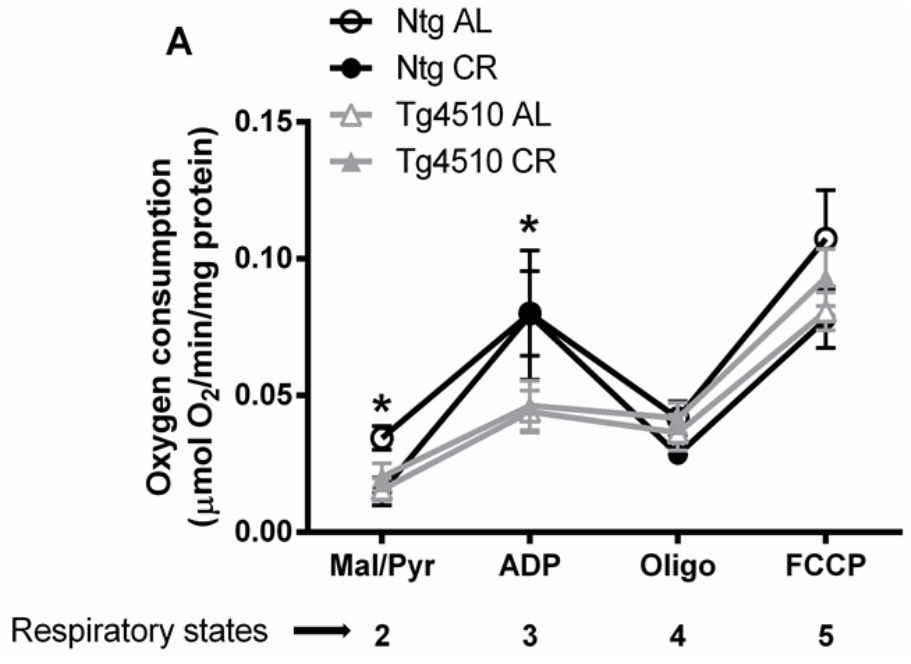
Table 1 Calorie restriction diet information

	Ad Lib Diet- NIH-31	Calorie Restriction Diet
	grams/kg	grams/kg
Casein	210	210
L-Cystine	3	4
Sucrose	200	199
Maltodextrin	100	99
Corn Starch	369	369
Cellulose (Fiber)	40	40
Flaxseed Oil	21	21
Canola Oil	19	19
79055 MM Ca-P Deficient	13.4	13.4
Calcium Phosphate Dibasic (CaHPO4)	7	7
Calcium Carbonate (CaCO3)	7.3	7.3
40060 VM, Teklad	10	14
Ethoxyquin (Liquid)	0.01	0.01
TOTAL	1000	1000
Protein, % by weight	17.9	17.9
Protein, % of kcal	23.8	23.8
Carbohydrate, % by weight	46.8	46.6
Carbohydrate, % of kcal	62.2	62.1
Fat, % by weight	4.7	4.7
Fat, % of kcal	14	14.1
kcal/g	3.0	3.0

Table 2 Definitions of mitochondrial respiratory states

State	Definition Note: RCR= State 3/State 4
2	Mitochondria and respiratory substrate present
3	ADP added
4	Oligomycin (ATP synthase inhibitor) added
5	FCCP (uncoupler) added



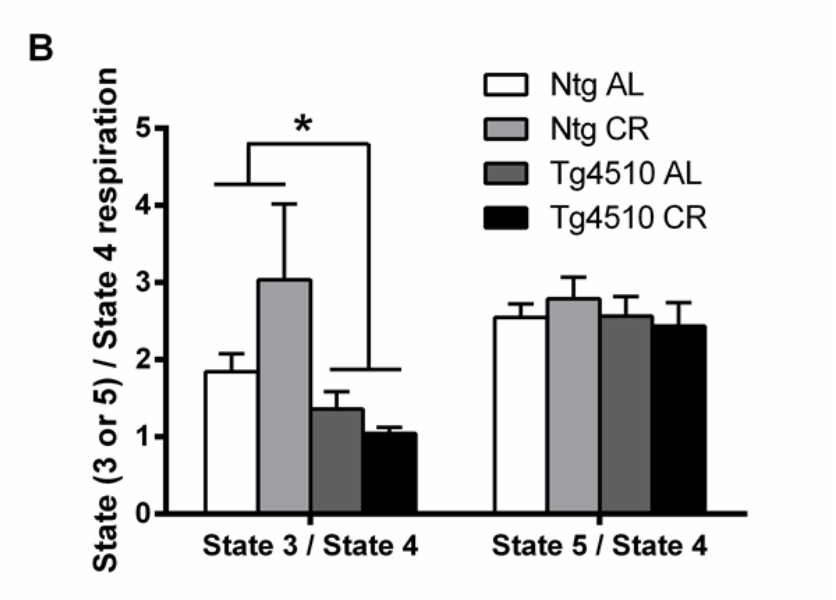


Figure 3 Respiratory states

Decreased state 2 and 3 respiratory rates and respiratory control ratio (RCR) in Tg4510 mitochondria. A) Respiratory rates and B) RCRs of isolated mouse cerebral mitochondria from Tg4510 and Ntg control mice. Mitochondria were suspended in a buffer containing malate and pyruvate (Mal/Pyr). 1 mM ADP, 11 μM oligomycin (Oligo), and 457 nM FCCP were added sequentially. The state 2 and state 3 respiratory rates of Tg4510 mitochondria were less than Ntg controls (ANOVA genotype effect, * $p < 0.05$). The RCR (state 3/state 4) of Tg4510 mitochondria was also less than Ntg controls (ANOVA genotype effect, * $p = 0.02$). AL =fed an *ad libitum* diet. CR= fed a calorie restricted diet.

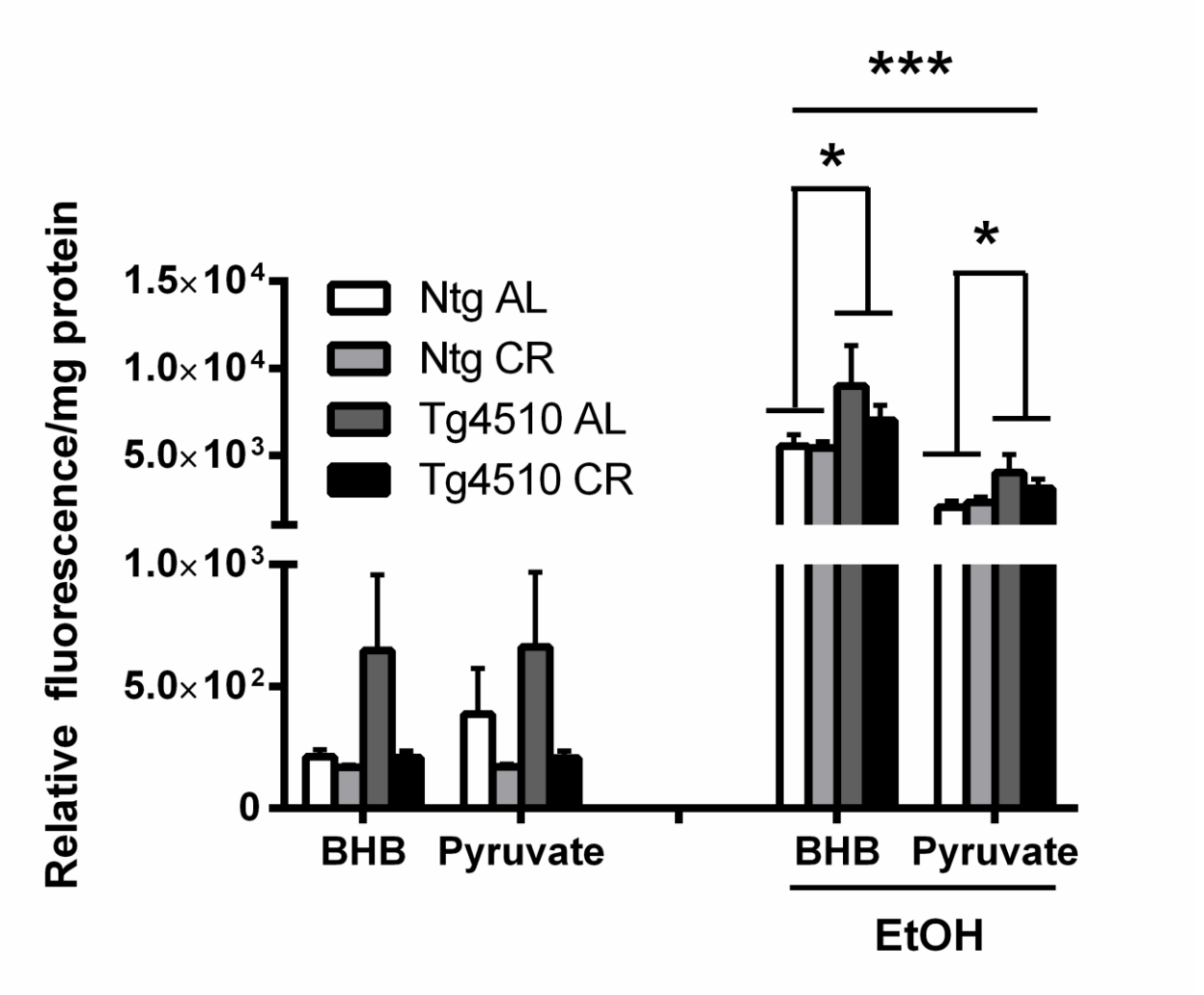


Figure 4 ROS production

ROS production from Tg4510 or Ntg cerebral mitochondria. Mitochondria were given either malate plus pyruvate or malate plus BHB as respiratory substrates in the presence or absence of 0.5% ethanol and DCF fluorescence was measured. Ethanol induces ROS production at complexes I and III. In the presence of ethanol, Tg4510 mitochondria produced more ROS irrespective of the respiratory substrates oxidized (ANOVA genotype effect, * $p < 0.05$). In the presence of ethanol, mitochondria oxidizing malate plus BHB produced more ROS than those oxidizing malate plus pyruvate (ANOVA respiratory substrate effect, *** $p < 0.001$).

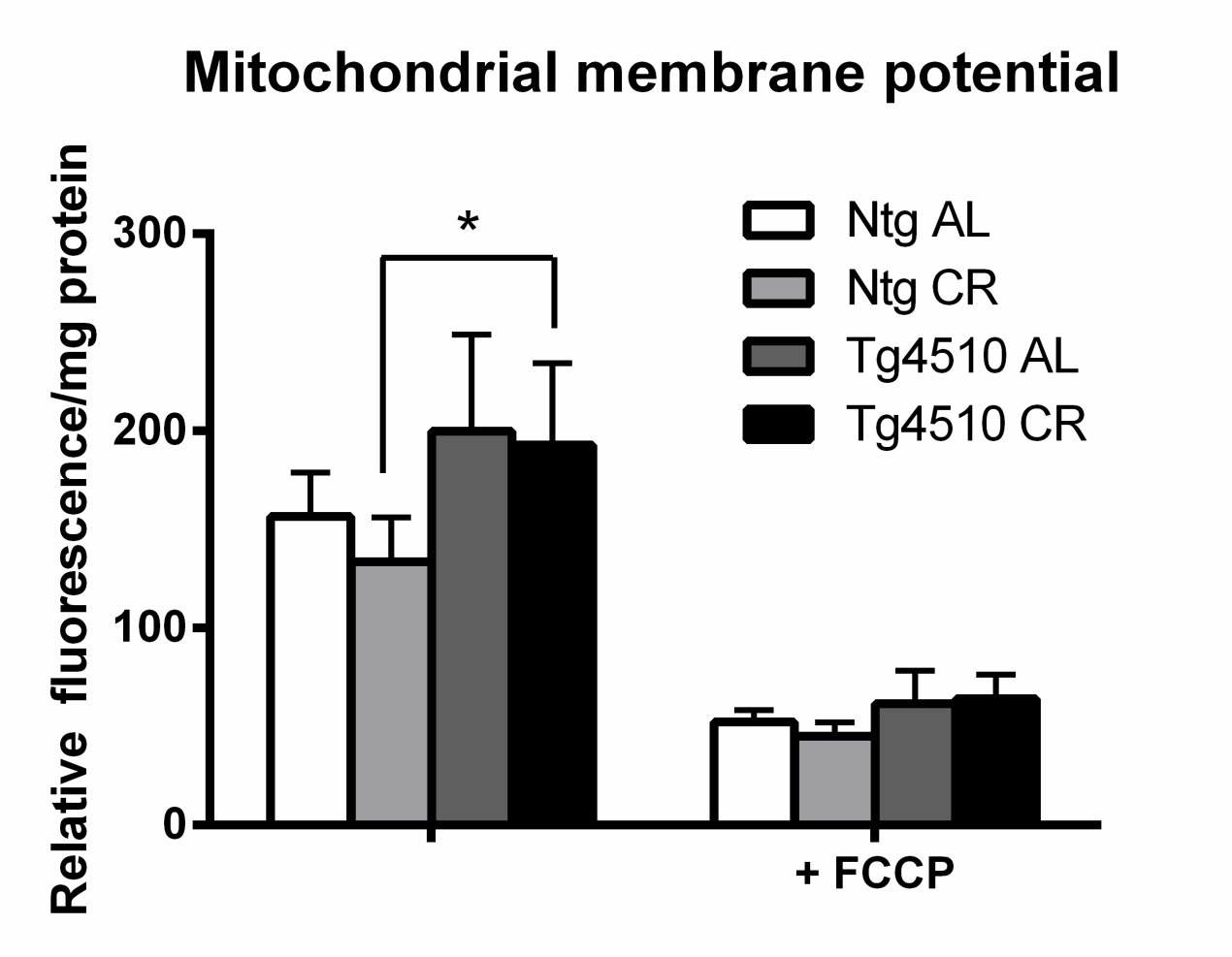


Figure 5 Mitochondrial membrane potential
Mitochondrial membrane potential of Tg4510 or Ntg cerebral mitochondria. Membrane potential was measured by JC-1 fluorescence either in the absence or presence of an uncoupler of oxidative phosphorylation, FCCP. There was an overall trend for increased membrane potential in Tg4510 mitochondria (ANOVA genotype effect, $p = 0.09$). And specifically, Tg4510 CR mitochondria had a higher membrane potential than Ntg CR mitochondria (t-test, * $p < 0.05$).

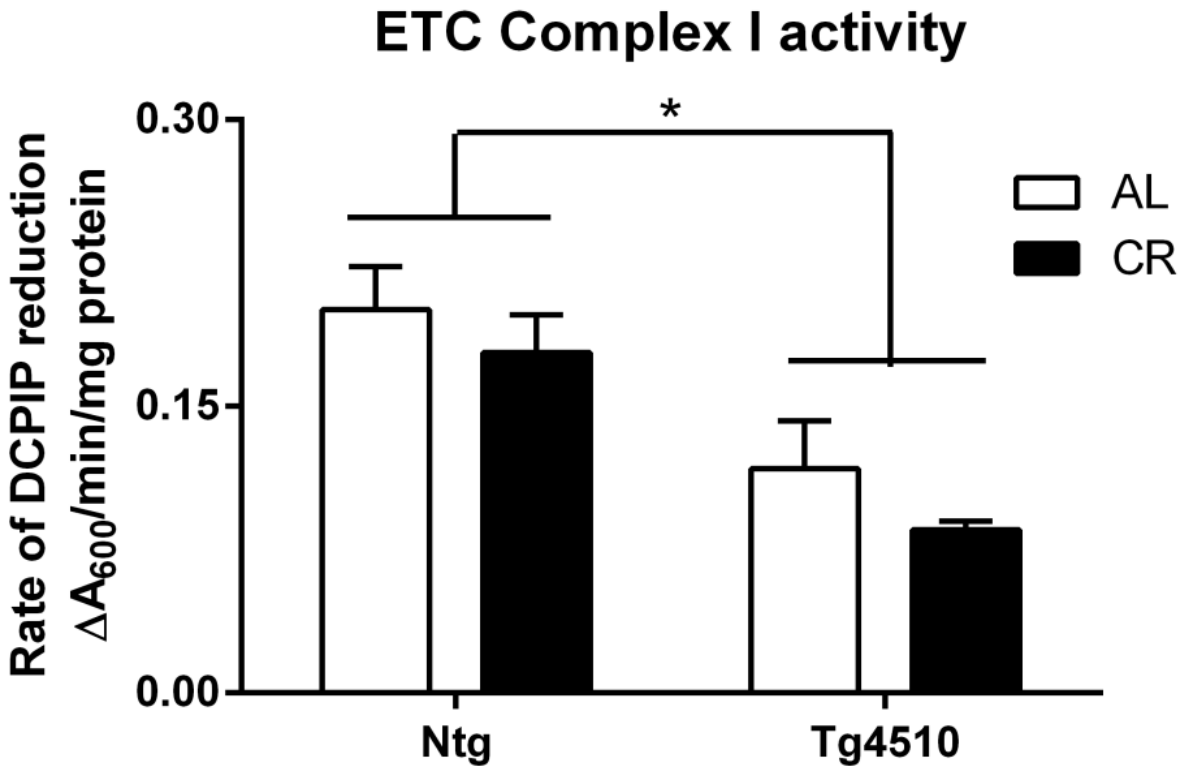


Figure 6 ETC complex I activity
CR does not restore decreased complex I activity in mitochondria from Tg4510 mice. Tg4510 mitochondria had lower complex I activity than Ntg mitochondria (ANOVA genotype effect, * $p < 0.05$).

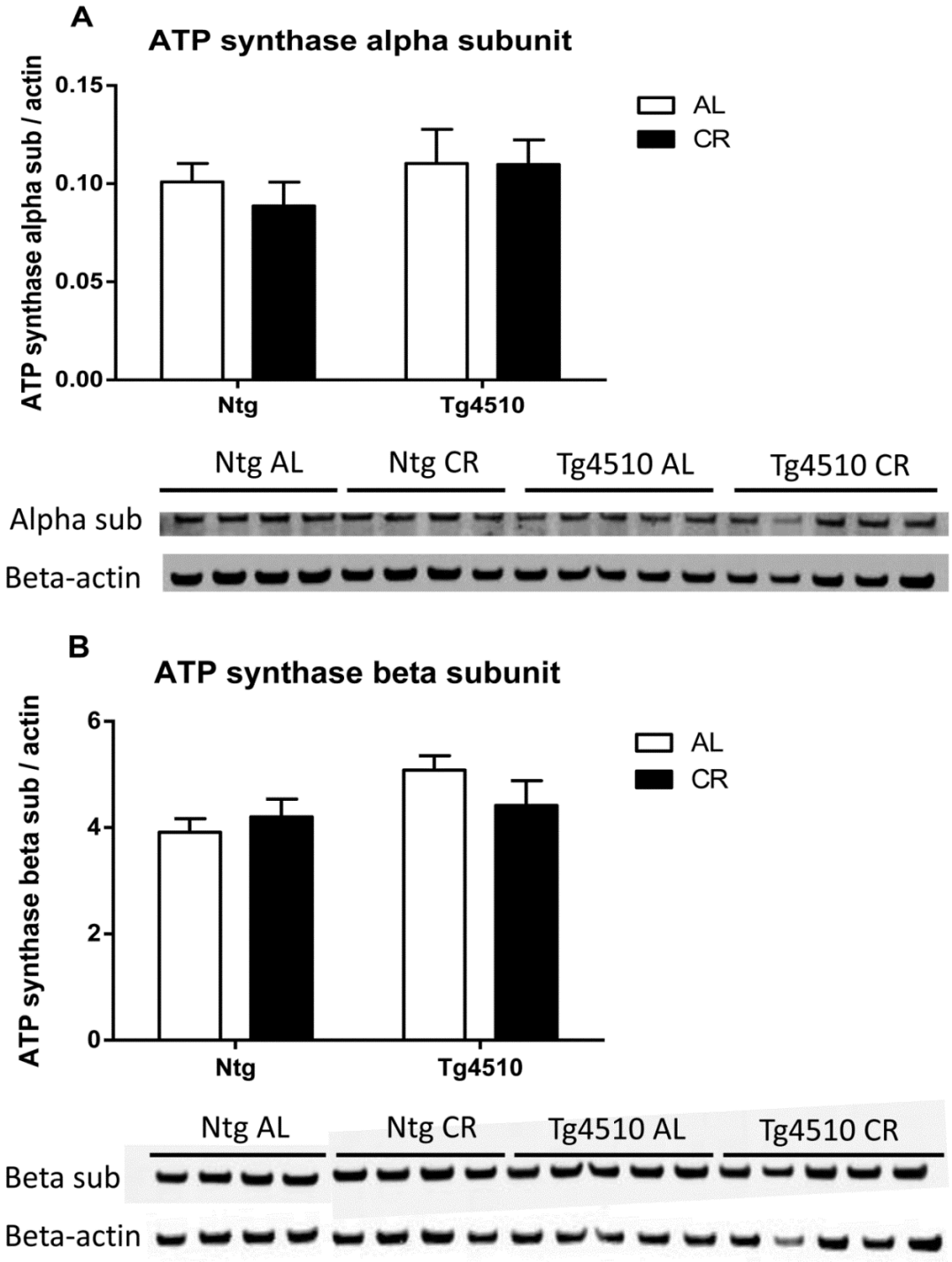


Figure 7 ATP synthase alpha and beta subunits
No change in the levels of ATP synthase subunits in Tg4510 mice. A) Western blot for the alpha-subunit of ATP synthase using cerebral mitochondrial lysates from Tg4510 and Ntg control mice. B) Western blot for the beta-subunit of ATP synthase using cerebral mitochondrial lysates from Tg4510 and Ntg control mice.

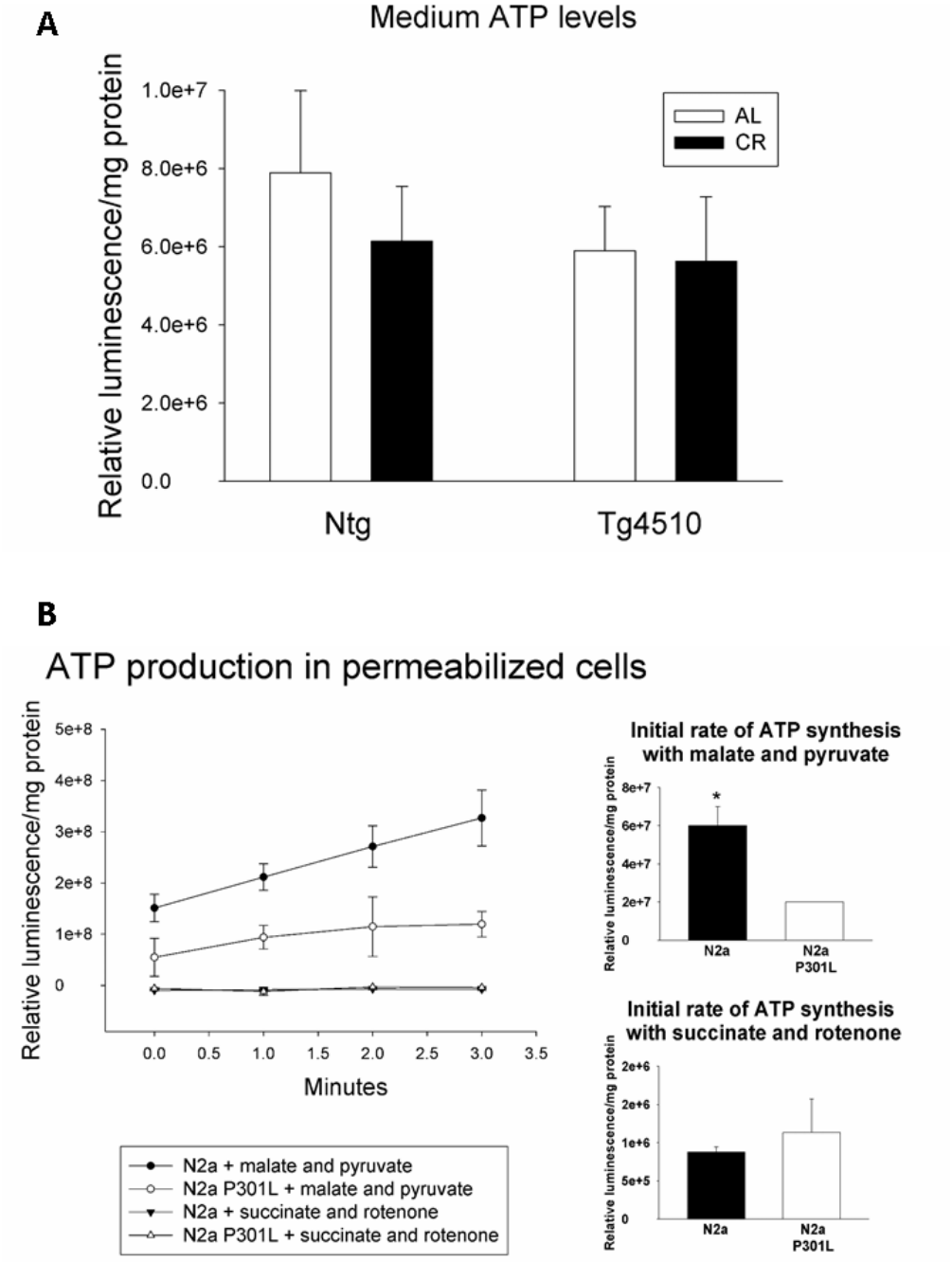


Figure 8 ATP levels and ATP production

The effect of P301 tau expression on mitochondrial ATP synthesis rates. (A) Medium ATP levels following administration of ADP to isolated Tg4510 and Ntg mouse cerebral mitochondria. (B) Medium ATP levels in the presence of digitonin-permeabilized stably transfected P301L tau and control N2a cells given ADP, Pi and either malate and pyruvate or succinate and rotenone. The 2 bar graphs on the right show the rates of ATP synthesis over the first 2 minutes of the reaction.

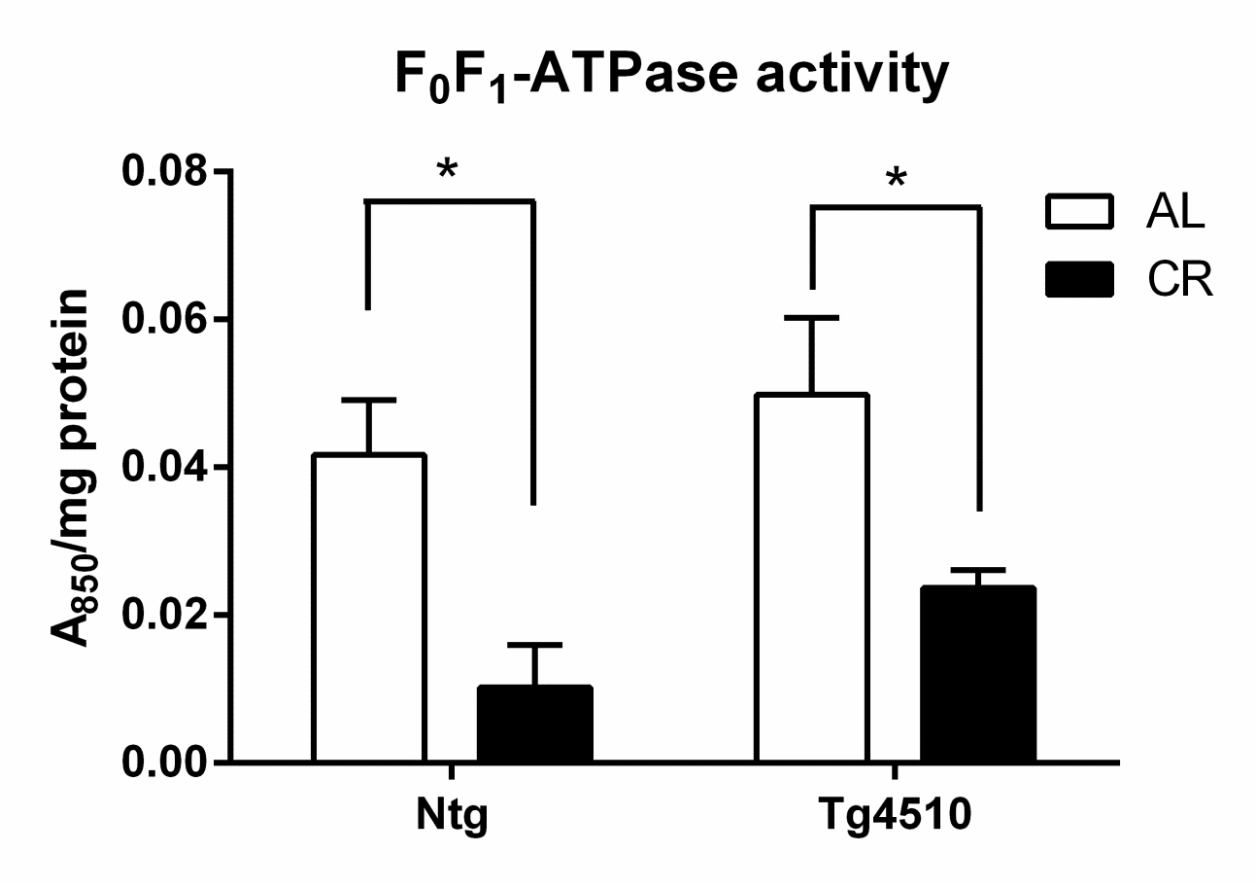
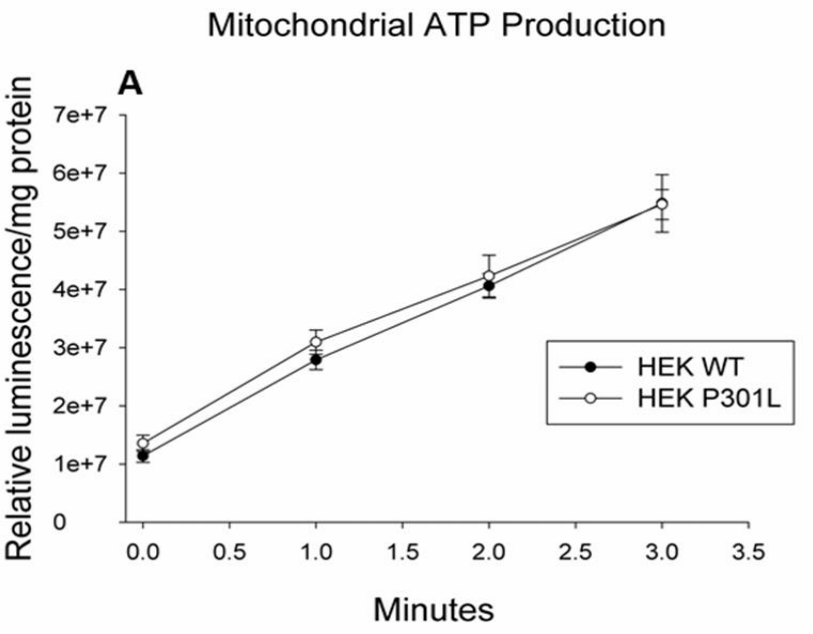


Figure 9 F_oF_1-ATPase activity
Decreased F_0F_1-ATPase activity in mitochondria from mice undergoing CR. Mice on a CR diet had decreased mitochondrial F_0F_1-ATPase activity (ANOVA diet effect, $p < 0.001$). When comparing genotypes separately, Ntg CR mice had lower F_0F_1-ATPase activity than Ntg AL controls (t-test, $p < 0.005$) and Tg4510 CR mice also had lower F_0F_1-ATPase activity than Tg4510 AL controls (t-test, $p < 0.05$).



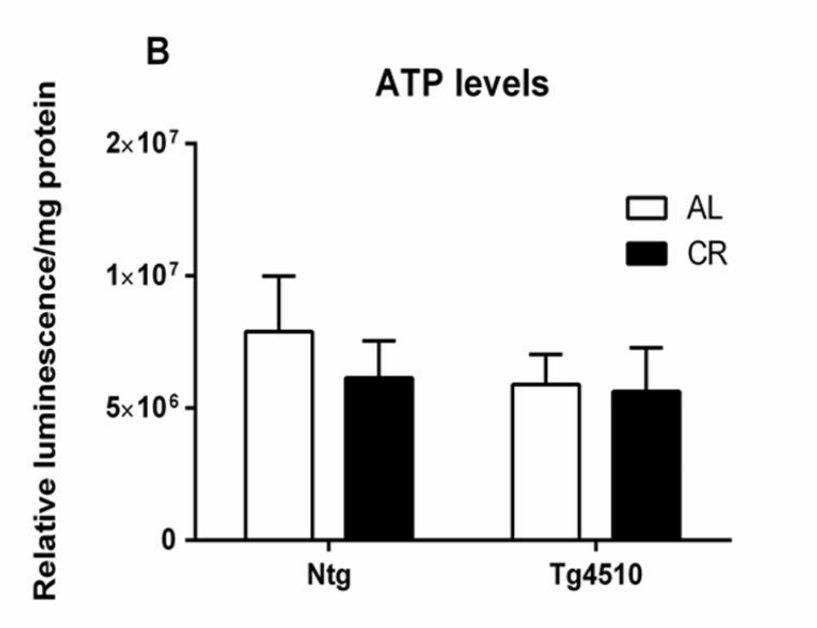


Figure 10 supplemental Mitochondrial ATP production
No effect of P301 tau expression on mitochondrial ATP synthesis in digitonin-permeabilized HEK293T cells. Levels of mitochondria-synthesized ATP in digitonin-permeabilized control and stably transfected P301L tau N2a cells administered ADP, Pi and malate and pyruvate as respiratory substrates. The complete method is described in the Material and Methods section. The amount of ATP measured in the presence of oligomycin, a mitochondrial F0F1-ATP synthase inhibitor, was subtracted off to obtain the level of mitochondria-synthesized ATP. No statistical difference was obtained between groups following calculation of the best fit straight lines through the data points as a measurement of the rate of ATP synthesis.

CHAPTER THREE: MELATONIN'S MITOCHONDRIAL PROTECTIVE ROLE IN ALZHEIMER'S MICE: ROLE OF MELATONIN RECEPTORS

Note to Reader: This chapter was published in Melatonin: Therapeutic Value and Neuroprotection CRC Press, Oct 31, 2014. This chapter has been reproduced with permission from the publisher. The lead author was responsible for enrichment of mouse brain samples for mitochondria and complex IV assay optimization. The lead author was also responsible for training and supervising the execution of 2 different complex IV assays in this study. The lead author was also responsible for generating figures, statistical analysis, and contribution to writing of the manuscript.

Abstract

Melatonin is a powerful antioxidant that has been shown to be cytoprotective in cell models and neuroprotective in mouse models of neurodegenerative diseases such as Alzheimer's disease (AD). But the role, if any that the MT1 and MT2 melatonin receptors play in this protection in AD models has not been well studied. Three to six months of treatment with the MT1/MT2 receptor agonist ramelteon (3 mg/kg/day) showed little to no benefit in the improvement of cognition or amyloid plaque pathology in AD mice. But piromelatine (Neu-P11), which functions both as an MT1/MT2 receptor agonist and as a serotonin 5-HT1A/1D receptor agonist, was even more protective than melatonin in the prevention of cognitive performance decline and the prevention of hippocampal cell loss in rats following hippocampal injection of amyloid-beta, the major component of plaques found in AD brain. Hippocampal and cortical brain regions from AD patients and animal models are often characterized by mitochondrial dysfunction, which may include cytochrome c oxidase deficiency, other oxidative phosphorylation (such as complex I and complex V) defects, dysregulation of components of the

TCA cycle, and impaired mitochondrial dynamics. Melatonin protected against the aging-induced decline and the amyloid-beta-mediated dysregulation of brain mitochondrial function in a mouse model of AD, with the protection being partly dependent and partly independent of the MT1 and MT2 melatonin receptors. Therefore melatonin receptors likely play a critical role in the preservation of mitochondrial function during aging and AD.

Alzheimer's Disease

Dementia affects 35 million people worldwide and this total is predicted to increase to over 60 million people in 2030 and over 100 million people by 2050. Due to this sharp increase in prevalence and the high cost for healthcare for these individuals, treatments that prevent or even delay the onset of dementia are highly sought. In this regard melatonin has been identified as a compound that may delay the onset of certain types of dementia. In this chapter we will discuss the potential of melatonin as a treatment for Alzheimer's disease (AD), the most prevalent form of dementia and the role that melatonin and its cellular receptors play in this process.

AD is characterized clinically by memory and functional deficits culminating in progressive cognitive decline. The vast majority of individuals with AD are 65 years of age or older. The likelihood of developing Alzheimer's doubles roughly every five years after age 65. The risk reaches nearly 50 percent after the age of 85. Another strong risk factor is family history as both genetics and environmental factors play a role in disease development.

In the brain, AD mostly affects the hippocampal and cerebral cortical regions and is characterized by the presence of extracellular amyloid beta (Aß) plaques, intracellular soluble Aß, and also by the presence of intracellular neurofibrillary tangles (NFTs) caused by the

hyperphosphorylated microtubule-associated tau protein. Familial or early onset forms of AD exist, make up around 5% of total AD cases, and are generally caused by mutations in by mutations in the presenilin-1 (PS1) or presenilin-2 (PS2) genes located on chromosome 14 or the amyloid precursor protein (APP) gene located on chromosome 21. The most prevalent (~ 85%) of these familial forms are caused by mutations in PS1. 95% of AD cases are sporadic, with age being the largest risk factor. The single largest genetic factor associated with late onset Alzheimer's disease (LOAD) is the ε4 allele of the apolipoprotein E (APOE) gene (Corder, Saunders et al. 1993; Strittmatter, Saunders et al. 1993). It likely contributes to 20-25 percent of LOAD cases. The *APOE* gene, coding for the major cholesterol binding protein in the human brain, apoliprotein E, exists as three polymorphic alleles (ε2, ε3 and ε4) resulting in 6 different genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4) (Mahley 1988; Bu 2009). The presence of one ε4 allele increases the likelihood of a person developing AD by three to four times (Corder, Saunders et al. 1993; Bertram and Tanzi 2008). Antecedent factors influencing AD progression are cardiovascular disease, smoking, hypertension, type II diabetes, obesity, and traumatic brain injury (TBI) (Mayeux and Stern 2012).

Mitochondrial dysfunction as a contributing factor in AD

Mitochondrial dysfunction occurs with aging (Harman 1972; Chomyn and Attardi 2003; Kujoth, Hiona et al. 2005; Wallace and Fan 2009) and also contributes to the pathophysiology of many diseases that occur as a normal part of aging. An association between mitochondrial dysfunction and AD is well established (Perry, Perry et al. 1980; Sorbi, Bird et al. 1983; Parker 1991). Redox imbalance and oxidative stress, which frequently develop as a result of mitochondrial dysfunction, have been observed in aging and neurodegenerative disorders such as

AD. This oxidative stress may lead to damage of important mitochondrial components such as mitochondrial DNA, mitochondrial proteins such as the electron transport chain (ETC) components, and mitochondrial phospholipids, especially to cardiolipin, which contains a high percentage of unsaturated fatty acids. Damage to the ETC proteins or cardiolipin, required for ETC complex function, may result in decreased ETC oxygen consumption, ATP production, and mitochondrial membrane potential, and also increased ROS production. This damage to mitochondria has also been shown to result in dysregulation of mitochondrial dynamics (e.g. fission, fusion, transport, mitochondrial biogenesis, and mitophagy) and if severe enough results in mitochondrial-mediated apoptosis. The mitochondrial damage has been shown to be most severe in areas adjacent to amyloid plaques (Xie, Guan et al. 2013).

Mitochondrial dysfunction can have varying effects depending on the degree. Small amounts of mitochondrial dysfunction, which typically slightly increase ROS production or decrease ATP levels can activate retrograde signaling and be compensated for by increased mitochondrial biogenesis. Two signals for mitochondrial biogenesis which occur as a result of mitochondrial dysfyunction include increased ROS production and AMP-activated protein kinase (AMPK) activation. Both of these signals can lead to the activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which binds nuclear respiratory factors-1 and 2, estrogen related receptor-alpha and other transcription factors which increase expression of proteins targeted to mitochondria. When damage to mitochondria becomes too great to be compensated by increased mitochondrial biogenesis and/ or degradation of damaged mitochondria through mitophagy, mitochondrial dysfunction arises that can lead to cell death.

Due to the fact that amyloid plaques are one of the two major phenotypic markers of AD, many strategies have been employed to pharmacologically decrease their formation, or once they have formed, to break them down and remove the plaque components from the brain. Unfortunately, anti-amyloid drugs have shown little success in AD treatment (Herrmann, Chau et al. 2011). Therefore, novel multi-pronged research strategies need to be employed that attack other phenotypes of AD. With this in mind, much scientific evidence links mitochondrial dysfunction with Alzheimer's disease (AD), yet therapies targeting mitochondrial dysfunction in AD have yet to be methodically tested in human patients. Several research groups have discovered altered activities of the mitochondrial citric acid cycle enzymes and electron transport chain complexes, predominately complex IV, in the brains from postmortem AD patients (Bubber, Haroutunian et al. 2005) and in transgenic mouse models of familial AD (Hauptmann, Scherping et al. 2008). Mitochondrial ATP synthase levels have also been found to be low in AD brain (Schagger and Ohm 1995). Specifically, mitochondrial dysfunction is one of the earliest symptoms in double mutant London and Swedish APP transgenic mice (Hauptmann, Scherping et al. 2008). Mitochondrial dysfunction occurs in these mice at 3 months of age, at an age before extracellular deposits of amyloid beta peptide (Aß), and much earlier than memory impairment. Therefore mitochondrial dysfunction may be an initiating event in AD, an idea which has been formalized in the mitochondrial cascade hypothesis of AD (Swerdlow and Khan 2004; Swerdlow, Burns et al. 2013).

An oligomeric form of Aß likely dimeric Aß has been described to inhibit cytochrome c oxidase of the mitochondrial electron transport chain (Crouch, Blake et al. 2005). Through this interaction, as well as through an interaction with complex I (Munguia, Govezensky et al. 2006), Aß stimulates increased reactive oxygen species production from the ETC, which may ultimately

lead to cell death through opening of the mitochondrial permeability transition pore in the inner mitochondrial membrane. This event uncouples mitochondrial oxidative phosphorylation and releases mitochondrial factors such as cytochrome c and apoptosis inducing factor (AIF) into the cytoplasm, where caspases execute the apoptotic program. Knockout of cyclophilin D, a mitochondrial matrix peptidylprolyl cis-trans isomerase and Aß-binding protein (Du, Guo et al. 2008) that facilitates mitochondrial permeability transition pore opening, increases learning and memory and synaptic function in a mouse model of AD (Du, Guo et al. 2008). In this regard, APP_{swe} overexpression upregulates mitochondrial cyclophilin D (Manczak, Mao et al. 2010), which facilitates mitochondrial permeability transition pore opening and cell death. High Aß levels, in addition to inhibiting the mitochondrial ETC, have been shown to decrease TCA cycle enzyme activities (Bubber, Haroutunian et al. 2005), alter the rates of mitochondrial fission and fusion (Manczak, Calkins et al. 2011), decrease the rate of mitophagy (Santos, Correia et al. 2010), and decrease axonal transport of mitochondria (Calkins, Manczak et al. 2011).

But whether mitochondrial dysfunction plays an initiating role in human AD disease pathogenesis, specifically in the synaptic or neuronal loss associated with the late onset form of AD is controversial. It may as yet prove that mitochondrial dysfunction is a result of other AD pathology. In this regard some reports of normal mitochondrial function in AD models have been published. For example, mitochondrial function appeared mostly normal in presynaptic nerve terminals in mouse models of AD (Choi, Gerencser et al. 2012). But the ability of Aß, a common component of AD plaques (Pereira, Santos et al. 1998); hyperphosphorylated tau, a component of neurofibrillary tangles (David, Hauptmann et al. 2005); and apolipoprotein E4 (Chang, ran Ma et al. 2005), a major risk factor for late-onset AD, to each cause mitochondrial dysfunction lends strong support for a mitochondrial etiology for AD.

Melatonin protects mitochondrial, cell, and brain function in AD mice in many ways.

Melatonin has long been used to alleviate jet lag and to modify circadian rhythms. However, its potential use as an antioxidant in the treatment of disease is gaining momentum, especially since controlled release formulas having a more favorable pharmacokinetic profile have been developed (Lemoine and Zisapel 2012). Melatonin can be used to increase the amount of total and REM sleep (Dijk and Cajochen 1997). Sleep itself can protect against AD as Aß and other metabolic waste products are more quickly cleared from brains in the sleeping state than during wakefulness (Xie, Kang et al. 2013). This has led some to suggest that disrupted circadian rhythms may be partly a cause instead of a result of AD (Bedrosian and Nelson 2012). Melatonin has an advantage over many other antioxidants such as Vitamin E in that it is freely permeable to the brain (Lahiri, Ge et al. 2004). Melatonin has been reported to extend the lifespan of APP transgenic mice (Matsubara, Bryant-Thomas et al. 2003) and prevent cognitive dysfunction in APP/PS1 mice (Olcese, Cao et al. 2009), although it failed to reverse cognitive dysfunction if melatonin treatment was initiated after the initiation of cognitive impairment (G. Arendash, unpublished data). Others also found little to no positive effect of melatonin on oxidative stress or amyloid burden when treatment was initiated late in life in a mouse AD model (Quinn, Kulhanek et al. 2005). Melatonin was also shown to decrease immunoreactive Aß levels (Olcese, Cao et al. 2009) and protein nitration (Matsubara, Bryant-Thomas et al. 2003) in the brain of AD mouse models. Melatonin is hypothesized to delay AD in mice through preventing Aß oligomerization (Pappolla, Bozner et al. 1998; Olcese, Cao et al. 2009), preventing reactive oxygen species-mediated damage (Ionov, Burchell et al. 2011), and by stabilizing mitochondrial function in the presence of increased levels of Alzheimer's amyloid (Dragicevic, Copes et al.

2011) or phosphorylated tau (Peng, Hu et al. 2013). One study found that supplementation of Tg2576 mutant amyloid precursor protein-expressing Alzheimer's mice with melatonin from 8-12 months of the lifespan was critical, because treating the mice with melatonin from 4-8 months of life was ineffective (Peng, Hu et al. 2013).

Melatonin receptors

In the plasma membrane, two receptors for melatonin, MT1 and MT2 have been identified. These melatonin receptors are seven-pass G-protein coupled receptors (GPCR) that can homo- or heterodimerize and can signal through multiple signaling pathways in different cells by using distinct G-protein alpha subunit isoforms. Melatonin has sub-nanomolar affinity for these receptors (Dubocovich and Markowska 2005), which allows the receptors to be activated when melatonin levels rise due to increased pineal gland secretion at night. The MT1 and MT2 genes, located on chromosomes 4q and 11q, respectively show 55% identity and encode proteins of 350 and 365 amino acids in length. The intracellular portions of the receptors contain casein kinase 1 and 2, protein kinase A, and protein kinase C phosphorylation sites. MT1 and MT2 are especially found at high density in the suprachiasmatic nucleus in the brain (Liu, Weaver et al. 1997) where they are bound by melatonin and influence the diurnal rhythm. mRNA levels of the MT1 receptor have also been shown to undergo diurnal variation (Guerrero, Gauer et al. 1999). The classic action of melatonin receptor signaling is to dissociate heterotrimeric G proteins where the Gα and Gßγ subunits interact with various signaling pathways. In the suprachiasmatic nucleus following melatonin binding, the Gαi2 and Gαi3 isoforms have been described to inhibit adenylate cyclase activity (Brydon, Roka et al. 1999). This event decreases cAMP levels and decreases pituitary adenylate cyclase-activating protein

(PACAP)-mediated CREB activation affecting the circadian clock (Travnickova-Bendova, Cermakian et al. 2002).

In addition to inhibiting cAMP mediated signaling, melatonin receptors can activate phospholipase C signaling through Gq-coupling (Chan, Lai et al. 2002). This activates Ca^{2+}-mediated and protein kinase C (PKC) phosphorylation cascades. These signals activate calmodulin kinase and mitogen-activated protein kinase (MAPK) signaling kinases including p38, JNK, and ERK. There is also evidence that melatonin receptor activation may lead to stimulation of the PI3 kinase/Akt pathway or opening of different ion channels such as voltage-gated calcium channels or large conductance calcium-activated potassium channels (Hardeland 2009). In addition to these effects mediated by GPCRs, melatonin can also interact directly with quinone reductase 2, orphan nuclear receptors such as retinoid Z receptor (RZR) and retinoid acid receptor-related orphan receptor (ROR), calmodulin, calreticulin, and other proteins (Reiter, Tan et al. 2010). The melatonin receptor-related protein GPR50, an orphan GPCR that is 45% identical to melatonin receptors, can bind to MT1 and function as an antagonist (Levoye, Dam et al. 2006). From this collection of data it is apparent that melatonin and melatonin receptors act through many different complex signaling mechanisms to affect cellular physiology.

The melatonin MT1 and MT2 receptors play complementary roles in cell function. MT1 receptors are found in similar, but not identical locations. MT1 is located in the suprachiasmatic nucleus, cerebellum, hippocampus, substantia nigra, and many other tissues of the body while MT2 receptor expression is mostly restricted to brain including the suprachiasmatic nucleus, but expression has been found in a few other tissues as well, but not to the extent of MT1 (Dubocovich and Markowska 2005). In the suprachiasmatic nucleus, MT1 and MT2 receptors

appear to play different roles. MT1 receptor activation leads to acute inhibition of neuronal activity, while MT2 activation leads to the phase shift of circadian rhythm (Liu, Weaver et al. 1997; Hunt, Al-Ghoul et al. 2001). Some of these studies using brain slices have also been confirmed in mice with specific knockout of the MT1 receptor or in mice administered 4P-PDOT, a specific inhibitor of MT2 (Dubocovich, Hudson et al. 2005). The phenotypes of the MT1 and MT2 receptor knockouts are subtle (Jin, von Gall et al. 2003) and phenotypes have mainly been found at the molecular level, with the exception of the lack of phase-shifting effect of melatonin in the MT1 receptor knockout mice and a deficit in learning and memory in the MT2 receptor knockout mice (Dubocovich, Hudson et al. 2005; Larson, Jessen et al. 2006).

Melatonin receptors and the hippocampus

The hippocampus plays a key role in memory and is the main site of dysfunction in AD. Both MT1 and MT2 receptors are expressed in the hippocampus, specifically in the CA1 and CA3 regions, the subiculum, and the dentate gyrus (Musshoff, Riewenherm et al. 2002). Administration of melatonin to hippocampal slices was shown to increase the neuronal firing rate, which was blocked by luzindole, which shows a slight selectivity for MT2 over MT1. Melatonin also showed an inhibition of long term potentiation in hippocampal slices, which was inhibited by both luzindole and 4P-PDOT, a more specific inhibitor of MT2 (Wang, Suthana et al. 2005). MT1 and MT2 knockout mice were used to confirm these observations (Dubocovich, Hudson et al. 2005; Larson, Jessen et al. 2006). To verify that MT2 is involved in learning and memory, MT2 knockout mice were tested in the elevated plus maze paradigm (Larson, Jessen et al. 2006). A marked inhibition of learning was measured indicating a deficiency in long term synaptic plasticity.

In human studies it was shown that the time of day that melatonin was administered drastically altered the effect of melatonin on learning behavior (Gorfine and Zisapel 2007). These studies implicated melatonin and the circadian clock in human memory processing and consolidation. In further neuroimaging studies, it was shown that melatonin administration was as effective as a 2-hour nap in increasing performance in a verbal association test (Gorfine, Yeshurun et al. 2007). Future studies should aim to confirm these initial human studies on the effects of melatonin on learning behavior and determine other paradigms where melatonin treatment may be beneficial.

Mitochondrial localization of the MT1 melatonin receptor in mice

Melatonin MT1 receptors have been localized to brain mitochondria in mice, whereas only a trace amount of MT2 receptors could be found in this subcellular localization (Wang, Sirianni et al. 2011). Interestingly, MT1 receptor levels declined in the R6/2 mouse model of Huntington's disease and this decline, including the decline in mitochondrial levels, was delayed by melatonin treatment, which also delayed disease pathology in these mice. Therefore, the decline in MT1 receptors induced by mutant huntingtin protein may be a contributing factor to the disease. It is currently unclear what role, if any, that mitochondrial MT1 receptors play in brain physiology and if these declines occur in other neurodegenerative disorders such as AD, but identifying the mitochondrial role of MT1 receptors will likely prove instrumental in understanding the protective effect of melatonin treatment for neurodegenerative diseases.

AD patients have decreased melatonin levels

Involvement of melatonin in the pathogenesis of AD has been suggested from studies indicating that AD patients have decreased blood and cerebrospinal fluid (CSF) levels of melatonin (Maurizi 1997). In AD patients ApoE4 allele status also plays a role in determining melatonin levels as APOE4 homozygotes had the lowest melatonin levels (Liu, Zhou et al. 1999). Unexpectedly, in C6 glioma cells, expression of APOE4, the allele most associated with development of AD, expression increased melatonin levels compared to expression of APOE3 or APOE2 (Liu, Meng et al. 2012). Perhaps different results would be obtained in neuronal cells or in primary, non-transformed cells. In addition, lower CSF levels of melatonin have been strongly correlated with progression of AD neuropathology, and preclinical AD subjects already have decreased CSF melatonin levels. In addition to these findings suggesting that decreased melatonin levels are an early event in AD pathogenesis, epidemiologic studies have reported that melatonin treatment provides cognitive benefit to patients with mild cognitive impairment (MCI) as well as AD patients. The decreased melatonin levels in AD brain may allow oxygen and nitrogen free radicals to damage sensitive neurons (Srinivasan, Kaur et al. 2010).

Changes in melatonin receptor levels in aging, Parkinson's disease, and AD

MT1 and MT2 receptor levels have been shown to decline with aging in many tissues (with the exception of the thymus) in rats (Sanchez-Hidalgo, Guerrero Montavez et al. 2009) and mice. There was also a report of the MT1 receptor increasing with age in mice in spleen (Bondy, Li et al. 2010). The level of the MT2 receptor declined in the aged suprachiasmatic nucleus in mice (von Gall and Weaver 2008) and humans (Wu, Zhou et al. 2007). But interestingly the

MT2 receptor increased with age in the hippocampus in gerbils (Lee, Choi et al. 2010). Therefore, regulation of melatonin receptor levels is likely species specific.

The MT1 receptor level was shown to increase in hippocampal sections CA-1-4 from AD patients (Savaskan, Olivieri et al. 2002), while the MT2 receptor levels declined in these and other regions such as the retina in AD patients (Savaskan, Ayoub et al. 2005; Savaskan, Jockers et al. 2007). It is possible that the increased MT1 receptor expression is a compensatory mechanism in response to the decreased melatonin levels, while the decreased MT2 receptor levels and/or decrease in melatonin levels may contribute to the onset of AD. In addition to the decline in melatonin receptor levels with aging, both MT1 and MT2 receptors have been shown to decrease in the substantia nigra and the amygdala in Parkinson's disease (PD) patients (Adi, Mash et al. 2010). Melatonin has been shown to delay movement problems associated with PD in rodent models (Mayo, Sainz et al. 2005). But the role that the MT1 and MT2 receptors play in this protection remains unclear.

Melatonin treatment has shown beneficial effects in AD patients

Preliminary data with human AD patients showed that melatonin supplementation decreased "sundowning", improved sleep, and slowed disease progression (Maurizi 2001). A slight improvement in cognitive function was observed when melatonin was given to AD patients (Brusco, Marquez et al. 1998; Brusco, Marquez et al. 2000; Asayama, Yamadera et al. 2003). In addition, a retrospective study showed that melatonin treatment improved cognitive performance and sleep quality in patients with MCI (Furio, Brusco et al. 2007). Several studies have also shown that melatonin increases sleep duration and quality in AD patients (Mishima, Okawa et al. 2000; Mahlberg, Kunz et al. 2004; Cardinali, Furio et al. 2011). This data suggests

that melatonin may be a beneficial add-on drug for the treatment of AD. However, there has also been a large clinical study showing that melatonin had no effect on sleep or agitation in AD patients (Gehrman, Connor et al. 2009). Therefore larger multi-center double-blind placebo controlled studies are needed to clarify these results.

Beneficial effects of melatonin in cell and mouse models of AD

Cell culture studies using AD model cells have also shown positive results following melatonin treatment. Most of the studies have been performed using mutant amyloid precursor protein (APP) overexpression, which is a model of familial AD. A drawback of these studies is that these cells might not address the driving force behind the pathology in LOAD patients. The ApoE4 allele appears to be the strongest determinant of LOAD. ApoE4 can enhance or inhibit toxic fibril formation when bound to Aß depending upon the concentration (Naiki, Gejyo et al. 1997). Cell culture studies have shown that melatonin can bind to ApoE and inhibit toxic Aß fibril formation to a much higher extent than melatonin can inhibit fibril formation by itself (Poeggeler, Miravalle et al. 2001). In culture, melatonin has also been shown to inhibit mitochondrial DNA damage and apoptosis induced by Aß (Pappolla, Sos et al. 1997; Pappolla, Chyan et al. 1999). Most of the culture and rodent studies attributed the beneficial effects of melatonin to a combination of direct anti-oxidant effects (Ionov, Burchell et al. 2011) and the ability of melatonin to prevent toxic Aß fibril formation (Olcese, Cao et al. 2009). However, neither of these explanations satisfactorily explain why melatonin was only able to prevent Aß-mediated mitochondrial dysfunction in young isolated hippocampal neurons of low (~10) passage number, but not in senescent hippocampal neurons of high (~25) passage number (Dong, Huang et al. 2010). A possible explanation for these results is that melatonin receptor expression

declined in the senescent neurons and that a portion of the protective effect is mediated by melatonin receptor signaling as well.

Melatonin has shown beneficial effects on cognitive function and plaque formation in many rodent studies of amyloid-ß toxicity (Pandi-Perumal, BaHammam et al. 2013). There are too many to discuss in this chapter, so we will mention some of the most relevant concerning the ability of melatonin to positively impact mitochondrial function. A more detailed examination of this topic can be found in the following reviews (Cheng, Feng et al. 2006; Rosales-Corral, Acuna-Castroviejo et al. 2012; Cardinali, Pagano et al. 2013; Lin, Huang et al. 2013; Pandi-Perumal, BaHammam et al. 2013) First a study showed that both exercise or melatonin treatment delayed many of the phenotypes in 3x-Tg AD mice. But only the combination of melatonin and exercise together was able to completely prevent the loss of mitochondrial ETC complex protein levels and increase levels of coenzyme Q9, the precursor to the ETC electron carrier CoQ10 (Garcia-Mesa, Gimenez-Llort et al. 2012). Another study showed that hippocampal injection of fibrillar Aß into mice led to cellular and mitochondrial uptake that increased ROS production and led to a decrease in the respiratory control ratio that was slightly increased by the presence of melatonin in the drinking water. However, an Aß-mediated inhibition of the mitochondrial F0F1-ATP synthase was not improved by melatonin administration (Rosales-Corral, Acuna-Castroviejo et al. 2012). Lastly we have shown that the ability of a one month melatonin treatment largely reverses mitochondrial dysfunction mediated by Aß in Alzheimer's mice and this restorative effect was blunted in mice receiving melatonin and caffeine (Dragicevic, Delic et al. 2012). The mechanism through which caffeine partially blocks the effect of melatonin remains unknown, but it is likely through inhibition of melatonin receptor signaling.

Indole-3-propionamide (IPAM) as an alternative to melatonin for the treatment of neurodegenerative disease.

Indole-3-propionic acid (IPA) (OXIGON^{TM}), a natural metabolite found in almost all organisms, and a related indole to melatonin, was reported to be a better anti-oxidant than melatonin and just like melatonin showed no pro-oxidant activity (Chyan, Poeggeler et al. 1999). However, being an acid, it is hydrophilic and is slow to permeate the blood brain barrier for the treatment of neurodegenerative diseases. Nonetheless, a phase II clinical trial using IPA to treat Friedreich's Ataxia was initiated in 2012 (Gomes and Santos 2013). The same group of researchers who discovered the strong antioxidant activity of IPA subsequently published that indole-3-propionamide (IPAM) was more effective as an antioxidant than melatonin, but retained the hydrophobic nature of melatonin (Poeggeler, Sambamurti et al. 2010). In addition, when i.p. injected into rats at 0.5 mg/kg, high IPAM levels could be measured in the brains for over 8 hours, but melatonin and IPA levels were not even measurable after 1 hour. IPAM also prevented Aß aggregation in a thioflavin T assay just like melatonin and IPA. Therefore IPAM may show the highest potential of any known indole antioxidant for the treatment of neurodegenerative diseases. IPAM was also shown to increase the lifespan of a rotifer species by 300% (Poeggeler, Sambamurti et al. 2010). IPA treatment had no effect on the lifespan of a separate rotifer species (Snell, Fields et al. 2012). We published that IPA-induced restoration of mitochondrial function in N2a-APP_{swe} cells was partially blocked by luzindole (Dragicevic, Copes et al. 2011). Therefore, melatonin-related indoles such as IPA and IPAM may be utilizing melatonin receptors to protect the cells from the damage-induced by Aß. However, these observations should be verified in animal models. These results suggest IPAM in addition to

controlled release melatonin may show beneficial effects in clinical trials for neurodegenerative diseases such as AD. Other melatonin-related compounds, such as the melatonin breakdown product AFMK, have also shown promise as a strong antioxidant in the protection of cells from oxidative stress induced by Aß (Poeggeler, Miravalle et al. 2001).

Direct effects of melatonin and IPAM on mitochondrial function.

Melatonin increases mitochondrial function through both direct and indirect mechanisms. Binding sites for melatonin have been found on mitochondrial membranes (Yuan and Pang 1991; Poon and Pang 1992). This may be partially explained by the recent localization of the melatonin MT1 receptor to mitochondria (Wang, Sirianni et al. 2011). But melatonin also binds mitochondrial complex I with an affinity of 150 pM (Hardeland 2009). Mitochondrial electron transport chain (ETC) complex I and IV activities are stimulated by melatonin in brain (Martin, Macias et al. 2000). Melatonin also preserves mitochondrial respiration in the aging mouse brain (Carretero, Escames et al. 2009), in mice given ruthenium red (Martin, Macias et al. 2000), or in mice given the ETC complex IV inhibitor cyanide (Yamamoto and Mohanan 2002). Like melatonin but more potently, IPAM stimulates complex I and IV activity, and stabilizes mitochondrial function in the presence of mitochondrial toxins such as FCCP, doxorubicin, and antimycin A (Poeggeler, Sambamurti et al. 2010).

Antioxidant signaling through melatonin receptors

Melatonin treatment has been shown to increase the mRNA levels of many antioxidants in rat brain including Mn-SOD (SOD2) Cu/Zn-SOD (SOD1), and catalase (Kotler, Rodriguez et al. 1998; Gunasingh, Philip et al. 2008; Garcia, Esparza et al. 2010). Glutathione peroxidase and

glutathione reductase have also been shown to be upregulated by melatonin in certain tissues (Carretero, Escames et al. 2009; Limon-Pacheco and Gonsebatt 2010; Pandi-Perumal, BaHammam et al. 2013). It is hypothesized that this upregulation of antioxidant gene expression occurs mainly through melatonin receptor signaling as luzindole, an inhibitor of melatonin receptors has been shown to partially or completely prevent upregulation of these antioxidant genes in various tissues and cell lines (Rezzani, Rodella et al. 2006; Choi, Dadakhujaev et al. 2011; Adamczyk-Sowa, Sowa et al. 2013).

Superoxide dismutase converts superoxide to hydrogen peroxide and then either catalase in the peroxisomes or glutathione peroxidase in the mitochondria or cytoplasm converts the hydrogen peroxide to water. It has been shown that melatonin treatment of rats increased mitochondrial superoxide dismutase activity in old rat brain (Ozturk, Akbulut et al. 2012). It was also shown that aging upregulated mitochondrial glutathione peroxidase activity and melatonin prevented this response. Lastly there was an aging-related decline in the superoxide dismutase to glutathione peroxidase ratio that was prevented by melatonin treatment. Therefore, melatonin treatment preserves the correct youthful ratio of these enzymes, preventing superoxide increases in the cell and oxidative damage.

Melatonin receptors are not always required for the antioxidant effect of melatonin on cells and tissues. In several cases luzindole did not prevent the protective effects of melatonin indicating that melatonin's antioxidant effects can frequently be receptor-independent (Behan, McDonald et al. 1999; Lahiri, Singh et al. 2009; Song, Kim et al. 2012). However, concerning the effect of melatonin on neural cells, melatonin was shown to prevent neural ischemic stroke injury, partially through a MT2-dependent mechanism as both luzindole and the more selective

MT2 antagonist 4P-PDOT (4-phenyl-2-propionamidotetralin) partially blocked melatonin's protective effect (Chern, Liao et al. 2012). Similarly luzindole blocked the protective effect of melatonin on the palmitic acid-induced increased in ROS levels and cell death in primary mouse astroglial cells (Wang, Liu et al. 2012). Therefore it will be important to determine if the protective effects of melatonin on mitochondrial dysfunction in aging and AD depends on the MT1 or MT2 receptors.

Melatonin receptor agonists for the treatment of AD

There has been *in vitro* data implicating a role for melatonin receptor signaling in mediating a partial protection from Aß-mediated mitochondrial dysfunction (Dragicevic, Copes et al. 2011), while two studies showed that AD model mice treated with the melatonin receptor agonist ramelteon showed little or no protection from AD pathology. In the first ramelteon treatment study, the AD model B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J transgenic mouse strain (APP/PS1 mice) was used. The mice were treated with ~3 mg/kg/day of ramelteon and no change in cognitive behavior as judged by performance in a water maze was observed after 3 months of treatment (McKenna, Christie et al. 2012). Even 6 months of ramelteon treatment failed to yield reductions in amyloid plaque burden. The second study, also using an APP/PS1 mouse strain, determined a lack of effect of ramelteon on spatial memory performance, but did find that ramelteon slightly decreased hippocampal protein oxidation in the APP/PS1 mice (Bano Otalora, Popovic et al. 2012). However, a study using the melatonin receptor agonist Neu-P11 (piromelatine) found that rats which had undergone intrahippocampal injection with Aß performed better in novel object recognition and Y-maze tasks and showed less CA1 hippocampal cell loss when i.p. injected with 50 mg/kg Neu-P11. The Neu-P11 injected group

even outperformed the melatonin injected group (He, Ouyang et al. 2013). One further piece of data that supports the use of melatonin receptor agonists clinically for cognitive dysfunction is that ramelteon was shown to improve delirium in five patients after only one day of treatment (Furuya, Miyaoka et al. 2012).

The role that melatonin receptors play in protecting AD-associated mitochondrial dysfunction

We have identified melatonin receptors, likely MT2 as being essential for the full mitochondrial protective effect of melatonin against Alzheimer's amyloid (Dragicevic, Copes et al. 2011). We also observed that a low concentration of caffeine or cAMP-dependent or cGMP-dependent phosphodiesterase inhibitors blocked melatonin from fully protecting against amyloid-mediated mitochondrial dysfunction. Therefore melatonin receptor signaling may decrease adenylate cyclase activity to decrease cAMP levels to protect mitochondrial function in the hippocampus in AD and inhibiting cAMP-dependent phosphodiesterases may antagonize this response by restoring cAMP levels. Although the mechanism has not yet been fully elucidated, direct oxidant scavenging and direct inhibition of amyloid fibril formation are likely not the sole mechanisms of melatonin-mediated protection in AD. Melatonin receptor signaling, therefore, likely contributes to the melatonin-mediated prevention of cognitive dysfunction in AD mice. Discovering the signal transduction pathway between melatonin receptors and mitochondria is an essential next step in determining the suitability of melatonin, melatonin-related indoles, or melatonin receptor agonists for clinical trials. The most straightforward hypothesis is that there is increased expression of antioxidant defense proteins as a result of MT2 receptor signaling that is protecting mitochondria from oxidative damage in AD model mice and cells.

The role that melatonin receptors play in protecting from aging-induced loss of cytochrome c oxidase activity in mice

In data just published at the time of print, we have performed experiments determining the role of melatonin receptors in preventing the decline in brain cytochrome c oxidase (COX) activity during aging (Fig. 11). We used striatal tissue from knockout mice deficient in both the MT1 and MT2 melatonin receptors. We found that mice deficient in both MT1 and MT2 melatonin receptors showed 33% more loss of COX activity at 16 months of age as compared to the activity in young mice (67% loss in MT1/MT2 knockout mice compared to 50% loss in WT controls). In addition melatonin treatment completely prevented the loss of COX activity in the WT mice, but the melatonin-treated MT1/MT2 receptor knockout mice lost 33% of COX activity by 16 months of age. These data indicate that roughly half of the effect of melatonin in preventing aging-related loss of COX activity is MT1 or MT2 receptor-dependent, while the other half of the protective effect is receptor-independent. Future studies will determine which of the melatonin receptors is required for the protection from the aging-related loss of COX activity.

The role that melatonin receptors play in protecting from AD-induced alteration of COX activity in mice

In further data just published at the time of print, we determined the role that melatonin receptors play in protection from APP_{swe}/PS1-induced alteration of COX activity in the striatum of mice (Fig. 12). In contrast to what we were expecting, we did not find a decrease in COX activity caused by APP_{swe} expression as we and others have observed when using mice of

different genetic backgrounds (Manczak, Anekonda et al. 2006; Dragicevic, Mamcarz et al. 2010). In fact we found a striking increase in COX activity from 13 to 16 months of age. Others have also found increased cytochrome c oxidase activity in APP-expressing mice, such as in the ventral striatum of APP23 mice partially backcrossed onto a C57B/6 background (Strazielle, Sturchler-Pierrat et al. 2003). This report showed that COX activity increased, but only in specific regions of the brain. COX activity was also shown to increase in Tg2576 mice at 5 months of age (Poirier, Amin et al. 2011) and in another report in these mice at 7 months of age (Cuadrado-Tejedor, Cabodevilla et al. 2013). Consistent with this data, mitochondrial electron transport genes are upregulated in Tg2576 mice (Reddy, McWeeney et al. 2004). However, many others have shown decreased COX activity in different brain regions of Tg2576 mice (Manczak, Anekonda et al. 2006; Valla, Schneider et al. 2007; Zhang, Xiong et al. 2010; Varghese, Zhao et al. 2011). Decreased COX activity has also been shown in double and triple transgenic mouse models of AD combining mutant APP overexpression with overexpression of presenilin-1 and/or tau (Rhein, Song et al. 2009; Wolf, Braden et al. 2012).

We used MT1/MT2 knockout mice crossed with APP_{swe}/PS1 transgenic mice and studied the effects on COX activity on striatal extracts from mice treated with or without melatonin. We found that melatonin treatment completely inhibited the APP_{swe}/PS1-mediated increase in COX activity under conditions of normal MT1 and MT2 expression and even led to a slight decrease in COX activity. However, in the MT1/MT2 knockout mice, melatonin treatment only led to a small non-statistically significant decrease in the APP_{swe}/PS1-mediated increase in COX activity ($p=0.3$). Therefore, the melatonin receptors play an important role in the ability of melatonin to prevent AD-related changes in COX activity. It will be important to repeat these experiments using hippocampal or cerebral cortical tissue or a different genetic background of mice to

determine if these trends hold under conditions where COX activity declines as a result of APP_{swe} expression.

Molecular mechanisms through which melatonin receptor signaling protects mitochondrial function in aging and disease

The signaling pathway through which melatonin receptor signaling induces antioxidant gene expression and protects mitochondrial function has yet to be convincingly identified in the central nervous system (CNS). However, it is likely to signal through one of the following pathways described below. There are at least 4 examples of melatonin stimulating the Nrf2 pathway to protect against oxidative damage in the nervous system. Melatonin was shown to decrease okadaic acid-induced memory dysfunction in rats through upregulation of the Nrf2 signaling pathway and prevention of NF- κB activation, which leads to neuroinflammation (Mendes, Lopes et al. 2013). Nrf2 is normally sequestered in the cytoplasm by Keap1. During oxidative and nitrosative stress, such as shown following ischemic brain injury, tyrosine 473 of Keap1 is nitrated, which prevents release of Nrf2 to the nucleus to induce transcription (Tao, Huang et al. 2013). Melatonin treatment prevents damage to Keap1, allowing Keap1 to release Nrf2 and Nrf2 to translocate to the nucleus, bind antioxidant response elements (ARE) in DNA and transcribe protective genes as a response to the stress. Melatonin was also shown to activate Nrf2 to provide protection from early brain injury in a subarachnoid hemorrhage model in rats (Wang, Ma et al. 2012), to protect against high linear energy transfer (LET) carbon ion radiation in mouse brains (Liu, Zhang et al. 2012), and to protect the sciatic nerve from increased levels of pro-inflammatory cytokines and cell death in streptozotocin-induced diabetic neuropathy in rats (Negi, Kumar et al. 2011).

Melatonin can also modulate expression of SIRT1, a nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase that deacetylates and activates many substrate proteins including PGC-1α, FoxO1, NF-κB, and p53. There have been several examples of melatonin downregulating SIRT1 in cancer cell lines (Cheng, Cai et al. 2013), while there have been at least 3 reports of melatonin upregulating or preventing the decline of SIRT1 expression in brain in response to a stress (Hardeland 2013). For example, melatonin prevented the decline in brain SIRT1 levels at 10 months of age in the SAMP8 mouse model of accelerated senescence (Gutierrez-Cuesta, Tajes et al. 2008), in sleep-deprived rat hippocampus (Chang, Wu et al. 2009), and in isolated aged neurons from rats (Tajes, Gutierrez-Cuesta et al. 2009).

Consistent with this activation of SIRT1 by melatonin, melatonin treatment has been shown to increase expression of the master mitochondrial transcriptional coactivator α in white adipocytes, turning a portion of them into brown adipocytes (Jimenez-Aranda, Fernandez-Vazquez et al. 2013). PGC-1α is known to induce gene expression of antioxidant genes such as SOD2 and glutathione peroxidase-1 (GPx1) in the brain (St-Pierre, Drori et al. 2006). However, the PGC-1α promoter has a CREB binding site for transcriptional activation (Ashabi, Ramin et al. 2012; Sheng, Wang et al. 2012). So it is possible that ability of melatonin to decrease cAMP levels through adenylate cyclase inhibition may lead to decreased PGC-1α transcription. However, treating cardiac cells with catecholamines, which increase adenylate cyclase activity to increase cAMP levels, decreases PGC-1α activity (Arany, Novikov et al. 2006). In addition there was also an inverse correlation between CREB activation and PGC-1α activation in the heart of spontaneously hypertensive rats. Therefore, there is not always a direct correlation between the activities of CREB and PGC-1α.

There is diurnal variation in the expression of SIRT1 and PGC-1α in some tissues (Asher and Schibler 2011) similar to the cyclic variation in melatonin synthesis in the pineal gland. In liver and skeletal muscle it has been shown that this diurnal expression pattern of PGC-1α stimulates the expression of clock genes through the co-activation of the ROR family of orphan nuclear receptors. Expression of the *Bmal1* and *Rev-erb-α* genes were notably induced. This diurnal expression of PGC-1α is likely influenced by the similar cyclic expression pattern of the NAD-dependent SIRT1 deacetylase (Asher, Gatfield et al. 2008; Nakahata, Kaluzova et al. 2008) and the diurnal variation in NAD levels and the NAD/NADH ratio caused by circadian expression of nicotinamide phosphoribosyltransferase (NAMPT) (Nakahata, Sahar et al. 2009; Ramsey, Yoshino et al. 2009). SIRT1 associates with the CLOCK-BMAL1 heterodimer and deacetylates BMAL1 and PER2, which destabilizes PER2 leading to its degradation. The rhythmic PGC-1α expression pattern is also likely influenced by the circadian oscillation of CREB activation as shown in both the suprachiasmatic nucleus (O'Neill, Maywood et al. 2008) as well as in peripheral tissues (Wang and Zhou 2010). The relation between the oscillations in these metabolic regulators, melatonin action, and AD remain relatively unexplored.

Since the enzymes that produce melatonin from tryptophan in the pineal gland are controlled by the circadian clock and because melatonin receptors are present in the suprachiasmatic nucleus in the hypothalamus, it has been speculated that melatonin binding to these receptors would influence the circadian clock machinery. Unexpectedly only a limited number of studies have examined the role of melatonin or melatonin receptors on the circadian clock genes (Jung-Hynes, Reiter et al. 2010). One study found that clock genes were downregulated in the adrenal cortex of a C57BL melatonin-deficient mouse strain compared to a C3H melatonin-proficient strain (Torres-Farfan, Seron-Ferre et al. 2006). Another study found

that melatonin, through binding to MT1 receptors, could decrease expression of PER1 and CLOCK, but had no effect on the levels of BMAL1 in primary striatal cultures from mice (Imbesi, Arslan et al. 2009). In addition, a phase-dependent effect of rhythmic melatonin administration was found on circadian clock gene expression (*Per2* and *Bmal1*) in the heart of hypertensive rats (Zeman, Szantoova et al. 2009), but no effect was observed in the suprachiasmatic nucleus (Poirel, Boggio et al. 2003). Specifically, the authors suggested that only melatonin applied during the dark phase of the 24-hour cycle allowed a strong synchronization of circadian clock expression in the heart. It will be interesting to determine if expression changes or activation of PGC-1α, SIRT1, or CREB play a role in these effects of melatonin.

AMP kinase (AMPK) works upstream and in parallel to SIRT1 in neuro-protective pathways. The two pathways intersect to increase PGC-1α activity and increase mitochondrial biogenesis. AMPK directly phosphorylates PGC-1α at threonine-177 and serine-538 to increase PGC-1α-induced activation of the PGC-1α promoter (Jager, Handschin et al. 2007). Phosphorylation of AMPK stimulates its kinase activity. Melatonin has been shown to have disparate effects on AMPK in different cancer cell lines (Hardeland 2013). In HT22 immortalized hippocampal cells, Aß treatment increased AMPK phosphorylation, while melatonin prevented this activation. This AMPK activation was interpreted as being mediated by oxidative stress and melatonin presumably prevented the oxidative damage from occurring to prevent AMPK activation. In primary tissues such as in livers undergoing steatosis (Zaouali, Boncompagni et al. 2013) and in muscle and livers from aged rats, especially when exercised (Mendes, Lopes et al. 2013), melatonin treatment lead to AMPK activation. The melatonin treatment led to increased physiological benefit from exercise in the aged rats. Studies should

also be performed in aged and AD brain to determine if melatonin treatment leads to activation of AMPK and if melatonin receptors play a role.

In aging and aging-related disease there is a strong correlation between increased inflammation and decreased mitochondrial function. These two factors appear to be most centrally linked to the aging process. Since these two phenomena are so intricately linked, it is likely that one induces the other. Melatonin treatment can decrease chronic and acute inflammation by transcriptionally inhibiting iNOS and cyclooxygenase-2 transcriptional activation (Cuzzocrea, Zingarelli et al. 1997; Costantino, Cuzzocrea et al. 1998; Deng, Tang et al. 2006). Melatonin has also been shown to directly act on immune cells to decrease production of IL-6, IL-8, TNF-α, and adhesion molecules (Esposito and Cuzzocrea 2010). Astrocytes become activiated in AD by proinflammatory cytokines and can upregulate iNOS to produce excess nitric oxide which binds and inhibits cytochrome c oxidase of the ETC in both neurons and glia leading to energy decline and tissue dysfunction. Melatonin prevents iNOS upregulation by inhibiting the p38 MAPK signaling pathway activated by cytokine binding (Vilar, de Lemos et al. 2014). Including melatonin in the diet at 200 ppm for 8 weeks normalized the expression of many pro-inflammatory genes that were upregulated in the aged mouse brain (Sharman, Sharman et al. 2004).

Melatonin has been shown to either inhibit or enhance autophagy dependent upon the tissue type and disease treatment (Coto-Montes, Boga et al. 2012). In many pathological conditions reactive oxygen species, which may be required for autophagy induction, are increased. Under these conditions, melatonin treatment may decrease reactive oxygen species levels to decrease autophagic flux. For example, autophagy increases in a rotenone induced Parkinson's disease model and melatonin treatment decreased autophagy markers and autophagic

cell death (Zhou, Chen et al. 2012). Similar observations were made in methamphetamine-induced autophagic cell death (Nopparat, Porter et al. 2010). However, in certain conditions melatonin treatment has also been shown to increase autophagy. One of the mechanisms through which melatonin receptor signaling may protect against AD is through modulation of the rate of autophagy. In this regard autophagy is defective in AD brain due to defective lysosomal acidification causing an accumulation of autophagosomes in certain AD neurons (Wolfe, Lee et al. 2013). This effect was also observed in presenilin-1 (PS1) knockout and mutant mouse neurons and was identified to be caused by a requirement for WT PS1 in the maturation and sorting of a v-ATPase subunit to the lysosome. In addition another group showed that the unfolded protein response increased autophagy in AD neurons (Scheper, Nijholt et al. 2011). Induction of autophagy relies on AMPK activation in many cell types (Meijer and Codogno 2007) and melatonin may activate AMPK under certain conditions as described above. Melatonin-induced autophagy has been shown to protect against neural cell death in early brain injury following a subarachnoid hemorrhage (Chen, Wang et al. 2013). Melatonin-induced autophagy has also been shown to provide neuroprotection from prion proteins (Jeong, Moon et al. 2012), and protect N2a cells from ischemia-reperfusion-induced cell death (Guo, Wang et al. 2010). In addition, melatonin prevented aging-related abnormalities in the autophagosomal-lysosomal system in brain from the SAMP8 senescence accelerated mouse model (Garcia, Pinol-Ripoll et al. 2011). Unfortunately, little is yet known on the effect of melatonin on autophagy in AD brain or AD model systems (Coto-Montes, Boga et al. 2012).

Conclusion

The very low toxicity of melatonin versus other potential AD therapeutics makes melatonin an obvious choice for human AD therapy if efficacy in slowing cognitive dysfunction can be convincingly demonstrated. Melatonin is more versatile than other antioxidants such as vitamin C or vitamin E because it can scavenge peroxynitrite as well as reactive oxygen species (Korkmaz, Reiter et al. 2009). Melatonin is also a candidate therapy for many other neurodegenerative and aging-associated disorders. Novel studies on the molecular mechanisms of mitochondrial protection mediated by melatonin receptor signaling will lead to a better understanding of how melatonin can be used to hinder disease progression in AD.

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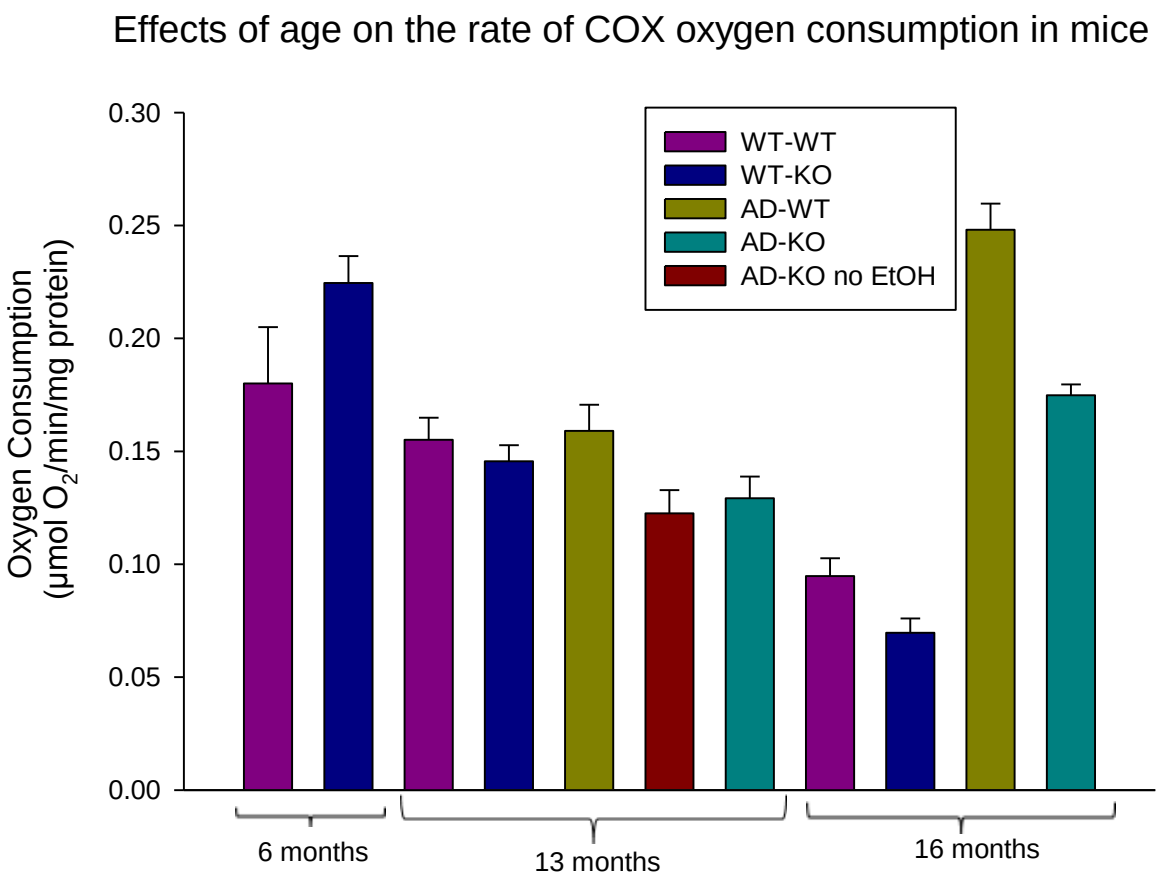

Figure 11 Effects of age on COX activity in mice
The rate of cytochrome c oxidase oxygen consumption was determined using the Clark type electrode, and buffer conditions containing reduced bovine cytochrome C. Members of the initial cohort of mice were sacrificed at 6 months, 13 months, and 16 months. There was an age dependent decline in COX activity across all genotypes, except at 16 months, where AD mice maintained high levels of COX activity.

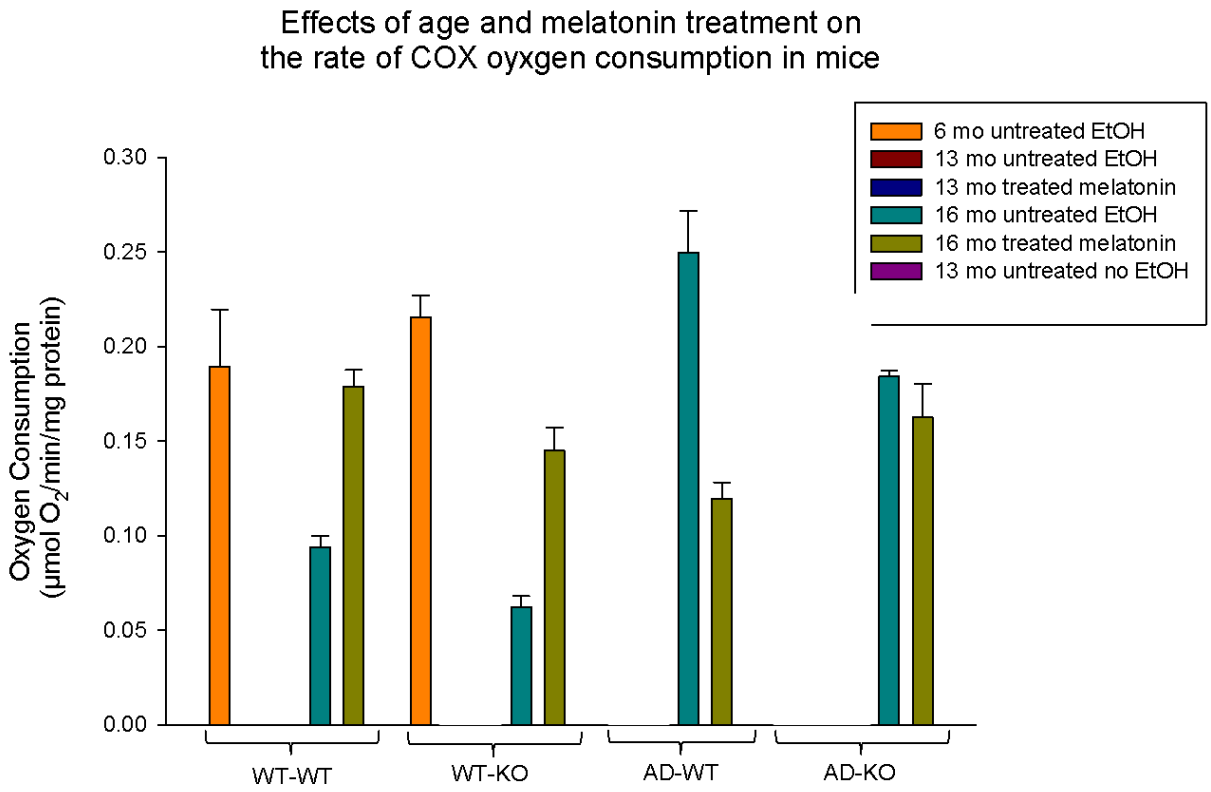

Figure 12 Effects of age and melatonin treatment on COX activity in mice
The rate of cytochrome c oxidase oxygen consumption was determined using the Clark type electrode, with the buffer containing reduced bovine cytochrome c and antimycin A to prevent a backflow of electrons. Members of the initial cohort of mice were sacrificed at 6 months, 13 months, and 16 months. There was an age-dependent decline in COX activity across all genotypes, except at 16 months, where AD mice maintained high levels of COX. Melatonin treatment fully restored COX activity only in the presence of melatonin receptors and only in in WT-WT, WT-KO, and AD-WT genotypes. No protective effect was observed in AD-KO genotype at 16 months.

CHAPTER FOUR: AMINO ACIDS PROTECT AGAINST OXIDATIVE STRESS IN CELLS OVEREXPRESSING ALPHA-SYNUCLEIN MODELING PARKINSON'S DISEASE

Abstract

Parkinson's (PD) disease is a neurodegenerative disorder characterized by alpha-synuclein (α-syn) accumulation and the loss of dopaminergic neurons in the substantia nigra (SN) region of the brain. Increased levels of α-syn in PD promote oxidative stress leading to mitochondrial electron transport chain (ETC) and tricarboxylic acid cycle (TCA) dysfunction. To model the high iron milieu of the SN and the increased levels of α-syn contributing to PD, WT α-syn was overexpressed in M17 neuroblastoma cells and the cells were supplemented with ferrous sulfate ($FeSO_4$). Mitochondrial oxygen consumption was greatly decreased by WT α-syn overexpression almost to the extent observed following overexpression of the pathogenic A30P, A53T, and E46K α-syn mutants. Overexpression of WT α-syn resulted in increased levels of a higher order α-syn conformer, but not monomer levels. Compared to control cells, cells overexpressing WT α-syn were more susceptible to oxidative stress caused by $FeSO_4$ treatment resulting in increased reactive oxygen species (ROS) production and decreased oxygen consumption and ATP levels. Overexpression of α-syn also resulted in decreased ATP levels in response to paraquat, a ROS inducer. Amino acids, TCA cycle intermediates and their ester forms were screened and several, such as serine, proline, aspartate, and succinate were found to

protect mitochondria against $FeSO_4$ or paraquat-induced ROS production, while valine addition consistently increased ATP levels. Mass spectrometry based proteome analysis indicated that monoamine oxidase-A was increased and superoxide dismutase-2 was decreased in abundance by α-syn overexpression. Potential mechanisms of iron and α-syn-mediated mitochondrial dysfunction in PD pathogenesis are discussed.

Introduction

In Parkinson's disease (PD) neurons in the substantia nigra region of the brain show aging-induced mitochondrial dysfunction resulting in increased reactive oxygen species (ROS) production leading to increased oxidative damage. Mitochondrial involvement in the pathophysiology of PD is well established (Martin, Pan et al. 2006; Beal 2007; Keane, Kurzawa et al. 2011). Although a genetic component exists in ~ 10 % of the PD cases, the disease is predominately idiopathic. Dysregulation of mitochondrial dynamics in neurons (e.g. fission, fusion, transport, biogenesis, and mitophagy) has been linked to both genetic and toxin-induced models of PD (Li, Yang et al. 2007; Dickey and Strack 2011; Gegg and Schapira 2011; Martinez and Greenamyre 2012). Dysregulation of these mitochondrial events is in part accompanied by reductions of ATP levels, increased ROS production, and decreased oxygen consumption.

Involvement of alpha-synuclein (α-syn) in both familial and sporadic types of PD is also well established (Zarranz, Alegre et al. 2004; International Parkinson Disease Genomics, Nalls et al. 2011). Common α-syn mutations known to cause the familial forms of PD and induce mitochondrial dysfunction are A30P, A53T, and E46K (Li, Uversky et al. 2001; Zarranz, Alegre et al. 2004; Protter, Lang et al. 2012; Cannon, Geghman et al. 2013). Recently, a dinucleotide polymorphism, REP1, found in the promoter region of the α-syn gene (SNCA) has been linked

with the sporadic form of PD (Chiba-Falek and Nussbaum 2001; Maraganore, de Andrade et al. 2006). Some polymorphisms in REP1 result in higher expression of α-syn. Even small increases in α-syn over a lifetime could potentially have detrimental effects that lead to PD or in the very least a predisposition to developing PD. Higher order oligomers and aggregates of α-syn form when α-syn levels increase (Parihar, Parihar et al. 2009). Certain oligomeric species of α-syn can form amyloid pores, which can disrupt the permeability properties of organelle and plasma membrane phospholipid bilayers (Volles and Lansbury 2002; Maries, Dass et al. 2003). α-syn can disrupt electron transport chain (ETC) function and permeabilize organelle membranes releasing iron and calcium into the cytoplasm, which can be taken up by mitochondria. This may promote the observed mitochondrial deficits in PD and lead to increased ROS production and eventual neuronal cell death. In addition to lysosomes (Bourdenx, Bezard et al. 2014), mitochondria are a major subcellular storage site for iron. Failure to chelate and process free iron properly can lead to production of reactive oxygen species (ROS) via Fenton chemistry. A detailed review on iron metabolism and its role in the progress of PD and other neurodegenerative diseases is found in (Horowitz and Greenamyre 2010). ROS can irreversibly oxidize proteins and membrane phospholipids, which compromise their function and promote Lewy body inclusion formation. (Zaleska and Floyd 1985; Keeney, Xie et al. 2006). In one proteomic study, approximately 550 different proteins were identified in the LB fraction, and of those 40 were verified as true components of LBs (Xia, Liao et al. 2008).

The mechanisms through which α-syn specifically decreases ETC function in the SN in PD are unknown, but increased α-syn levels are associated with decreased activity of ETC complex I (Stichel, Zhu et al. 2007). A recent study has also found that α-syn gets inserted into the mitochondrial inner membrane directly inhibiting ETC complex I function promoting ROS

production that, together with the high calcium and iron levels in the substantia nigra, induce opening of the mitochondrial permeability transition pore releasing cytochrome c to activate caspases and induce cell death (Luth, Stavrovskaya et al. 2014). Overexpression of α-syn in a transgenic mouse model has also been shown to decrease the activities of ETC complexes II, IV, and V (Subramaniam, Vergnes et al. 2014), perhaps from ROS-mediated damage as a consequence of complex I inhibition.

Deficits in TCA cycle enzyme activities have been shown to occur in both Alzheimer's disease and PD (Gibson, Kingsbury et al. 2003; Gibson, Karuppagounder et al. 2008). Decreased TCA cycle enzyme activities are most likely secondary to the oxidative damage of the ETC proteins and to cardiolipin, a phospholipid in the mitochondrial inner membrane required for ETC function. The TCA cycle enzyme aconitase is an iron-containing enzyme, especially sensitive to oxidative damage (Lushchak, Piroddi et al. 2014). ETC complexes are the main producers of free radicals in neurons, producing over 90% of cellular ROS. Decreased TCA cycle enzyme activities yield lower amounts of NADH affecting oxidative phosphorylation leading to imbalanced levels of TCA cycle intermediates affecting the many anabolic pathways relying on TCA cycle intermediates (i.e. amino acid and fatty acid biosynthesis). Although therapeutic treatments to lower α-syn levels in PD patients have not yet been clinically approved, the bioenergetic deficits and increased ROS production from overexpression of α-syn could potentially be treated through supplementation with antioxidants and TCA cycle intermediates. In this regard succinate bypasses complex I deficits and deliver electrons to complex II of the ETC, which showed benefits in a PD model (Ved, Saha et al. 2005). Restoring bioenergetic function while simultaneously reducing ROS may be the most effective way of delaying neuronal cell death in PD. In particular, ester forms of metabolic intermediates have a lower

charge and increased hydrophobicity so they more readily diffuse across the blood brain barrier, and enter cells in the brain without the need for active transport. Upon cell entry these compounds are processed by esterases back to their physiological form to be metabolized. Another possible treatment is to supplement with melatonin, a strong antioxidant previously described to delay α-syn aggregation and protect in several rodent models of PD (Singhal, Srivastava et al. 2012).

To model sporadic PD we stably overexpressed wild type α-syn in human immortalized M17 neuroblastoma cells. The cells were grown in an iron-rich environment to model the environment of the substantia nigra (SN). TCA cycle intermediates, their ester forms, amino acids, and other compounds known to stimulate metabolism were supplemented to the cells and assays of mitochondrial function were performed. Metabolites found to be protective against $FeSO_4$ insult were further tested for protection against paraquat, another inducer of ROS production.

Materials and methods

Cell culture

M17 cells stably overexpressing human α-syn using a cytomegalovirus (CMV) promoter were grown using M10 culture media (DMEM with 10% FBS, 30 µg/ml penicillin, 50 µg/ml streptomycin, and 2 mM glutamine). Stably transfected M17 cell lines overexpressing WT or mutant α-syn were created using the pcDNA3a vector containing a neomycin resistance cassette (Life Technologies®) following selection with G418. Cells were grown in either 96 well plates or 10 cm culture dishes. Cells were grown in media containing ferrous sulfate ($FeSO_4$) for 24, 48 or 72 hours with and without metabolite treatments. Treatments consisted of either TCA cycle

intermediates, esterified TCA cycle intermediates, amino acids, or compounds such as melatonin, rapamycin, and AICAR previously found to be neuroprotective. In some cases titrations were performed for the most effective concentrations. Compounds found to be protective against $FeSO_4$ insult in the initial screen were also tested for evidence of mitochondrial protection using a 24 hour insult with 600 μM paraquat.

ATP assays

CellTiter-Glo (Promega, Madison, WI) ATP detection reagent was used to determine the levels of medium ATP following a 10 minute 22°C incubation of cells with CellTiter-Glo reagent. CellTiter-Glo reagent was combined with a cell suspension in a 1:1 ratio in a 96-well microplate and then shaken for 2 minutes. Luminescence was read using a Biotek Synergy 2 microplate reader after the contents of the 96 well plates were transferred to white opaque bottom plates for increased signal and incubated at room temperature for 10 minutes.

Oxygen consumption analysis

Following growth in 10 cm culture dishes, cells were trypsinized, the trypsin was deactivated with five times the volume of culture media, and the cells were spun down and resuspended in 1 mL of fresh culture media. From the 1 ml cell suspension, 350 μl was placed into a Strathkelvin Mitocell MT200A respiratory chamber with a Clark type electrode at 37^0C with a stir bar. The basal respiratory rate was recorded for 4 minutes. The middle 1 minute was chosen for slope analysis. Oxygen consumption was normalized to the total cell number in the chamber. Cell counts were obtained using a hemocytometer.

Reactive oxygen species measurements

2',7'-dichlorodihydrofluorescein diacetate was used to detect the rate of reactive oxygen species (ROS) production. Following hydrolysis of the acetate esters by intracellular esterases, dichlorodihydrofluorescein is produced. Dichlorodihydrofluorescein, when oxidized by ROS becomes the highly fluorescent compound dichlorofluorescein (DCF). A 2',7'-dichlorodihydrofluorescein diacetate stock solution of 500 µM was made up in 2% DMSO in PBS and the tube was wrapped in foil to prevent exposure to light. Media was aspirated from the cells and the cells were washed once with 100 µl PBS. An assay concentration of 50 µM was obtained by mixing 10 µl of the stock solution with 90 µl of PBS and added to the monolayer of cells in a 96-well plate. The plate was incubated in the dark at 37°C for 30 minutes. The fluorescence was then measured using a Biotek Synergy 2 microplate reader with an excitation filter of 485/20 nm and emission filter of 528/15 nm. Values were normalized to the total amount of protein in each well. Cells were lysed using RIPA buffer without SDS.

Protein assays

A determination of the protein concentration was performed using the BCA protein assay (Thermo Scientific Pierce). All results were normalized using the protein concentrations present for the assays.

Western blot analysis

Cells were solubilized in (non-denaturing) RIPA (Tris-HCl 50 mM, 150 mM NaCl, sodium deoxycholate 12 µM, 1 % Triton X-100) buffer without SDS. Invitrogen- Novex 4-20% tris-glycine standard protein gels were used for polyacrylamide gel electrophoresis. Novex®

Tris-Glycine gels do not contain SDS and were used to run proteins under denaturing conditions. To run denatured proteins, Novex® Tris-Glycine SDS Sample Buffer and Novex® Tris-Glycine SDS Running Buffer were used. The gel was transferred to a PVDF membrane. The membrane was blocked in blotto blocking solution with 1x TBS (Fisher scientific) for 1 hour. The membrane was probed with the mouse anti-alpha- synuclein IgG from BD Biosciences antibody using new blotto solution. The GAPDH loading control antibody was from Meridian Life Science Inc. LumiGOLD ECL detection kit was used for secondary antibody luminescence and the signal was captured on autoradiography film. The densitometry values obtained using imageJ software were normalized to those of the GAPDH loading control.

SILAC Labeling and LC-MS/MS Analysis

M17 control and WT α-syn stably transfected cell lines were thawed in standard M10 culture media described above. The next day the media was changed and M17 cells were grown in SILAC media supplemented with "heavy" $^{13}C_6$-$^{15}N_4$-Arginine and $^{13}C_6$ L-Lysine, and WT α-syn cells were grown in SILAC media supplemented with normal "light" L-Arginine and L-Lysine. Cells were grown for approximately 6 doublings in order to maximize the incorporation of labeled amino acids. Cells were lysed in 100mM Tris-HCl (pH 7.6) with 4% SDS, 100mM DTT, and Halt protease inhibitor cocktail (Pierce) at 95˚C for 5 minutes, and briefly sonicated. Protein concentrations were determined using the Pierce 660 nm protein assay with ionic detergent compatibility reagent (Pierce). Cell lysates were processed using filter-aided sample preparation (FASP) (Wisniewski, Zougman et al. 2009; Chaput, Kirouac et al. 2012). Proteins were digested with Trypsin/Lys-C (Promega) at 1:50 (w:w; protease:protein) overnight at 37˚C. Peptides were desalted using Pierce C-18 SPE columns with a Supelco vacuum manifold and

dried using a vacuum concentrator (Thermo). Peptides were then fractionated on a Dionex U3000 HPLC system with a 15cm x 1.0mm i.d. strong cation-exchange (SCX) column (PolyLC Inc.) packed with 5µm 300Å polySULFOETHYL A-SCX material. Two minute fractions were collected using a 30 minute gradient (15-200mM ammonium formate in 25% acetonitrile, ten fractions selected for LC-MS/MS analysis based on the UV 280nm trace were again dried in a vacuum concentrator and resuspended in 0.1% formic acid in H_2O. Peptides were analyzed on a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific) with a 75um x 50cm UPLC column (Dionex) using a 2 hour gradient (2-40% acetonitrile) on an EASY-nLC 1000 system. Raw files were searched against the 2014 UniprotKB human protein sequence database using Maxquant software (version 1.5.0.30, maxquant.org). Statistically significant differentially expressed proteins were determined using Significance A, an outlier test with a p value < 0.05, in Perseus (version 1.5.031, http://141.61.102.17/perseus_doku/). Statistically significant differentially expressed proteins were used for bioinformatic analysis (Ingenuity Pathway Analysis) to determine over-represented biological functions and predicted pathway/upstream regulator activity changes.

Results

Overexpression of alpha-synuclein results in decreased oxygen consumption.

Following the generation of stably transfected WT α-syn and A30P α-syn, A53T α-syn, E46K α-syn M17 cell lines, oxygen consumption was determined using cells suspended in DMEM-based culture media. In all cases, the stable transfection led to large declines in oxygen consumption (Figure 4.1) (one-way ANOVA, $P<0.005$). The rate of oxygen consumption was decreased by 56% in cells overexpressing wild type α-syn. The reduction in oxygen consumption

in the α-syn mutants A30P, A53T, E46K was 80.1%, 93.1%, and 66.0% percent respectively. Therefore, overexpression of the WT α-syn had a slightly less severe effect on mitochondrial function than overexpression of the mutant forms (Fig. 13). Overexpression of the α-syn mutants even led to a greatly decreased rate of cell division in standard high glucose DMEM media. The cells were observed to have a doubling rate of approximately 4 days (data not shown). Cells overexpressing WT α-syn did not show this greatly decreased rate of cell division, therefore, we decided to characterize other phenotypes in these cells.

Alpha-synuclein aggregates into tetramers when overexpressed

We next wished to determine the level of WT α-syn overexpression that led to the decreased oxygen consumption. Therefore we performed Western blot analysis of the cells using mild sample solubilization conditions able to identify α-syn oligomers. Small α-syn A53T mutant oligomers were previously reported to be SDS, temperature and detergent stable (Tsika, Moysidou et al. 2010; Marques and Outeiro 2012). Therefore, we wanted to determine if over expression of WT α-syn resulted in the same type of aggregation as in α-syn A53T. We were also interested in determining if limiting SDS exposure and excluding heating from sample prep would result in a clearer picture of which higher order oligomers are also most likely to be thermodynamically stable in vivo. Following overexpression of WT α-syn, there was, surprisingly, no increase in the intensity of the α-syn band running at 16 kDa on the gel representing monomeric α-syn, but a multimeric form of α-syn running at approximately 60 kD was increased by 55% (Fig. 14). The human brain contains both monomers and other conformers of α-syn such as dimers, trimers, tetramers, and perhaps pentamers (Gould, Mor et al. 2014), with the tetrameric form of α-syn being very stable in the human brain (Bartels, Choi et al.

2011). The tetramer may be stabilized by a lipid or other small molecule, as the monomer re-forms upon full purification of the protein (Luth, Bartels et al. 2015).

$FeSO_4$ decreases oxygen consumption

As the substantia nigra contains high levels of iron, we incubated the M17 control and WT α-syn-expressing cells with 4 mM $FeSO_4$ and determined the rate of oxygen consumption. This concentration was determined by titrating $FeSO_4$ until deficits in WT α-syn were observed and M17 control cell remained unaffected. As expected, the rate of oxygen consumption declined following incubation with 4 mM $FeSO_4$ in WT α-syn-expressing cells. Oxygen consumption also declined in M17 control cells, although this effect did not quite reach statistical significance (Fig. 15).

α-syn overexpression decreases ROS production in the absence of $FeSO_4$, but increases ROS production in the presence of $FeSO_4$

It has long been known that α-syn overexpression sensitizes to peroxide and iron-mediated cell death and iron and free radicals stimulate α-syn aggregate formation (Ostrerova-Golts, Petrucelli et al. 2000). So we next determined the effect of WT α-syn overexpression on ROS production in the absence and presence of $FeSO_4$ treatment to the cells. As shown in Fig. 16, in the absence of $FeSO_4$ WT α-syn-overexpressing cells produce less ROS than M17 controls. However, once $FeSO_4$ is introduced, ROS levels of α-syn-overexpressing cells exceed those of cells not overexpressing α-syn.

Overexpression of a-syn results in $FeSO_4$ or paraquat-induced decreases in ATP levels

We next determined the effect of WT α-syn overexpression and $FeSO_4$ treatment on cellular ATP levels. As shown in Fig. 17, WT α-syn overexpression, increased ATP levels in the untreated M17 cells, which is surprising considering that the overexpression also reduced mitochondrial oxygen consumption. However, WT α-syn-overexpressing cells showed a 45% decrease in ATP levels upon $FeSO_4$ treatment, while there was no change in ATP levels in M17 control cells following $FeSO_4$ treatment. Therefore, it appears that there is a compensatory increase in glycolysis or a decrease in ATP consumption that compensates for the α-syn-induced decrease in oxygen consumption to maintain or even slightly increase cellular ATP levels in the absence of $FeSO_4$. However, this compensatory mechanism is unable to be maintained in the presence of $FeSO_4$ leading to a large decrease in ATP levels.

We also performed a similar experiment, but this time instead of treating the cells with FeSO4, we treated the cells with paraquat, an herbicide and ROS generator known to cause Parkinson's-like symptoms when ingested by humans. As shown in Fig. 18, very similar results were obtained as with $FeSO_4$ treatment, paraquat treatment decreased ATP levels in the α-syn-overexpressing cells and slightly increased ATP in WT cells.

Some amino acids and TCA cycle intermediates rescue ATP production deficits and decrease ROS following oxidative insults

Since α-syn overexpression leads to mitochondrial dysfunction, especially in the presence of $FeSO_4$, we decided to perform a small-scale screen of 32 compounds, mostly metabolites or

cell-permeable metabolite esters that may be able to delay or compensate for the decreased mitochondrial function. We first tested the compounds on ATP levels and ROS production in the absence and presence of $FeSO_4$. We then went on to test the best candidates on α-syn-expressing cells in the presence of paraquat. We performed this second analysis as it was likely that many of the hits from the $FeSO_4$ screen were decreasing ROS by chelating the iron in the culture media and we wished to determine which compounds decreased ROS production independently of their chelating abilities. Amino acids and metabolic intermediates found to statistically increase ATP and/or decrease ROS, including the results from the paraquat insult, are shown in Table 3 and presented as percent increase.

We believe that criteria for a useful therapeutic compound include increasing ATP levels without increasing ROS production or decreasing ROS production without decreasing ATP levels. A compound that satisfied these criteria under all 3 conditions tested is serine. Serine decreased ROS production without affecting ATP levels. When examining the results of the paraquat data, it is shown that succinate and melatonin, a tryptophan-related hormone known to be a strong antioxidant, also satisfied these criteria by decreasing ROS production without decreasing ATP levels. Although not satisfying the criteria due to increased ROS production, the compounds that increased ATP levels to the greatest extent in the presence of paraquat were valine and diethyl oxaloacetate, likely through increasing mitochondrial biogenesis (Valerio, D'Antona et al. 2011; Wilkins, Harris et al. 2014). Likely due to their chelating ability, many of the compounds satisfied these criteria when examining the data from the $FeSO_4$ treatment. Valine, serine, fumarate, aspartate, rapamycin (an mTOR kinase inhibitor), and proline treatments both increased ATP levels and decreased ROS production, while glutaric acid,

alanine, asparagine, succinate, and isoleucine increased ATP levels without affecting ROS levels. Glutaric acid was particularly potent, as it may be the best iron chelator administered.

Proteomic analysis of α-syn overexpression following SILAC labeling

In attempt to understand the mechanisms of mitochondrial dysfunction in WT α-syn-overexpressing cells, we performed proteome analysis on cells lysates following SILAC labeling. A total of 211 proteins were identified with significant fold changes in at least 2 of the 3 biological replicates (appendix A). The entire data set was analyzed by Perseus software using the significance A setting. Several proteins relevant to mitochondrial function showed either significantly increased or decreased abundance (Fig. 19). For example, the mitochondrial ETC complex I subunits NDUFA13 and NDUFB4 and the mitochondrial ETC complex IV assembly protein SURF1 showed increased abundance. α-syn has been shown to directly interact with ETC complex IV (Elkon, Don et al. 2002), although the significance of this interaction is still unknown. NDUFA13 can bind the transcription factor STAT3 and serve as a tumor suppressor. In addition monoamine oxidase-A, MAO-A, a mitochondrial outer membrane protein involved in the degradation of dopamine and other amine neurotransmitters was increased in abundance in α-syn overexpressing cells. This increased expression of MAO-A may in part lead to the decreased dopamine levels observed in PD patients as well as the increased toxic levels of DOPAL, the aldehyde product formed from MAO catabolism of dopamine, contributing to the death of substantia nigra neurons in PD patients (Goldstein, Sullivan et al. 2013). A significant decrease in abundance was observed for mitochondrial superoxide dismutase SOD2 (Fig. 19). The decreased expression of SOD2 was surprising given that α-syn overexpressing cells showed decreased ROS production in the absence of $FeSO_4$. However, the decreased ROS production

was likely due to the decreased entry of electrons into the ETC due to the decreased complex I activity. The decreased SOD2 levels may play a role in the higher ROS levels observed when the α-syn expressing cells are exposed to oxidative stressors such as $FeSO_4$.

Discussion

We found that overexpression of WT α-syn in M17 human neuroblastoma cells resulted in an increased susceptibility to mitochondrial dysfunction characterized by increased ROS production and decreased ATP levels following $FeSO_4$ treatment. This susceptibility may in part be caused by the increased levels of monoamine oxidase A (MAO-A), and the decreased levels of mitochondrial superoxide dismutase (SOD2). We also report several natural metabolites such as serine, succinate, and melatonin that partially rescue the increased ROS production induced by paraquat addition to α-syn overexpressing cells without decreasing ATP levels. We also showed that α-syn overexpression resulted in increased levels of oligomeric α-syn. These oligomers may interact with mitochondria to inhibit complex I to make the cells more susceptible to cell death in the iron-rich, oxidative environment of the SN.

The results from the oxygen consumption measurements described here correspond very well with the results obtained when WT α-syn and the A30P and A53T α-syn mutants were expressed in SHSY cells (Parihar, Parihar et al. 2009). The results of the ROS production measurements reported here are also consistent with the results from two other groups. In the previous reports, the authors treated either SH-SY5Y neuroblastoma cells (Perfeito, Lazaro et al. 2014) or SK-N-SH neuroblastoma cells (He, Song et al. 2011) expressing WT α-syn with iron. However, these other groups did not measure ATP levels. In addition, neither of the groups used paraquat treatment as an oxidative stressor, although one of the groups made a similar

observation using rotenone, another generator of ROS production (Perfeito, Lazaro et al. 2014). One of the groups used ferric ammonium citrate (FAC) as the iron source when measuring ROS production (He, Song et al. 2011) as ferric ion more readily stimulates oligomerization of α-syn than ferrous ion (Levin, Hogen et al. 2011). However, when we used FAC as an iron source, we found an increase in oxygen consumption (data not shown) as the citrate was likely used a mitochondrial respiratory substrate confounding the results, so we switched to using $FeSO_4$ as the iron source. Other major novel aspects of our study are the mitochondrial protective effects observed with amino acid and succinate supplementation.

Overexpression of α-syn may predispose cells to $FeSO_4$ toxicity by 4 mechanisms converging on aberrant iron metabolism and ROS production

1. Endosomal/lysosomal membrane disruption resulting in iron leakage. Even small increases in α-syn levels may increase dopaminergic neuron susceptibility to mitochondrial stress given the highly oxidizing environment of the SN. We showed that overexpression of α-syn induced increased levels of an α-syn multimer, which could possibly lead to the formation of amyloid pores and altered ion homeostasis (Pacheco, Morales et al. 2015). α-syn may insert into endosomes/lysosomes destabilizing the membranes allowing iron to leak (Zhu, Li et al. 2003). Unsequestered iron can promote free radical damage through Fenton chemistry. α-syn has also been shown to form pores in the plasma membrane allowing extracellular Ca^{2+} leak into the cytoplasm (Pacheco, Morales et al. 2015), which may also cause mitochondrial dysfunction.

2. Increased MOA-A levels increasing ROS production. MOA-A is a drug target for many psychiatric disorders as MAO-A degrades not only dopamine, but other amine neurotransmitters as well. In the brain MAO-A is the main MAO isoform in neurons (Fitzgerald, Ugun-Klusek et al. 2014). MAO-B, the product of a separate gene, is a specific target for Parkinson's disease as MAO-B function is specific for dopamine degradation and not other biogenic amines. In the brain MAO-B is expressed largely in astrocytes (Fitzgerald, Ugun-Klusek et al. 2014). MAO-B activity predominates in the aged brain (Fowler, Wiberg et al. 1980), but MAO-A is the major isoform expressed in the SN (Westlund, Denney et al. 1988). MAO levels are hypothesized to increase in PD brain, in part because MAO-B levels have been measured to increase in PD patient platelets (Zhou, Miura et al. 2001) and in part because Parkin, an E3 ubiquitin ligase mutated in familial PD negatively regulates MAO levels by targeting estrogen-related receptors (ERRs), which induce their expression, for degradation (Ren, Jiang et al. 2011).

A metabolic byproduct of normal MAO activity is hydrogen peroxide, which can be readily converted into more damaging oxygen radicals by interaction with iron, present at high levels in the cytoplasm of the SN (Edmondson 2014). Because of this peroxide production, MAO activity has been shown to damage ETC function (Cohen, Farooqui et al. 1997). α-syn could also possibly increase MAO-A by inhibiting the proper degradation of mitochondrial outer membrane proteins by the proteasome (Taylor and Rutter 2011) or by slowing down degradation of the entire organelle by mitophagy (Xilouri, Brekk et al. 2013), although one report has shown increased α-syn expression to damage mitochondria and increase mitophagy (Chen, Xie et al. 2015). Interestingly, upregulation of MAO-B has been shown to disrupt the ability of Parkin to signal for mitophagy of damaged mitochondria (Siddiqui, Hanson et al. 2012).

Increased MAO-B levels have been shown to increase oxidative stress to damage mitochondrial DNA (Hauptmann, Grimsby et al. 1996) and inhibit the TCA cycle enzyme alpha-ketoglutarate dehydrogenase leading to deficiencies in spare respiratory capacity (Kumar, Nicholls et al. 2003). Most strikingly, transgenic overexpression of MAO-B in astrocytes has been shown to induce Parkinson's-like pathology in the mid-brain of mice (Mallajosyula, Kaur et al. 2008). Not as much attention in PD research has been given to MAO-A. But it is known that MAO-A levels are upregulated by oxidative stress (De Zutter and Davis 2001; Fitzgerald, Ufer et al. 2007). In addition it was recently shown that MAO-A was upregulated during rotenone-(an inhibitor of ETC complex I) mediated cell death of neuroblastoma cells and knockdown of MAO-A partially restored viability (Fitzgerald, Ugun-Klusek et al. 2014). Furthermore, knockdown of MAO-A under these conditions substantially decreased ROS levels showing that MAO-A is an important contributor to neuronal cellular ROS generation. Under basal conditions knockdown of MAO-A increased ETC complex I activity and cellular ATP levels showing how normal MAO-A function may be detrimental to cellular energetics (Fitzgerald, Ugun-Klusek et al. 2014).

3. Decreased abundance of SOD2. α-syn has many properties in common with prion proteins (Olanow and Brundin 2013). SOD2, which is normally localized to mitochondria, can be mislocalized to the cytoplasm, where it aggregates with caspases and gets ubiquitinated and degraded as previously reported in scrapie PrP^{Sc} models (Sinclair, Lewis et al. 2013). Like the PrP^{Sc} prion aggregates, α-syn aggregates could be promoting the same type of mislocalization of SOD2, resulting in decreased SOD2 levels and further ROS susceptibility. This explanation is in line with the recent descriptions of the prion hypothesis of PD (Chu and Kordower 2015).

Molecular similarities between α-syn and prion protein could lead to some pathological overlaps, perhaps like the decreased levels of SOD2 reported here.

Specific alleles of SOD2 confer increased risk for developing PD (Shimoda-Matsubayashi, Hattori et al. 1997; Fong, Wu et al. 2007). It has previously been shown that there was a large decrease in SOD2 activity in a rotenone-induced model of PD in rats (Thakur and Nehru 2015), and a small decrease in SOD2 levels in either a MPTP-induced model (Liu, Peritore et al. 2015) or a dichlorvos pesticide-induced PD model in mice (Binukumar, Bal et al. 2010). Levels of SOD2 were increased in the cortex of PD patients (Marttila, Lorentz et al. 1988; Radunovic, Porto et al. 1997) and also in the blood (Santiago, Scherzer et al. 2014). SOD2 was also shown to be oxidatively modified in PD patient brain (Dalfo, Portero-Otin et al. 2005). However, since SOD2 levels have yet to be measured in human SN, it is too early to determine if our observation of decreased SOD2 levels following α-syn overexpression are clinically relevant.

4. Decreased abundance of brain derived neurotrophic factor (BDNF). BDNF was decreased in abundance in the α-syn overexpressing cells. BDNF has been shown to increase mitochondrial biogenesis and antioxidant defenses by upregulating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Markham, Cameron et al. 2004; Yuan, Sun et al. 2010). For example, PGC-1α interacts with FOX3a to induce SOD2 (Olmos, Valle et al. 2009). So downregulation of BDNF could greatly exacerbate the MAO-A and α-syn-induced oxidative stress through decreasing SOD2 levels. In addition α-syn was shown to bind to the promoter and inhibit expression of PGC-1α (Siddiqui, Chinta et al. 2012), which may also contribute to the decreased complex I activity and SOD2 levels occurring when α-syn is overexpressed. In addition PGC-1α levels have been shown to be decreased in PD patient brain

(Eschbach, von Einem et al. 2015). But, since MAO-A was upregulated by α-syn overexpression and since PGC-1α is a required coactivator for ERR-mediated upregulation of gene expression (Giguere 2008), it is likely that PGC-1α is still expressed and at least partially functional.

Further investigations into the mechanisms of α-syn-induced PD pathology as discussed above are warranted. Ultimately, in the oxidative environment of the SN, the changes induced by increased α-syn levels may contribute to increased ROS production, which results in ETC damage and damage to other proteins of central metabolism, specifically those involved in glycolysis and the TCA cycle. TCA cycle enzyme dysfunction due to oxidative damage would affect both energy production and biosynthetic processes relying on TCA cycle intermediates. Mechanisms that could be acting in concert to damage mitochondria in PD are summarized in Figure 4.8.

Increased expression of ETC complex I subunits and complex IV assembly factor as a possible compensatory mechanism to reduce ROS production

ROS production is governed by thermodynamic conditions within the mitochondria, namely those pertaining to the $NADH/NAD^+$ ratio and oxygen tension (Murphy 2009). High membrane potential and high $NADH/NAD^+$ ratio predispose ETC complex I to ROS production. Additionally, ETC complex I inhibition mediated by α-syn or lipid peroxidation caused by excessive MAO-A activity and free iron levels could also lead to decreased flux of electrons down the ETC. Ground state oxygen can only accept one electron at a time according to Hund's rule. Increasing the amount of time it takes the second electron necessary to reduce oxygen completely allows more time for an oxygen with an unpaired electron to exist, creating a free

oxygen radical. Increasing the expression of ETC complexes I and IV (Fig. 19) could potentially decrease ROS production by increasing the flux of electrons down the ETC decreasing the amount of time oxygen exists with an unpaired electron.

Supplementation with metabolic intermediates as a possible treatment for PD

The TCA cycle integrates catabolic and anabolic pathways. Metabolic intermediates generated are siphoned off for synthetic reactions and high energy molecules such as NADH and $FADH_2$, which donate electrons for oxidative phosphorylation, are generated in the process. Damage to TCA cycle enzymes would decrease their activity and result in energy deficits and imbalances in intermediates essential for anabolic reactions. These energetic deficits may result in decreased altered levels of amino acids, fatty acids, and other metabolites and altered activity of the molecular target of rapamycin (mTOR) kinase, responsible for nutrient sensing and metabolic integration. mTOR is inhibited when nutrients are low resulting in increased autophagy leading to degradation of proteins to increase free amino acid and other nutrient levels, decreased cell cycle progression, and decreased mitochondrial function (Schieke, Phillips et al. 2006). Indeed, when we inhibited mTOR through rapamycin treatment (Table 3) we saw an increase in ATP levels, and decrease in ROS production (Table 3). However, in another study rotenone inhibition of ETC complex I increased ROS production inhibiting mTOR function leading to neuronal apoptosis (Zhou, Liu et al. 2015). α-syn overexpression was shown to increase mTOR activity (Gao, Duan et al. 2015), perhaps as a compensatory response to attempt to increase mitochondrial function in response to inhibition of ETC complex I.

Supplementation with amino acids such as serine that decrease ROS production in concert with TCA cycle intermediates such as succinate is a possible strategy to address this

metabolic demand, because of their generally regarded as safe (GRAS) classification by the FDA. Several other metabolic intermediates and amino acids were also found to partially restore ATP levels and decrease ROS production (Table 3). Since the efficacy of amino acid or TCA cycle metabolite therapy for the treatment of neurodegenerative diseases may be limited by their rate of transport, treatment with TCA cycle and amino acid esters may increase their efficacy. Consistent with this, we found a TCA cycle ester diethyl oxaloacetate to greatly increase ATP levels. Others have found similar results with ethyl pyruvate or alpha-ketoglutarate showing protection against oxidative damage and motor deficits in an MPTP-induced mouse model of PD (Satpute, Lomash et al. 2013).

Somewhat consistent with our results, a study using the Caco-2 intestinal epithelial cell line found 8 out of the 20 amino acids tested protected against hydrogen peroxide-induced oxidative stress (Katayama and Mine 2007). However, that study found alanine, cysteine, histidine, isoleucine, leucine, lysine, tryptophan, and valine to protect from oxidative stress. In the M17 cells overexpressing α-syn, we tested all the amino acids except glutamate, glycine, threonine, and tyrosine. In the untreated cells, we found serine, proline, and phenylalanine to decrease ROS production. In the paraquat-treated cells we found alanine, aspartate, proline, and serine to decrease ROS production, while valine and isoleucine increased ROS production. In the $FeSO_4$-treated cells we found aspartate, valine, proline, and serine to decrease ROS production, while methionine and phenylalanine increased ROS production. In the M17 neuronal cell line used here, the amino acids most consistently able to decrease ROS production were serine, proline, and aspartate, which were not protective in the Caco-2 cells. Using the nematode *C. elegans* we have recently shown that 18 of the 20 amino acids (exceptions were aspartate and phenylalanine) extended lifespan with proline and serine having the largest effects

(Edwards, Canfield et al. 2015). Lifespan extension relied upon activation of DAF-16/FOXO, SKN-1/Nrf2, and AMP kinase signaling pathways. In the study using the Caco-2 cells, the authors found that 5 of the amino acids increased glutathione levels, 6 of the amino acids increased glutathione-S-transferase activity, and the 3 branched chain amino acids increased catalase activity (Katayama and Mine 2007). This suggests that certain amino acids may activate the Nrf2-antioxidant response element signaling pathway to protect against oxidative stress. Future plans are to test this hypothesis.

Conclusions

Overexpression of α-syn resulted in mitochondrial susceptibility to $FeSO_4$ insult. $FeSO_4$ was used in this study to approximate the iron rich physiological milieu of dopaminergic neurons and because of the ability of iron to oligomerize α-syn into a higher order structures that can permeabilize phospholipid bilayers (Kostka, Hogen et al. 2008). The observed $FeSO_4$-induced increase in mitochondrial sensitivity in α-syn-overexpressing cells is likely due to oxidative damage to ETC complex I and TCA cycle enzymes. Our proteomics analysis suggests one possible mechanism of action is through an α-syn-induced increase in MAO-A levels, which in turn increases hydrogen peroxide production and increases levels of the toxic aldehyde DOPAL. Hydrogen peroxide is readily converted to more harmful free radicals via Fenton chemistry, possibly due to α-syn disruption of iron trafficking. Increased ROS and α-syn levels may decrease SOD2 levels, which may lead to oxidative damage of ETC complexes and TCA cycle enzymes. Succinate and amino acids were found to be partially protective against this oxidative stress, while valine or diethyl oxaloacetate greatly increased ATP levels. These findings merit

further investigation into the mechanisms through which amino acid and other metabolic therapies decrease ROS production, increase ATP levels, and possibly protect against PD.

Highlights:

α-synuclein overexpression increases mitochondrial susceptibility to iron or paraquat

α-synuclein overexpression leads to an increase in MAO-A and a decrease in SOD2

Succinate or aspartate decrease ROS in paraquat-treated α-synuclein-expressing cells

Serine, proline, or alanine decrease ROS in paraquat-treated α-syn-expressing cells

Valine or isoleucine increase ATP in paraquat treated α-synuclein-expressing cells

Acknowledgments

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Table 3 Effects of metabolites on ROS production and ATP levels

	No insult				Iron insult 4.0 mM				Paraquat insult 600 µM			
	ATP		ROS		ATP		ROS		ATP		ROS	
Compound	% increase	p-value	% increase	p-value	% increase	p-value	% increase	p-value	% increase	p-value	% increase	p-value
Glutaric Acid 1 mM	14	0.0578	-3	0.782	410	0.0001	4	0.5429	-37	0.0002	-19	0.1219
Valine 5mM	1	0.8851	-12	0.172	143	0.0001	-48	0.0026	76	0.0008	56	0.0047
Serine 5 mM	3	0.2297	-18	0.043	99	0.0001	-46	0.0034	3	0.5797	-44	0.0025
Fumarate 1mM	-52	0.0001	-11	0.280	98	0.0030	-25	0.0001	-3	0.0001	-10	0.0001
Aspartate 5 mM	10	0.0053	-10	0.291	82	0.0001	-56	0.0011	-35	0.0004	-60	0.0018
Rapamycin 10 nM	-50	0.0001	-18	0.108	76	0.0004	-19	0.0024	-21	0.0004	-15	0.0111
Methionine 1 mM	10	0.0040	2	0.623	61	0.0001	34	0.0318	N/P	N/P	N/P	N/P
L-Alanine 1 mM	-1	0.6075	-7	0.126	55	0.0001	-15	0.2034	-38	0.0008	-70	0.0004
Melatonin 10 µM	7	0.2303	7	0.004	40	0.0646	-32	0.0001	-6	0.0787	-19	0.0216
Proline 5mM	-11	0.0012	-26	0.008	39	0.0001	-46	0.0033	-22	0.0058	-50	0.0017
Phenylalanine 1mM	-19	0.0003	-14	0.025	26	0.0065	112	0.0001	N/P	N/P	N/P	N/P
Asparagine 1 mM	-2	0.5654	-4	0.566	26	0.0073	28	0.0873	N/P	N/P	N/P	N/P
Succinate 1 mM	13	0.0001	29	0.014	20	0.0231	0	0.9927	30	0.3209	-136	0.0011
Diethyl oxaloacetate 1 mM	-7	0.6338	41	0.042	20	0.0600	16	0.1545	89	0.0001	69	0.0001
Isoleucine 5 mM	0	0.9808	8	0.096	20	0.0070	-2	0.9160	35	0.0001	34	0.0001

N/P=not performed

The effect of select metabolites on ROS production and ATP levels following a 72 hour treatment. $FeSO_4$ and paraquat were titrated until ~ 50% reduction in ATP and ~50% increase in ROS was achieved in WT21 cells but not in M17 control cells. Metabolic intermediates were also titrated, starting with high, but physiologically attainable millimolar concentrations. Compounds shown in this table were the 15 hits that either increased ATP levels or decreased ROS production in a larger screen of 32 compounds. Compounds found to significantly increase ROS production in the presence of $FeSO_4$ were excluded from the paraquat screen. ATP was measured as relative luminescence using CellTiter-Glo (Promega) and ROS as relative DCF fluorescence in 96 well plates. The percent increase was determined comparing cells receiving $FeSO_4$ only and cells receiving $FeSO_4$ and the treatment. This allowed for internal normalization and allowed us to combine experiments performed on different days. A statistically significant increase in ATP or decrease in ROS production from the untreated $FeSO_4$ insulted control was determined using one-way ANOVA.

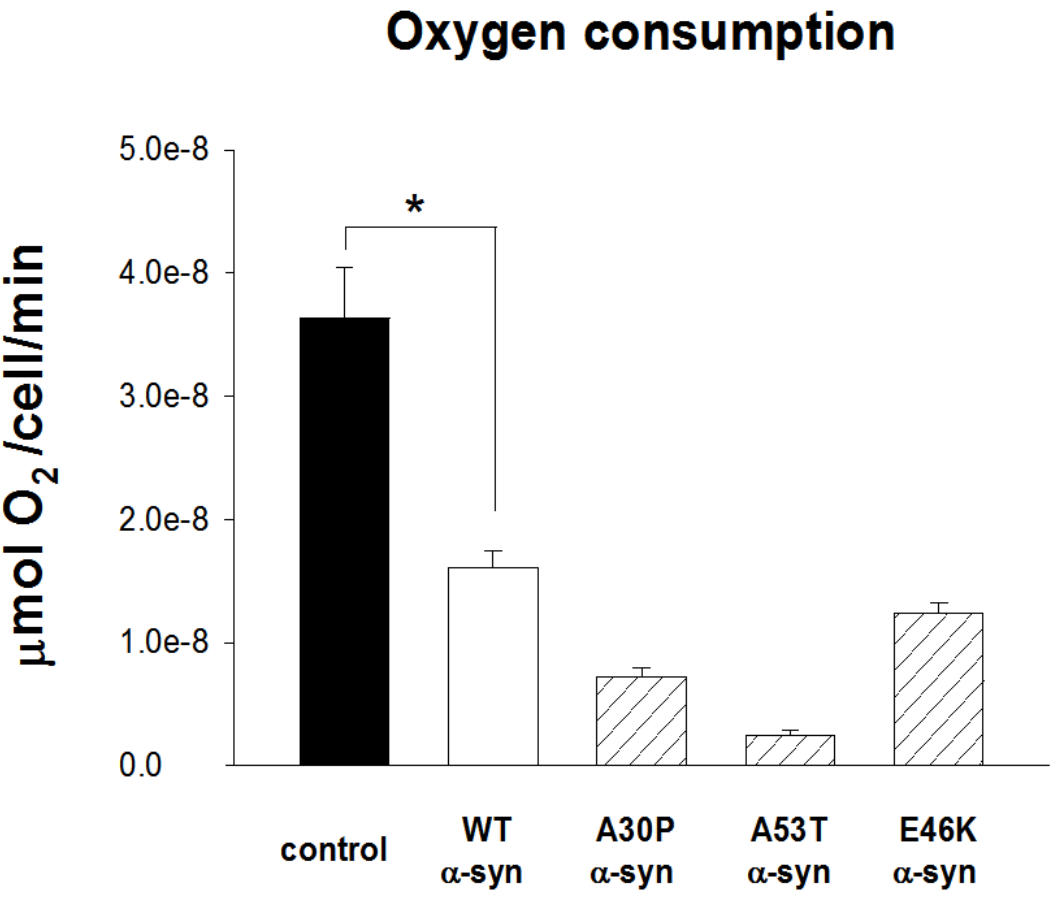


Figure 13 Oxygen consumption
Overexpression of WT or mutant forms of α-syn decreases oxygen consumption. Oxygen consumption was determined using a Clark type oxygen electrode. Cells were trypsinized from a confluent 10 cm plate (*n*=4 per genotype). Trypsin was neutralized by addition of 5 ml of DMEM containing FBS. Cells were counted in a 6 ml suspension with a hemocytometer before being pelleted, and resuspended in 1 ml of media. 350 µl of the 1 ml cell suspension was transferred to a respiratory chamber. O_2 consumption was measured for 4 minutes and the respiratory rate was normalized per cell. Statistical significance was determined using one-way ANOVA, * $p < 0.005$ compared to control.

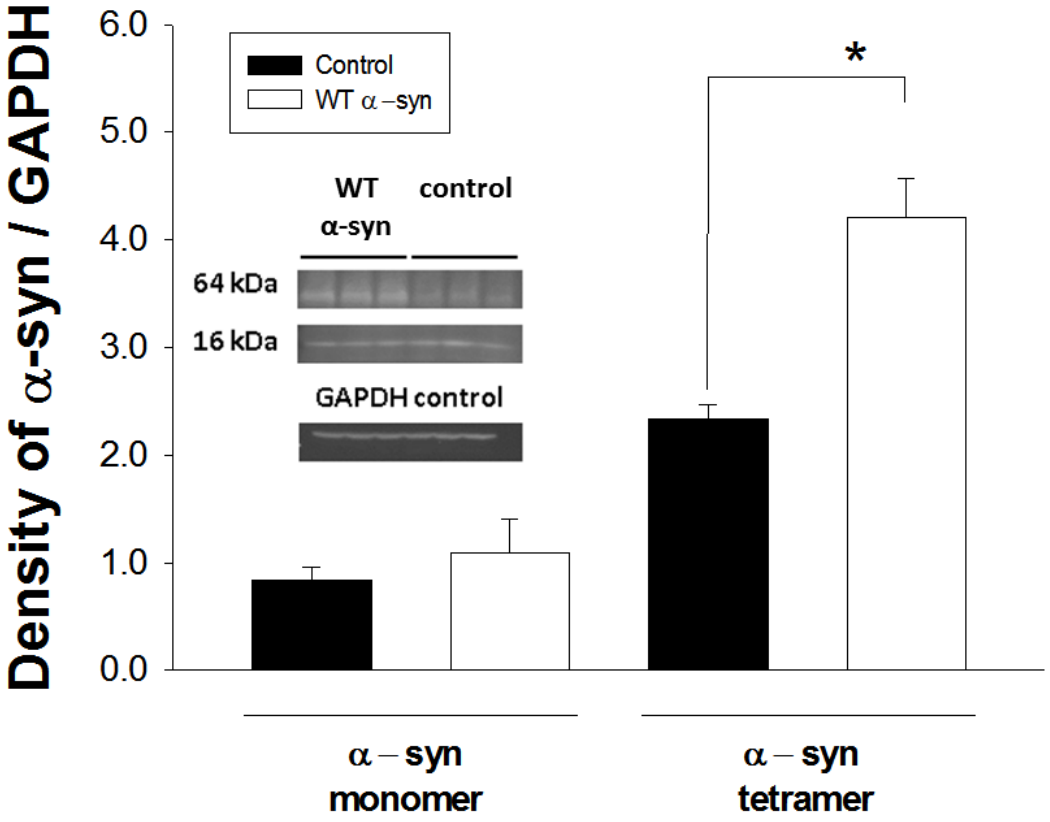


Figure 14 Densitometric analysis of α syn levels

Densitometric analysis of α-syn levels in cells stably overexpressing WT α-syn show increased amounts of an α-syn multimer. Western blot band quantification was performed using NIH ImageJ software. Values were combined from 3 biological replicates after they were normalized to the GAPDH loading control. The values were compared using one-way ANOVA, * $p < 0.05$.

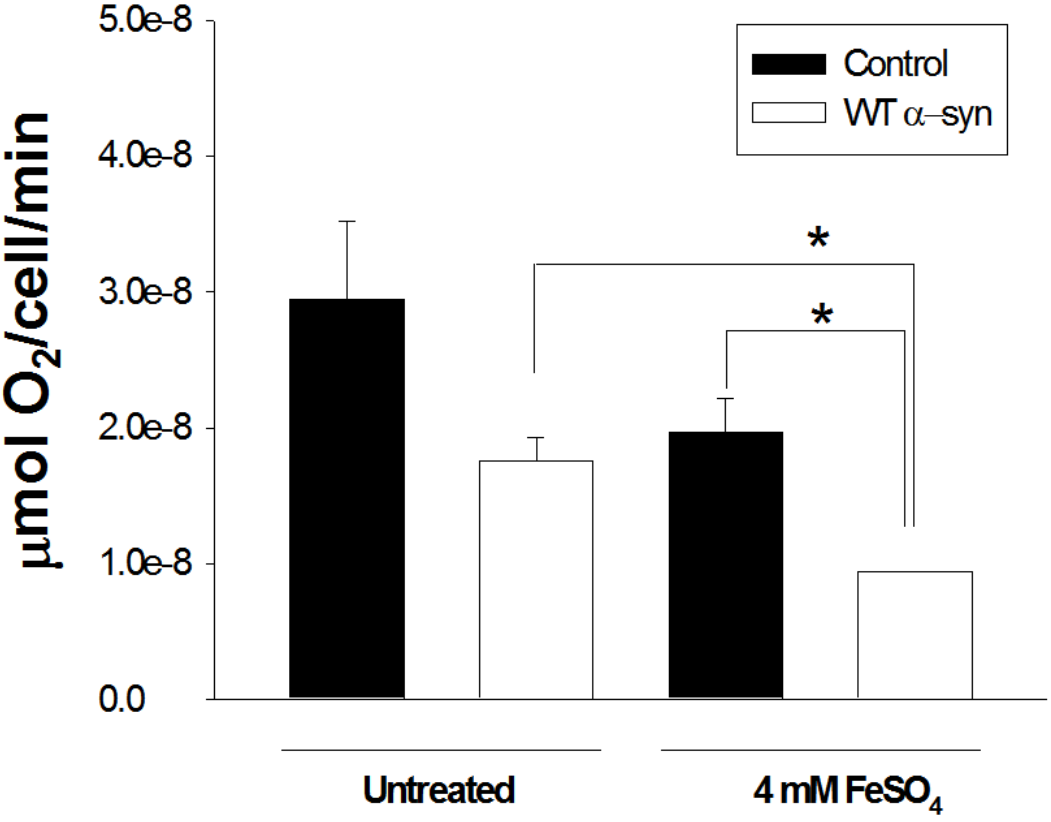


Figure 15 Oxygen consumption in the presence of $FeSO_4$
$FeSO_4$ treatment decreases oxygen consumption. Oxygen consumption was determined in WT α-syn-overexpressing or control cells using a Clark oxygen electrode following incubation for 48 hours with or without 4 mM $FeSO_4$. Cells were trypsinized from confluent 10 cm plates (*n*=4 for each treatment). The oxygen consumption rate for WT α-syn cells in the presence of iron was below what could be detected from one plate of cells. Biological replicates of 4 were pooled into 2 biological replicates, which had identical values. Trypsin was neutralized by the addition of 5 ml of DMEM containing FBS. Cells were counted in a 6 ml suspension with a hemocytometer before being pelleted and re-suspended in 1 ml of media. 350 µl of a 1 ml cell suspension was transferred to a respiratory chamber. O_2 consumption was measured for 4 minutes and the respiratory rate was normalized per cell. The values were compared using two-way ANOVA, * $p < 0.05$.

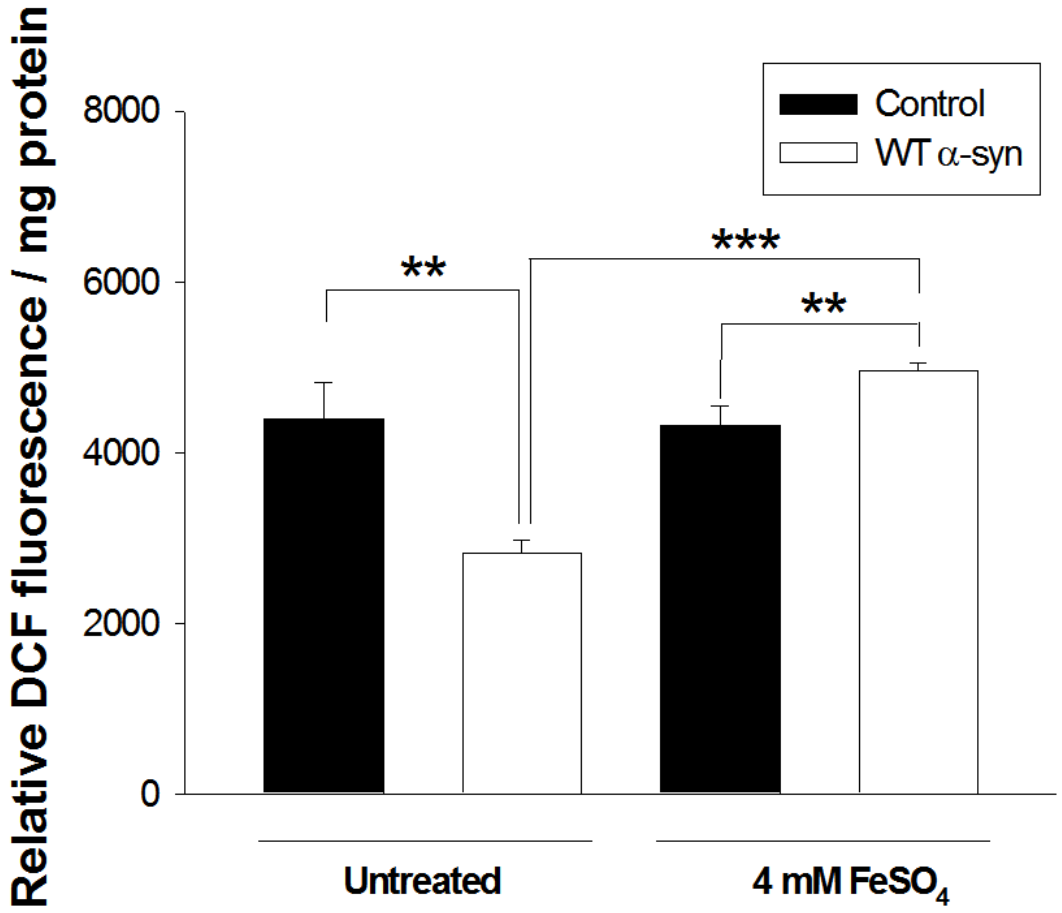


Figure 16 ROS levels
WT α-syn-overexpressing cells only showed higher ROS production than control cells in the presence of $FeSO_4$. In the absence of $FeSO_4$ WT α-syn-overexpressing cells showed less ROS production than control cells (two-way ANOVA $p < 0.005$). ROS production was measured following a 48 hour incubation with or without 4 mM $FeSO_4$.

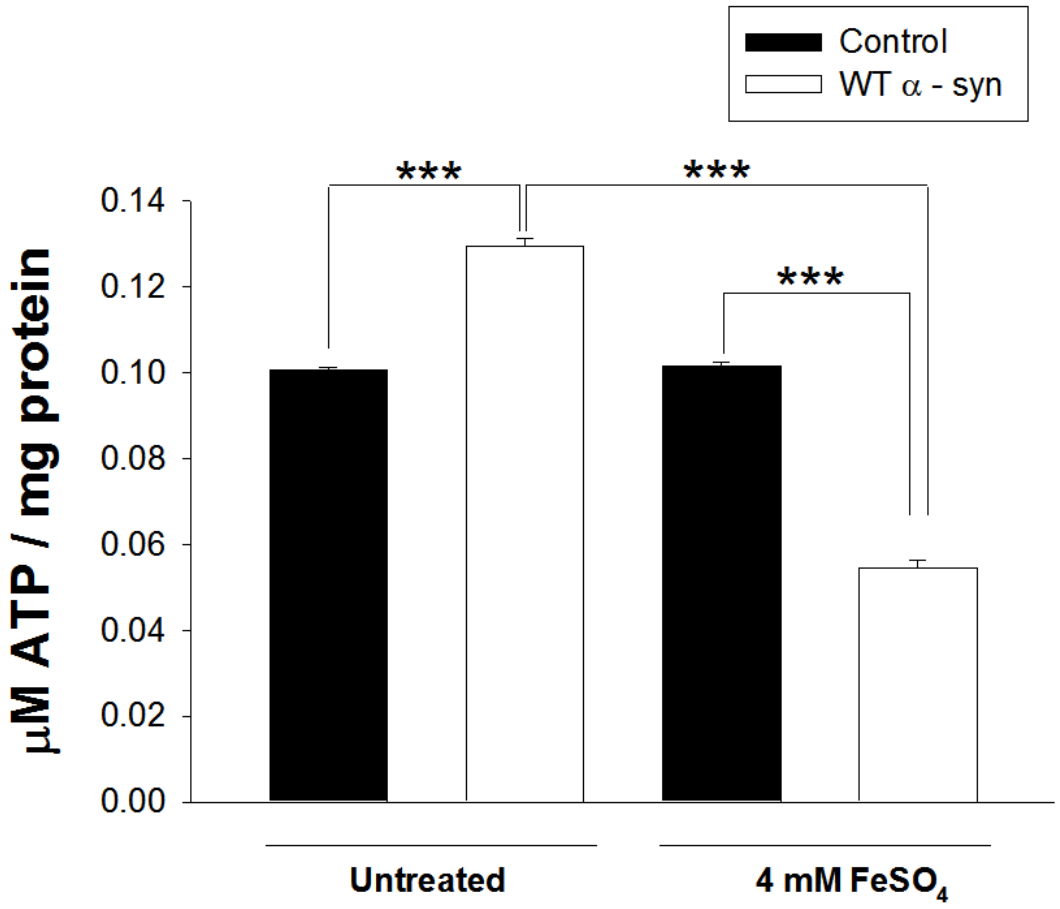


Figure 17 ATP levels in the presence of iron
WT α-syn-overexpressing cells only showed lower ATP levels than control cells in the presence of $FeSO_4$. In the absence of $FeSO_4$ WT α-syn-overexpressing cells showed higher ATP levels than control cells (two-way ANOVA $p < 0.005$). ROS production was measured following a 48 hour incubation with or without 4 mM $FeSO_4$.

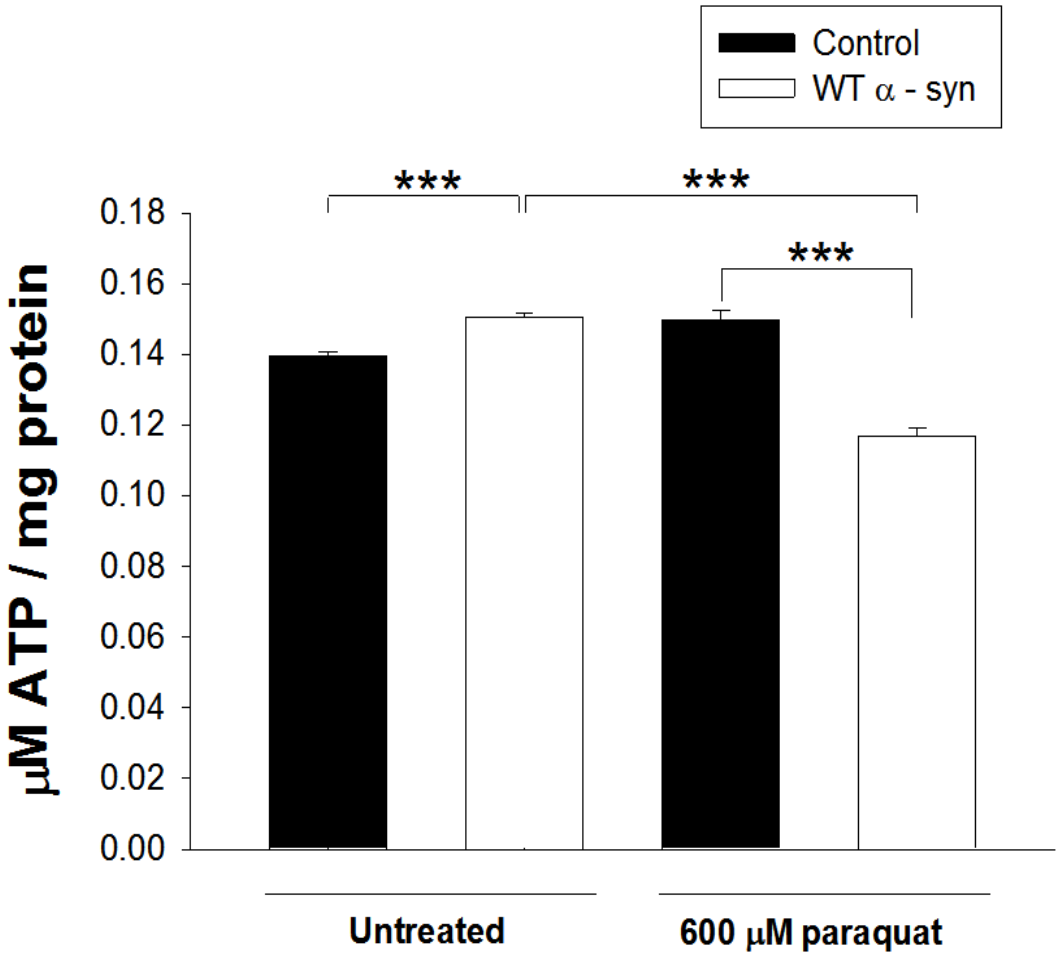


Figure 18 ATP levels in the presence of paraquat
WT α-syn-overexpressing cells only showed lower ATP levels than control cells in the presence of paraquat. In the absence of paraquat WT α-syn-overexpressing cells showed higher ATP levels than control cells (two-way ANOVA $p < 0.005$). Paraquat also increased ATP levels significantly albeit slightly in control cells; statistical indicators were omitted for clarity. ATP levels were measured following a 48 hour incubation with or without 600 □M paraquat.

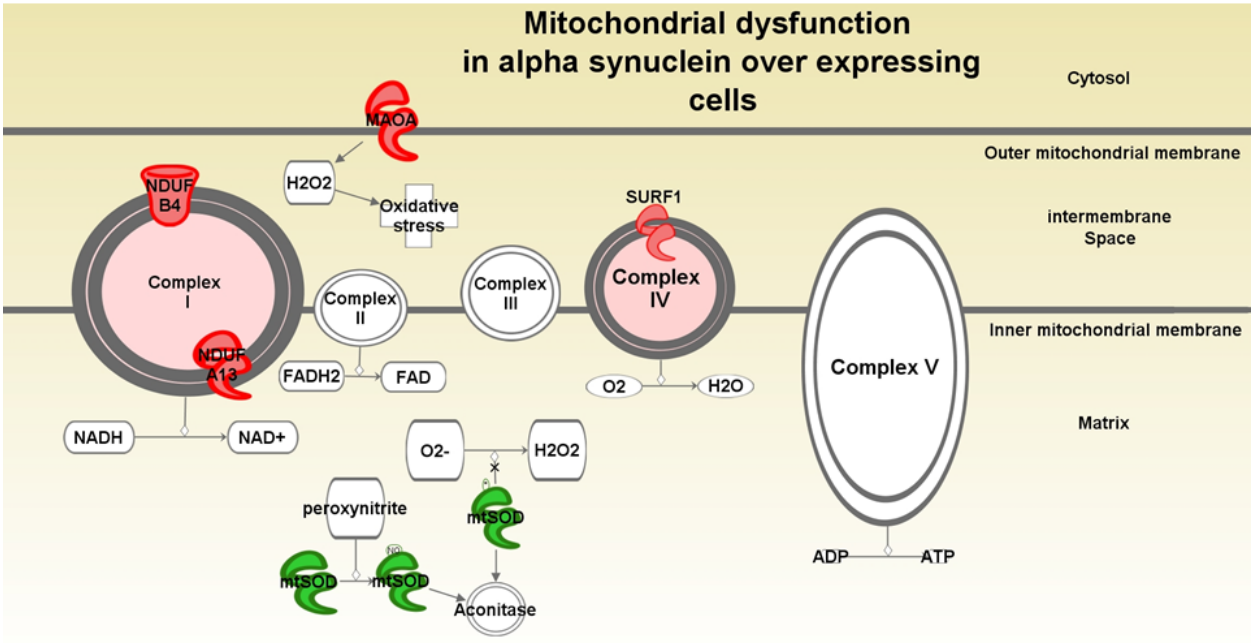


Figure 19 Mitochondrial protein level changes

Figure 4.7 Select proteome changes accompanying overexpression of α-syn. Red indicates increased abundance, while green indicates decreased abundance. Monoamine oxidase-A (MAO-A), NADH dehydrogenase (ubiquinone) 1 alpha sub-complex, 13 (NDUFA13), NADH dehydrogenase (ubiquinone) 1 beta sub-complex 4 (NDUFB4), and surfeit 1 (SURF1) show $\log_2$ fold increases of 1.6, 1.4, and 1.7, while superoxide dismutase 2 (SOD2) showed a $\log_2$ fold decrease of 1.4.

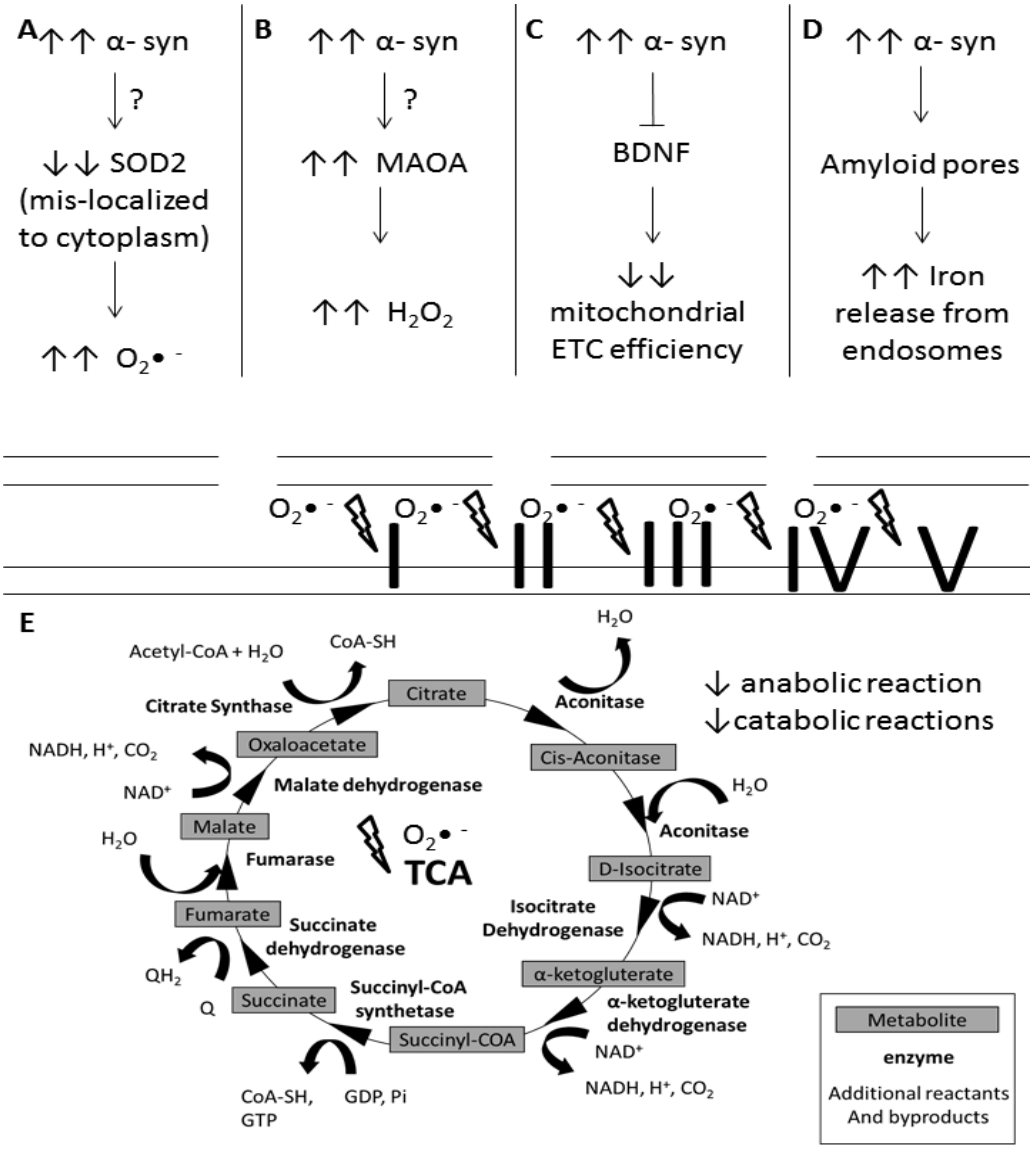


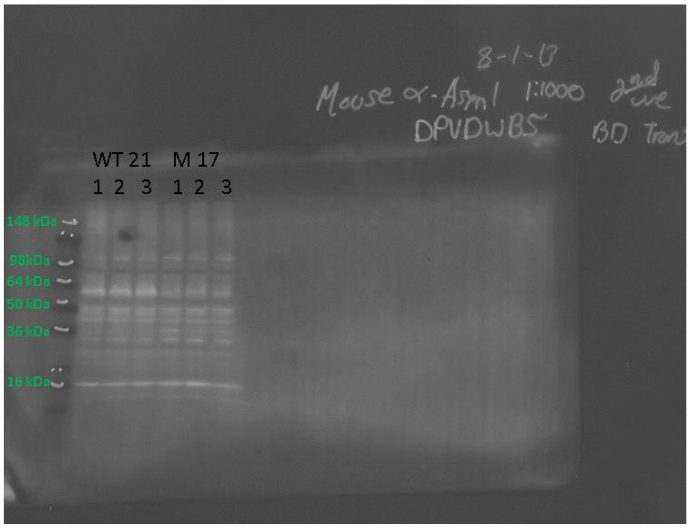
Figure 20 Mechanisms leading to ROS mediated damage

Mechanisms leading to ROS mediated damage of ETC complexes and TCA cycle enzymes. (A) Overexpression of α-syn results in a decreased abundance of SOD2 preventing adequate superoxide conversion to the less toxic hydrogen peroxide. (B) Overexpression of α-syn likely leads to increased MAO-A activity and increased hydrogen peroxide production. (C) Decreased BDNF signaling may decrease mitochondrial biogenesis and SOD2 levels leading to increased ROS production. (D) When α-syn levels increase α-syn aggregates into amyloid pores which penetrate lysosomes and endosomes transporting iron releasing it into the cytoplasm where it can stimulate α-syn aggregation. Free iron can convert hydrogen peroxide to more harmful oxygen free radicals. (E) Tricarboxylic acid cycle enzymes and electron transport chain complexes are damaged by increased ROS levels decreasing energy production, and unbalancing metabolites essential for key anabolic processes.

Table 4 List of all the compounds screened

Drugs	Metabolic intermediates	Ester metabolic intermediates	Amino acids	
AICAR	Succinate	Diethyl fumurate	**essential**	**non-essential**
Melatonin	Beta-hydroxybutyrate	Diethyl malate	L-isoleucine	L-asparagine
Rapamycin	Citrate	Diethyl oxaloacetate	L-leucine	L-cysteine
	Fumurate	Dimethyl malate	L-histidine	L-glutamine
	Glutaric acid	Dimethyl succinate	L-lysine	L-alanine
	L-Malate		L-methionine	L-arginine
	Malonic acid		L-phenylalanine	L-proline
	Pyruvate		L-tryptophan	L-serine
			L-valine	L-aspartate

Analysis of whole cell lysate from M17 and WT α-syn neuroblastoma cells



GAPDH blot loading ctrl

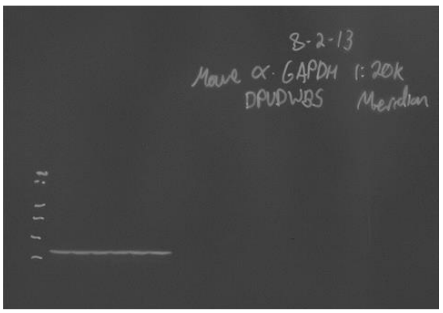


Figure 21Full Western blot and GAPDH loading control

Western blots were not cropped for clarity. The blot on the left shows the most intense banding at approximately 58 kDa indicating that most of the alpha synuclein is present in the oligomers smaller than 98 kDa but larger than 36 kDa.

CHAPTER FIVE: MITOCHONDRIAL DYSFUNCTION IN THE NEUROVASCULAR UNIT OF THE SPINAL CORD IN SPORADIC ALS PATIENTS

Abstract

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by progressive motor neuron degeneration in the brain and spinal cord, leading to muscle atrophy, paralysis and death, typically around four-five years after the initial symptoms. Mitochondrial dysfunction is a major contributor to cell damage promoting the progression of ALS. Mitochondrial function abnormalities are characterized in the spinal cords of ALS animal models and ALS patients. However, the role of mitochondria is still unclear in the context of the neurovascular unit in the spinal cord of sporadic ALS patients. In the present study, activities of mitochondrial electron transport chain (ETC) complexes were analyzed in post-mortem gray and white matter cervical and lumbar spinal cords from sporadic ALS patients. Also, immunohistochemical analysis of mitochondrial density in lateral funiculus, spinal cord motor neurons, and capillaries in gray and white matter was performed. Our results demonstrated differences between activities of mitochondrial ETC complexes. Complex IV activity, but not Complex I, was significantly decreased only in the gray matter in both cervical and lumbar spinal cords, Quantitative analyses of mitochondrial density showed mitochondrial aggregation mainly in cervical motor neurons and white matter capillaries in cervical and lumbar spinal cords.

Findings herein suggest for the first time that aberrant mitochondrial dynamics affect the neurovascular unit of the spinal cord in ALS patients likely due to mitochondrial complex IV deficits

Introduction

Sporadic amyotrophic lateral sclerosis (sALS) is an adult onset neurodegenerative disorder with no known cure or effective treatment. It is characterized by progressive motor neuron degeneration in the brain and spinal cord, leading to muscle atrophy and eventual death (Kinsley and Siddique 1993). Mitochondrial dysfunction is a well-established contributor to the progression of many neurodegenerative disorder including Alzheimer's disease, Parkinson's disease, and ALS (Cozzolino and Carri 2012; Cozzolino, Rossi et al. 2015; Delic, Brownlow et al. 2015). Mitochondrial is characterized by a decrease in adenosine triphosphate (ATP) production, an increase in reactive oxygen species (ROS) production. Mitochondrial dysfunction is also characterized by a decrease in oxygen consumption and a decrease in mitochondrial membrane potential (de Moura, dos Santos et al. 2010). These mitochondrial defects are directly linked to improper function of the electron transport chain (ETC).

A diagram of the mitochondrial ETC is shown in Figure 1. Nicotinamide adenine dinucleotide (NADH) dehydrogenase or ETC Complex I contributes to around 40% of the proton gradient across the mitochondrial inner membrane and its dysfunction has been implicated in several neurodegenerative disorders including ALS, Alzheimer's disease, Parkinson's disease, and Huntington's disease (Marella, Seo et al. 2009; Moreira, Carvalho et al. 2010; Ghiasi, Hosseinkhani et al. 2012; Papa and De Rasmo 2013). It has been recently determined that mitochondrial complex I function is modulated by the cAMP/PKA signaling pathway (Piccoli,

Scacco et al. 2006; Papa, Scacco et al. 2010). Succinate dehydrogenase (Complex II), is the only ETC complex that also participates in the tricarboxylic acid (TCA) cycle and is entirely encoded by genes in the nucleus, having no mitochondrial DNA-encoded subunits. Complex II enzymatic activity functions to convert succinate to fumarate, and it has been implicated in early-onset Parkinson's disease and ALS (Haas, Nasirian et al. 1995; Wiedemann, Manfredi et al. 2002). The cytochrome bc_1 complex (ETC Complex III) contributes to the proton gradient by the translocation of protons and the reduction of the lipid soluble electron carrier cytochrome c. Dysfunction in ETC Complex III is associated with mitochondrial myopathy and lactic acidosis (Darley-Usmar, Kennaway et al. 1983). Complex II and III activity has also been shown to decrease in the spinal cords of ALS patients (Wiedemann, Manfredi et al. 2002). Cytochrome c oxidase (ETC Complex IV) is the last complex in the electron transport chain and it oxidizes cytochromes by sequential transfer of electrons to the terminal electron acceptor oxygen. Complex IV deficiency has also been shown to contribute to the progress of many neurodegenerative diseases, including sporadic Alzheimer disease, and sporadic ALS (Fujita, Yamauchi et al. 1996; Mosconi, de Leon et al. 2011; Hroudova, Singh et al. 2014). Unfortunately, these studies did not delineate between complex IV activity of the cervical and lumbar white and gray matter mitochondrial activity. Early death in ALS is associated with degeneration of the phrenic nerve, innervating the diaphragm. It is possible that mitochondrial dysfunction is more sever in the cervical compared to lumbar spinal cord. F_0-F_1-ATP synthase (Complex V), utilizes the electrochemical gradient generated by the ETC complexes I-IV to phosphorylate ADP to ATP. Dysfunction of complex V has been implicated in Alzheimer's disease, and Parkinson's disease (Martinez-Cano, Ortiz-Genaro et al. 2005; Ferrer, Perez et al. 2007). But the activity of complex V in spinal cords of ALS patients remains unexplored.

Mutations in the gene coding for superoxide dismutase 1 (SOD1) were shown to contribute to the development of familial ALS (fALS), which is presented with the same symptoms as sALS. Currently, mutations in 19 genes are known to cause fALS , with the most recently identified being TANK-binding kinase 1(TBK1) (2015; Cirulli, Lasseigne et al. 2015). Proteins coded for by these 19 genes range in function from RNA stability in the case of TAR DNA binding protein, to conversion of free radicals to less reactive oxygen species as in the case of SOD1. Yet these mutations are not present in sALS, although they yield the same mitochondrial phenotypes. Recently, a mitochondrial contribution to ALS pathology has been contested (Parone, Da Cruz et al. 2013). Using an SOD 1 mutant mouse, Parone et al showed that improving mitochondrial functions, does not improve ALS associated muscle atrophy and death. Establishing a clearer picture of the mitochondrial involvement in the pathology of sALS in patients is necessary in order to advance our current knowledge.

The neurovascular unit (NVU) provides a stage for a complex interplay between neuronal energy demand and nutrient supply. It encompasses interaction of neurons, astrocytes, endothelial cells, myocytes, pericytes and extracellular matrix components (Neuwelt, Bauer et al. 2011; Stanimirovic and Friedman 2012; Abbott 2013; Muoio, Persson et al. 2014). Disruption of proper NVU function contributes to the progress of neurodegenerative disorders and to the pathology following traumatic brain injury (Zlokovic 2005; Desai, Monahan et al. 2007; Khan, Im et al. 2009). Mitochondrial involvement in the breakdown of NVU in neurodegenerative disorders remains poorly understood.

Recently, Garbuzova-Davis et al. described sALS as a disease characterized by dysfunction in endothelial cells and capillaries that comprise the neurovascular unit (Garbuzova-Davis, Haller et al. 2007; Garbuzova-Davis and Sanberg 2014). We therefore wanted to

determine if mitochondrial dysfunction contributed to the collapse of the NVU in addition to contributing directly to neuronal degeneration. We determined the ETC complex IV activity in gray and white matter of cervical and lumbar spinal cord. We also assayed ETC complex I activity and mitochondrial aggregation in the capillaries of gray and white matter, lateral funiculus, and motor neurons.

Methods

Mitochondrial Isolation

The gray and white matters were dissected in frozen post-mortem cervical and lumbar spinal cords from ALS patients and controls. Homogenate was enriched for mitochondria by first placing tissue samples in 2.5 mL of ice cold mitochondrial isolation buffer (75 mM sucrose, 0.1% BSA, 1 mM 152 EGTA, 215 mM mannitol, 20 mM K 153 + HEPES, pH 7.2). The tissue samples were homogenized in the isolation buffer with seven strokes in a dounce homogenizer with a tight fitting teflon pestle on ice. The homogenates were then centrifuged at 13,000 x g for 5 minutes at 4℃. The supernatant was removed and the pellet was re-suspended in 150 µL of ice cold mitochondrial isolation buffer with 1 mM n-dodecyl-β-D-maltoside.

ETC complex I analysis

ETC complex I activity was measured by first adding 10 µL of mitochondrial suspension to 495 µL of reaction buffer (25 mmol/L Potassium Phosphate, 3.5 g/L BSA, 60 µM DCPIP (2,6-dichlorophenol indophenol), 70 µmM decylubiquinone, and 1.0 µM antimycin-A). A 96 well plate was used and 98 µL of the reaction buffer with mitochondria was added to 3 wells and incubated at 37℃ for 3 minutes. The absorbance was then measured at 600 nm and readings

were taken for 2 minutes at 30 second intervals. A multi-channel pipette was used to inject 2 µL of 10 mM NADH for a final concentration of 0.2 mM. The absorbance was then was measured at 600 nm and readings were taken for 20 minutes at 2 minute and 30 second intervals. Afterwards 1 µL of 10 mM rotenone (in DMSO) and the absorbance was measured at 600 nm for the same length of time and interval of time as the NADH reading.

ETC complex IV analysis

A Clark electrode was used to measure ETC complex IV activity. The electrode chamber was filled with 35 µL of mitochondrial suspension. The volume was brought up to 350 µL with COX buffer having a final concentration of 1 µM FCCP, 50 µM cytochrome c, 2 mM ascorbate, 1 µM antimycin A and 5 mM TMPD (N,N,N',N'-tetramethyl-*p*-phenylenediamine). The oxygen concentration was recorded for at least 6 minutes and then 10 mM of KCN was added and the oxygen concentration was recorded for 2 more minutes.

ETC complex V analysis

As previously described by Delic et al (Delic, Brownlow et al. 2015) A molybdenum-based assay measuring free phosphate was used to determine the F_0F_1-ATPase hydrolytic activity of frozen-thawed human spinal cord post mortem tissue enriched for mitochondria. The reaction buffer consisted of 80 mM KCl, 3 mM $MgCl_2$, 5 mM sodium azide, 0.2 mM EGTA, 0.2 mM $CaCl_2$, and 25 mM MOPS. The pH was adjusted to 7.0 using Tris base. 2 µM oligomycin was added to control samples to obtain the F_0F_1-ATPase-independent rate of phosphate release, which was subtracted off. The coloring solution consisted of 0.7 mM ammonium heptamolybdate, 3 mM ascorbic acid, 100 µM potassium antimony tartrate, and 140 mM H_2SO_4

made from stock concentrations of 0.3 M ascorbic acid, 4 mM potassium antimony tartrate and 24 mM ammonium heptamolybdate. 1 mM ATP and 10 μg/ml of mitochondrial protein were added to the reaction buffer at 37°C. After 20 minute incubation, 270 μL of coloring solution was added to 30 μL of reaction buffer (containing ATP and mitochondrial protein) and the absorbance was read at 850 nm on a Biotek Synergy 2 microplate reader 4 minutes later.

Immunohistochemistry

A series of spinal cord tissue sections from ALS patients and controls, previously cut on a cryostat, were labeled with a specific marker for mitochondria. Briefly, after blocking tissue for 60 minutes, the mouse monoclonal anti-VDAC1 antibody (1:50, Millipore) was applied on tissues overnight at 4°C. The next day the slides were washed with PBS and then incubated with goat anti-mouse secondary antibody conjugated to FITC (1:700, Alexa, molecular Probes) for 2 hours. A coverslip was added to the sections with Vectashield containing DAPI and examined using epifluorescence with an Olympus BX60 microscope. Integral density of mitochondria was analyzed using NIH ImageJ software.

Protein assay

Protein levels were measured using a BCA protein assay (Thermo Scientific).

Statistical analysis:

Error bars represent the standard error of the mean. One-way ANOVA tests were performed with control and ALS patient tissue as variables, * indicates a p value of < 0.05. Analysis was performed using SigmaPlot version 11.

Results

Human spinal cord autopsied samples from ALS and aged-matched controls were obtained. Gray and white matter were dissected out and enriched for mitochondria. Since there have been mixed reports on mitochondrial ETC complex dysfunction in ALS patients, we measured complex I and complex IV activity in the gray and white matter homogenates. We found no statistical difference in the activity of complex I between the ALS patients and the aged matched controls or between gray matter and white matter (Fig. 22 A). However, we found that complex IV activity was higher in gray matter than in white matter in control samples (Fig. 22 B). In addition we found that complex IV activity was reduced in gray matter, but not in white matter of ALS patient cervical and lumbar spinal cord samples.

To examine any possible changes in mitochondrial morphology or number in ALS immunostaining of mitochondria in spinal cord sections was performed (Fig. 23 A- 23 P). Nuclear counterstaining was performed with DAPI. Micrographs were taken of capillaries in white and gray matter, regions of lateral funiculus, and individual motor neurons. Surprisingly, the density of staining appeared stronger in ALS cervical and lumbar sections compared to controls. It is important to note that although consistent and statistically significant, the differences were minor. More sensitive methods of quantifying the micrographs are currently being explored. Excluding hemosiderin staining artifacts, larger mitochondrial aggregates were

observed in lumbar and cervical ALS motor neurons (Fig. 23 I, 23 M) than age matched controls (Fig. 23 A, 23 E). More dense mitochondrial aggregates were also present in ALS white matter (Fig. 23 J, 23 N) compared to control white matter (Fig. 23 B, 23 F). Mitochondrial aggregation and density in white matter capillaries of ALS patient spinal cord also appear higher (Fig. 23 K, 23 O) than in control white matter capillaries (Fig. 23 C, 23 G). Mitochondrial aggregation and density also appeared higher in the gray matter capillaries of cervical and lumbar ALS patient spinal cord (Fig. 23 L, 23 P) compared to controls (Fig. 23 D, 23 H).

Mitochondrial density from the images in Figure 23 was quantified (Fig. 24 A - 24 D). The threshold was adjusted to exclude hemosiderin. As expected from the manual inspection of the images, the mitochondrial density was higher in motor neurons than in capillaries or the lateral funiculus (Fig. 24 A). Interestingly, ALS cervical motor neurons showed higher mitochondrial density than those of controls (Fig. 24 A). Similarly, the increased mitochondrial density observed in ALS patients compared to controls was also observed when examining the lateral funiculus sections (Fig. 24 B) or white matter capillaries (Figure 24 D), but there was no statistical difference in gray matter capillaries (Figure 24 C).

Discussion

In this study we showed that there is decreased ETC complex IV activity in the gray matter of both lumbar and cervical spinal cord samples from sALS patients. We also showed that there appears to be greater amount of mitochondrial aggregation in the capillaries of gray and white matter, the lateral funiculus, and motor neurons in the samples from the ALS patients. Although the activities of ETC complexes I, IV, and V were assayed in our samples, we only report complex I and IV activities as complex V activity in the spinal cord lysates lost the ability

to be inhibited by oligomycin and therefore we could not distinguish between complex V ATPase activity and non-complex V ATPase activity present in the samples. Several autolysis studies have shown that ETC complex V was the most susceptible complex of oxidative phosphorylation to breakdown and its function was completely lost 20 hours following mitochondrial isolation (Rouslin 1983; Gribanov, Pankrushina et al. 1994; Vincent, Gratton et al. 1995).

ETC complex IV is decreased in both cervical and lumbar spinal cord.

Progression of sALS varies greatly, with the majority of patients dying within five years of diagnosis. Respiratory problems are associated with negative prognosis and patient outcome (Shoesmith, Findlater et al. 2007). Deterioration of the neurons located in the cervical spine leads to loss of innervation of chest wall, diaphragm, and muscles involved with swallowing. We therefore originally hypothesized that cervical mitochondria would have more profound dysfunction than those in the motor neurons of the lumbar spine. Deficits in complex IV activity were, however, of the same magnitude in both regions, suggesting that cervical mitochondria are not more damaged. It is possible that motor neurons of the cervical spine are more susceptible to neuronal cell death and loss at the same level of mitochondrial damage. No complex IV deficits were observed in white matter. The reasons for this are unclear at present, but it is possible that mitochondria in the cell bodies are in close proximity to the ER, where they are exposed to higher levels of released Ca^{2+} that stimulates mitochondrial citric acid cycle metabolism and ROS production leading to more oxidative damage of complex IV.

Increased mitochondrial aggregation in the neurovascular unit of ALS patient spinal cord

Several groups have shown decreased levels of mitochondrial DNA and complex I+III, II+III, and IV activities in ALS patient spinal cord or muscle (Vielhaber, Kunz et al. 2000; Wiedemann, Manfredi et al. 2002). These results are consistent with our results assuming there was a complex III deficit in our ALS patient samples that we did not measure. However, a decreased amount of the mitochondrial marker citrate synthase led one of the groups to conclude there were decreased amounts of mitochondria in the ALS patient spinal cord (Vielhaber, Kunz et al. 2000; Wiedemann, Manfredi et al. 2002) . We show what appears to be increased mitochondrial density following IHC mitochondrial staining and subsequent micrograph density analysis in spinal cord from our ALS patient samples. Increased mitochondrial density was observed in lateral funiculus, and capillaries. This apparent increase in mitochondrial density could be due to mitochondrial aggregation or impaired fission (Shi, Gal et al. 2010). Dysfunctional mitochondria fuse with healthy mitochondria in an attempt to correct deficits (Schon and Gilkerson 2010). When this process collapses, mitochondria are unable to undergo fission, resulting in poorly functioning mega-mitochondria. This appears to be a problem not only in neurons, but the entire NVU (see Fig. 24).

Future studies will focus on assaying ETC complex III and mitochondrial DNA levels in our human spinal cord samples and analyzing mitochondrial ETC complex activities in each of the components of the NVU in a mouse model of ALS. Once the degree of mitochondrial dysfunction is established, potential therapies can be administered to the mice to determine if they improve ETC complex function.

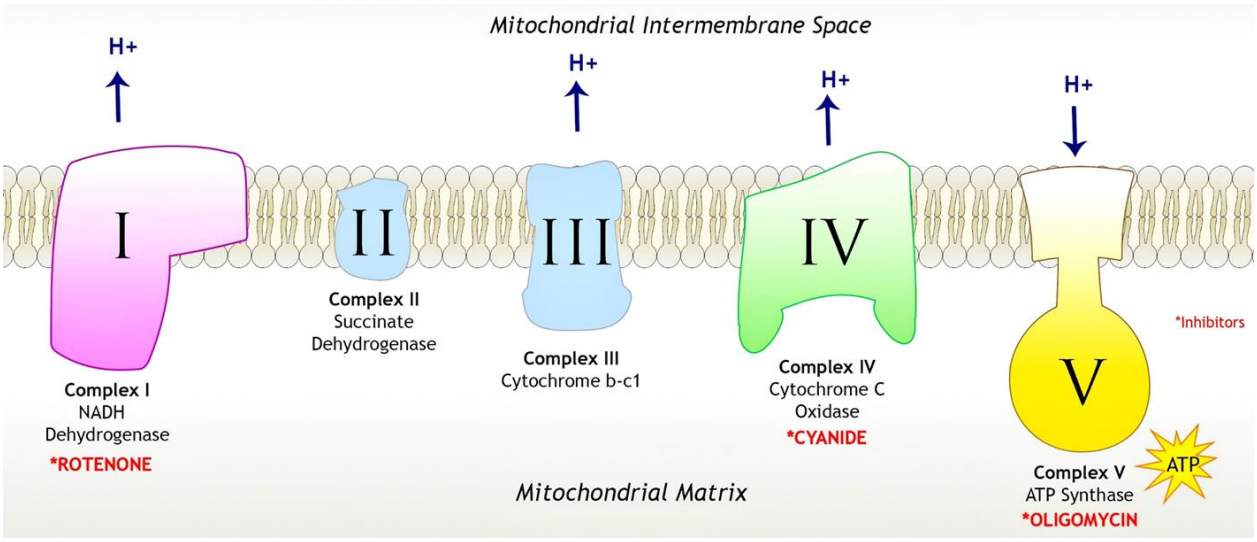


Figure 22 Diagram of the ETC

NADH from the citric acid cycle is oxidized by the removal of two electrons, which are donated to complex I and then transferred to the lipid-soluble carrier, ubiquinone, converting it to ubiquinol. Complex II, or succinate dehydrogenase, also contributes to the ubiquinol pool by reduction of succinate. Succinate dehydrogenase reduction of succinate to fumarate is an essential step in the citric acid cycle as well. In complex III, also known as the cytochrome bc_1 complex, hydrogens are translocated to the intermembrane space, by oxidation of ubiquinol to ubiquinone. The electrons are transferred to the water-soluble electron carrier protein cytochrome c in the inner membrane space, and then finally shuttled to cytochrome c oxidase (complex IV). Complex IV sequentially transfers electrons on to a molecule of oxygen. In response, oxygen is converted to two molecules of water, further consuming hydrogens in the mitochondrial matrix. During this process, NAD^+ is regenerated for use in the citric acid cycle, and the electrochemical gradient is generated by protons pumped out of the matrix. This gradient is then used by complex V, ATP synthase, to generate ATP.

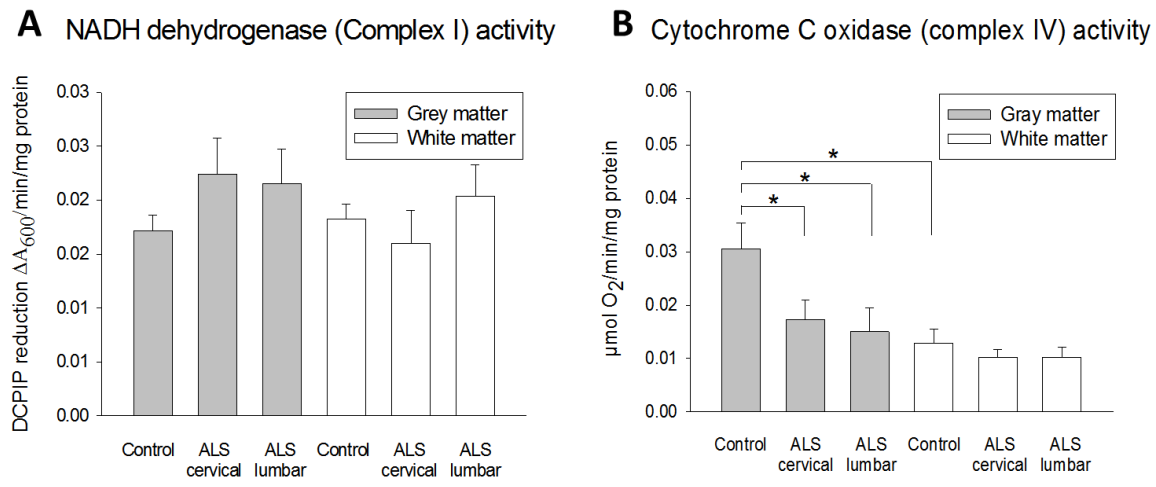


Figure 23 Mitochondrial complex I and complex IV activity

Decreased complex IV activity, but not complex I activity in ALS spinal cord.
(A) ETC complex I activity in mitochondria from post-mortem spinal cords of ALS patients and controls. Mitochondria were isolated and suspended in reaction buffer with DCPIP. The absorbance was read at 800 nm. The reduction of (DCPIP) changed its color from blue to clear. The reaction was inhibited by 1μM rotenone. (B) Decreased activity of mitochondrial ETC complex IV was observed in ALS gray matter compared to controls.

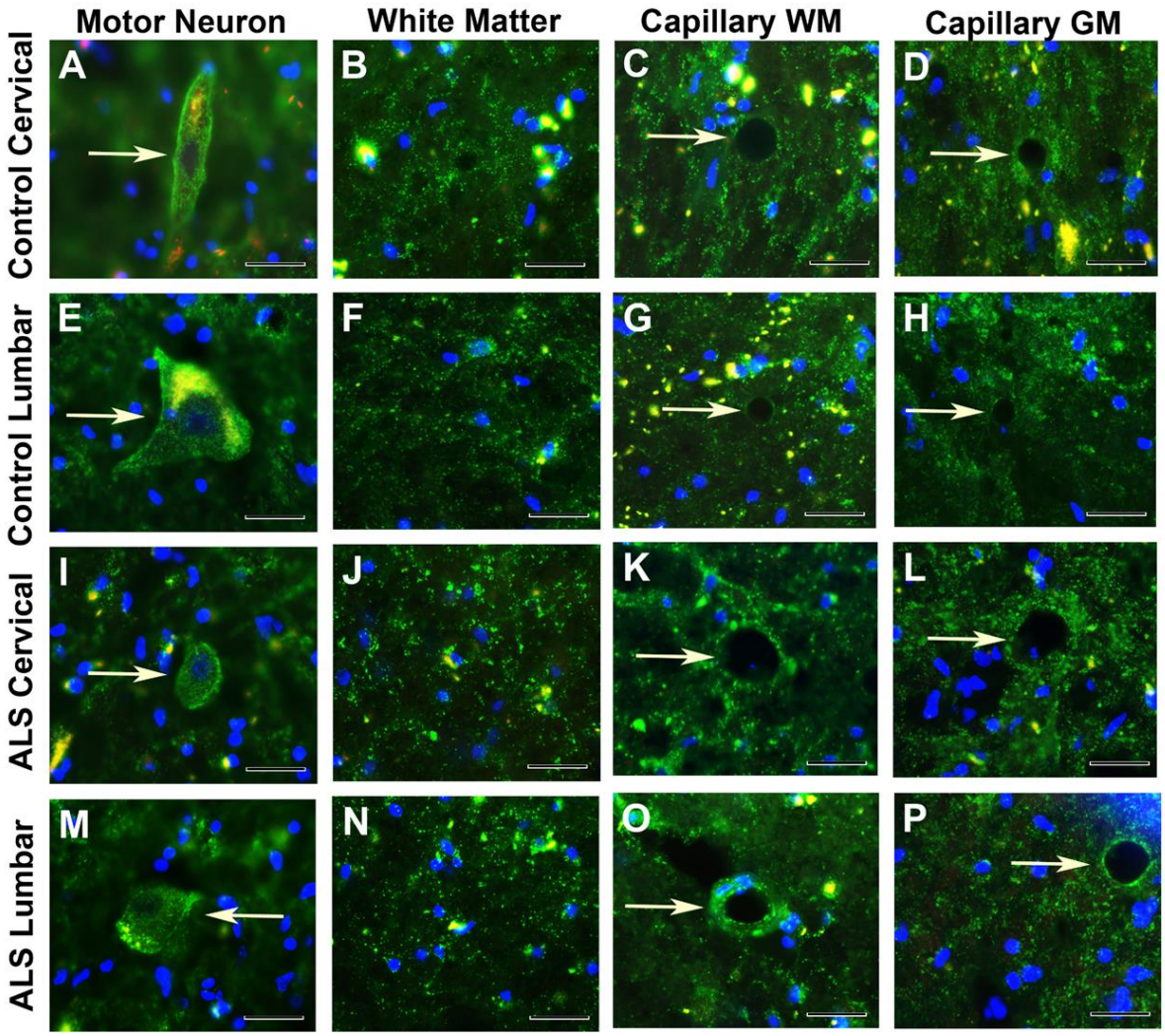


Figure 24 Immunohistochemistry staining of mitochondria
Immunohistochemistry staining of mitochondria (green) in post-mortem spinal cords of ALS patients and controls. The scale bar in panels A-P is 25 μM. Mitochondrial aggregation is indicated with an arrow. DAPI stained nuclei are shown in blue.

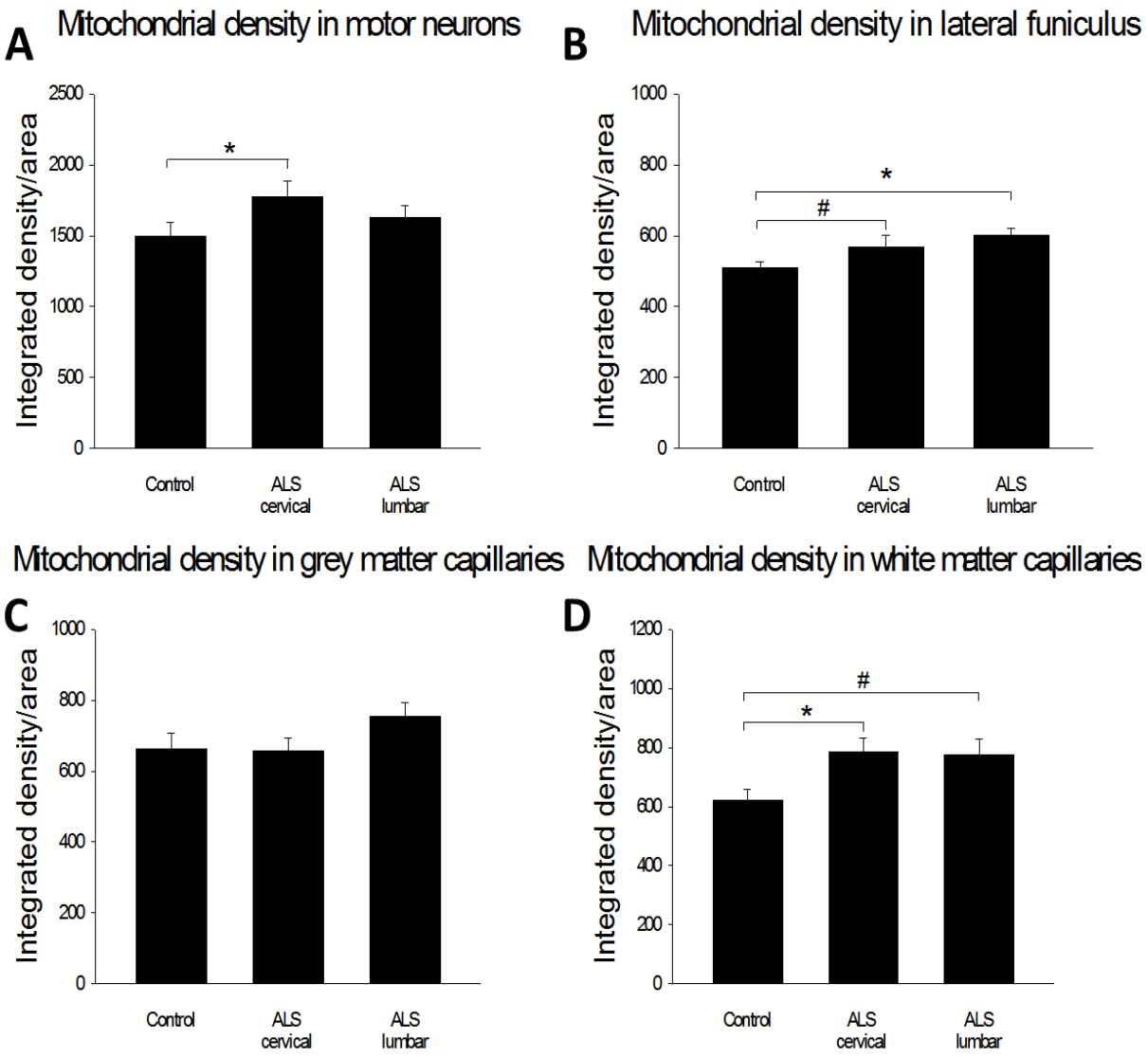


Figure 25 Integrated density analysis of mitochondria area in spinal cord micrographs
Figure 5.4 Integrated density analysis of mitochondrial area in spinal cord micrographs. ImageJ software was used to quantitate mitochondrial areas from the panels in figure 3. 10 ALS and 4 control sections were analyzed.

CHAPTER SIX: THE EFFECTS OF AICAR AND RAPAMYCIN ON MITOCHONDRIAL FUNCTION IN TRANSFORMED MTDNA MUTATOR MEFS

Abstract

Mitochondrial DNA point mutations and deletions accumulate with age and may play a role in aging as suggested by the premature aging phenotype in mitochondrial DNA polymerase gamma (POLG) exonuclease-deficient mice. Increased AMP-activated protein kinase (AMPK) signaling and decreased mammalian target of rapamycin (mTOR) signaling delay aging and influence mitochondrial function. Transformed murine embryonic fibroblasts (MEFs) from POLG proofreading-deficient mice were more sensitive to most mitochondrial inhibitors than WT MEFs. When cultured in high glucose media (HGM), POLG exonuclease-deficient MEFs had a similar respiratory rate, but higher reactive oxygen species (ROS) production and higher ATP levels than WT MEFs due to an upregulation of glycolysis. Large deficits in oxygen consumption were revealed when POLG exonuclease-deficient MEFs were cultured in low glucose, pyruvate-containing media (LGPM). The effects of AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide), an AMPK activator, and rapamycin, an inhibitor of mTOR, on mitochondrial function and ATP levels were tested. Treatment with rapamycin led to a transient increase in oxygen consumption only in WT MEFs. Rapamycin treatment generally increased ATP levels without affecting ROS production. Short term treatment with AICAR increased ATP levels in POLG exonuclease-deficient MEFs only when cultured in LGPM. AICAR treatment

did not affect oxygen consumption, decreased ATP levels, and increased ROS production when MEFs were cultured in HGM. In contrast, long term AICAR treatment in HGM with pyruvate and uridine decreased oxygen consumption and ROS production without decreasing ATP levels. These results demonstrate the complex interactions between mitochondrial function, nutrient levels, cellular energy status, and mTOR and AMPK signaling.

Introduction

Several lines of evidence suggest the involvement of mitochondrial dysfunction in the development of mammalian aging (Navarro and Boveris 2010; Park and Larsson 2011; Dai, Rabinovitch et al. 2012; Prolla and Denu 2014). With aging, most eukaryotic organisms studied to date have been shown to accumulate mitochondrial DNA (mtDNA) point mutations and/or deletions (Melov, Lithgow et al. 1995; Yui, Ohno et al. 2003; Kazachkova, Ramos et al. 2013). Whether these mtDNA mutations in aged individuals are primarily caused by reactive oxygen species (ROS)-mediated mtDNA damage in mid to late life or are due to mtDNA polymerase gamma (POLG) errors early in development that clonally expand with age has been debated (Larsson 2010), but accumulating evidence from mammalian studies have shown minimal levels of mitochondrial DNA heteroplasmy in aged cells suggesting the latter. The degree to which the accumulation of mtDNA mutations contributes to normal human aging is still unknown (Trifunovic, Wredenberg et al. 2004; Kujoth, Hiona et al. 2005; Khrapko and Vijg 2007). Mitochondria DNA mutations may not accumulate to an extent to play a detrimental role in most tissues, but they can buildup to deleterious levels in specific tissues or tissue regions such as in muscle fibers (McKenzie, Bua et al. 2002), the substantia nigra region of the brain (Kraytsberg,

Kudryavtseva et al. 2006), and stem cells, such as those present in the colonic crypts (McDonald, Preston et al. 2006).

If clonal expansion of mtDNA mutations that occur due to polymerase errors in young organisms is responsible for the majority of mutations in aged mitochondrial genomes, than modulating anti-oxidant defense mechanisms may have little effect on aging outcomes, as was recently proposed in a study examining muscle from SOD1 knockout mice (Wanagat, Ahmadieh et al. 2015). Instead therapies that stimulate mitochondrial turnover through increased mitophagy and mitochondrial biogenesis may prove effective at delaying mtDNA mutation accumulation. This strategy is promising as mitophagy has been shown to selectively degrade damaged or dysfunctional mitochondria with a low membrane potential. The low membrane potential stabilizes the PINK1 protein on the outer mitochondrial membrane to recruit the Parkin E3 ubiquitin ligase necessary for recruitment of autophagosomes and mitochondrial degradation (Koyano and Matsuda 2015). However, studies using human cell lines have suggested that deleted mitochondrial DNA molecules may have a replicative advantage that counters the increased rate of degradation of dysfunctional mitochondria (Diaz, Bayona-Bafaluy et al. 2002). But data from human muscle fibers (Campbell, Krishnan et al. 2014) did not support the findings that mtDNA molecules with deletions have a replicative advantage. Two methods for inducing autophagy and mitophagy are activation of the AMP-dependent protein kinase (AMPK) pathway and inhibition of the mechanistic target of rapamycin (mTOR) signaling pathway and so modulating the activity of theses pathways may be able to protect mitochondrial function and delay aging.

AMPK is one of the main metabolic sensors responding to increases in ADP and AMP levels in the cell as a result of ATP consumption (Steinberg and Kemp 2009; Hardie 2011).

AMPK is a conserved heterotrimer (αβγ) and overexpression of an alpha subunit has been shown to extend lifespan in *C. elegans* (Apfeld, O'Connor et al. 2004). More recent studies have shown that activation of AMPK by lipoic acid (LA), which is also a strong antioxidant, improves skeletal muscle energy metabolism possibly through increased expression of peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) to increase mitochondrial biogenesis and glucose transporter-4 (GLUT4) to increase glycolysis, while decreasing the phosphorylation of mTOR to stimulate autophagy (Wang, Li et al. 2010). Activation of AMPK by AICAR was blunted in aged rats resulting in reduced mitochondrial biogenesis (Reznick, Zong et al. 2007). This result suggests that aging-related declines in AMPK activity could be a contributing factor in the impaired mitochondrial function in aged organisms.

mTOR is a kinase that forms two distinctive complexes, mTORC1 and mTORC2. In the mTORC1complex, mTOR associates with raptor and other proteins. mTORC1 is stimulated by increased oxygen levels, amino acid levels, energy levels, and nutrient availability to promote increased metabolism and cell cycle progression (Halloran, Hussong et al. 2012; Laplante and Sabatini 2012). mTORC1 is inhibited by nutrient stress such as calorie restriction (CR), and is potently inhibited by the compound rapamycin (Laplante and Sabatini 2012), although long term rapamycin treatment also inhibits mTORC2 function (Schreiber, Ortiz et al. 2015). Decreasing mTOR activity with rapamycin has been shown to increase lifespan in mice possibly through enhanced proteostasis due to a decreased rate of protein synthesis and an increase in autophagy (Harrison, Strong et al. 2009).

CR leads to the activation of AMPK and sirtuins, which are NAD^+ dependent protein deacetylases. Together, sirtuins and AMPK inhibit the mTORC1 pathway leading to a decreased rate of protein synthesis. mTORC1 has also been reported to have a significant positive effect on

mitochondrial activity, increasing resting oxygen consumption and oxidative capacity. Rapamycin treatment to inhibit mTOR has been shown to lower mitochondrial membrane potential, oxygen consumption, and ATP synthesis in cultured cells (Schieke, Phillips et al. 2006). Short term (12-hour) inhibition of mTORC1 did not affect mitochondrial function (Morita, Gravel et al. 2013), but longer term inhibition lowered the levels of the transcriptional regulator yin-yang 1 (YY1), resulting in downregulated expression of PGC-1α, ERR-α, and other regulators of mitochondrial function (Cunningham, Rodgers et al. 2007). Consistent with this, mTOR has been shown to translocate from the cytoplasm to the outer mitochondrial membrane in response to radiation stress to inhibit hexokinase II to inhibit glycolysis and increase the reliance of the cell on oxidative metabolism (Lu, Qin et al. 2015).

mTORC2 is composed of mTOR and other proteins such as rictor. Knockdown of rictor increased mTORC1 levels and the rate of oxygen consumption, while knockdown of raptor decreased the rate of oxygen consumption (Schieke, Phillips et al. 2006). mTORC2 associates with mitochondria associated ER membranes (MAM). mTORC2 inhibition led to increased Ca^{2+} transfer between the organelles, stimulating mitochondrial metabolism (Betz, Stracka et al. 2013). Contrary to expectations, chronic rapamycin treatment led to increased mitochondrial biogenesis in adipose tissue of obese db/db mice (Deepa, Walsh et al. 2013). Therefore, the effect of rapamycin on mitochondrial function may be tissue-specific or depend upon the duration of the treatment (Fang, Westbrook et al. 2013).

TOR signaling has also been shown to alter mitochondrial function through effects on protein synthesis (Morita, Gravel et al. 2013). In dietarily restricted *Drosophila melanogaster*, there was increased expression of eukaryotic translation initiation factor 4E (eIF4E)-binding protein (d4E-BP), a target of TOR kinase that globally decreases the rate of translation, but

specifically increases the translation of genes involved in mitochondrial ETC function (Zid, Rogers et al. 2009). In mammalian cells, mTOR has been shown to upregulate translation of mitochondrial transcription factor A (TFAM) and several subunits of mitochondrial ETC complexes I and V (ATP synthase) (Morita, Gravel et al. 2013). Therefore, decreased respiration was observed following mTOR inhibition. Conversely, hyperactivation of mTORC1 by a PTEN mutation greatly stimulated respiration (Goo, Lim et al. 2012).

The degree to which mTOR or AMPK signaling is modified by different sugars or respiratory substrates remains relatively unexplored. In the studies described here, we specifically examine how altering glucose and pyruvate levels in the culture media modify the effects of AICAR and rapamycin treatment on mitochondrial function in WT and mtDNA mutator murine embryonic fibroblast (MEF) cell lines. The mtDNA mutator cell line was established as a culture model to study the effects of random mtDNA mutations, similar to those that occur with aging, on cellular energetics under different nutrient conditions, which we varied by altering the media glucose and pyruvate levels. The experiments were also designed to compare the effects of anti-aging rapamycin and AICAR treatments on cellular respiration, ROS production, and ATP levels in WT and mtDNA mutator cells.

Methods

Generation of stable cell lines

Murine embryonic fibroblasts (MEFs) were obtained from WT and homozygous $POLG^{D257A/D257A}$ mitochondrial DNA mutator (Kujoth, Hiona et al. 2005) embryos following removal of the head according to standard protocols and frozen down (Lei 2013). To generate stable cell lines, we thawed the MEFs, plated them, and then stably transfected them with a

plasmid containing the viral E1A gene and a puromycin selectable marker using a retroviral transfection system (Swift, Lorens et al. 2001). E1A-induced transformation was used instead of spontaneous transformation in attempt to preserve the function of genes in signaling pathways that commonly mutate during spontaneous transformation.

Cell culture

WT and POLG$^{D257A/D257A}$ MEFs were grown in either 96 well plates or in 10 cm culture dishes using HyClone® Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 30 µg/ml penicillin, 50 µg/ml streptomycin, and 4 mM L-glutamine. Cells were grown using either high (4.5 g/L) or low (1 g/L) glucose DMEM with or without 1 mM pyruvate for 24 hours or 48 hours. For longitudinal studies, high glucose media with 1 mM pyruvate and 50 µg/ml uridine (HGPUM) was used. Cells with mtDNA mutations require uridine and pyruvate to survive due to insufficient NADH oxidation and pyrimidine synthesis. Longitudinal (10, 20, and 29 days of treatment) studies were only performed in HGPUM since MEFs cultured in low glucose media for the measurement of oxygen consumption frequently did not survive the trypsinization step prior to placing the cells in the Clark electrode chamber. The media was changed every 2 days for cells grown for the 10, 20, and 29 day longitudinal studies. For the respiratory, ROS production, and ATP measurements cells were treated with 1 mM 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) or with 10 nM rapamycin.

ATP assays

CellTiter-Glo (Promega, Madison, WI) ATP detection reagent was used to determine the levels of ATP following a 10 minute 22°C incubation of cells with CellTiter-Glo reagent.

CellTiter-Glo reagent was combined with the cell suspension in a 1:1 ratio in a 96-well microplate and then shaken for 2 minutes. Luminescence was read using a Biotek Synergy 2 microplate reader after the contents of the 96 well plate were transferred to a white opaque bottom plate for increased signal.

Oxygen consumption analysis

Cells were trypsinized from the 10 cm dishes, the trypsin was deactivated with culture media, and the cells were spun down and resuspended in 1 mL of the appropriate fresh culture media. 350 µl of the suspension was then placed into a Strathkelvin Mitocell MT200A respiratory chamber with a Clark type electrode at 37^0C with a stir bar. The cellular respiratory rate was recorded for 4 minutes. The middle 1 minute was chosen for slope analysis. Oxygen consumption was normalized to the total cell number in the chamber. Cell counts were obtained using a hemocytometer. Cells were only grown in low glucose conditions for up to 30 hours due to the low O_2 consumption signal obtained at further timepoints.

Reactive oxygen species level measurements

2',7'-dichlorodihydrofluorescein diacetate was used to detect reactive oxygen species (ROS) production. Following hydrolysis of the acetate esters by intracellular esterases, dichlorodihydrofluorescein is produced. Dichlorodihydrofluorescein, when oxidized by ROS becomes the highly fluorescent compound dichlorofluorescein (DCF). A 2',7'-dichlorodihydrofluorescein diacetate stock solution of 500 µM was made up in 2% DMSO in PBS and the tube was wrapped in foil to prevent exposure to light. Media was aspirated from the cells grown in 96-well plates and the cells were washed once with 100 µl PBS. An assay

concentration of 50 µM was obtained by mixing 10 µl of the stock solution with 90 µl of PBS and added to the monolayer of cells. As a positive control, 0.5 µl of denatured 100% EtOH was added, as it can greatly stimulate mitochondrial ROS production at electron transport chain complexes I and III (Cunningham and Bailey, 2001). The plate was incubated in the dark at 37°C for 30 minutes. The fluorescence was then measured using a Biotek Synergy 2 microplate reader with an excitation filter of 485/20 nm and emission filter of 528/15 nm. Values were normalized to the total amount of protein in each well.

Protein assays

Cells were lysed and extracts were solubilized using modified RIPA buffer without SDS. A determination of the protein concentration was performed using the BCA protein assay (Thermo Scientific Pierce). Results were normalized using the protein concentrations present for the assays.

Colony formation drug toxicity assays

The E1A-transformed MEFs were cultured in high glucose media (HGM) consisting of high glucose DMEM with the addition of 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM). Three distinct $POLG^{D257A/D257A}$ MEF clones and three distinct WT MEF clones were used for each experiment. 2,000 cells were placed in each well of a 6-well plate in HGM. For each 6-well plate, 5 different concentrations of a drug were added one day after the cells were plated leaving one untreated well per plate. Drug concentrations were doubled or tripled from one well to the next depending upon the specific compound tested. Cells were grown for 8-10 days with one change of media. Then the

culture media was removed and the cells were washed twice with PBS. A 5% crystal violet stock solution was made in 95% ethanol. 500 mL of a 1x 0.5% crystal violet staining solution was made by combining 50 mL of the 5% crystal violet stock solution with 350 mL of methanol, 50 mL of 35% formaldehyde, and 50 mL of deionized water. The cells were fixed and stained for 10 minutes. Then the solution was removed and the wells were washed several times with deionized water until the colonies became easily visible, the wells were allowed to dry, and the colonies were manually counted with a colony counting pen. * indicates a p-value < 0.05. The IC_{50} is the stressor concentration that allows half the maximal number of colonies to grow. The IC_{50} values were calculated from the dose response data using GraphPad Prism software.

Assays for determining compounds that alter the rate of pyruvate addiction

The E1A transformed MEFs were cultured in triplicate in 6-well plates using high glucose, pyruvate and uridine-containing media (HGPUM) consisting of high glucose DMEM media with 10 % fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), pyruvate (1 mM), uridine (50 μg/mL), and a compound to be tested. Several autophagy inhibitors including bafilomycin A1, chloroquine, and ammonium chloride were toxic to the cells at the normal doses used to inhibit autophagy, so concentrations were titrated down to allow for long term viability. Every 3-4 days, cells would become confluent and so were washed in PBS and trypsinized, 90% of the cells were discarded and the remaining 10% of the cells returned to the same well for continued culture. Every 9-12 days the 90% of the cells that were normally discarded were counted using a Coulter Z1 Particle Counter and 2,000 cells from each well were seeded into a well in each of two new 6-well plates. One 6-well plate was cultured using the (HGPUM) pyruvate and uridine containing culture media without drug treatment and

the second 6-well plate was cultured in the same media lacking pyruvate and uridine (HGM). Cells were grown for 8-10 days with one change of media. Culture media was removed and the cells were washed twice with PBS, and fixed and stained with 0.5% crystal violet staining solution as described above, de-stained with deionized water, and colonies were counted manually. The percent of the number of colonies that formed in the absence of pyruvate and uridine (HGM) compared to the number of colonies that formed in the presence of pyruvate and uridine (HGPUM) was plotted each 9-12 days for 3 weeks to 3 months depending upon the rate of the loss of colony formation of the cells seeded in media lacking pyruvate and uridine (HGM).

Statistical analysis

Error bars represent the standard error of the mean. Two-way ANOVA tests were performed with genotype and treatment variables, followed by Fisher's LSD post hoc means comparison. * indicates a p value of < 0.05, ** indicates a p-value < 0.005, and *** indicates a p-value < 0.001. SigmaPlot 11.0 was used for statistical analysis and graph generation.

Results

$POLG^{D257A/D257A}$ cells are more sensitive to some but not all mitochondrial inhibitors

We first generated stable E1A-transfected WT and $POLG^{D257A/D257A}$ MEFs and characterized their ability to form colonies when grown in the presence of various concentrations of mitochondrial inhibitors, oxidants, or nucleoside analogs that could potentially affect mitochondrial function. The results showed that the cell growth and colony formation of $POLG^{D257A/D257A}$ MEFs was more susceptible than WT MEFs to the mitochondrial ETC complex I inhibitor rotenone, the ETC complex IV inhibitor azide, the uncoupler FCCP, the mitochondrial

protein synthesis inhibitor chloramphenicol, and the DNA intercalating agent ethidium, which because of its positive charge accumulates in mitochondria (two tailed t-test $p < 0.05$). No statistical difference in sensitivity was found for treatment with the ETC complex III inhibitor antimycin A, the mitochondrial F_0F_1-ATP synthase inhibitor oligomycin, or the oxidants tert-butylhydroperoxide or hydrogen peroxide (Fig. 25 A-I). There was also no statistical difference for the prevention of colony formation between the two cell lines when the ETC complex III inhibitor myxothiazol was added (data not shown). It is not entirely clear why the $POLG^{D257A/D257A}$ MEFs were more sensitive than WT MEFs to complex I and complex IV inhibitors, but not to complex III or complex V (ATP synthase) inhibitors, but it is likely due to the fewer number of genes present in mtDNA in complexes III and V. Mammalian mtDNA only encodes 1 gene for complex III and 2 genes for complex V, while mtDNA encodes 3 genes for complex IV and 7 genes for complex I. The greater the number of genes present in mtDNA, the more likely that the genes will be disrupted by mutation to sensitize the cells to the toxic effects of the ETC inhibitors. This also likely explains why the mtDNA mutator MEFs were more sensitive to the mitochondrial protein synthesis inhibitor as mitochondrial ribosomal function requires 2 large ribosomal RNAs as well as 22 small tRNAs encoded in mtDNA.

Since some nucleoside analog antivirals such as azidothymidine (AZT), dideoxycytidine (ddC), and fialuridine (FIAU) are suspected to cause mitochondrial dysfunction by incorporation into mtDNA (Johnson, Ray et al. 2001), we tested the toxicity of several nucleoside drugs on WT and $POLG^{D257A/D257A}$ MEFs. Results showed no significant differences in sensitivity for the prevention of colony formation following the administration of any of the nucleoside analogs including AZT, ddC, FIAU, cytarabine (Ara-C), or bromodeoxyuridine (BrdU) (Supplementary Fig. 31 A-E) suggesting that the murine POLG exonuclease domain does not play a major role in

protecting cells from the toxicity of any of these nucleoside analogs. This is consistent with the very slow removal of chain-terminating AZT monophosphate and ddC monophosphate from the end of a DNA strand by the human mitochondrial polymerase gamma exonuclease site (Johnson, Ray et al. 2001). Although FIAU monophosphate was shown to be a good substrate for POLG exonuclease activity (Johnson, Ray et al. 2001), murine POLG exonuclease-deficient MEFs did not show increased sensitivity to FIAU toxicity. It is known that FIAU causes mitochondrial toxicity in humans, but not in mice (Xu, Nishimura et al. 2014) and this is likely due to a nucleoside transporter targeted to human mitochondria that lacks the targeting signal in mice (Lee, Lai et al. 2006).

Long term culture of MEFs in pyruvate-containing media can induce pyruvate addiction

It is known that many mammalian cell lines without functional mtDNA can grow in a high glucose medium (HGM) with the supplementation of pyruvate and uridine to the culture (King and Attardi 1989). Pyruvate reduction to lactate oxidizes NADH to NAD to maintain the NAD needed for glycolytic flux in the absence of ETC activity. ETC function is also needed for pyrimidine synthesis, which can be bypassed by uridine supplementation. We hypothesized that the transformed $POLG^{D257A/D257A}$ MEFs could only accumulate a small amount of mtDNA mutations before cell death when cultured in normal HGM because of the cell's reliance on mitochondrial function under this condition, but a larger mitochondrial phenotype may be revealed if the MEFs were cultured in HGM with pyruvate and uridine (HGPUM) that is permissive for the loss of mtDNA. Therefore, we cultured the MEFs for two to three months in HGPUM and roughly every ten days seeded some cells into wells on plates containing HGM to

determine if colonies would form. The same number of cells was also seeded into wells on plates containing HGPUM (the same media as the long term culture media) as a positive control for plating efficiency. One E1A transformed $POLG^{D257A/D257A}$ MEF clone slowly lost the ability to form colonies in HGM over two months of culture while two others did not, giving variable results. In contrast we didn't find any of three spontaneously transformed $POLG^{D257A/D257A}$ cell lines to lose the ability to form colonies in HGM after being cultured for two months in HGPUM Data from one clone is shown in Supp. Fig. 32 A. Surprisingly, one of three E1A-transfromed WT MEFs also showed a decreased ability to form colonies when taken from HGPUM and seeded in HGM (Supp. Fig. 32 B). We also found this slow loss of colony formation to occur when uridine was removed from the media, but not when pyruvate was removed (data not shown), and so we call the phenomenon "pyruvate addiction".

To learn more about the pyruvate addiction phenotype, we performed the same types of long term culture experiments, but this time supplemented the $POLG^{D257A/D257A}$ MEF line that previously showed the pyruvate addiction phenotype with compounds known to protect mitochondria or stimulate mitochondrial biogenesis and then assayed the ability of the cells to form colonies in HGM without the added compound. As shown in Supp. Fig. 33 A, AICAR treatment delayed the pyruvate addiction, while rosiglitazone, metformin, nicotinamide, resveratrol, or a high (50 mM) pyruvate concentration did not. Treatment with a high pyruvate concentration has been shown to induce mitochondrial biogenesis (Wilson, Yang et al. 2007). Since autophagy of damaged mitochondria maintains a healthy mitochondrial population and is required for lifespan extension, we tested the effect of autophagy modulators on pyruvate addiction. In contrast to AICAR, we found that rapamycin (Supp. Fig. 33 B and 33 C) or low dose ammonium chloride addition (Supp. Fig. 34 A and 34 B) to the HGPUM media greatly

stimulated the rate of onset of the pyruvate addiction. Rapamycin treatment stimulates autophagy, while ammonium chloride treatment is known to interfere with lysosome acidification to inhibit autophagy. We also found rapamycin and ammonium chloride to greatly stimulate pyruvate addiction in WT MEFs (Supp Fig. 34 C).

Since we observed effects with the 2 autophagy modulators, we performed similar long term experiments using both mtDNA mutator and WT MEFs supplementing the media with the autophagy inhibitors 3-methyladenine, bafilomycin A1, or chloroquine (Supp Fig. 34 A-C). 3-methyladenine and bafilomycin A1 affected pyruvate addiction, but the results varied depending upon the specific cell line or clone used, while chloroquine, like AICAR, consistently delayed pyruvate addicition. Of the 2 compounds consistently shown to stimulate pyruvate addiction (rapamycin and ammonium chloride) one stimulates and one inhibits autophagy. Of the 2 compounds (chloroquine and AICAR) shown to consistently inhibit pyruvate addiction, chloroquine is an established inhibitor of autophagy, while AICAR either stimulates or inhibits autophagy depending upon the specific cell line tested (Meley, Bauvy et al. 2006; Sanchez, Csibi et al. 2012). Therefore, we conclude that the relationship between pyruvate addiction and autophagy is likely indirect. Because of the effects of rapamycin and AICAR on pyruvate addiction and because of the known longevity-inducing effects of these compounds in model organisms, we decided to thoroughly characterize their effects on mitochondrial function and cellular ATP levels in $POLG^{D257A/D257A}$ and WT MEF cell lines.

Pyruvate addition reveals large deficits in oxygen consumption in POLG$^{D257A/D257A}$ MEFs

To determine if altering the carbohydrate source in the media affected mitochondrial function we cultured the MEFs in either low glucose media in the presence of 1 mM pyruvate (LGPM) or in HGM. We also chose to culture cells for up to 29 days in HGPUM, the same conditions used to stimulate pyruvate addiction. When cultured in HGM, surprisingly, we found no difference in the respiratory rate between WT and POLG$^{D257A/D257A}$ MEFs after 24 hours and only an insignificant trend toward decreased respiration for POLG$^{D257A/D257A}$ MEFs after 48 hours. However, large deficits in oxygen consumption were revealed for POLG$^{D257A/D257A}$ MEFs when they were cultured in pyruvate-containing media. For example, POLG$^{D257A/D257A}$ MEFs showed large deficits in oxygen consumption after 24 hours (Fig. 26 C) or 30 hours (Fig. 26 D) of culture in LGPM or after 10 (Fig. 26 E), 20 (Fig. 26F), or 29 (Supp. Fig. 35) days of culture in HGPUM. Both the POLG$^{D257A/D257A}$ and WT MEFs greatly lost the ability to respire upon trypsinization when cultured in LGPM for 36 hours or longer and so time points for oxygen consumption were not obtained beyond 30 hours when the MEFs were cultured in LGPM. There was a slight increase in respiration between the 10 and 20 day time points for WT MEFs cultured in HGPUM, perhaps as a metabolic adaptation to the culture media. The POLG$^{D257A/D257A}$ MEFs did not further lose respiratory capacity over the 29 days when cultured in HGPUM suggesting that increased levels of mtDNA mutations were not accumulating to any great extent in this limited time frame.

Long term AICAR treatment decreases O_2 consumption in WT MEFs

Although AICAR treatment has been described to increase mitochondrial biogenesis in rodents (Komen and Thorburn 2014), we like others studying mitochondrial function in hepatocytes (Guigas, Taleux et al. 2007) or myocytes (Spangenburg, Jackson et al. 2013) did not find a statistically significant increase in oxygen consumption upon AICAR treatment. This was true using either WT or $POLG^{D257A/D257A}$ MEFs irrespective of glucose or pyruvate levels or the duration of treatment (Fig. 26 A-D). However, we did find that AICAR treatment of WT MEFs for 10 or 20 days in HGPUM led to a strong decrease in the respiratory rate (Fig. 26 E) that dissipated by 29 days of culture (Supp. Fig. 35). The AICAR-mediated decrease in respiratory rate observed following long term treatment could be mediated by the slow buildup of an AICAR metabolite that either inhibits mitochondrial ETC complex I (Guigas, Taleux et al. 2007) or that down-regulates mitochondrial ETC components as has been shown for AICAR treatment of certain cancer lines (Jose, Hebert-Chatelain et al. 2011).

Rapamycin transiently increases O_2 consumption in WT but not $POLG^{D257A/D257A}$ MEFs

Rapamycin treatment increased the rate of oxygen consumption in WT MEFs after 24 hours (Figure 6.2A and 6.2C), but not after 48 hours (Fig. 26 B and 26 D) of treatment in HGM or LGPM or after 10 (Fig. 26 E), 20, (Fig. 26 F) or 29 (Supp. Fig. 35) days of treatment in HGPUM. Rapamycin did not significantly increase oxygen consumption under any of these conditions in $POLG^{D257A/D257A}$ MEFs. The mtDNA mutations in the $POLG^{D257A/D257A}$ MEFs likely cause impaired mitochondrial biogenesis preventing the transient increase in respiration observed in WT MEFs.

In POLG$^{D257A/D257A}$ MEFs ROS production increases in HGM, but decreases following long term culture in HGPUM

We next determined the rate of ROS production of POLG$^{D257A/D257A}$ and WT MEFs. In LGM and LGPM depending upon the duration of treatment and the presence of pyruvate, there were trends for or very small increases in ROS production in POLG$^{D257A/D257A}$ MEFs compared to WT MEFs (Figure 28 A-D). However, upon culture in HGM, large increases in ROS production were observed in the POLG$^{D257A/D257A}$ MEFs that declined substantially when cultured in HGPM, where pyruvate is present. Pyruvate is known to have direct oxidant scavenging properties (Kladna, Marchlewicz et al. 2015) and can also prevent decreased anti-oxidant gene expression induced by the presence of high sugar levels (Varma and Chandrasekaran 2015). In contrast to these results from short term culture studies, culture for 10 days in HGPUM led to decreased ROS production in POLG$^{D257A/D257A}$ MEFs compared to WT MEFs (Fig. 27E and Fig. 27F) likely due to the lower ETC activity in POLG$^{D257A/D257A}$ MEFs compared to WT MEFs at this time point as indicated by the respiratory studies. Decreased ETC activity can decrease ROS production when fewer electrons enter and are passed down the ETC leading to fewer electrons bound by O_2 to form superoxide.

AICAR treatment for 48 hours increases, but for 10 days decreases ROS production

After 24 hours of AICAR treatment there was little to no effect on ROS production depending upon the exact media composition (Fig. 27 A and 27C and Fig. 28 A and 28 C). AICAR treatment caused a slight decrease in ROS production in WT MEFs in low glucose media (LGM) (Fig. 27 A), while a slight increase in ROS production occurred in

POLG$^{D257A/D257A}$ MEFs in HGM (Fig. 28 A). After 48 hours of treatment, AICAR almost invariably increased ROS production in both POLG$^{D257A/D257A}$ and WT MEFs (Fig. 27 B and 27 D and Fig. 28 B and 28 D) irrespective of glucose and pyruvate levels. The AICAR-induced increase in ROS production likely either reflects the initial buildup of an AICAR metabolite that inhibits mitochondrial ETC complex I (Guigas, Taleux et al. 2007) or a change in the expression of ETC genes (Jose, Hebert-Chatelain et al. 2011). However, after 10 or 20 days of AICAR treatment in HGPUM (Fig. 28 E and Fig. 28 F), ROS levels were decreased, likely reflecting the low ETC activity induced by AICAR limiting the passage of electrons and generation of superoxide under these conditions.

Rapamycin treatment does not affect the rate of ROS production in WT or POLG$^{D257A/D257A}$ MEFs

Under almost every culture condition tested, we found that rapamycin treatment had no statistically significant effect on ROS production (Fig. 27 A-D and Fig. 28 A-F). The only exception to this was a very small decrease in ROS production in POLG$^{D257A/D257A}$ MEFs after 24 hours of treatment in HGM (Fig. 28A).

POLG$^{D257A/D257A}$ MEFs frequently show decreased ATP levels in low glucose conditions, but increased ATP levels in high glucose conditions

We next monitored ATP levels in the POLG$^{D257A/D257A}$ and WT MEFs. As expected in LGM and LGPM (Fig. 29A-D), POLG$^{D257A/D257A}$ MEFs most frequently showed decreased ATP levels, except at the 24 hour time point in LGPM, where ATP levels were increased (Fig. 29 C). But surprisingly, ATP levels were increased when POLG$^{D257A/D257A}$ MEFs were cultured in HGM, HGPM, or HGPUM (Fig. 30 A-E) with the exception of the 24 hour timepoint in HGM (Fig. 30

A) and the 20 day timepoint in HGPUM (Fig. 30 F) where ATP levels were nearly the same as those in WT MEFs. Therefore, there is a compensatory upregulation of glycolysis in the $POLG^{D257A/D257A}$ MEFs that leads to increased ATP levels in the presence of high glucose. To verify this, we measured the rate of acidification of fresh media on confluent cultures grown in HGM and found that $POLG^{D257A/D257A}$ MEFs acidified the medium more rapidly, having a medium pH of 6.92 +/- 0.10 after 48 hours and a pH of 6.05 +/-0.02 after 72 hours, while WT MEFs had a medium pH of 7.24 +/- 0.04 after 48 hours and a pH of 6.84 +/- 0.05 after 72 hours. Similar findings of enhanced glycolysis were reported for $POLG^{D257A/D257A}$ primary MEFs (Kukat, Edgar et al. 2011), dermal fibroblasts (Saleem, Safdar et al. 2015), and induced pluripotent stem cells following differentiation (Wahlestedt, Ameur et al. 2014). But induced pluripotent stem cells from $POLG^{D257A/D257A}$ mice in their non-differentiated state were shown to maintain a normal glycolytic rate (Wahlestedt, Ameur et al. 2014).

AICAR treatment decreases ATP levels under high glucose conditions, except for in WT MEFs cultured for long term in HGPUM

We next treated the WT and $POLG^{D257A/D257A}$ MEFs with AICAR and measured cellular ATP levels. Under most conditions, especially when using WT MEFs in LGM and LGPM, AICAR treatment decreased ATP levels, but there were exceptions due to the use of the 2 different cell genotypes and 2 different durations of treatment (Fig. 29 A-D). For example, a 24 hour AICAR treatment of WT MEFs in LGPM slightly increased ATP levels (Fig. 29 C), while a 48 hour treatment of $POLG^{D257A/D257A}$ MEFs slightly increased ATP levels in LGM (Fig. 29 B) and greatly increased ATP levels in LGPM (Fig. 29 D). The results of AICAR treatment in HGM and HGPM were very consistent. Under all conditions AICAR treatment decreased ATP

levels (Fig. 30 A-D). This was also true for the 10 and 20 day treatments of $POLG^{D257A/D257A}$ MEFs in HGPUM (Fig. 30 E and Fig. 30F). But in contrast to this, WT MEFs cultured for 10 days in HGPUM showed increased ATP levels (Fig. 30 E), but this increase dissipated by 20 days of treatment (Fig. 30 F).

Rapamycin greatly increases ATP levels following 48 hours of treatment in low glucose media

We next determined the effects of rapamycin on cellular ATP levels in the MEFs. When cultured in LGM and LGPM, rapamycin treatment for 24 hours (Fig. 29 A and C) generally had no statistically significant effect on ATP levels, except for an increase in $POLG^{D257A/D257A}$ MEFs cultured in LGM. In contrast, when MEFs were cultured for 48 hours in LGM or LGPM rapamycin treatment led to a consistent increase in ATP levels (Fig. 29 B and 29D). In HGM and HGPM (Fig. 30 A-D), the cells showed a quicker, but greatly blunted response to the rapamycin treatment, especially the $POLG^{D257A/D257A}$ MEFs, which only showed increased ATP levels at the 24 hour timepoint in HGM. The WT MEFs showed small to moderate increases in ATP levels with rapamycin treatment under all high glucose culture conditions including 10 days in HGPUM, where mtDNA mutator MEFs only showed a small increase in ATP levels (Fig. 30 E). In both cell types the increased ATP levels dissipated by 20 days of treatment (Fig. 30 F). It was surprising that rapamycin treatment did not lead to consistently larger increases in ATP levels as mTOR inhibition both stimulates autophagy to increase respiratory substrate levels and slows the rate of translation, a very energy intensive process (Morita, Gravel et al. 2015).

Discussion

We found that E1A-transformed MEFs from POLG$^{D257A/D257A}$ mice were more sensitive than WT MEFs to growth inhibition and toxicity caused by a mitochondrial uncoupler or an mtDNA intercalating agent, or to inhibition of mitochondrial ETC complexes I or IV, or to inhibition of mitochondrial protein synthesis. The POLG$^{D257A/D257A}$ MEFs possessed oxygen consumption deficits and increased ROS production that were accentuated in LGPM compared to HGM. We also showed that rapamycin treatment transiently increased the rate of oxygen consumption. Interestingly, isolated mitochondria from heart or liver of aged mtDNA mutator mice (Kujoth, Hiona et al. 2005) or primary MEFs from these mice (Kukat, Edgar et al. 2011) showed oxygen consumption deficits without increased ROS production, whereas the transformed MEFs showed increased ROS production. This highlights the utility of this cell model where an important phenotype was revealed that was not present when initially analyzing primary tissues. Recently however, increased ROS production has been found in Leydig cells from mtDNA mutator mice (Shabalina, Landreh et al. 2015) and increased oxidative damage has been found in muscle from these animals (Kolesar, Safdar et al. 2014). In addition the antioxidant N-acetyl-L-cysteine was able to rescue deficits in neuronal and hematopoetic stem cell differentiation (Ahlqvist, Hamalainen et al. 2012) and mitochondrial-targeted catalase was able to restore age-dependent cardiomyopathy (Dai, Chen et al. 2010). So therefore increased ROS production and oxidative damage likely contribute to the premature aging phenotype in certain tissues of these mice.

It is somewhat surprising that AICAR and rapamycin gave different respiratory responses as AMPK activation mediates many of its effects through inhibition of the mTOR pathway (Bolster, Crozier et al. 2002). AMPK mediates this inhibition in part through phosphorylation of

the mTORC1-specific protein raptor (Gwinn, Shackelford et al. 2008). AMPK activation (Faubert, Boily et al. 2013) or mTOR inhibition (Kittipongdaja, Wu et al. 2015) have both been shown to inhibit glycolytic flux under normal physiological conditions, which may limit toxic methylglyoxyl formation (Allaman, Belanger et al. 2015), as well as limit pyruvate production important for fueling the citric acid cycle and oxidative phosphorylation. But AICAR treatment has many non-specific effects on cells including inhibition of mitochondrial ETC complex I that may have prevented AICAR treatment from transiently increasing respiration. The transient increase in respiration following rapamycin treatment was surprising given other authors studying myotubes (Cunningham, Rodgers et al. 2007; Ye, Varamini et al. 2012), leukemic Jurkat T cells (Schieke, Phillips et al. 2006; Ramanathan and Schreiber 2009), or T-cell lymphoma (Kittipongdaja, Wu et al. 2015) did not find mTOR inhibition to stimulate respiration and in most cases found decreased respiration. However, when studying adipose tissue it was found that mTOR inhibition is associated with increased mitochondrial biogenesis (Deepa, Walsh et al. 2013) or enhanced respiration (Polak, Cybulski et al. 2008). Importantly, the Bartke group found that the effects of rapamycin on oxygen consumption in mice depend upon the duration of rapamycin treatment. Mice treated for 2 weeks showed decreased oxygen consumption, while those treated for 20 weeks showed increased oxygen consumption (Fang, Westbrook et al. 2013). The mechanisms behind these findings are currently unclear. But if rapamycin treatment gradually mimics the longevity-promoting effects of calorie restriction, then mitochondrial transcripts may slowly become preferentially translated by the ribosome through increased expression or activity of 4E-BPs (Zid, Rogers et al. 2009) to increase mitochondrial biogenesis over time. The kinetics of these events are likely much different in cell culture and a rapamycin-induced preferential translation of mitochondrial-targeted transcripts could be

responsible for the transient increase in oxygen consumption of WT MEFs. Oxygen consumption likely returned to control rates due to the slower kinetics of rapamycin-induced decreases in transcription of YY1 and PGC-1α, leading to decreased mitochondrial biogenesis as has been previously described following mTORC1 inhibition in myotubes (Cunningham, Rodgers et al. 2007).

Transformed POLG$^{D257A/D257A}$ MEFs are a convenient model to study therapies for aging-induced mitochondrial dysfunction

POLG$^{D257A/D257A}$ MEFs did not show significant mitochondrial respiratory dysfunction in standard HGM conditions. The presence of pyruvate in either high or low glucose conditions revealed the respiratory dysfunction. We did not perform respiratory experiments in LGM without pyruvate. But we believe it likely that this condition would also reveal the respiratory dysfunction. Low glucose conditions are known to shift cultured cells from a primarily glycolytic metabolic state to a more oxidative state, stimulating mitochondrial biogenesis to maintain adequate levels of cellular ATP (Elkalaf, Andel et al. 2013). Moderate mitochondrial dysfunction was found in postmitotic tissues from the POLG$^{D257A/D257A}$ mice, but cell and tissue dysfunction in mitotic cells and tissues such as blood cells and intestine appeared more severe leading to mortality of the mice (Kujoth, Hiona et al. 2005). To model the large degree of mitochondrial dysfunction *in vivo*, we find it critical to culture the POLG$^{D257A/D257A}$ MEFs in LGPM or at least in HGPUM, but not in HGM. When the MEFs are grown in LGPM, they are well suited for use in high throughput screens attempting to identify compounds that improve mitochondrial function. Others have identified the requirement to culture mitochondrial ETC complex I-deficient patient fibroblasts in low glucose or galactose-containing media when

screening for drugs that may increase mitochondrial biogenesis (Golubitzky, Dan et al. 2011). Out of ten candidate compounds in their small screen, they identified AICAR as the most promising compound to increase mitochondrial function in these cells, even though AICAR did not increase the respiratory rate.

Mitochondrial inhibitor sensitivity of WT and POLG$^{D257A/D257A}$ MEFs

POLG$^{D257A/D257A}$ MEFs were more susceptible to the mitochondrial toxins FCCP, ethidium, chloramphenicol, rotenone, and azide, but not to the mitochondrial inhibitors antimycin A or oligomycin or to the oxidizing agents hydrogen peroxide or tert-butyl hydroperoxide (Fig. 25). Greater susceptibility of POLG$^{D257A/D257A}$ MEFs to rotenone and azide suggests ETC complex I and complex IV deficits in the MEFs. This leads to either decreased respiration when the mtDNA mutator MEFs are cultured in LGPM or to lower complex I and complex V reserve capacity, such as what likely occurs when the mtDNA mutator MEFs are grown in HGM.

Effects of AICAR on oxygen consumption rates

An increase in oxygen consumption was not observed in response to AICAR treatment of the MEFs. Our results are consistent with many other groups that failed to find an increase in oxygen consumption following AICAR treatment (Guigas, Taleux et al. 2007; Golubitzky, Dan et al. 2011; Spangenburg, Jackson et al. 2013), even though AICAR treatment was shown to induce PGC-1α expression and mitochondrial biogenesis in rodent muscle (Suwa, Nakano et al. 2015). After 10 days of AICAR treatment in HGPUM, there was decreased respiration of the WT MEFs and after 20 days there was decreased respiration in the POLG$^{D257A/D257A}$ MEFS, but

neither of these declines was observed after 29 days of treatment. Therefore it appears that AICAR treatment causes transient inhibition of mitochondrial respiration. AICAR has many non-specific effects on cells due to the many enzymes that respond to altered AMP levels, but AICAR as well as the highly specific AMPK inhibitor A-769662 have both been shown to inhibit oxygen consumption in cell culture, with A-769662 specifically inhibiting respiration in SV40-transformed MEFS (Vincent, Coelho et al. 2014). The transient inhibition of respiration could be due to changes in AICAR levels in the media due to cellular metabolism of AICAR.

Effects of AICAR on ATP levels

Pharmacological stimulation of AMPK activity has been shown to either increase or decrease the rate of glycolysis depending upon the specific culture conditions (Dagher, Ruderman et al. 1999; Marsin, Bouzin et al. 2002). Under conditions of oxidative stress AMPK activation has been shown to increase glycolysis through phosphorylation and activation of the glycolytic enzyme phosphofructokinase 2 (PFK2) (Wu and Wei 2012). During mitochondrial inhibition AMPK activation has been shown to upregulate the glucose transporters GLUT1 or GLUT4 (Chen, Ye et al. 2010; Pinheiro, Silveira et al. 2010). AMPK has also been shown to phosphorylate and activate the glycolytic enzyme hexokinase (Abnous and Storey 2008). We hypothesize that activation of glycolysis by AMPK only occurs during very low energy levels (and perhaps also during high ROS conditions) as we found AICAR, with the one exception noted above, to only increase ATP levels when MEFs were cultured in LGM or LGPM, and most consistently following a 48 hour AICAR treatment of $POLG^{D257A/D257A}$ MEFs, a condition where glucose would nearly be depleted from the media.

More commonly, however, such as during well-fed, high nutrient conditions it has been shown that specific activation of AMPK decreases glycolysis and lactate production (Martinez-Martin, Blas-Garcia et al. 2012; Faubert, Boily et al. 2013). But strikingly, AICAR treatment has been shown to decrease glycolysis in AMPK-deficient MEFS, suggesting that it functions through an independent, unidentified mechanism (Vincent, Coelho et al. 2014). The ability of AICAR treatment and specific AMPK activation to inhibit or downregulate electron transport chain activity and to inhibit glycolysis likely explains the decreased ATP levels measured when using a high glucose culture medium following 24 or 48 hour treatments. Under high glucose conditions, AICAR only increased ATP levels in WT MEFs after a 10 day incubation in HGPUM. The decreases in ATP levels at the 48 hour time points, before the strong downregulation of oxygen consumption observed at 10 days, may be caused in part by AICAR-mediated upregulation of pyruvate dehydrogenase kinase 4 (PDK-4) gene expression (Houten, Chegary et al. 2009), which leads to inhibition of pyruvate conversion to acetyl-CoA. The overall effect of this is a metabolic switch away from glucose utilization to oxidation of fatty acids and amino acids for energy production that likely led to the decreased ATP levels measured as these other respiratory substrates are present at lower concentrations in culture media. But it is difficult to explain why long term 10 and 20 day AICAR treatment in HGPUM did not lead to decreased ATP levels in WT MEFs since respiratory rates declined. It is possible that long term AICAR treatment to WT MEFs led to a buildup of cells in the culture population that evolved to divide slightly more rapidly than the rest due to resisting the AICAR-mediated decrease in glycolysis. This could explain why the WT MEFs did not show decreased ATP levels, even though respiratory rates greatly declined.

Pyruvate addiction in E1A-transformed MEFs

While culturing the E1A-transformed MEFs long term in HGPUM and then re-seeding them at equal concentrations in HGM and HGPUM, we found that roughly one-third of the WT and POLG cell lines isolated gradually became addicted to pyruvate and eventually could no longer form colonies without it added to the media. In addition it was found that compounds that modulate the rate of autophagy altered the rate at which the cells became addicted to pyruvate, with rapamycin and ammonium chloride greatly facilitating the rate and AICAR and chloroquine decreasing the rate. The molecular mechanisms behind this phenomenon remain uncharacterized, but one possibility includes that when the E1A-transformed MEFs are grown long term in the presence of pyruvate they downregulate mechanisms for oxidizing NADH to NAD, so they cannot divide due to reductive stress. Adding pyruvate back to the media then allows for a re-oxidation of NADH through the action of lactate dehydrogenase enzymes. Alternatively, when grown for long periods of time in the presence of pyruvate, the cells could downregulate their antioxidant defense mechanisms since pyruvate is a strong antioxidant. Then once the cells are grown without pyruvate, they could die from oxidative damage, being unable to upregulate their antioxidant defense mechanisms in time. Future studies could test these hypotheses.

Conclusions

Mitochondrial dysfunction may be a major contributing factor for aging and age-related diseases underscoring the need for cell models of mitochondrial dysfunction to conveniently study this phenomenon. E1A-transformed $POLG^{D257A/D257A}$ MEFs are a model of mitochondrial dysfunction that shows enhanced sensitivity to mitochondrial ETC complex I and IV inhibitors, a

mitochondrial uncoupler, a mitochondrial protein synthesis inhibitor, and a DNA intercalating agent that accumulates in mitochondria. But $POLG^{D257A/D257A}$ MEFs did not show enhanced sensitivity to mitochondrial complex III or complex V (ATP synthase) inhibitors, peroxides, or nucleoside analogs. $POLG^{D257A/D257A}$ MEFs showed decreased respiratory rates and ATP levels and enhanced ROS production when grown under low glucose conditions. Rapamycin treatment of WT MEFS resulted in a transient increase in respiration that did not occur following treatment of $POLG^{D257A/D257A}$ MEFs. AICAR treatment affected $POLG^{D257A/D257A}$ and WT MEFs similarly. For example, under high glucose conditions AICAR treatment decreased ATP levels and increased ROS levels, which upon a longer duration of treatment transitioned into decreased respiratory rates. Under low glucose conditions short term AICAR treatment increased ATP levels when they were low and decreased ATP levels when they were higher suggesting that AMPK activation normalizes cellular ATP levels around a set threshold level. As the respiratory rate was not altered significantly under these conditions, the rate of glycolysis was likely adjusted by AICAR to mediate this effect.

Acknowledgments

We would like to acknowledge Dr. Greg Kujoth for generating the mice and Dr. Radhika Punttagunta for isolating the primary MEFs. We would like to thank Neil Copes and Clare Edwards for helpful discussion. This study was supported by University of South Florida startup funds awarded to Dr. Patrick Bradshaw.

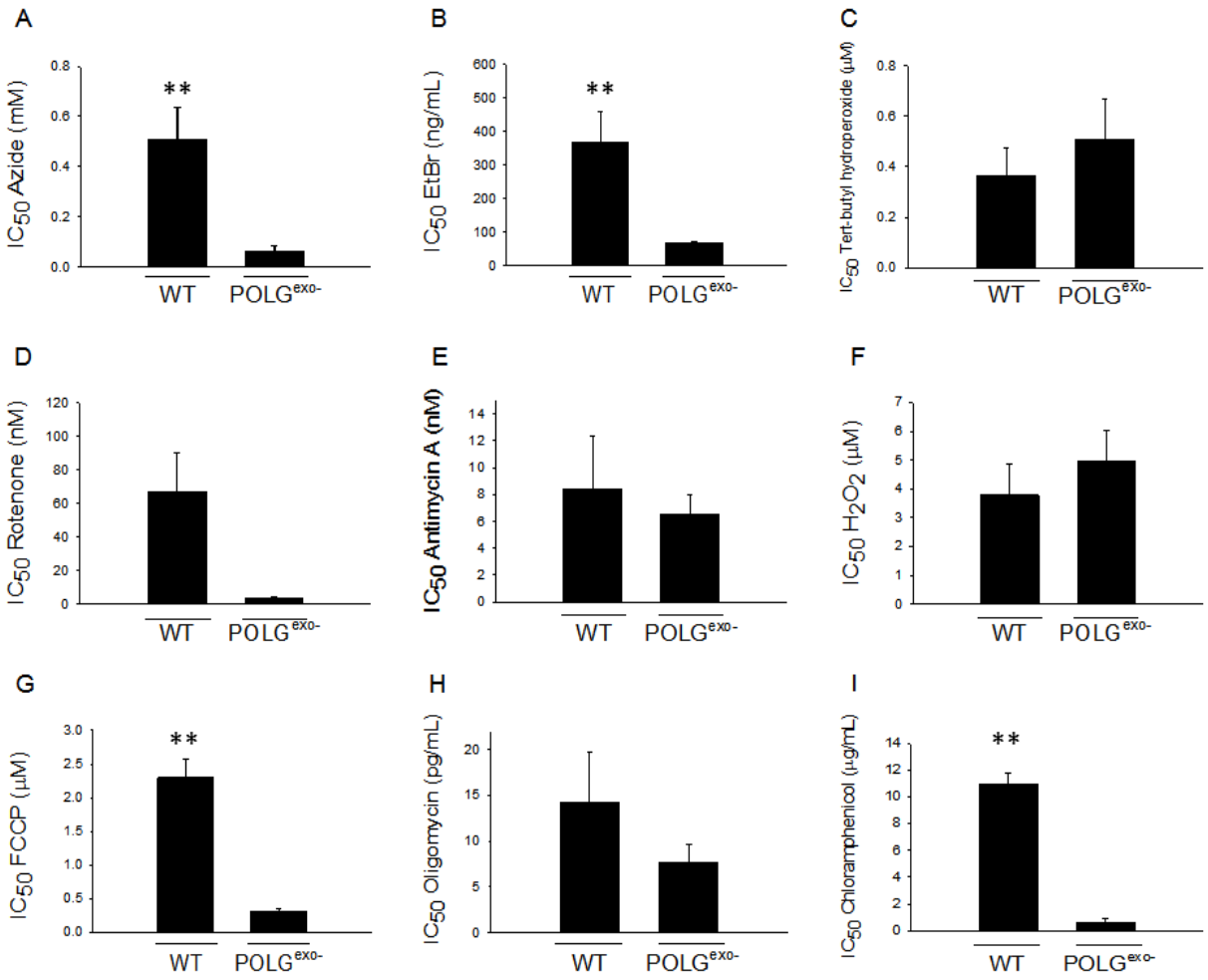


Figure 26 IC_{50} values of WT and POLG exonuclease-deficienct MEFs
$POLG^{D257A/D257A}$ MEFS are more sensitive than WT MEFs to growth inhibition by FCCP, azide, chloramphenicol, and ethidium bromide. Error bars represent the standard error of the mean * $p<0.05$, ** $p<0.001$.

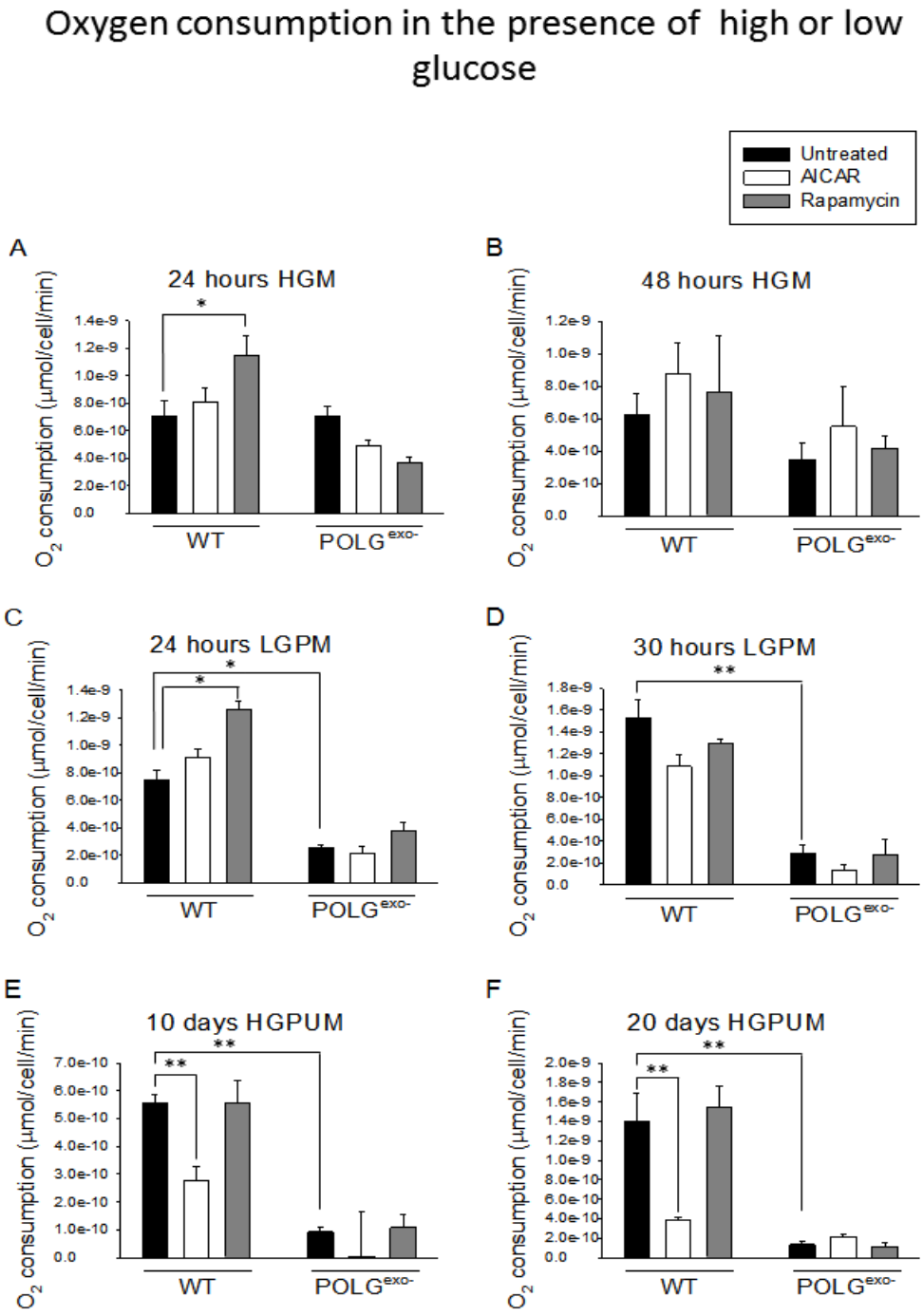


Figure 27 Oxygen consumption in high or low glucose

O_2 consumption rates of WT and $POLG^{D257A/D257A}$ MEFs. Rapamycin transiently increases oxygen consumption in WT MEFs at the 24 hour timepoint either in the absence or presence of pyruvate. Error bars represent the standard error of the mean * $p<0.05$, ** $p<0.001$.

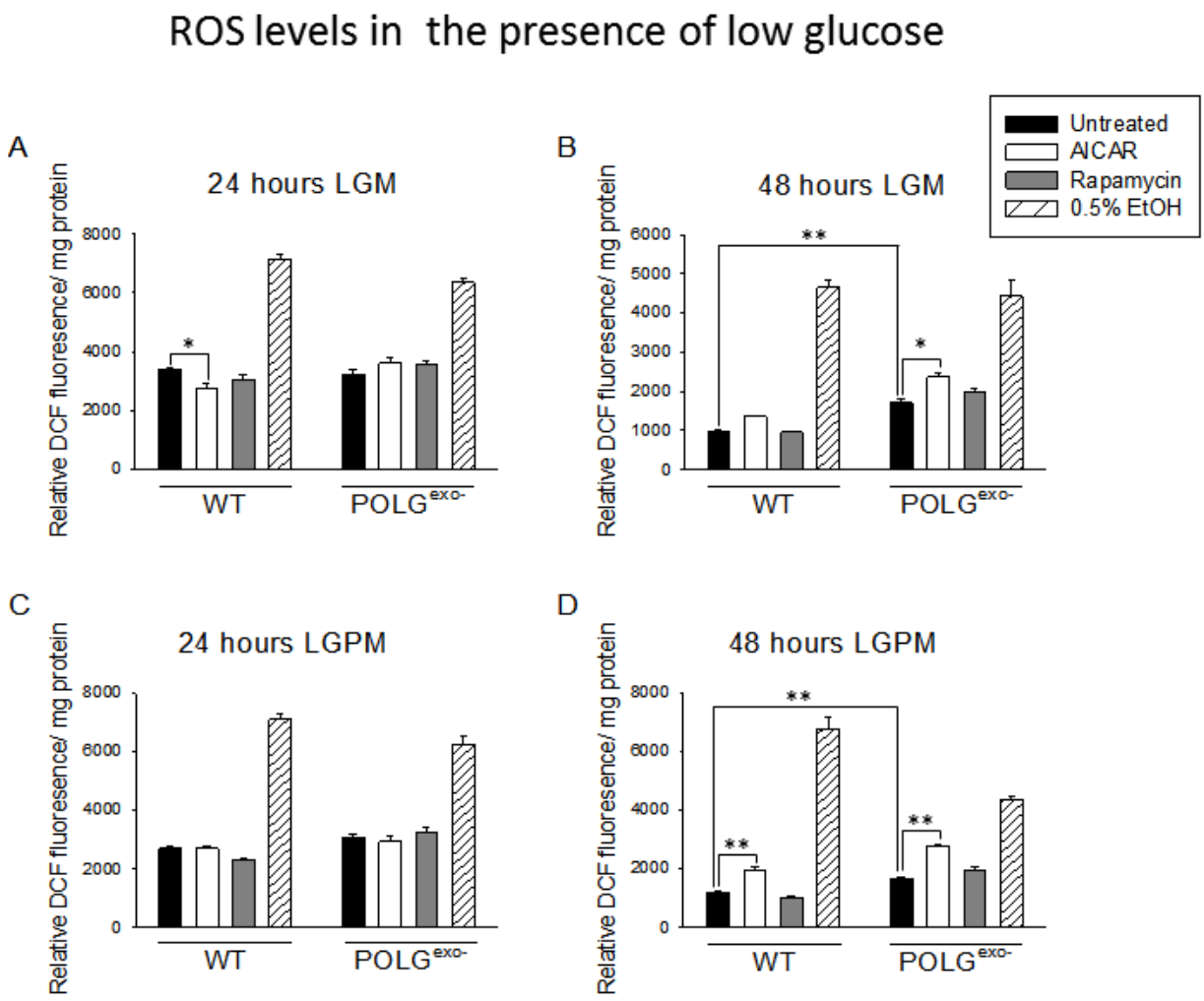


Figure 28 ROS levels in low glucose

ROS production of WT and POLG$^{D257A/D257A}$ MEFs was altered by AICAR under low glucose conditions. Rapamycin treatment in LGM or LGPM had no effect on ROS production. Error bars represent the standard error of the mean * $p<0.05$, ** $p<0.001$. Lines indicate two groups being compared. (A) ROS levels after 24 hours in LGM. (B) ROS levels after 48 hours in LGM. (C) ROS levels after 24 hours in LGPM. (D) ROS levels after 48 hours in LGPM.

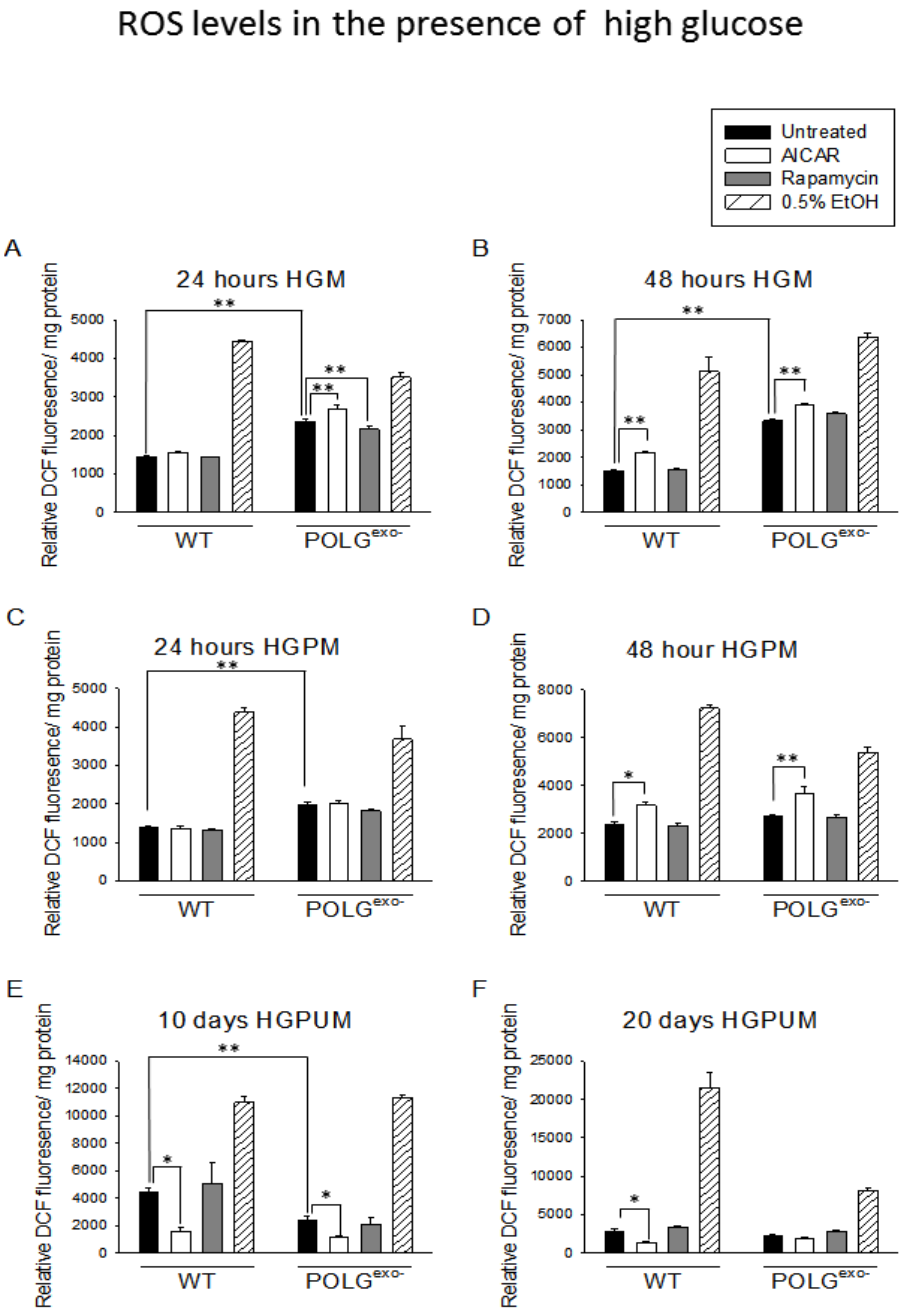


Figure 29 ROS levels in high glucose

ROS production of WT and POLG$^{D257A/D257A}$ MEFs was altered by AICAR or rapamycin treatment under high glucose conditions. Error bars represent the standard error of the mean * $p<0.05$, ** $p<0.001$. Lines indicate two groups being compared. (A) ROS levels after 24 hours in HGM. (B) ROS levels after 48 hours in HGM. (C) ROS levels after 24 hours in HGPM. (D) ROS levels after 48 hours in HGPM. (E) ROS levels after 10 days in HGPUM. (F) ROS levels after 20 days in HGPUM.

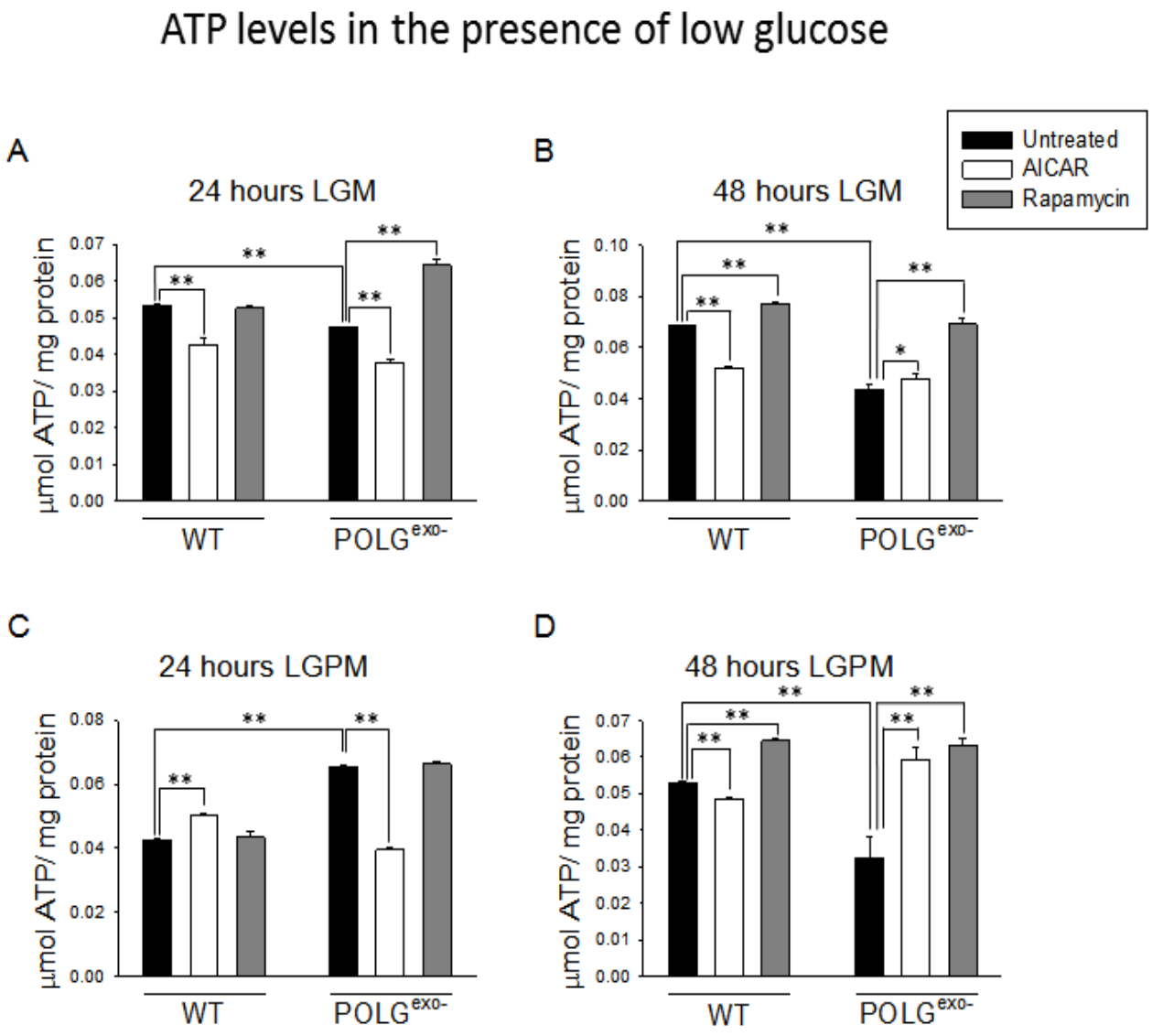


Figure 30 ATP levels in low glucose
ATP levels of WT and POLG$^{D257A/D257A}$ MEFs were altered by AICAR or rapamycin treatment under low glucose conditions. Error bars represent the standard error of the mean * $p<0.05$, ** $p<0.001$. Lines indicate two groups being compared. (A) ATP levels after 24 hours in LGM. (B) ATP levels after 48 hours in LGM. (C) ATP levels after 24 hours in LGPM. (D) ATP levels after 48 hours in LGPM.

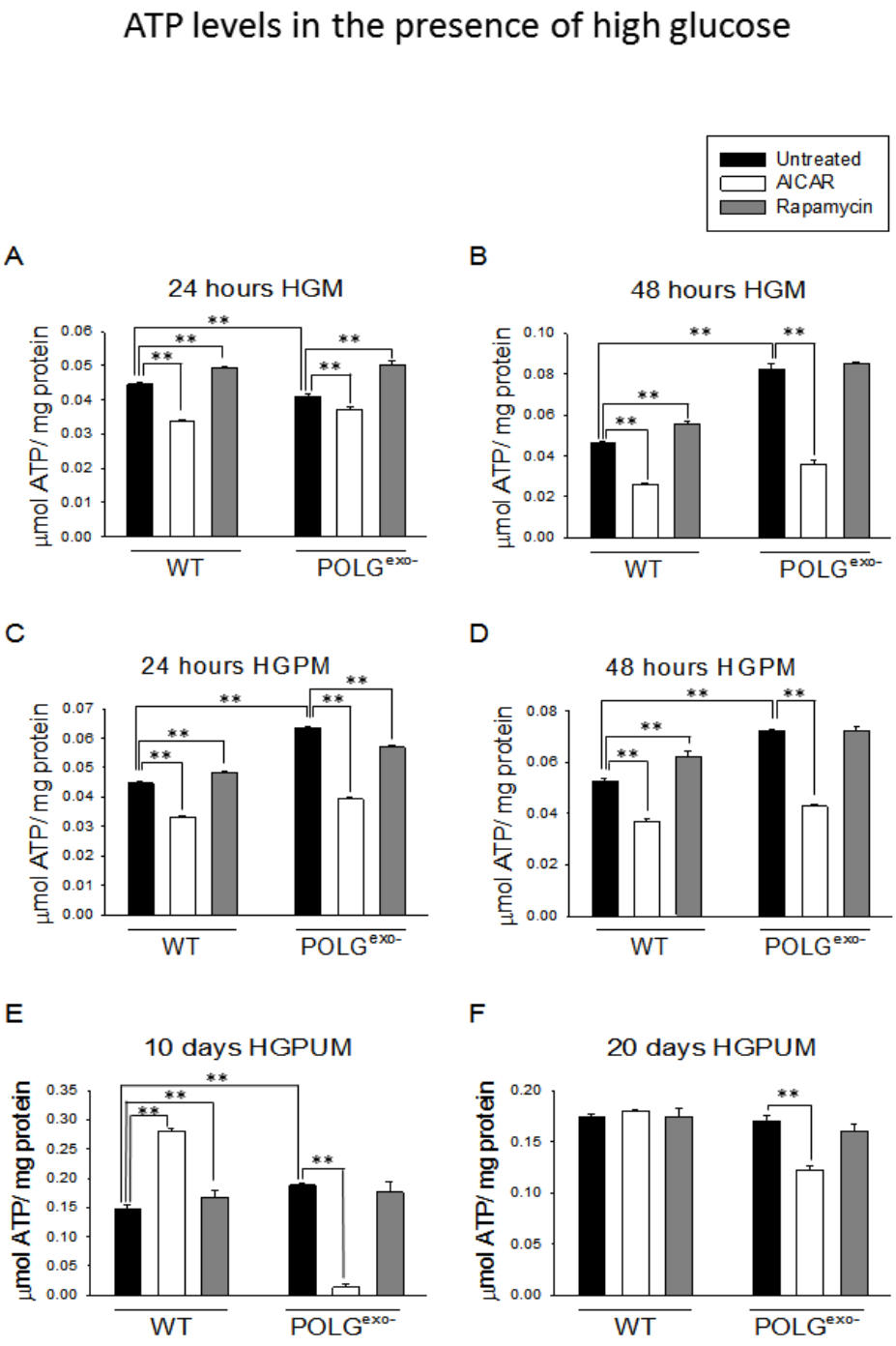


Figure 31 ATP levels in high glucose
ATP levels of WT and POLG$^{D257A/D257A}$ MEFs were altered by AICAR or rapamycin treatment under high glucose conditions. Error bars represent the standard error of the mean * $p<0.05$, ** $p<0.001$. (A) ATP levels after 24 hours in HGM. (B) ATP levels after 48 hours in HGM. (C) ATP levels after 24 hours in HGPM. (D) ATP levels after 48 hours in HGPM. (E) ATP levels after 10 days in HGPUM. (F) ATP levels after 20 days in HGPUM.

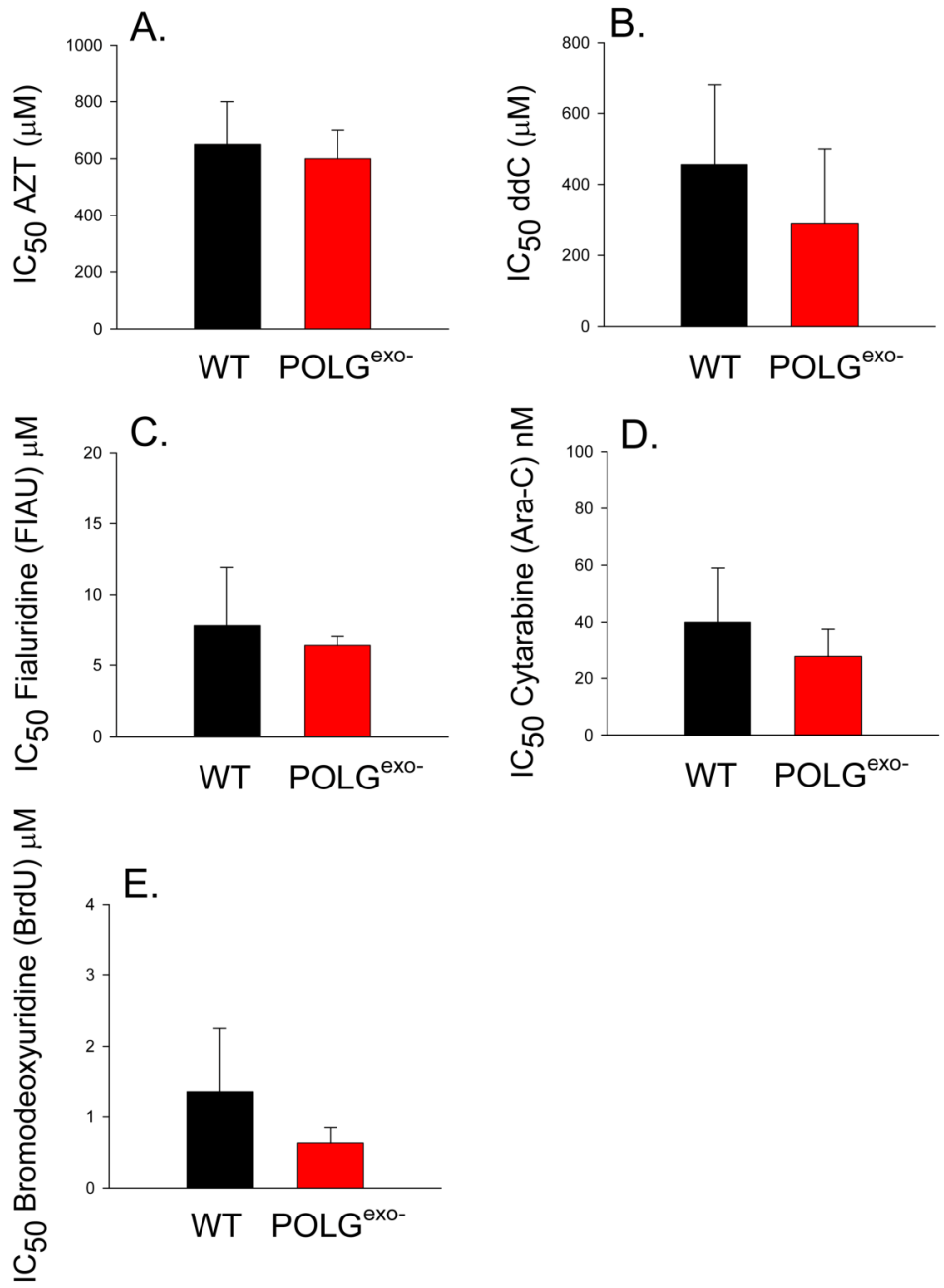


Figure 32 supplemental IC_{50} values when given nucleoside analogs
$POLG^{D257A/D257A}$ MEFs do not show increased sensitivity to the toxicity of nucleoside analogs. A colony counting assay was performed to determine IC_{50} values for the nucleoside analogs. The nucleoside anlogs tested were (A) AZT, (B) ddC, (C) FIAU, (D) Ara-C, and (E) BrdU. Error bars represent the standard error of the mean * $p<0.05$.

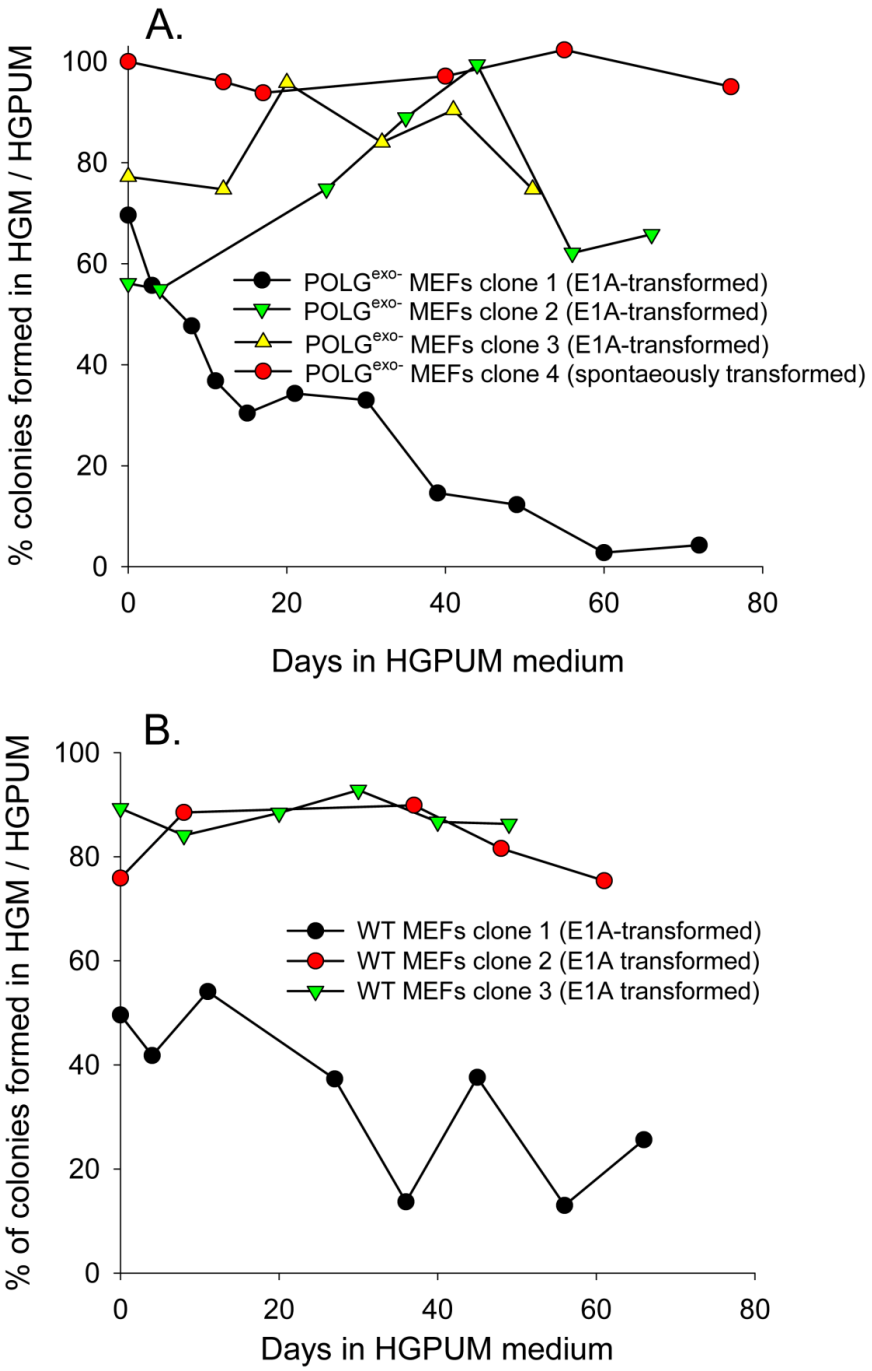


Figure 33 % colony formation
Select E1A-transformed MEF clones become addicted to pyruvate when cultured long term in HGPUM. (A) POLG$^{D257A/D257A}$ MEFs (B) WT MEFs. Roughly every 10 days equal numbers of cells were seeded in both HGM and HGPUM media and the percentage of cells that form colonies in HGM compared to those that form colonies in HGPUM was plotted.

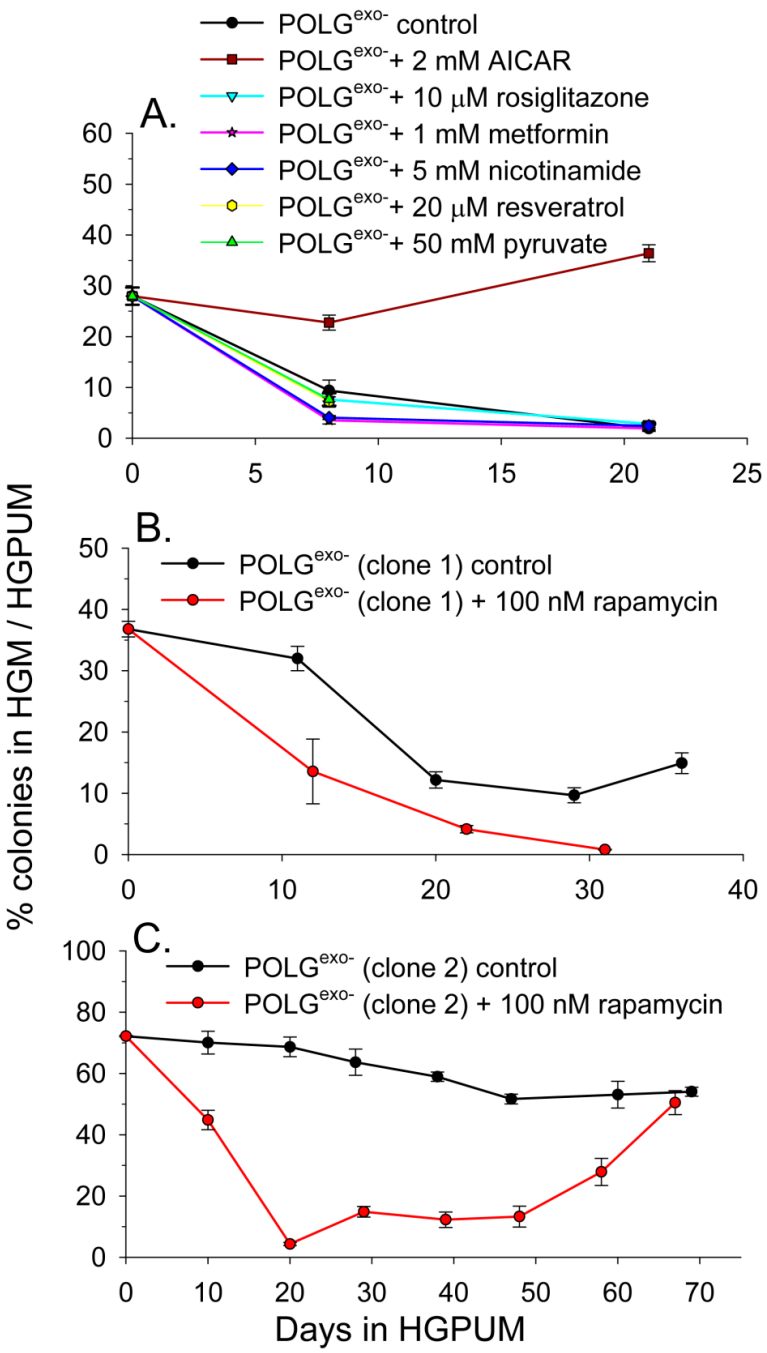


Figure 34 supplemental % colony formation in HGM/HGPUM
AICAR treatment delays pyruvate addiction, while rapamycin treatment stimulates it. Colony counting assays were performed following long term growth of the POLG$^{D257A/D257A}$ MEFs in HGPUM in the presence of (A) AICAR, rosiglitazone, metformin, nicotinamide, resveratrol, or pyruvate at the concentration indicated or in the presence of (B) 100 nM rapamycin (POLG$^{D257A/D257A}$ clone 1) or (C) 100 nM rapamycin (POLG$^{D257A/D257A}$ clone 2).

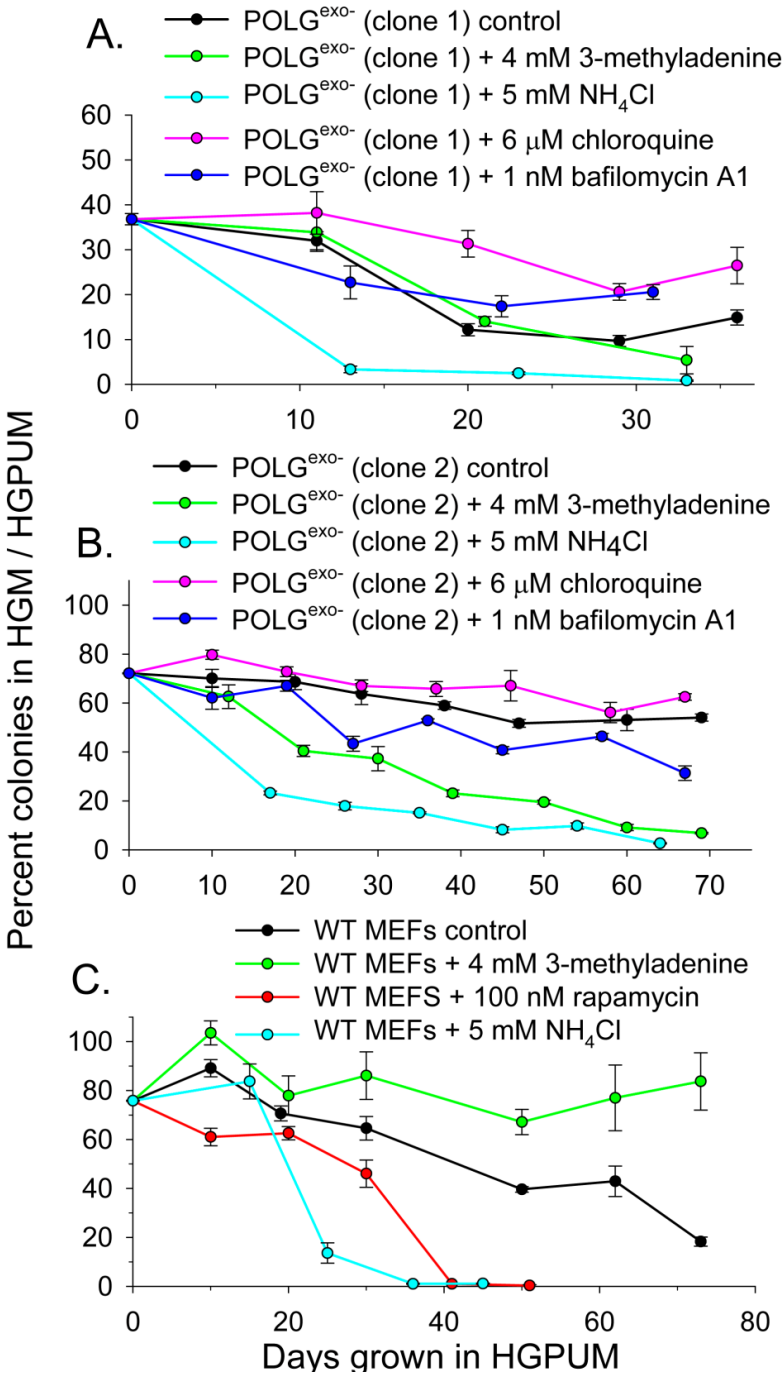


Figure 35 supplemental % colony formation in HGM/HGPUM
Ammonium chloride treatment stimulates pyruvate addiction while chloroquine treatment delays it. Colony counting assays were performed following long term growth in HGPUM of either (A) $POLG^{D257A/D257A}$ MEFs (clone 1) or (B) $POLG^{D257A/D257A}$ MEFs (clone 2) in the presence of 3-methyladenine, ammonium chloride, chloroquine, or bafilomycin A1 or (C) WT MEFs in the presence of 3-methyladenine, rapamycin, or ammonium chloride.

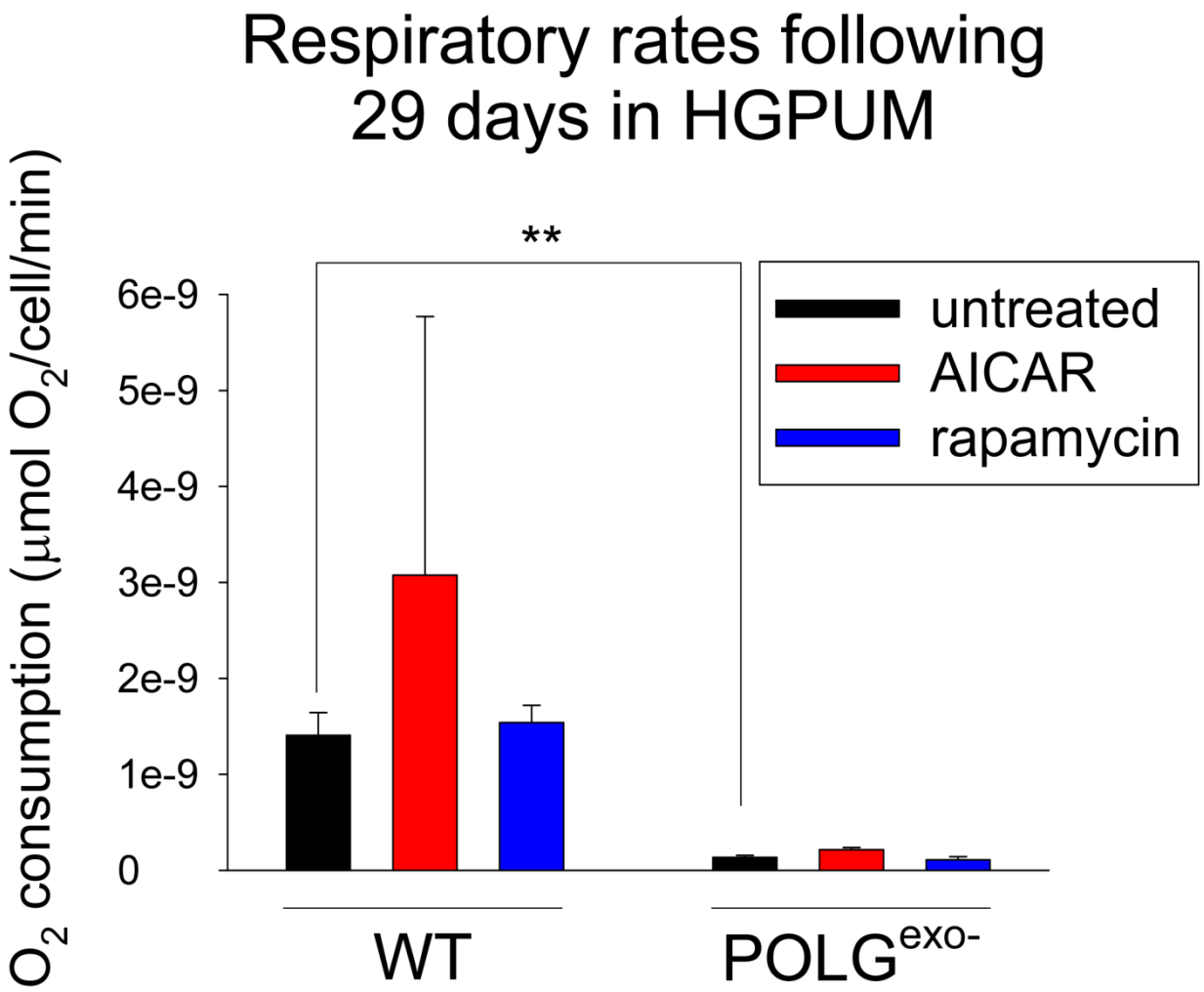


Figure 36 O_2 consumption following culture in HGPUM for 29 days
AICAR or rapamycin treatment for 29 days in HGPUM did not significantly alter the rate of oxygen consumption. Untreated $POLG^{D257A/D257A}$ MEFs showed a lower rate of oxygen consumption than untreated WT MEFs. Error bars represent the standard error of the mean ** $p < 0.005$.

CHAPTER SEVEN: FUTURE DIRECTIONS

Calorie restriction

Although we measured brain mitochondrial function following calorie restriction (CR) of P301L tau transgenic mice, several lines of inquiry merit additional research. For instance, deficits of ADP driven oxygen consumption still remain incompletely explained. We have eliminated complex V as a culprit, at least in cells, for causing reduced ADP-driven oxygen consumption, but we did not rule out dysfunction of adenine nucleotide translocase (ANT-1), which transports ADP into the mitochondrial matrix or voltage dependent anion channel (VDAC), which transports ADP across the outer mitochondrial membrane. Deficits in ANT-1 or VDAC1 could explain the decreased ADP driven oxygen consumption observed in our studies.

In our studies, we determined global neural mitochondrial dysfunction in P301L tau model of AD. We did not discriminate between neuronal and glial mitochondria. It would be of interest to determine if CR has a differential effect on the mitochondria of neurons compared to glia in AD. It would also be of interest to see how mitochondrial function of brain stem cells in the hippocampus and the sub-ventricular zone is affected by CR. Synaptic mitochondria could be isolated to specifically examine mitochondria from neurons. In addition microglia, astrocytes, or neural stem cells could be isolated and mitochondrial function could be studied in these primary cells.

Melatonin receptors

In these studies it was determined that melatonin receptors are necessary for full restoration of normal cytochrome c oxidase (COX) activity with melatonin treatment in aged WT or APP/PS1 mice (figures 3.1, 3.2), however the downstream signaling mechanisms from the receptors were not determined in these studies. In aged WT mice, melatonin completely restored the age-dependent decrease of COX activity in the presence of melatonin receptors and only partially restored the activity in the absence of melatonin receptors (figure 3.1). The SIRT1 deacetylase activates the mitochondrial master regulator, PGC1-α, which increases the transcription of both antioxidant response element containing genes and genes coding for mitochondrial proteins. SIRT1 has been shown to decrease with age (Quintas, de Solis et al. 2012). Future studies will determine if increased melatonin levels results in increased transcription of SIRT1. Melatonin has also been shown to inhibit the ubiquitin-dependent proteasome pathway (Kwon, Kim et al. 2011). Future studies will also determine if the partial rescue of COX activity in the absence of melatonin receptors in aged WT mice is achieved by melatonin's ability to inhibit the proteasome.

Higher levels of ROS associated with AD could increase the direct phosphorylation of PGC1-α by activating AMPK (Jager, Handschin et al. 2007). Melatonin levels are significantly decreased in the CSF of AD patients (Liu, Zhou et al. 1999; Zhou, Liu et al. 2003). Critically low levels of melatonin signaling could prevent proper SIRT1 deacetylation of PGC1-α by decreasing SIRT1 expression. Future studies will determine if both deacetylation and phosphorylation are necessary for PGC1-α activation of Nrf2 and if NRF1 and NRF-2 activation by PGC1-α can occur with only phosphorylation by AMPK. Future experiments will also

determine whether MT1 and MT2 are necessary for decreasing the transcription of proinflammatory mediators.

Amino acids and metabolic intermediates as therapeutics for neurodegenerative disease

In our PD study, we determined that cells overexpressing alpha-synuclein were susceptible to mitochondrial dysfunction caused by $FeSO_4$ treatment. These mitochondrial deficits may be in part due to decreased levels of mitochondrial superoxide dismutase 2 (SOD2), and increased levels of monoamine oxidase type A MAO-A, as shown in chapter 4. We were able to rescue some of these mitochondrial deficits by supplementation with amino acids and other metabolic intermediates. It would be important to determine if additive or synergistic effects can be achieved by co-supplementation with multiple amino acids, since they are frequently co-transported across cell membranes. Ester forms of amino acids were not tested, but like the ester forms of TCA cycle intermediates administered, they would not require active transport. This could potentially increase their effectiveness by increasing the rate of delivery and decreasing the requirement for active transport.

Using proteomics we determined significant fold change differences in protein expression. The top canonical signaling pathways that were significantly changed were: protein ubiquitination pathway, thrombin signaling, tight junction signaling, regulation of eIF3 and p70S6K signaling, and of most interest to us mitochondrial dysfunction signaling (appendix A figure 1). Mitochondrial proteins in cells overexpressing alpha-synuclein (table 4.1). These findings must be confirmed using Western blotting. Furthermore, it would be of interest to determine if the rescue was achieved by improving function of existing mitochondria and/or by increasing mitochondrial biogenesis. To test this we will measure total mitochondria using the

mitochondria-specific dye MitoTracker (MT) Green® (Life Technologies). MT Green has been described to bind to all mitochondria regardless of their health. To test if the increased total mitochondria are also healthier, we will use a cationic dye JC-1 that is imported into mitochondria in response to the mitochondrial membrane potential.

Leuicine-rich repeat kinase 2 (LRRK2) has been implicated in the pathology of sporadic PD and may prove to be a viable therapeutic target (Sen and West 2009). LRRK2 has also been shown to interact with Parkin, an E3 ubiquitin ligase, important in directing mitophagy (Smith, Pei et al. 2005). It would be of great interest to determine if aberrant interaction of LRRK2 with Parkin prevents proper association of Parkin and Pink1, a mitochondrial kinase associated with PD. This would result in the buildup of mitochondria with low membrane potential that would normally be directed toward mitophagy through proper Parkin and Pink1 interaction. This would potentially be a novel mechanism of mitochondria-related PD pathology.

Role of mitochondrial dysfunction in the collapse of the NVU in ALS

Future studies will focus on assaying ETC complex III and mitochondrial DNA levels in our human spinal cord samples. To further study the mechanisms of mitochondrial dysfunction and their contribution to the collapse of the neurovascular unit, we will analyze mitochondrial ETC complex activities in each of the components of the NVU (e.g. astrocytes, neurons, oligodendrocytes, and endothelial cells) in a mouse model of ALS. Once the degree of mitochondrial dysfunction is established, potential therapies can be administered to the mice to determine if ETC complex function improves. Strategies we found protective in our other studies could be explored as potential therapeutic approaches for ALS as well.

Effects of AICAR and rapamycin on mitochondrial function

In our studies we have found that AICAR treatment decreased mitochondrial function in both WT and $POLG^{D257A/D257A}$ MEFs. It would be important to confirm that AMPK is indeed activated by performing western blots for phosphorylated AMPK. Conversely, non-phosphorylated mTOR would be indicative of mTOR activation. Future studies should also determine other effects of AICAR and rapamycin on metabolism, particularly on glycolysis, to help elucidate the transient effects of rapamycin and the persistent decrease in ATP levels by AICAR. Similar studies should also be performed on other lineages of cells obtained from polymerase gamma exonuclease-deficient mice. As previously discussed, mitochondrial dysfunction can vary between different tissues in POLG exonuclease-deficient mice.

Potential clinical and translational applications of the current research

This body of work has demonstrated the existence of mitochondrial dysfunction in major neurodegenerative diseases, namely the contribution of mitochondrial dysfunction to the progress of: amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Parkinson's disease (PD). We have also shown herein, that some aspects of mitochondrial dysfunction can be rescued by neuroprotective nutritional interventions. Many of these compounds, like TCA cycle intermediates and essential amino acids are generally regarded as safe (GRAS) by the food and drug administration (FDA). This makes metabolic intermediates and amino acids attractive treatments to explore, since they could be fast-tracked into human trials. At present, we have no effective way of gauging the progress of mitochondrial dysfunction clinically. This would make the study of potential mitochondrial beneficial, like TCA intermediates and amino acids impossible. Below we outline how current ideas on the metabolic health index (MHI) could be improved upon by advent of databases, use of standardized mitochondrial analytic techniques,

and proteomic approaches that would overcome current hurdles to furthering our knowledge of cellular metabolism and neurodegenerative diseases. We will also propose how these new approaches could be used to test the efficacy of GRAS compounds, which could be protective on human mitochondria via minimally invasive approaches.

Development of biomarkers for neurodegenerative diseases

In addition to the previously discussed neurodegenerative diseases Friedreich's Ataxia (FA) and Huntington's disease (HD) are also some of the most common neurological diseases. Although their etiology varies, a common underlying contributor to progression of all these diseases is mitochondrial dysfunction (Quintanilla and Johnson 2009; Shi, Wei et al. 2010; Exner, Lutz et al. 2012; Puccio, Anheim et al. 2014; Swerdlow, Burns et al. 2014). Their economic and societal impact is alarming. Alzheimer's disease alone is estimated to cost $100 billion a year (Meek, McKeithan et al. 1998). The economic impact of PD is estimated to be $14.4 billion a year, and the prevalence is projected to more than double by 2040 (Kowal, Dall et al. 2013). In 2010 cost of all brain disorders in Europe was estimated to be €798 billion (Gustavsson, Svensson et al. 2011). At present, no cures or effective treatments exists for the above neurodegenerative diseases. Improved diagnostic tools for diseases characterized by mitochondrial dysfunction would greatly improve early detection, intervention, and aid insight into the disease mechanisms.

Hurdles to research and diagnosis

Neither detailed disease progression nor effectiveness of drugs on mitochondrial function can be adequately measured using currently available clinical methods. These were identified as

major hurdles in the way of progress toward effective treatments by NIH intramural and extramural scientists and industry and foundation representatives (Moraes, Anderson et al. 2013). For example, the best current tool available for diagnosis of Alzheimer's disease (AD) is brain imaging (magnetic resonance imaging or positron emission tomography). These procedures can only detect damage that has advanced to the tissue level. For diseases like HD and FA genetic diagnostic tests are available. However, the ability to effectively gauge the progress of these diseases and their impact on mitochondria does not exist. Many neurodegenerative diseases have established metabolic and mitochondrial dysfunctions that also affect the platelets in peripheral blood (Zharikov and Shiva 2013). In addition to modulating mitochondrial activity in the brain, AD, ALS, FA, HD, and PD can also affect mitochondrial activity in peripheral blood cells. Ability to measure these metabolic dysfunctions from patient derived platelets would be a powerful clinical diagnostic and research tool. Furthermore, curating these mitochondrial disease state phenotypes in databases would create a powerful research and eventually diagnostic tool. Analysis of the patient's biological sample for proteomic, metabolomics, and transcriptomic changes, coupled with carefully curated mitochondrial disease state phenotypes could potentially usher in a new field of research in "transmetabolomics." Mitochondrial phenotypic changes could be cross referenced with proteomic level changes at unprecedented levels. This inevitable new field of research would overcome the hurdles discussed above that are preventing discoveries of cures and effective treatments for mitochondria related diseases.

Assessing mitochondrial health

The rate of adenosine triphosphate (ATP) production is an important indicator of mitochondrial health. Mitochondria are after all the "powerhouse of the cell". ATP production is

accomplished by the use of the electrochemical potential across the mitochondrial inner membrane or the mitochondrial membrane potential (MMP). MMP is maintained by a constant flux of electrons down the electron transport chain (ETC) and their deposition onto oxygen, reducing it to water. These high energy electrons and accompanying hydrogens are obtained from glycolysis and the tricarboxyclic acid cycle (TCA). The reduction of oxygen is coupled to phosphorylation of adenosine diphosphate (ADP) into ATP. This is ADP-driven oxygen consumption, resulting in oxidative phosphorylation (OXPHOS). If the flux of electrons down the ETC is interrupted or other mitochondrial perturbation are caused by a diseased state, levels of reactive oxygen species (ROS) may increase, further damaging the mitochondria. In order to determine many of these parameters mitochondria have to be isolated from tissue and rapidly analyzed. Once frozen, the mitochondrial membrane is damaged and only analysis of individual electron transport chain complexes can be performed. Mitochondria can be analyzed without removal from the cytoplasm, in monolayered cultured cells or from non-adherent cells in suspension. Analyzing whole cells for mitochondrial function will be considerably less labor intensive if phenotypic differences are detectable. Established methods of mitochondrial analysis were never applied together in a cohesive way, in order to create an index for mitochondrial health. Furthermore, no efforts were made to establish databases, both of mitochondrial disease state phenotypes using standardized methods, and proteomic changes under the same disease conditions.

Hopefully, future grant funding will allow for rapid creation of a model database with standardized mitochondrial values for each of these specific diseases. This data would then allow for the creation of a health index for the first time in history that would aggregate multiple

indicators of mitochondrial function i.e. ATP production, OXOPHOS, MMP, ROS levels and proteomic level changes under the same conditions using peripheral blood.

Why use platelets as surrogates

Over the past couple of decades, platelets have been used as surrogates of mitochondrial dysfunction in other tissues in several patient populations (Parker, Boyson et al. 1989; Parker, Boyson et al. 1990; Parker 1991; Sangiorgi, Mochi et al. 1994; Blandini, Nappi et al. 1998; Sjovall, Morota et al. 2010; Zharikov and Shiva 2013). However, standardized methods were not used in the experiments over the years and due to the absence of centralized repositories for this type of data, creation of a meaningful clinical score is currently impossible (Moraes, Anderson et al. 2013). Furthermore, some mitochondrial diseases like Freidreich's ataxia have conflicting findings on whether blood cells are suitable surrogates for an inference into mitochondrial dysfunction of post-mitotic cells with a higher degree of heteroplasmy (Selak, Lyver et al. 2011). Studies also show that transformed peripheral blood mononuclear cells exhibit mitochondrial dysfunction phenotypes (Napoli, Taroni et al. 2006). This suggests that although cells might be harboring mitochondrial defects the phenotype is below the threshold of detection in certain cases and depends on the detection methods used.

Recently, in a hypothesis paper Balu Chacko et al, proposed a bioenergetics health index (BHI) and have verified the potential for the use of platelets as a "canary in a coal mine" for mitochondria in less accessible areas like the brain (Chacko, Kramer et al. 2014). Using Seahorse Bioscience's extracellular flux analyzer they were able to demonstrate the feasibility of platelets as surrogates for mitochondrial function in other tissues.

Innovation

Their approach however, lacks key components that significantly diminish its application to establishing an effective BHI. Using a flux analyzer, extracellular changes in dissolved oxygen concentration and acidity are used to determine mitochondrial function. Thesemeasurements are only part of the story. Their BHI does not measure mitochondrial membrane potential (MMP), reactive oxygen species (ROS) production, adenosine triphosphate (ATP) production, nor individual mitochondrial electron transport chain (ETC) complex activity. These are key indicators of mitochondrial function and any integrated score would be served well to include them. Furthermore, the BHI proposed by Chacko et al. relies only on patients own baseline, which only allows measurements of disease progress from that point in time. Comparing the individual's BHI to a database of values from both healthy individuals and from various disease states would be more telling. Patients could be informed as to the progress of the disease and what can be expected with the disease progression. Their progress can also be monitored. Obtaining and curating proteomic level changes under these same conditions and cross-referencing them with phenotypic changes would allow for an unprecedented level of treatment, research, and innovation.

The environment of the blood is highly dynamic. This can have considerable impact on the variability of any blood cell's metabolic activity and protein abundance levels. Sampling blood from the same patient at different times of the day and pre and post meal could mask phenotypic differences present as previously discussed. Growing cells in a standard culture medium for 2 days could streamline metabolic activity between individuals and allow for finer detection of phenotypic differences. As with our studies of mouse embryonic fibroblasts with a defective mitochondrial DNA polymerase, phenotypic differences can be unmasked by the

growth of cells for 24-48 hour under limited nutrient conditions. Using these approaches will significantly increase the sensitivity of the detection of potential differences between control donors within an age group (i.e. 20-30 year olds) and patients that are pre-clinical for a disease. This may also allow for the detection of fine proteome level changes that can be used as a diagnostic tool or as biomarkers for the progress of the disease or efficacy of the treatment regimen in pre-clinical patients. Moving away from a single indicator of disease to a concert of phenotypic and proteomic level changes (that themselves could only work for certain segments of the population) will allow for more rapid drug design and treatment innovations and personalized medicine. The feasibility of this approach could be demonstrated by obtaining blood from 10 healthy donors (20-30 years old) and 10 age matched donors with HD and/or FA at the earliest or pre-clinical stages of the disease. Using the data from all of the mitochondrial health parameters previously discussed, databases could be created to curate the data. An algorithm could be written that integrates phenotypic values into a single score and cross references the score with proteomic level changes.

Commercial opportunities and societal benefits

Once streamlined, the database and the process can be licensed out to hospitals, research institutes, and government agencies for diagnostic and research purposes. The reagents necessary to perform the assays at any hospital lab would be pre-prepared for quality control purposes and provided as part of the package along with access to the score and the database. This would also significantly cut down on the diagnostics time and the cost of the procedure. The ability to diagnose mitochondrial dysfunction and gauge the effectiveness of potential therapeutics will eliminate the biggest hurdle identified by the NIH to development of treatment of mitochondria-

related diseases. Access to the growing database would allow scientists to compare mitochondrial function across a multitude of diseases, age groups, treatments, and accompanying proteomic level changes in unprecedented numbers. This would lead to a new era of rapid discovery.

Maintaining these mitochondrial values for each patient and monitoring progress over time will result in more personalized medicine. The same approach can also be used to gauge improvements in mitochondria of diabetics, as they undergo a diet or exercise regimen. Furthermore, professional athletes, those under specific diets, or multidrug regiments, which can impact central metabolism can also benefit from having access to the database. As the metabolic health index becomes a standard staple of blood tests available to physicians, it can serve as a forecast of patients declining health, or show improvement in response to a treatment before physical symptoms improve.

Compounds we previously found to be protective in our in vitro PD model are generally regarded as safe (GRAS) by the FDA. This would allow us to move quickly toward approval for human trials, where we could measure the efficacy of these drugs on the patient population used to established BHI and the database. Proteomic and metabolomic analysis combined with phenotypic improvements would allow us to rapidly map out mechanisms by which these compounds improve metabolic function, leading to more effective rational drug design. This new approach to metabolic research would revolutionize the current drug discovery process and likely decrease bench to bedside time by orders of magnitude and in the process decrease the massive drug discovery costs. Benefits of developing this technology can have far reaching effects well beyond medicine. For instance, similar metabolic health indices and markers could be developed for agricultural and wildlife conservation purposes. Fitness' of animal populations we rely on for

food could be determined. If prey species metabolic health index drops, so would their fitness measured by their ability to reproduce. We would have a rapid, robust, and inexpensive forecast for their health in response to environmental changes. We could measure the impact of pollutants, natural disasters, and global warming on metabolic health. The development of metabolic health indices that draw upon mitochondrial phenotypic values obtained using standardized methods and nutrient limiting conditions, and cross referencing them with proteomic and metabolomics changes under the same conditions would usher in a new field of transmetabolomics.

CONCLUSION

The research presented here describes the presence of mitochondrial dysfunction in several neurodegenerative disease models and a model of aging. It also describes several strategies for improving mitochondrial function that may be used in attempt to improve mitochondrial function. Through targeting mitochondrial function in combination with the more traditional targets in neurodegenerative diseases, new therapies may one day be developed that profoundly delay the onset and progression of these devastating diseases.

REFERENCES

(2015, April 20th, 2015). "National Library of Medicine (US). Genetics Home Reference ", from http://ghr.nlm.nih.gov/condition/amyotrophic-lateral-sclerosis/show/Related+Gene(s).

Abbott, N. J. (2013). "Blood-brain barrier structure and function and the challenges for CNS drug delivery." J Inherit Metab Dis **36**(3): 437-449.

Abnous, K. and K. B. Storey (2008). "Skeletal muscle hexokinase: regulation in mammalian hibernation." Mol Cell Biochem **319**(1-2): 41-50.

Adamczyk-Sowa, M., P. Sowa, et al. (2013). "Role of melatonin receptor MT(2) and quinone reductase II in the regulation of the redox status of 3T3-L1 preadipocytes in vitro." Cell Biol Int **37**(8): 835-842.

Adi, N., D. C. Mash, et al. (2010). "Melatonin MT1 and MT2 receptor expression in Parkinson's disease." Med Sci Monit **16**(2): BR61-67.

Ahlqvist, K. J., R. H. Hamalainen, et al. (2012). "Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in Polg mutator mice." Cell Metab **15**(1): 100-109.

Ajioka, R. S., J. D. Phillips, et al. (2006). "Biosynthesis of heme in mammals." Biochim Biophys Acta **1763**(7): 723-736.

Allaman, I., M. Belanger, et al. (2015). "Methylglyoxal, the dark side of glycolysis." Front Neurosci **9**: 23.

Amadoro, G., V. Corsetti, et al. (2012). "Interaction between NH(2)-tau fragment and Abeta in Alzheimer's disease mitochondria contributes to the synaptic deterioration." Neurobiol Aging **33**(4): 833 e831-825.

Apfeld, J., G. O'Connor, et al. (2004). "The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans." Genes Dev **18**(24): 3004-3009.

Arany, Z., M. Novikov, et al. (2006). "Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha." Proc Natl Acad Sci U S A **103**(26): 10086-10091.

Asayama, K., H. Yamadera, et al. (2003). "Double blind study of melatonin effects on the sleep-wake rhythm, cognitive and non-cognitive functions in Alzheimer type dementia." J Nippon Med Sch **70**(4): 334-341.

Ashabi, G., M. Ramin, et al. (2012). "ERK and p38 inhibitors attenuate memory deficits and increase CREB phosphorylation and PGC-1alpha levels in Abeta-injected rats." Behav Brain Res **232**(1): 165-173.

Asher, G., D. Gatfield, et al. (2008). "SIRT1 regulates circadian clock gene expression through PER2 deacetylation." Cell **134**(2): 317-328.

Asher, G. and U. Schibler (2011). "Crosstalk between components of circadian and metabolic cycles in mammals." Cell Metab **13**(2): 125-137.

Bailey, S. M., E. C. Pietsch, et al. (1999). "Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III." Free Radic Biol Med **27**(7-8): 891-900.

Baker, M. R. (2014). "ALS--dying forward, backward or outward?" Nat Rev Neurol **10**(11): 660.

Balsa, E., R. Marco, et al. (2012). "NDUFA4 is a subunit of complex IV of the mammalian electron transport chain." Cell Metab **16**(3): 378-386.
Bano Otalora, B., N. Popovic, et al. (2012). "Circadian system functionality, hippocampal oxidative stress, and spatial memory in the APPswe/PS1dE9 transgenic model of Alzheimer disease: effects of melatonin or ramelteon." Chronobiol Int **29**(7): 822-834.
Bartels, T., J. G. Choi, et al. (2011). "alpha-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation." Nature **477**(7362): 107-110.
Beal, M. F. (2007). "Mitochondria and neurodegeneration." Novartis Found Symp **287**: 183-192; discussion 192-186.
Bedrosian, T. A. and R. J. Nelson (2012). "Pro: Alzheimer's disease and circadian dysfunction: chicken or egg?" Alzheimers Res Ther **4**(4): 25.
Behan, W. M., M. McDonald, et al. (1999). "Oxidative stress as a mechanism for quinolinic acid-induced hippocampal damage: protection by melatonin and deprenyl." Br J Pharmacol **128**(8): 1754-1760.
Benit, P., S. Lebon, et al. (2009). "Respiratory-chain diseases related to complex III deficiency." Biochim Biophys Acta **1793**(1): 181-185.
Bertram, L. and R. E. Tanzi (2008). "Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses." Nat Rev Neurosci **9**(10): 768-778.
Betz, C., D. Stracka, et al. (2013). "Feature Article: mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology." Proc Natl Acad Sci U S A **110**(31): 12526-12534.
Binukumar, B. K., A. Bal, et al. (2010). "Nigrostriatal neuronal death following chronic dichlorvos exposure: crosstalk between mitochondrial impairments, alpha synuclein aggregation, oxidative damage and behavioral changes." Mol Brain **3**: 35.
Blandini, F., G. Nappi, et al. (1998). "Quantitative study of mitochondrial complex I in platelets of parkinsonian patients." Mov Disord **13**(1): 11-15.
Bolender, N., A. Sickmann, et al. (2008). "Multiple pathways for sorting mitochondrial precursor proteins." EMBO Rep **9**(1): 42-49.
Bolster, D. R., S. J. Crozier, et al. (2002). "AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling." J Biol Chem **277**(27): 23977-23980.
Bondy, S. C., H. Li, et al. (2010). "Melatonin alters age-related changes in transcription factors and kinase activation." Neurochem Res **35**(12): 2035-2042.
Bourdenx, M., E. Bezard, et al. (2014). "Lysosomes and alpha-synuclein form a dangerous duet leading to neuronal cell death." Front Neuroanat **8**: 83.
Brandt, U. (2006). "Energy converting NADH:quinone oxidoreductase (complex I)." Annu Rev Biochem **75**: 69-92.
Brownlow, M. L., A. Joly-Amado, et al. (2014). "Partial rescue of memory deficits induced by calorie restriction in a mouse model of tau deposition." Behav Brain Res **271C**: 79-88.
Brusco, L. I., M. Marquez, et al. (1998). "Monozygotic twins with Alzheimer's disease treated with melatonin: Case report." J Pineal Res **25**(4): 260-263.
Brusco, L. I., M. Marquez, et al. (2000). "Melatonin treatment stabilizes chronobiologic and cognitive symptoms in Alzheimer's disease." Neuro Endocrinol Lett **21**(1): 39-42.
Brustovetsky, N. and M. Klingenberg (1994). "The reconstituted ADP/ATP carrier can mediate H+ transport by free fatty acids, which is further stimulated by mersalyl." J Biol Chem **269**(44): 27329-27336.

Brydon, L., F. Roka, et al. (1999). "Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins." Mol Endocrinol **13**(12): 2025-2038.
Bu, G. (2009). "Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy." Nat Rev Neurosci **10**(5): 333-344.
Bubber, P., V. Haroutunian, et al. (2005). "Mitochondrial abnormalities in Alzheimer brain: mechanistic implications." Ann Neurol **57**(5): 695-703.
Calkins, M. J., M. Manczak, et al. (2011). "Impaired mitochondrial biogenesis, defective axonal transport of mitochondria, abnormal mitochondrial dynamics and synaptic degeneration in a mouse model of Alzheimer's disease." Hum Mol Genet **20**(23): 4515-4529.
Campbell, G., K. J. Krishnan, et al. (2014). "Dissecting the mechanisms underlying the accumulation of mitochondrial DNA deletions in human skeletal muscle." Hum Mol Genet **23**(17): 4612-4620.
Cannon, J. R., K. D. Geghman, et al. (2013). "Expression of human E46K-mutated alpha-synuclein in BAC-transgenic rats replicates early-stage Parkinson's disease features and enhances vulnerability to mitochondrial impairment." Exp Neurol **240**: 44-56.
Cardinali, D. P., A. M. Furio, et al. (2011). "The use of chronobiotics in the resynchronization of the sleep/wake cycle. Therapeutical application in the early phases of Alzheimer's disease." Recent Pat Endocr Metab Immune Drug Discov **5**(2): 80-90.
Cardinali, D. P., E. S. Pagano, et al. (2013). "Melatonin and mitochondrial dysfunction in the central nervous system." Horm Behav **63**(2): 322-330.
Carretero, M., G. Escames, et al. (2009). "Long-term melatonin administration protects brain mitochondria from aging." J Pineal Res.
Carretero, M., G. Escames, et al. (2009). "Long-term melatonin administration protects brain mitochondria from aging." J Pineal Res **47**(2): 192-200.
Carroll, J., I. M. Fearnley, et al. (2006). "Bovine complex I is a complex of 45 different subunits." J Biol Chem **281**(43): 32724-32727.
Cerqueira, F. M., F. M. Cunha, et al. (2012). "Calorie restriction increases cerebral mitochondrial respiratory capacity in a NO*-mediated mechanism: impact on neuronal survival." Free Radic Biol Med **52**(7): 1236-1241.
Chacko, B. K., P. A. Kramer, et al. (2014). "The Bioenergetic Health Index: a new concept in mitochondrial translational research." Clin Sci (Lond) **127**(6): 367-373.
Chan, A. S., F. P. Lai, et al. (2002). "Melatonin mt1 and MT2 receptors stimulate c-Jun N-terminal kinase via pertussis toxin-sensitive and -insensitive G proteins." Cell Signal **14**(3): 249-257.
Chang, H. M., U. I. Wu, et al. (2009). "Melatonin preserves longevity protein (sirtuin 1) expression in the hippocampus of total sleep-deprived rats." J Pineal Res **47**(3): 211-220.
Chang, S., T. ran Ma, et al. (2005). "Lipid- and receptor-binding regions of apolipoprotein E4 fragments act in concert to cause mitochondrial dysfunction and neurotoxicity." Proc Natl Acad Sci U S A **102**(51): 18694-18699.
Chaput, D., L. H. Kirouac, et al. (2012). "SILAC-based proteomic analysis to investigate the impact of amyloid precursor protein expression in neuronal-like B103 cells." Electrophoresis **33**(24): 3728-3737.
Chen, J., L. Wang, et al. (2013). "Melatonin-enhanced autophagy protects against neural apoptosis via a mitochondrial pathway in early brain injury following a subarachnoid hemorrhage." J Pineal Res **56**: 12-19.

Chen, L., Z. Xie, et al. (2015). "A53T human alpha-synuclein overexpression in transgenic mice induces pervasive mitochondria macroautophagy defects preceding dopamine neuron degeneration." J Neurosci **35**(3): 890-905.
Chen, Y., J. Ye, et al. (2010). "Myostatin regulates glucose metabolism via the AMP-activated protein kinase pathway in skeletal muscle cells." Int J Biochem Cell Biol **42**(12): 2072-2081.
Cheng, Y., L. Cai, et al. (2013). "SIRT1 inhibition by melatonin exerts antitumor activity in human osteosarcoma cells." Eur J Pharmacol **715**(1-3): 219-229.
Cheng, Y., Z. Feng, et al. (2006). "Beneficial effects of melatonin in experimental models of Alzheimer disease." Acta Pharmacol Sin **27**(2): 129-139.
Chern, C. M., J. F. Liao, et al. (2012). "Melatonin ameliorates neural function by promoting endogenous neurogenesis through the MT2 melatonin receptor in ischemic-stroke mice." Free Radic Biol Med **52**(9): 1634-1647.
Chiba-Falek, O. and R. L. Nussbaum (2001). "Effect of allelic variation at the NACP-Rep1 repeat upstream of the alpha-synuclein gene (SNCA) on transcription in a cell culture luciferase reporter system." Hum Mol Genet **10**(26): 3101-3109.
Choi, S. I., S. Dadakhujaev, et al. (2011). "Melatonin protects against oxidative stress in granular corneal dystrophy type 2 corneal fibroblasts by mechanisms that involve membrane melatonin receptors." J Pineal Res **51**(1): 94-103.
Choi, S. W., A. A. Gerencser, et al. (2012). "No consistent bioenergetic defects in presynaptic nerve terminals isolated from mouse models of Alzheimer's disease." J Neurosci **32**(47): 16775-16784.
Chomyn, A. and G. Attardi (2003). "MtDNA mutations in aging and apoptosis." Biochem Biophys Res Commun **304**(3): 519-529.
Chu, Y. and J. H. Kordower (2015). "The prion hypothesis of Parkinson's disease." Curr Neurol Neurosci Rep **15**(5): 28.
Chyan, Y. J., B. Poeggeler, et al. (1999). "Potent neuroprotective properties against the Alzheimer beta-amyloid by an endogenous melatonin-related indole structure, indole-3-propionic acid." J Biol Chem **274**(31): 21937-21942.
Cirulli, E. T., B. N. Lasseigne, et al. (2015). "Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways." Science **347**(6229): 1436-1441.
Cohen, G., R. Farooqui, et al. (1997). "Parkinson disease: a new link between monoamine oxidase and mitochondrial electron flow." Proc Natl Acad Sci U S A **94**(10): 4890-4894.
Cohen, T. J., J. L. Guo, et al. (2011). "The acetylation of tau inhibits its function and promotes pathological tau aggregation." Nat Commun **2**: 252.
Colman, R. J., R. M. Anderson, et al. (2009). "Caloric restriction delays disease onset and mortality in rhesus monkeys." Science **325**(5937): 201-204.
Colman, R. J., T. M. Beasley, et al. (2014). "Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys." Nat Commun **5**: 3557.
Colom, B., J. Oliver, et al. (2007). "Caloric restriction and gender modulate cardiac muscle mitochondrial H2O2 production and oxidative damage." Cardiovasc Res **74**(3): 456-465.
Corder, E. H., A. M. Saunders, et al. (1993). "Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families." Science **261**(5123): 921-923.
Costantino, G., S. Cuzzocrea, et al. (1998). "Protective effects of melatonin in zymosan-activated plasma-induced paw inflammation." Eur J Pharmacol **363**(1): 57-63.

Coto-Montes, A., J. A. Boga, et al. (2012). "Role of melatonin in the regulation of autophagy and mitophagy: a review." Mol Cell Endocrinol **361**(1-2): 12-23.
Cozzolino, M. and M. T. Carri (2012). "Mitochondrial dysfunction in ALS." Prog Neurobiol **97**(2): 54-66.
Cozzolino, M., S. Rossi, et al. (2015). "Mitochondrial dynamism and the pathogenesis of Amyotrophic Lateral Sclerosis." Front Cell Neurosci **9**: 31.
Crouch, P. J., R. Blake, et al. (2005). "Copper-dependent inhibition of human cytochrome c oxidase by a dimeric conformer of amyloid-beta1-42." J Neurosci **25**(3): 672-679.
Cuadrado-Tejedor, M., J. F. Cabodevilla, et al. (2013). "Age-related mitochondrial alterations without neuronal loss in the hippocampus of a transgenic model of Alzheimer's disease." Curr Alzheimer Res **10**(4): 390-405.
Cunningham, C. C. and S. M. Bailey (2001). "Ethanol consumption and liver mitochondria function." Biol Signals Recept **10**(3-4): 271-282.
Cunningham, J. T., J. T. Rodgers, et al. (2007). "mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex." Nature **450**(7170): 736-740.
Cuzzocrea, S., B. Zingarelli, et al. (1997). "Protective effect of melatonin in carrageenan-induced models of local inflammation: relationship to its inhibitory effect on nitric oxide production and its peroxynitrite scavenging activity." J Pineal Res **23**(2): 106-116.
Dagher, Z., N. Ruderman, et al. (1999). "The effect of AMP-activated protein kinase and its activator AICAR on the metabolism of human umbilical vein endothelial cells." Biochem Biophys Res Commun **265**(1): 112-115.
Dai, D. F., T. Chen, et al. (2010). "Age-dependent cardiomyopathy in mitochondrial mutator mice is attenuated by overexpression of catalase targeted to mitochondria." Aging Cell **9**(4): 536-544.
Dai, D. F., P. S. Rabinovitch, et al. (2012). "Mitochondria and cardiovascular aging." Circ Res **110**(8): 1109-1124.
Dalfo, E., M. Portero-Otin, et al. (2005). "Evidence of oxidative stress in the neocortex in incidental Lewy body disease." J Neuropathol Exp Neurol **64**(9): 816-830.
Darley-Usmar, V. M., N. G. Kennaway, et al. (1983). "Deficiency in ubiquinone cytochrome c reductase in a patient with mitochondrial myopathy and lactic acidosis." Proc Natl Acad Sci U S A **80**(16): 5103-5106.
David, D. C., S. Hauptmann, et al. (2005). "Proteomic and functional analyses reveal a mitochondrial dysfunction in P301L tau transgenic mice." J Biol Chem **280**(25): 23802-23814.
Davie, C. A. (2008). "A review of Parkinson's disease." Br Med Bull **86**: 109-127.
Dawson, G. R., G. R. Seabrook, et al. (1999). "Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein." Neuroscience **90**(1): 1-13.
de Lau, L. M. and M. M. Breteler (2006). "Epidemiology of Parkinson's disease." Lancet Neurol **5**(6): 525-535.
de Moura, M. B., L. S. dos Santos, et al. (2010). "Mitochondrial dysfunction in neurodegenerative diseases and cancer." Environ Mol Mutagen **51**(5): 391-405.
De Zutter, G. S. and R. J. Davis (2001). "Pro-apoptotic gene expression mediated by the p38 mitogen-activated protein kinase signal transduction pathway." Proc Natl Acad Sci U S A **98**(11): 6168-6173.

Deepa, S. S., M. E. Walsh, et al. (2013). "Rapamycin Modulates Markers of Mitochondrial Biogenesis and Fatty Acid Oxidation in the Adipose Tissue of db/db Mice." J Biochem Pharmacol Res **1**(2): 114-123.

Delic, V., M. Brownlow, et al. (2015). "Calorie restriction does not restore brain mitochondrial function in P301L tau mice, but it does decrease mitochondrial FF-ATPase activity." Mol Cell Neurosci.

Deng, W. G., S. T. Tang, et al. (2006). "Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding." Blood **108**(2): 518-524.

Desai, B. S., A. J. Monahan, et al. (2007). "Blood-brain barrier pathology in Alzheimer's and Parkinson's disease: implications for drug therapy." Cell Transplant **16**(3): 285-299.

Diaz, F., M. P. Bayona-Bafaluy, et al. (2002). "Human mitochondrial DNA with large deletions repopulates organelles faster than full-length genomes under relaxed copy number control." Nucleic Acids Res **30**(21): 4626-4633.

Dickey, A. S. and S. Strack (2011). "PKA/AKAP1 and PP2A/Bbeta2 regulate neuronal morphogenesis via Drp1 phosphorylation and mitochondrial bioenergetics." J Neurosci **31**(44): 15716-15726.

Dijk, D. J. and C. Cajochen (1997). "Melatonin and the circadian regulation of sleep initiation, consolidation, structure, and the sleep EEG." J Biol Rhythms **12**(6): 627-635.

Dimmer, K. S., S. Fritz, et al. (2002). "Genetic basis of mitochondrial function and morphology in Saccharomyces cerevisiae." Mol Biol Cell **13**(3): 847-853.

Dong, W., F. Huang, et al. (2010). "Differential effects of melatonin on amyloid-beta peptide 25-35-induced mitochondrial dysfunction in hippocampal neurons at different stages of culture." J Pineal Res **48**(2): 117-125.

Dragicevic, N., N. Copes, et al. (2011). "Melatonin treatment restores mitochondrial function in Alzheimer's mice: a mitochondrial protective role of melatonin membrane receptor signaling." J Pineal Res **51**: 75-86.

Dragicevic, N., V. Delic, et al. (2012). "Caffeine increases mitochondrial function and blocks melatonin signaling to mitochondria in Alzheimer's mice and cells." Neuropharmacology **63**(8): 1368-1379.

Dragicevic, N., M. Mamcarz, et al. (2010). "Mitochondrial amyloid-beta levels are associated with the extent of mitochondrial dysfunction in different brain regions and the degree of cognitive impairment in Alzheimer's transgenic mice." J Alzheimers Dis **20 Suppl 2**(2): S535-550.

Du, H., L. Guo, et al. (2008). "Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease." Nat Med **14**(10): 1097-1105.

Du, H., L. Guo, et al. (2010). "Early deficits in synaptic mitochondria in an Alzheimer's disease mouse model." Proc Natl Acad Sci U S A **107**(43): 18670-18675.

Dubocovich, M. L., R. L. Hudson, et al. (2005). "Effect of MT1 melatonin receptor deletion on melatonin-mediated phase shift of circadian rhythms in the C57BL/6 mouse." J Pineal Res **39**(2): 113-120.

Dubocovich, M. L. and M. Markowska (2005). "Functional MT1 and MT2 melatonin receptors in mammals." Endocrine **27**(2): 101-110.

Eckert, A., R. Nisbet, et al. (2014). "March separate, strike together - Role of phosphorylated TAU in mitochondrial dysfunction in Alzheimer's disease." Biochim Biophys Acta **1842**(8): 1258-1266.
Edmondson, D. E. (2014). "Hydrogen peroxide produced by mitochondrial monoamine oxidase catalysis: biological implications." Curr Pharm Des **20**(2): 155-160.
Edwards, C., J. Canfield, et al. (2015). "Mechanisms of amino acid-mediated lifespan extension in Caenorhabditis elegans." BMC Genet **16**: 8.
Egan, D. F., D. B. Shackelford, et al. (2011). "Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy." Science **331**(6016): 456-461.
Elkalaf, M., M. Andel, et al. (2013). "Low glucose but not galactose enhances oxidative mitochondrial metabolism in C2C12 myoblasts and myotubes." PLoS One **8**(8): e70772.
Elkon, H., J. Don, et al. (2002). "Mutant and wild-type alpha-synuclein interact with mitochondrial cytochrome C oxidase." J Mol Neurosci **18**(3): 229-238.
Eschbach, J., B. von Einem, et al. (2015). "Mutual exacerbation of peroxisome proliferator-activated receptor gamma coactivator 1alpha deregulation and alpha-synuclein oligomerization." Ann Neurol **77**(1): 15-32.
Esposito, E. and S. Cuzzocrea (2010). "Antiinflammatory activity of melatonin in central nervous system." Curr Neuropharmacol **8**(3): 228-242.
Exner, N., A. K. Lutz, et al. (2012). "Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences." EMBO J **31**(14): 3038-3062.
Faccenda, D., C. H. Tan, et al. (2013). "IF1 limits the apoptotic-signalling cascade by preventing mitochondrial remodelling." Cell Death Differ **20**(5): 686-697.
Fang, Y., R. Westbrook, et al. (2013). "Duration of rapamycin treatment has differential effects on metabolism in mice." Cell Metab **17**(3): 456-462.
Faubert, B., G. Boily, et al. (2013). "AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo." Cell Metab **17**(1): 113-124.
Ferrer, I., E. Perez, et al. (2007). "Abnormal levels of prohibitin and ATP synthase in the substantia nigra and frontal cortex in Parkinson's disease." Neurosci Lett **415**(3): 205-209.
Fitzgerald, J. C., C. Ufer, et al. (2007). "Monoamine oxidase-A modulates apoptotic cell death induced by staurosporine in human neuroblastoma cells." J Neurochem **103**(6): 2189-2199.
Fitzgerald, J. C., A. Ugun-Klusek, et al. (2014). "Monoamine oxidase-A knockdown in human neuroblastoma cells reveals protection against mitochondrial toxins." Faseb J **28**(1): 218-229.
Fong, C. S., R. M. Wu, et al. (2007). "Pesticide exposure on southwestern Taiwanese with MnSOD and NQO1 polymorphisms is associated with increased risk of Parkinson's disease." Clin Chim Acta **378**(1-2): 136-141.
Formentini, L., M. Sanchez-Arago, et al. (2012). "The mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde prosurvival and proliferative response." Mol Cell **45**(6): 731-742.
Foster, D. B., T. Liu, et al. (2013). "The cardiac acetyl-lysine proteome." PLoS One **8**(7): e67513.

Fowler, C. J., A. Wiberg, et al. (1980). "The effect of age on the activity and molecular properties of human brain monoamine oxidase." J Neural Transm **49**(1-2): 1-20.
Fujita, K., M. Yamauchi, et al. (1996). "Decreased cytochrome c oxidase activity but unchanged superoxide dismutase and glutathione peroxidase activities in the spinal cords of patients with amyotrophic lateral sclerosis." J Neurosci Res **45**(3): 276-281.
Furio, A. M., L. I. Brusco, et al. (2007). "Possible therapeutic value of melatonin in mild cognitive impairment: a retrospective study." J Pineal Res **43**(4): 404-409.
Furuya, M., T. Miyaoka, et al. (2012). "Marked improvement in delirium with ramelteon: five case reports." Psychogeriatrics **12**(4): 259-262.
Gao, S., C. Duan, et al. (2015). "Alpha-synuclein overexpression negatively regulates insulin receptor substrate 1 by activating mTORC1/S6K1 signaling." Int J Biochem Cell Biol **64**: 25-33.
Garbuzova-Davis, S., E. Haller, et al. (2007). "Ultrastructure of blood-brain barrier and blood-spinal cord barrier in SOD1 mice modeling ALS." Brain Res **1157**: 126-137.
Garbuzova-Davis, S. and P. R. Sanberg (2014). "Blood-CNS Barrier Impairment in ALS patients versus an animal model." Front Cell Neurosci **8**: 21.
Garcia-Escudero, V., P. Martin-Maestro, et al. (2013). "Deconstructing mitochondrial dysfunction in Alzheimer disease." Oxid Med Cell Longev **2013**: 162152.
Garcia-Mesa, Y., L. Gimenez-Llort, et al. (2012). "Melatonin plus physical exercise are highly neuroprotective in the 3xTg-AD mouse." Neurobiol Aging **33**(6): 1124 e1113-1129.
Garcia-Roves, P. M., M. E. Osler, et al. (2008). "Gain-of-function R225Q mutation in AMP-activated protein kinase gamma3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle." J Biol Chem **283**(51): 35724-35734.
Garcia, J. J., G. Pinol-Ripoll, et al. (2011). "Melatonin reduces membrane rigidity and oxidative damage in the brain of SAMP8 mice." Neurobiol Aging **32**(11): 2045-2054.
Garcia, T., J. L. Esparza, et al. (2010). "Oxidative stress status and RNA expression in hippocampus of an animal model of Alzheimer's disease after chronic exposure to aluminum." Hippocampus **20**(1): 218-225.
Gegg, M. E. and A. H. Schapira (2011). "PINK1-parkin-dependent mitophagy involves ubiquitination of mitofusins 1 and 2: Implications for Parkinson disease pathogenesis." Autophagy **7**(2): 243-245.
Gehrman, P. R., D. J. Connor, et al. (2009). "Melatonin fails to improve sleep or agitation in double-blind randomized placebo-controlled trial of institutionalized patients with Alzheimer disease." Am J Geriatr Psychiatry **17**(2): 166-169.
Ghiasi, P., S. Hosseinkhani, et al. (2012). "Mitochondrial complex I deficiency and ATP/ADP ratio in lymphocytes of amyotrophic lateral sclerosis patients." Neurol Res **34**(3): 297-303.
Gibson, G. E., S. S. Karuppagounder, et al. (2008). "Oxidant-induced changes in mitochondria and calcium dynamics in the pathophysiology of Alzheimer's disease." Ann N Y Acad Sci **1147**: 221-232.
Gibson, G. E., A. E. Kingsbury, et al. (2003). "Deficits in a tricarboxylic acid cycle enzyme in brains from patients with Parkinson's disease." Neurochem Int **43**(2): 129-135.
Giguere, V. (2008). "Transcriptional control of energy homeostasis by the estrogen-related receptors." Endocr Rev **29**(6): 677-696.
Goldstein, D. S., P. Sullivan, et al. (2013). "Determinants of buildup of the toxic dopamine metabolite DOPAL in Parkinson's disease." J Neurochem **126**(5): 591-603.

Golubitzky, A., P. Dan, et al. (2011). "Screening for active small molecules in mitochondrial complex I deficient patient's fibroblasts, reveals AICAR as the most beneficial compound." PLoS One **6**(10): e26883.
Gomes, C. M. and R. Santos (2013). "Neurodegeneration in Friedreich's ataxia: from defective frataxin to oxidative stress." Oxid Med Cell Longev **2013**: 487534.
Goo, C. K., H. Y. Lim, et al. (2012). "PTEN/Akt signaling controls mitochondrial respiratory capacity through 4E-BP1." PLoS One **7**(9): e45806.
Gorfine, T., Y. Yeshurun, et al. (2007). "Nap and melatonin-induced changes in hippocampal activation and their role in verbal memory consolidation." J Pineal Res **43**(4): 336-342.
Gorfine, T. and N. Zisapel (2007). "Melatonin and the human hippocampus, a time dependent interplay." J Pineal Res **43**(1): 80-86.
Gould, N., D. E. Mor, et al. (2014). "Evidence of native alpha-synuclein conformers in the human brain." J Biol Chem **289**(11): 7929-7934.
Gribanov, G. A., A. N. Pankrushina, et al. (1994). "[Changes in ATPase activity in tissues and mitochondria of some steroid-producing rat organs during autolysis]." Vopr Med Khim **40**(6): 35-37.
Grundke-Iqbal, I., K. Iqbal, et al. (1986). "Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology." Proc Natl Acad Sci U S A **83**(13): 4913-4917.
Guerrero, H. Y., F. Gauer, et al. (1999). "Daily and circadian expression patterns of mt1 melatonin receptor mRNA in the rat pars tuberalis." Adv Exp Med Biol **460**: 175-179.
Guigas, B., N. Taleux, et al. (2007). "AMP-activated protein kinase-independent inhibition of hepatic mitochondrial oxidative phosphorylation by AICA riboside." Biochem J **404**(3): 499-507.
Gunasingh, M. J., J. E. Philip, et al. (2008). "Melatonin prevents amyloid protofibrillar induced oxidative imbalance and biogenic amine catabolism." Life Sci **83**(3-4): 96-102.
Guo, Y., J. Wang, et al. (2010). "Melatonin protects N2a against ischemia/reperfusion injury through autophagy enhancement." Journal of Huazhong University of Science and Technology -- Medical Sciences -- **30**(1): 1-7.
Gustavsson, A., M. Svensson, et al. (2011). "Cost of disorders of the brain in Europe 2010." Eur Neuropsychopharmacol **21**(10): 718-779.
Gutierrez-Cuesta, J., M. Tajes, et al. (2008). "Evaluation of potential pro-survival pathways regulated by melatonin in a murine senescence model." J Pineal Res **45**(4): 497-505.
Gwinn, D. M., D. B. Shackelford, et al. (2008). "AMPK phosphorylation of raptor mediates a metabolic checkpoint." Mol Cell **30**(2): 214-226.
Haas, R. H., F. Nasirian, et al. (1995). "Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease." Ann Neurol **37**(6): 714-722.
Halagappa, V. K., Z. Guo, et al. (2007). "Intermittent fasting and caloric restriction ameliorate age-related behavioral deficits in the triple-transgenic mouse model of Alzheimer's disease." Neurobiol Dis **26**(1): 212-220.
Halloran, J., S. A. Hussong, et al. (2012). "Chronic inhibition of mammalian target of rapamycin by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice." Neuroscience **223**: 102-113.
Hancock, C. R., D. H. Han, et al. (2011). "Does calorie restriction induce mitochondrial biogenesis? A reevaluation." Faseb J **25**(2): 785-791.

Hardeland, R. (2009). "Melatonin, Mitochondrial Electron Flux and Leakage: Recent Findings and Resolution of Contradictory Results." Advanced Studies in Biology **1**(5): 207-230.
Hardeland, R. (2009). "Melatonin: signaling mechanisms of a pleiotropic agent." Biofactors **35**(2): 183-192.
Hardeland, R. (2013). "Melatonin and the theories of aging: a critical appraisal of melatonin's role in antiaging mechanisms." J Pineal Res **55**(4): 325-356.
Hardie, D. G. (2011). "AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function." Genes Dev **25**(18): 1895-1908.
Hardy, J. A. and G. A. Higgins (1992). "Alzheimer's disease: the amyloid cascade hypothesis." Science **256**(5054): 184-185.
Harman, D. (1972). "The biologic clock: the mitochondria?" J Am Geriatr Soc **20**(4): 145-147.
Harrison, D. E., R. Strong, et al. (2009). "Rapamycin fed late in life extends lifespan in genetically heterogeneous mice." Nature **460**(7253): 392-395.
Hauptmann, N., J. Grimsby, et al. (1996). "The metabolism of tyramine by monoamine oxidase A/B causes oxidative damage to mitochondrial DNA." Arch Biochem Biophys **335**(2): 295-304.
Hauptmann, S., I. Scherping, et al. (2008). "Mitochondrial dysfunction: An early event in Alzheimer pathology accumulates with age in AD transgenic mice." Neurobiol Aging.
He, P., X. Ouyang, et al. (2013). "A novel melatonin agonist Neu-P11 facilitates memory performance and improves cognitive impairment in a rat model of Alzheimer' disease." Horm Behav **64**(1): 1-7.
He, Q., N. Song, et al. (2011). "Alpha-synuclein aggregation is involved in the toxicity induced by ferric iron to SK-N-SH neuroblastoma cells." J Neural Transm **118**(3): 397-406.
Hebert, A. S., K. E. Dittenhafer-Reed, et al. (2013). "Calorie restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome." Mol Cell **49**(1): 186-199.
Hederstedt, L. and L. Rutberg (1981). "Succinate dehydrogenase--a comparative review." Microbiol Rev **45**(4): 542-555.
Heitman, J., N. R. Movva, et al. (1991). "Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast." Science **253**(5022): 905-909.
Hepple, R. T., D. J. Baker, et al. (2006). "Caloric restriction protects mitochondrial function with aging in skeletal and cardiac muscles." Rejuvenation Res **9**(2): 219-222.
Herrmann, N., S. A. Chau, et al. (2011). "Current and emerging drug treatment options for Alzheimer's disease: a systematic review." Drugs **71**(15): 2031-2065.
Hirst, J. (2013). "Mitochondrial complex I." Annu Rev Biochem **82**: 551-575.
Hoppins, S., L. Lackner, et al. (2007). "The machines that divide and fuse mitochondria." Annu Rev Biochem **76**: 751-780.
Horowitz, M. P. and J. T. Greenamyre (2010). "Mitochondrial iron metabolism and its role in neurodegeneration." J Alzheimers Dis **20 Suppl 2**: S551-568.
Hossain, M. B., P. Ji, et al. (2009). "Poly(ADP-ribose) Polymerase 1 Interacts with Nuclear Respiratory Factor 1 (NRF-1) and Plays a Role in NRF-1 Transcriptional Regulation." J Biol Chem **284**(13): 8621-8632.
Houten, S. M., M. Chegary, et al. (2009). "Pyruvate dehydrogenase kinase 4 expression is synergistically induced by AMP-activated protein kinase and fatty acids." Cell Mol Life Sci **66**(7): 1283-1294.
Hroudova, J., N. Singh, et al. (2014). "Mitochondrial dysfunctions in neurodegenerative diseases: relevance to Alzheimer's disease." Biomed Res Int **2014**: 175062.

Huang, Y. and L. Mucke (2012). "Alzheimer mechanisms and therapeutic strategies." Cell **148**(6): 1204-1222.
Hunt, A. E., W. M. Al-Ghoul, et al. (2001). "Activation of MT(2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock." Am J Physiol Cell Physiol **280**(1): C110-118.
Imbesi, M., A. D. Arslan, et al. (2009). "The melatonin receptor MT1 is required for the differential regulatory actions of melatonin on neuronal 'clock' gene expression in striatal neurons in vitro." J Pineal Res **46**(1): 87-94.
International Parkinson Disease Genomics, C., M. A. Nalls, et al. (2011). "Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies." Lancet **377**(9766): 641-649.
Ionov, M., V. Burchell, et al. (2011). "Mechanism of neuroprotection of melatonin against beta-amyloid neurotoxicity." Neuroscience **180**: 229-237.
Itoh, K., N. Wakabayashi, et al. (1999). "Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain." Genes Dev **13**(1): 76-86.
Iwata, S., J. W. Lee, et al. (1998). "Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex." Science **281**(5373): 64-71.
Jager, S., C. Handschin, et al. (2007). "AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha." Proc Natl Acad Sci U S A **104**(29): 12017-12022.
Jeong, J. K., M. H. Moon, et al. (2012). "Melatonin-induced autophagy protects against human prion protein-mediated neurotoxicity." J Pineal Res **53**(2): 138-146.
Jimenez-Aranda, A., G. Fernandez-Vazquez, et al. (2013). "Melatonin induces browning of inguinal white adipose tissue in Zucker diabetic fatty rats." J Pineal Res **55**(4): 416-423.
Jin, X., C. von Gall, et al. (2003). "Targeted disruption of the mouse Mel(1b) melatonin receptor." Mol Cell Biol **23**(3): 1054-1060.
Johnson, A. A., A. S. Ray, et al. (2001). "Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase." J Biol Chem **276**(44): 40847-40857.
Jose, C., E. Hebert-Chatelain, et al. (2011). "AICAR inhibits cancer cell growth and triggers cell-type distinct effects on OXPHOS biogenesis, oxidative stress and Akt activation." Biochim Biophys Acta **1807**(6): 707-718.
Jung-Hynes, B., R. J. Reiter, et al. (2010). "Sirtuins, melatonin and circadian rhythms: building a bridge between aging and cancer." J Pineal Res **48**(1): 9-19.
Katayama, S. and Y. Mine (2007). "Antioxidative activity of amino acids on tissue oxidative stress in human intestinal epithelial cell model." J Agric Food Chem **55**(21): 8458-8464.
Kazachkova, N., A. Ramos, et al. (2013). "Mitochondrial DNA damage patterns and aging: revising the evidences for humans and mice." Aging Dis **4**(6): 337-350.
Keane, P. C., M. Kurzawa, et al. (2011). "Mitochondrial dysfunction in Parkinson's disease." Parkinsons Dis **2011**: 716871.
Keeney, P. M., J. Xie, et al. (2006). "Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled." J Neurosci **26**(19): 5256-5264.
Kelly, D. P. and R. C. Scarpulla (2004). "Transcriptional regulatory circuits controlling mitochondrial biogenesis and function." Genes Dev **18**(4): 357-368.

Kensler, T. W., N. Wakabayashi, et al. (2007). "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway." Annu Rev Pharmacol Toxicol **47**: 89-116.
Khan, M., Y. B. Im, et al. (2009). "Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact." J Neuroinflammation **6**: 32.
Khrapko, K. and J. Vijg (2007). "Mitochondrial DNA mutations and aging: a case closed?" Nat Genet **39**(4): 445-446.
Kim, J., Y. N. Cha, et al. (2010). "A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders." Mutat Res **690**(1-2): 12-23.
King, M. P. and G. Attardi (1989). "Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation." Science **246**(4929): 500-503.
Kinsley, L. and T. Siddique (1993). Amyotrophic Lateral Sclerosis Overview. GeneReviews(R). R. A. Pagon, M. P. Adam, H. H. Ardingeret al. Seattle (WA).
Kitada, T., S. Asakawa, et al. (1998). "Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism." Nature **392**(6676): 605-608.
Kittipongdaja, W., X. Wu, et al. (2015). "Rapamycin Suppresses Tumor Growth and Alters the Metabolic Phenotype in T-Cell Lymphoma." J Invest Dermatol.
Kladna, A., M. Marchlewicz, et al. (2015). "Reactivity of pyruvic acid and its derivatives towards reactive oxygen species." Luminescence.
Kolesar, J. E., A. Safdar, et al. (2014). "Defects in mitochondrial DNA replication and oxidative damage in muscle of mtDNA mutator mice." Free Radic Biol Med **75**: 241-251.
Komen, J. C. and D. R. Thorburn (2014). "Turn up the power - pharmacological activation of mitochondrial biogenesis in mouse models." Br J Pharmacol **171**(8): 1818-1836.
Korkmaz, A., R. J. Reiter, et al. (2009). "Melatonin: an established antioxidant worthy of use in clinical trials." Mol Med **15**(1-2): 43-50.
Kostka, M., T. Hogen, et al. (2008). "Single particle characterization of iron-induced pore-forming alpha-synuclein oligomers." J Biol Chem **283**(16): 10992-11003.
Kotler, M., C. Rodriguez, et al. (1998). "Melatonin increases gene expression for antioxidant enzymes in rat brain cortex." J Pineal Res **24**(2): 83-89.
Kowal, S. L., T. M. Dall, et al. (2013). "The current and projected economic burden of Parkinson's disease in the United States." Mov Disord **28**(3): 311-318.
Koyano, F. and N. Matsuda (2015). "Molecular mechanisms underlying PINK1 and Parkin catalyzed ubiquitylation of substrates on damaged mitochondria." Biochim Biophys Acta.
Kraytsberg, Y., E. Kudryavtseva, et al. (2006). "Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons." Nat Genet **38**(5): 518-520.
Krebs, H. A. and W. A. Johnson (1980). "The role of citric acid in intermediate metabolism in animal tissues." FEBS Lett **117 Suppl**: K1-10.
Kujoth, G. C., A. Hiona, et al. (2005). "Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging." Science **309**(5733): 481-484.
Kukat, A., D. Edgar, et al. (2011). "Random mtDNA mutations modulate proliferation capacity in mouse embryonic fibroblasts." Biochem Biophys Res Commun **409**(3): 394-399.
Kumar, M. J., D. G. Nicholls, et al. (2003). "Oxidative alpha-ketoglutarate dehydrogenase inhibition via subtle elevations in monoamine oxidase B levels results in loss of spare respiratory capacity: implications for Parkinson's disease." J Biol Chem **278**(47): 46432-46439.

Kwon, K. J., J. N. Kim, et al. (2011). "Melatonin synergistically increases resveratrol-induced heme oxygenase-1 expression through the inhibition of ubiquitin-dependent proteasome pathway: a possible role in neuroprotection." J Pineal Res **50**(2): 110-123.
La Morgia, C., L. Caporali, et al. (2014). "Association of the mtDNA m.4171C>A/MT-ND1 mutation with both optic neuropathy and bilateral brainstem lesions." BMC Neurol **14**: 116.
LaFerla, F. M., K. N. Green, et al. (2007). "Intracellular amyloid-beta in Alzheimer's disease." Nat Rev Neurosci **8**(7): 499-509.
Lahiri, D. K., Y. W. Ge, et al. (2004). "Age-related changes in serum melatonin in mice: higher levels of combined melatonin and 6-hydroxymelatonin sulfate in the cerebral cortex than serum, heart, liver and kidney tissues." J Pineal Res **36**(4): 217-223.
Lahiri, S., P. Singh, et al. (2009). "Melatonin protects against experimental reflux esophagitis." J Pineal Res **46**(2): 207-213.
Lambert, A. J., B. Wang, et al. (2004). "The effect of aging and caloric restriction on mitochondrial protein density and oxygen consumption." Exp Gerontol **39**(3): 289-295.
Laplante, M. and D. M. Sabatini (2012). "mTOR signaling in growth control and disease." Cell **149**(2): 274-293.
Larson, J., R. E. Jessen, et al. (2006). "Impaired hippocampal long-term potentiation in melatonin MT2 receptor-deficient mice." Neurosci Lett **393**(1): 23-26.
Larsson, N. G. (2010). "Somatic mitochondrial DNA mutations in mammalian aging." Annu Rev Biochem **79**: 683-706.
Lasagna-Reeves, C. A., D. L. Castillo-Carranza, et al. (2011). "Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice." Mol Neurodegener **6**: 39.
Lee, C. H., J. H. Choi, et al. (2010). "MT2 melatonin receptor immunoreactivity in neurons is very high in the aged hippocampal formation in gerbils." Cell Mol Neurobiol **30**(2): 255-263.
Lee, E. W., Y. Lai, et al. (2006). "Identification of the mitochondrial targeting signal of the human equilibrative nucleoside transporter 1 (hENT1): implications for interspecies differences in mitochondrial toxicity of fialuridine." J Biol Chem **281**(24): 16700-16706.
Lee, J., K. B. Seroogy, et al. (2002). "Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice." J Neurochem **80**(3): 539-547.
Lee, J. M. and J. A. Johnson (2004). "An important role of Nrf2-ARE pathway in the cellular defense mechanism." J Biochem Mol Biol **37**(2): 139-143.
Lei, Y. (2013). "Generation and culture of mouse embryonic fibroblasts." Methods Mol Biol **1031**: 59-64.
Lemoine, P. and N. Zisapel (2012). "Prolonged-release formulation of melatonin (Circadin) for the treatment of insomnia." Expert Opin Pharmacother **13**(6): 895-905.
Lerner, C., A. Bitto, et al. (2013). "Reduced mammalian target of rapamycin activity facilitates mitochondrial retrograde signaling and increases life span in normal human fibroblasts." Aging Cell **12**(6): 966-977.
Levenson, C. W. and N. J. Rich (2007). "Eat less, live longer? New insights into the role of caloric restriction in the brain." Nutr Rev **65**(9): 412-415.
Levin, J., T. Hogen, et al. (2011). "Generation of ferric iron links oxidative stress to alpha-synuclein oligomer formation." J Parkinsons Dis **1**(2): 205-216.

Levoye, A., J. Dam, et al. (2006). "The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization." Embo J **25**(13): 3012-3023.
Lewis, J., E. McGowan, et al. (2000). "Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein." Nat Genet **25**(4): 402-405.
Li, J., V. N. Uversky, et al. (2001). "Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human alpha-synuclein." Biochemistry **40**(38): 11604-11613.
Li, W. W., R. Yang, et al. (2007). "Localization of alpha-synuclein to mitochondria within midbrain of mice." Neuroreport **18**(15): 1543-1546.
Lill, R. and U. Muhlenhoff (2008). "Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases." Annu Rev Biochem **77**: 669-700.
Limon-Pacheco, J. H. and M. E. Gonsebatt (2010). "The glutathione system and its regulation by neurohormone melatonin in the central nervous system." Cent Nerv Syst Agents Med Chem **10**(4): 287-297.
Lin, L., Q. X. Huang, et al. (2013). "Melatonin in Alzheimer's disease." Int J Mol Sci **14**(7): 14575-14593.
Liu, C., D. R. Weaver, et al. (1997). "Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock." Neuron **19**(1): 91-102.
Liu, L., C. Peritore, et al. (2015). "Protective role of SIRT5 against motor deficit and dopaminergic degeneration in MPTP-induced mice model of Parkinson's disease." Behav Brain Res **281**: 215-221.
Liu, R. Y., J. N. Zhou, et al. (1999). "Decreased melatonin levels in postmortem cerebrospinal fluid in relation to aging, Alzheimer's disease, and apolipoprotein E-epsilon4/4 genotype." J Clin Endocrinol Metab **84**(1): 323-327.
Liu, Y. and X. J. Chen (2013). "Adenine nucleotide translocase, mitochondrial stress, and degenerative cell death." Oxid Med Cell Longev **2013**: 146860.
Liu, Y., L. Zhang, et al. (2012). "Exogenous melatonin modulates apoptosis in the mouse brain induced by high-LET carbon ion irradiation." J Pineal Res **52**(1): 47-56.
Liu, Y. J., F. T. Meng, et al. (2012). "Apolipoprotein E influences melatonin biosynthesis by regulating NAT and MAOA expression in C6 cells." J Pineal Res **52**(4): 397-402.
Loeb, L. A., D. C. Wallace, et al. (2005). "The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations." Proc Natl Acad Sci U S A **102**(52): 18769-18770.
Lu, C. L., L. Qin, et al. (2015). "Tumor Cells Switch to Mitochondrial Oxidative Phosphorylation under Radiation via mTOR-Mediated Hexokinase II Inhibition - A Warburg-Reversing Effect." PLoS One **10**(3): e0121046.
Lushchak, O. V., M. Piroddi, et al. (2014). "Aconitase post-translational modification as a key in linkage between Krebs cycle, iron homeostasis, redox signaling, and metabolism of reactive oxygen species." Redox Rep **19**(1): 8-15.
Luth, E. S., T. Bartels, et al. (2015). "Purification of alpha-synuclein from human brain reveals an instability of endogenous multimers as the protein approaches purity." Biochemistry **54**(2): 279-292.
Luth, E. S., I. G. Stavrovskaya, et al. (2014). "Soluble, prefibrillar alpha-synuclein oligomers promote complex I-dependent, Ca2+-induced mitochondrial dysfunction." J Biol Chem **289**(31): 21490-21507.

MacKenzie, E. L., K. Iwasaki, et al. (2008). "Intracellular iron transport and storage: from molecular mechanisms to health implications." Antioxid Redox Signal **10**(6): 997-1030.
Magnuson, B., B. Ekim, et al. (2012). "Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks." Biochem J **441**(1): 1-21.
Mahlberg, R., D. Kunz, et al. (2004). "Melatonin treatment of day-night rhythm disturbances and sundowning in Alzheimer disease: an open-label pilot study using actigraphy." J Clin Psychopharmacol **24**(4): 456-459.
Mahley, R. W. (1988). "Apolipoprotein E: cholesterol transport protein with expanding role in cell biology." Science **240**(4852): 622-630.
Mallajosyula, J. K., D. Kaur, et al. (2008). "MAO-B elevation in mouse brain astrocytes results in Parkinson's pathology." PLoS One **3**(2): e1616.
Manczak, M., T. S. Anekonda, et al. (2006). "Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression." Hum Mol Genet **15**(9): 1437-1449.
Manczak, M., M. J. Calkins, et al. (2011). "Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: implications for neuronal damage." Hum Mol Genet **20**(13): 2495-2509.
Manczak, M., P. Mao, et al. (2010). "Mitochondria-targeted antioxidants protect against amyloid-beta toxicity in Alzheimer's disease neurons." J Alzheimers Dis **20 Suppl 2**(2): S609-631.
Manczak, M. and P. H. Reddy (2012). "Abnormal interaction of VDAC1 with amyloid beta and phosphorylated tau causes mitochondrial dysfunction in Alzheimer's disease." Hum Mol Genet **21**(23): 5131-5146.
Maraganore, D. M., M. de Andrade, et al. (2006). "Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease." JAMA **296**(6): 661-670.
Marella, M., B. B. Seo, et al. (2009). "Parkinson's disease and mitochondrial complex I: a perspective on the Ndi1 therapy." J Bioenerg Biomembr **41**(6): 493-497.
Maries, E., B. Dass, et al. (2003). "The role of alpha-synuclein in Parkinson's disease: insights from animal models." Nat Rev Neurosci **4**(9): 727-738.
Markham, A., I. Cameron, et al. (2004). "BDNF increases rat brain mitochondrial respiratory coupling at complex I, but not complex II." Eur J Neurosci **20**(5): 1189-1196.
Marques, O. and T. F. Outeiro (2012). "Alpha-synuclein: from secretion to dysfunction and death." Cell Death Dis **3**: e350.
Marsin, A. S., C. Bouzin, et al. (2002). "The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase." J Biol Chem **277**(34): 30778-30783.
Martin, L. J., Y. Pan, et al. (2006). "Parkinson's disease alpha-synuclein transgenic mice develop neuronal mitochondrial degeneration and cell death." J Neurosci **26**(1): 41-50.
Martin, M., M. Macias, et al. (2000). "Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo." J Pineal Res **28**(4): 242-248.
Martinez-Cano, E., G. Ortiz-Genaro, et al. (2005). "[Functional disorders of FOF1-ATPase in submitochondrial particles obtained from platelets of patients with a diagnosis of probable Alzheimer's disease]." Rev Neurol **40**(2): 81-85.

Martinez-Martin, N., A. Blas-Garcia, et al. (2012). "Metabolomics of the effect of AMPK activation by AICAR on human umbilical vein endothelial cells." Int J Mol Med **29**(1): 88-94.
Martinez, T. N. and J. T. Greenamyre (2012). "Toxin models of mitochondrial dysfunction in Parkinson's disease." Antioxid Redox Signal **16**(9): 920-934.
Marttila, R. J., H. Lorentz, et al. (1988). "Oxygen toxicity protecting enzymes in Parkinson's disease. Increase of superoxide dismutase-like activity in the substantia nigra and basal nucleus." J Neurol Sci **86**(2-3): 321-331.
Marzetti, E., R. Calvani, et al. (2013). "Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials." Int J Biochem Cell Biol **45**(10): 2288-2301.
Matsubara, E., T. Bryant-Thomas, et al. (2003). "Melatonin increases survival and inhibits oxidative and amyloid pathology in a transgenic model of Alzheimer's disease." J Neurochem **85**(5): 1101-1108.
Maurizi, C. P. (1997). "Loss of intraventricular fluid melatonin can explain the neuropathology of Alzheimer's disease." Med Hypotheses **49**(2): 153-158.
Maurizi, C. P. (2001). "Alzheimer's disease: roles for mitochondrial damage, the hydroxyl radical, and cerebrospinal fluid deficiency of melatonin." Med Hypotheses **57**(2): 156-160.
Mayeux, R. and Y. Stern (2012). "Epidemiology of Alzheimer disease." Cold Spring Harb Perspect Med **2**(8).
Mayo, J. C., R. M. Sainz, et al. (2005). "Melatonin and Parkinson's disease." Endocrine **27**(2): 169-178.
Mbefo, M. K., M. B. Fares, et al. (2015). "Parkinson Disease Mutant E46K Enhances alpha-Synuclein Phosphorylation in Mammalian Cell Lines, in Yeast, and in Vivo." J Biol Chem **290**(15): 9412-9427.
McDonald, S. A., S. L. Preston, et al. (2006). "Clonal expansion in the human gut: mitochondrial DNA mutations show us the way." Cell Cycle **5**(8): 808-811.
McKenna, J. T., M. A. Christie, et al. (2012). "Chronic ramelteon treatment in a mouse model of Alzheimer's disease." Arch Ital Biol **150**(1): 5-14.
McKenzie, D., E. Bua, et al. (2002). "Mitochondrial DNA deletion mutations: a causal role in sarcopenia." Eur J Biochem **269**(8): 2010-2015.
Meek, P. D., K. McKeithan, et al. (1998). "Economic considerations in Alzheimer's disease." Pharmacotherapy **18**(2 Pt 2): 68-73; discussion 79-82.
Meijer, A. J. and P. Codogno (2007). "AMP-activated protein kinase and autophagy." Autophagy **3**(3): 238-240.
Meley, D., C. Bauvy, et al. (2006). "AMP-activated protein kinase and the regulation of autophagic proteolysis." J Biol Chem **281**(46): 34870-34879.
Melov, S., G. J. Lithgow, et al. (1995). "Increased frequency of deletions in the mitochondrial genome with age of Caenorhabditis elegans." Nucleic Acids Res **23**(8): 1419-1425.
Mendes, C., A. M. Lopes, et al. (2013). "Adaptations of the aging animal to exercise: role of daily supplementation with melatonin." J Pineal Res **55**(3): 229-239.
Mirra, S. S., J. R. Murrell, et al. (1999). "Tau pathology in a family with dementia and a P301L mutation in tau." J Neuropathol Exp Neurol **58**(4): 335-345.

Mishima, K., M. Okawa, et al. (2000). "Supplementary administration of artificial bright light and melatonin as potent treatment for disorganized circadian rest-activity and dysfunctional autonomic and neuroendocrine systems in institutionalized demented elderly persons." Chronobiol Int **17**(3): 419-432.
Moraes, C. T., V. Anderson, et al. (2013). "Translational research in primary mitochondrial diseases: challenges and opportunities." Mitochondrion **13**(6): 945-952.
Moreira, P. I., C. Carvalho, et al. (2010). "Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology." Biochim Biophys Acta **1802**(1): 2-10.
Moretto, A. and C. Colosio (2013). "The role of pesticide exposure in the genesis of Parkinson's disease: epidemiological studies and experimental data." Toxicology **307**: 24-34.
Morgan, T. E., A. M. Wong, et al. (2007). "Anti-inflammatory mechanisms of dietary restriction in slowing aging processes." Interdiscip Top Gerontol **35**: 83-97.
Morita, M., S. P. Gravel, et al. (2013). "mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation." Cell Metab **18**(5): 698-711.
Morita, M., S. P. Gravel, et al. (2015). "mTOR coordinates protein synthesis, mitochondrial activity and proliferation." Cell Cycle **14**(4): 473-480.
Mosconi, L., M. de Leon, et al. (2011). "Reduced mitochondria cytochrome oxidase activity in adult children of mothers with Alzheimer's disease." J Alzheimers Dis **27**(3): 483-490.
Mouton, P. R., M. E. Chachich, et al. (2009). "Caloric restriction attenuates amyloid deposition in middle-aged dtg APP/PS1 mice." Neurosci Lett **464**(3): 184-187.
Munguia, M. E., T. Govezensky, et al. (2006). "Identification of amyloid-beta 1-42 binding protein fragments by screening of a human brain cDNA library." Neurosci Lett **397**(1-2): 79-82.
Muoio, V., P. B. Persson, et al. (2014). "The neurovascular unit - concept review." Acta Physiol (Oxf) **210**(4): 790-798.
Murphy, M. P. (2009). "How mitochondria produce reactive oxygen species." Biochem J **417**(1): 1-13.
Musshoff, U., D. Riewenherm, et al. (2002). "Melatonin receptors in rat hippocampus: molecular and functional investigations." Hippocampus **12**(2): 165-173.
Naiki, H., F. Gejyo, et al. (1997). "Concentration-dependent inhibitory effects of apolipoprotein E on Alzheimer's beta-amyloid fibril formation in vitro." Biochemistry **36**(20): 6243-6250.
Nakahata, Y., M. Kaluzova, et al. (2008). "The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control." Cell **134**(2): 329-340.
Nakahata, Y., S. Sahar, et al. (2009). "Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1." Science **324**(5927): 654-657.
Nakamaru-Ogiso, E., H. Han, et al. (2010). "The ND2 subunit is labeled by a photoaffinity analogue of asimicin, a potent complex I inhibitor." FEBS Lett **584**(5): 883-888.
Nakamura, A., K. Kawakami, et al. (2013). "Dietary restriction increases protein acetylation in the livers of aged rats." Gerontology **59**(6): 542-548.
Napoli, E., F. Taroni, et al. (2006). "Frataxin, iron-sulfur clusters, heme, ROS, and aging." Antioxid Redox Signal **8**(3-4): 506-516.
Navarro, A. and A. Boveris (2010). "Brain mitochondrial dysfunction in aging, neurodegeneration, and Parkinson's disease." Front Aging Neurosci **2**.

Negi, G., A. Kumar, et al. (2011). "Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-kappaB and Nrf2 cascades." J Pineal Res **50**(2): 124-131.
Neuwelt, E. A., B. Bauer, et al. (2011). "Engaging neuroscience to advance translational research in brain barrier biology." Nat Rev Neurosci **12**(3): 169-182.
Nguyen, T., P. Nioi, et al. (2009). "The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress." J Biol Chem **284**(20): 13291-13295.
Nisoli, E., C. Tonello, et al. (2005). "Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS." Science **310**(5746): 314-317.
Nistico, R., B. Mehdawy, et al. (2011). "Paraquat- and rotenone-induced models of Parkinson's disease." Int J Immunopathol Pharmacol **24**(2): 313-322.
Nopparat, C., J. E. Porter, et al. (2010). "The mechanism for the neuroprotective effect of melatonin against methamphetamine-induced autophagy." J Pineal Res **49**(4): 382-389.
O'Neill, J. S., E. S. Maywood, et al. (2008). "cAMP-dependent signaling as a core component of the mammalian circadian pacemaker." Science **320**(5878): 949-953.
Oddo, S., A. Caccamo, et al. (2003). "Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction." Neuron **39**(3): 409-421.
Ohgami, R. S., D. R. Campagna, et al. (2005). "Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells." Nat Genet **37**(11): 1264-1269.
Olanow, C. W. and P. Brundin (2013). "Parkinson's disease and alpha synuclein: is Parkinson's disease a prion-like disorder?" Mov Disord **28**(1): 31-40.
Olcese, J. M., C. Cao, et al. (2009). "Protection against cognitive deficits and markers of neurodegeneration by long-term oral administration of melatonin in a transgenic model of Alzheimer disease." J Pineal Res **47**(1): 82-96.
Olmos, Y., I. Valle, et al. (2009). "Mutual dependence of Foxo3a and PGC-1alpha in the induction of oxidative stress genes." J Biol Chem **284**(21): 14476-14484.
Orrenius, S. and B. Zhivotovsky (2005). "Cardiolipin oxidation sets cytochrome c free." Nat Chem Biol **1**(4): 188-189.
Osiewacz, H. D. (2010). "Role of mitochondria in aging and age-related disease." Exp Gerontol **45**(7-8): 465.
Ostrerova-Golts, N., L. Petrucelli, et al. (2000). "The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity." J Neurosci **20**(16): 6048-6054.
Ott, M. O. and S. L. Bullock (2001). "A gene trap insertion reveals that amyloid precursor protein expression is a very early event in murine embryogenesis." Dev Genes Evol **211**(7): 355-357.
Ozturk, G., K. G. Akbulut, et al. (2012). "Age-related changes in the rat brain mitochondrial antioxidative enzyme ratios: modulation by melatonin." Exp Gerontol **47**(9): 706-711.
Pacheco, C. R., C. N. Morales, et al. (2015). "Extracellular alpha-synuclein alters synaptic transmission in brain neurons by perforating the neuronal plasma membrane." J Neurochem **132**(6): 731-741.
Pandi-Perumal, S. R., A. S. BaHammam, et al. (2013). "Melatonin antioxidative defense: therapeutical implications for aging and neurodegenerative processes." Neurotox Res **23**(3): 267-300.

Papa, S. and D. De Rasmo (2013). "Complex I deficiencies in neurological disorders." Trends Mol Med **19**(1): 61-69.
Papa, S., S. Scacco, et al. (2010). "cAMP-dependent protein kinase regulates post-translational processing and expression of complex I subunits in mammalian cells." Biochim Biophys Acta **1797**(6-7): 649-658.
Pappolla, M., P. Bozner, et al. (1998). "Inhibition of Alzheimer beta-fibrillogenesis by melatonin." J Biol Chem **273**(13): 7185-7188.
Pappolla, M. A., Y. J. Chyan, et al. (1999). "Alzheimer beta protein mediated oxidative damage of mitochondrial DNA: prevention by melatonin." J Pineal Res **27**(4): 226-229.
Pappolla, M. A., M. Sos, et al. (1997). "Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide." J Neurosci **17**(5): 1683-1690.
Parihar, M. S., A. Parihar, et al. (2009). "Alpha-synuclein overexpression and aggregation exacerbates impairment of mitochondrial functions by augmenting oxidative stress in human neuroblastoma cells." Int J Biochem Cell Biol **41**(10): 2015-2024.
Park, C. B. and N. G. Larsson (2011). "Mitochondrial DNA mutations in disease and aging." J Cell Biol **193**(5): 809-818.
Parker, W. D., Jr. (1991). "Cytochrome oxidase deficiency in Alzheimer's disease." Ann N Y Acad Sci **640**: 59-64.
Parker, W. D., Jr., S. J. Boyson, et al. (1990). "Evidence for a defect in NADH: ubiquinone oxidoreductase (complex I) in Huntington's disease." Neurology **40**(8): 1231-1234.
Parker, W. D., Jr., S. J. Boyson, et al. (1989). "Abnormalities of the electron transport chain in idiopathic Parkinson's disease." Ann Neurol **26**(6): 719-723.
Parone, P. A., S. Da Cruz, et al. (2013). "Enhancing mitochondrial calcium buffering capacity reduces aggregation of misfolded SOD1 and motor neuron cell death without extending survival in mouse models of inherited amyotrophic lateral sclerosis." J Neurosci **33**(11): 4657-4671.
Parrella, E., T. Maxim, et al. (2013). "Protein restriction cycles reduce IGF-1 and phosphorylated Tau, and improve behavioral performance in an Alzheimer's disease mouse model." Aging Cell **12**(2): 257-268.
Peng, C. X., J. Hu, et al. (2013). "Disease-modified glycogen synthase kinase-3beta intervention by melatonin arrests the pathology and memory deficits in an Alzheimer's animal model." Neurobiol Aging **34**(6): 1555-1563.
Pereira, C., M. S. Santos, et al. (1998). "Mitochondrial function impairment induced by amyloid beta-peptide on PC12 cells." Neuroreport **9**(8): 1749-1755.
Perfeito, R., D. F. Lazaro, et al. (2014). "Linking alpha-synuclein phosphorylation to reactive oxygen species formation and mitochondrial dysfunction in SH-SY5Y cells." Mol Cell Neurosci **62**: 51-59.
Perry, E. K., R. H. Perry, et al. (1980). "Coenzyme A-acetylating enzymes in Alzheimer's disease: possible cholinergic 'compartment' of pyruvate dehydrogenase." Neurosci Lett **18**(1): 105-110.
Piccoli, C., S. Scacco, et al. (2006). "cAMP controls oxygen metabolism in mammalian cells." FEBS Lett **580**(18): 4539-4543.
Pinheiro, C. H., L. R. Silveira, et al. (2010). "Regulation of glycolysis and expression of glucose metabolism-related genes by reactive oxygen species in contracting skeletal muscle cells." Free Radic Biol Med **48**(7): 953-960.

Poeggeler, B., L. Miravalle, et al. (2001). "Melatonin reverses the profibrillogenic activity of apolipoprotein E4 on the Alzheimer amyloid Abeta peptide." Biochemistry **40**(49): 14995-15001.
Poeggeler, B., K. Sambamurti, et al. (2010). "A novel endogenous indole protects rodent mitochondria and extends rotifer lifespan." PLoS One **5**(4): e10206.
Poirel, V. J., V. Boggio, et al. (2003). "Contrary to other non-photic cues, acute melatonin injection does not induce immediate changes of clock gene mRNA expression in the rat suprachiasmatic nuclei." Neuroscience **120**(3): 745-755.
Poirier, G. L., E. Amin, et al. (2011). "Early-onset dysfunction of retrosplenial cortex precedes overt amyloid plaque formation in Tg2576 mice." Neuroscience **174**: 71-83.
Polak, P., N. Cybulski, et al. (2008). "Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration." Cell Metab **8**(5): 399-410.
Poon, A. M. and S. F. Pang (1992). "2[125I]iodomelatonin binding sites in spleens of guinea pigs." Life Sci **50**(22): 1719-1726.
Prolla, T. A. and J. M. Denu (2014). "NAD+ deficiency in age-related mitochondrial dysfunction." Cell Metab **19**(2): 178-180.
Protter, D., C. Lang, et al. (2012). "alphaSynuclein and Mitochondrial Dysfunction: A Pathogenic Partnership in Parkinson's Disease?" Parkinsons Dis **2012**: 829207.
Puccio, H., M. Anheim, et al. (2014). "Pathophysiogical and therapeutic progress in Friedreich ataxia." Rev Neurol (Paris) **170**(5): 355-365.
Puigserver, P., Z. Wu, et al. (1998). "A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis." Cell **92**(6): 829-839.
Quinn, J., D. Kulhanek, et al. (2005). "Chronic melatonin therapy fails to alter amyloid burden or oxidative damage in old Tg2576 mice: implications for clinical trials." Brain Res **1037**(1-2): 209-213.
Quintanilla, R. A. and G. V. Johnson (2009). "Role of mitochondrial dysfunction in the pathogenesis of Huntington's disease." Brain Res Bull **80**(4-5): 242-247.
Quintas, A., A. J. de Solis, et al. (2012). "Age-associated decrease of SIRT1 expression in rat hippocampus: prevention by late onset caloric restriction." Exp Gerontol **47**(2): 198-201.
Radunovic, A., W. G. Porto, et al. (1997). "Increased mitochondrial superoxide dismutase activity in Parkinson's disease but not amyotrophic lateral sclerosis motor cortex." Neurosci Lett **239**(2-3): 105-108.
Rahman, M., N. K. Nirala, et al. (2014). "Drosophila Sirt2/mammalian SIRT3 deacetylates ATP synthase beta and regulates complex V activity." J Cell Biol **206**(2): 289-305.
Ramanathan, A. and S. L. Schreiber (2009). "Direct control of mitochondrial function by mTOR." Proc Natl Acad Sci U S A **106**(52): 22229-22232.
Ramsey, K. M., J. Yoshino, et al. (2009). "Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis." Science **324**(5927): 651-654.
Reddy, P. H., S. McWeeney, et al. (2004). "Gene expression profiles of transcripts in amyloid precursor protein transgenic mice: up-regulation of mitochondrial metabolism and apoptotic genes is an early cellular change in Alzheimer's disease." Hum Mol Genet **13**(12): 1225-1240.
Reiter, R. J., D. X. Tan, et al. (2010). "Melatonin: a multitasking molecule." Prog Brain Res **181**: 127-151.
Ren, Y., H. Jiang, et al. (2011). "Parkin degrades estrogen-related receptors to limit the expression of monoamine oxidases." Hum Mol Genet **20**(6): 1074-1083.

Reznick, R. M., H. Zong, et al. (2007). "Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis." Cell Metab **5**(2): 151-156.
Rezzani, R., L. F. Rodella, et al. (2006). "Beneficial effects of melatonin in protecting against cyclosporine A-induced cardiotoxicity are receptor mediated." J Pineal Res **41**(3): 288-295.
Rhein, V., X. Song, et al. (2009). "Amyloid-beta and tau synergistically impair the oxidative phosphorylation system in triple transgenic Alzheimer's disease mice." Proc Natl Acad Sci U S A **106**(47): 20057-20062.
Romano, A. D., E. Greco, et al. (2014). "Bioenergetics and mitochondrial dysfunction in aging: recent insights for a therapeutical approach." Curr Pharm Des **20**(18): 2978-2992.
Rosales-Corral, S., D. Acuna-Castroviejo, et al. (2012). "Accumulation of exogenous amyloid-beta peptide in hippocampal mitochondria causes their dysfunction: a protective role for melatonin." Oxid Med Cell Longev **2012**: 843649.
Rosales-Corral, S. A., D. Acuna-Castroviejo, et al. (2012). "Alzheimer's disease: pathological mechanisms and the beneficial role of melatonin." J Pineal Res **52**(2): 167-202.
Rouslin, W. (1983). "Mitochondrial complexes I, II, III, IV, and V in myocardial ischemia and autolysis." Am J Physiol **244**(6): H743-748.
Saleem, A., A. Safdar, et al. (2015). "Polymerase gamma mutator mice rely on increased glycolytic flux for energy production." Mitochondrion **21**: 19-26.
Sanchez-Hidalgo, M., J. M. Guerrero Montavez, et al. (2009). "Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging." J Pineal Res **46**(1): 29-35.
Sanchez, A. M., A. Csibi, et al. (2012). "AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1." J Cell Biochem **113**(2): 695-710.
Sangiorgi, S., M. Mochi, et al. (1994). "Abnormal platelet mitochondrial function in patients affected by migraine with and without aura." Cephalalgia **14**(1): 21-23.
Santacruz, K., J. Lewis, et al. (2005). "Tau suppression in a neurodegenerative mouse model improves memory function." Science **309**(5733): 476-481.
Santiago, J. A., C. R. Scherzer, et al. (2014). "Network analysis identifies SOD2 mRNA as a potential biomarker for Parkinson's disease." PLoS One **9**(10): e109042.
Santos, R. X., S. C. Correia, et al. (2010). "A synergistic dysfunction of mitochondrial fission/fusion dynamics and mitophagy in Alzheimer's disease." J Alzheimers Dis **20 Suppl 2**: S401-412.
Sanz, A., P. Caro, et al. (2005). "Dietary restriction at old age lowers mitochondrial oxygen radical production and leak at complex I and oxidative DNA damage in rat brain." J Bioenerg Biomembr **37**(2): 83-90.
Satpute, R., V. Lomash, et al. (2013). "Neuroprotective effects of alpha-ketoglutarate and ethyl pyruvate against motor dysfunction and oxidative changes caused by repeated 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine exposure in mice." Hum Exp Toxicol **32**(7): 747-758.
Savaskan, E., M. A. Ayoub, et al. (2005). "Reduced hippocampal MT2 melatonin receptor expression in Alzheimer's disease." J Pineal Res **38**(1): 10-16.
Savaskan, E., R. Jockers, et al. (2007). "The MT2 melatonin receptor subtype is present in human retina and decreases in Alzheimer's disease." Curr Alzheimer Res **4**(1): 47-51.

Savaskan, E., G. Olivieri, et al. (2002). "Increased melatonin 1a-receptor immunoreactivity in the hippocampus of Alzheimer's disease patients." J Pineal Res **32**(1): 59-62.
Scarpulla, R. C. (1997). "Nuclear control of respiratory chain expression in mammalian cells." J Bioenerg Biomembr **29**(2): 109-119.
Schagger, H. and T. G. Ohm (1995). "Human diseases with defects in oxidative phosphorylation. 2. F1F0 ATP-synthase defects in Alzheimer disease revealed by blue native polyacrylamide gel electrophoresis." Eur J Biochem **227**(3): 916-921.
Scheper, W., D. A. Nijholt, et al. (2011). "The unfolded protein response and proteostasis in Alzheimer disease: preferential activation of autophagy by endoplasmic reticulum stress." Autophagy **7**(8): 910-911.
Schieke, S. M., D. Phillips, et al. (2006). "The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity." Journal of Biological Chemistry **281**(37): 27643-27652.
Schieke, S. M., D. Phillips, et al. (2006). "The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity." J Biol Chem **281**(37): 27643-27652.
Schon, E. A., S. DiMauro, et al. (2012). "Human mitochondrial DNA: roles of inherited and somatic mutations." Nat Rev Genet **13**(12): 878-890.
Schon, E. A. and R. W. Gilkerson (2010). "Functional complementation of mitochondrial DNAs: mobilizing mitochondrial genetics against dysfunction." Biochim Biophys Acta **1800**(3): 245-249.
Schreiber, K. H., D. Ortiz, et al. (2015). "Rapamycin-mediated mTORC2 inhibition is determined by the relative expression of FK506-binding proteins." Aging Cell **14**(2): 265-273.
Selak, M. A., S. M. Armour, et al. (2005). "Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase." Cancer Cell **7**(1): 77-85.
Selak, M. A., E. Lyver, et al. (2011). "Blood cells from Friedreich ataxia patients harbor frataxin deficiency without a loss of mitochondrial function." Mitochondrion **11**(2): 342-350.
Sen, S. and A. B. West (2009). "The therapeutic potential of LRRK2 and alpha-synuclein in Parkinson's disease." Antioxid Redox Signal **11**(9): 2167-2187.
Sergeant, N., A. Wattez, et al. (2003). "Association of ATP synthase alpha-chain with neurofibrillary degeneration in Alzheimer's disease." Neuroscience **117**(2): 293-303.
Shabalina, I. G., T. V. Kramarova, et al. (2006). "Carboxyatractyloside effects on brown-fat mitochondria imply that the adenine nucleotide translocator isoforms ANT1 and ANT2 may be responsible for basal and fatty-acid-induced uncoupling respectively." Biochem J **399**(3): 405-414.
Shabalina, I. G., L. Landreh, et al. (2015). "Leydig cell steroidogenesis unexpectedly escapes mitochondrial dysfunction in prematurely aging mice." Faseb J.
Sharman, E. H., K. G. Sharman, et al. (2004). "Age-related changes in murine CNS mRNA gene expression are modulated by dietary melatonin." J Pineal Res **36**(3): 165-170.
Shaw, G. C., J. J. Cope, et al. (2006). "Mitoferrin is essential for erythroid iron assimilation." Nature **440**(7080): 96-100.
Sheftel, A., O. Stehling, et al. (2010). "Iron-sulfur proteins in health and disease." Trends Endocrinol Metab **21**(5): 302-314.
Sheftel, A. D., A. S. Zhang, et al. (2007). "Direct interorganellar transfer of iron from endosome to mitochondrion." Blood **110**(1): 125-132.

Sheng, B., X. Wang, et al. (2012). "Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease." J Neurochem **120**(3): 419-429.
Shi, P., J. Gal, et al. (2010). "Mitochondrial dysfunction in amyotrophic lateral sclerosis." Biochim Biophys Acta **1802**(1): 45-51.
Shi, P., Y. Wei, et al. (2010). "Mitochondrial dysfunction is a converging point of multiple pathological pathways in amyotrophic lateral sclerosis." J Alzheimers Dis **20 Suppl 2**: S311-324.
Shimoda-Matsubayashi, S., T. Hattori, et al. (1997). "Mn SOD activity and protein in a patient with chromosome 6-linked autosomal recessive parkinsonism in comparison with Parkinson's disease and control." Neurology **49**(5): 1257-1262.
Shoesmith, C. L., K. Findlater, et al. (2007). "Prognosis of amyotrophic lateral sclerosis with respiratory onset." J Neurol Neurosurg Psychiatry **78**(6): 629-631.
Siddiqui, A., S. J. Chinta, et al. (2012). "Selective binding of nuclear alpha-synuclein to the PGC1alpha promoter under conditions of oxidative stress may contribute to losses in mitochondrial function: implications for Parkinson's disease." Free Radic Biol Med **53**(4): 993-1003.
Siddiqui, A., I. Hanson, et al. (2012). "Mao-B elevation decreases parkin's ability to efficiently clear damaged mitochondria: protective effects of rapamycin." Free Radic Res **46**(8): 1011-1018.
Sinclair, L., V. Lewis, et al. (2013). "Cytosolic caspases mediate mislocalised SOD2 depletion in an in vitro model of chronic prion infection." Dis Model Mech **6**(4): 952-963.
Singh, R., D. Lakhanpal, et al. (2012). "Late-onset intermittent fasting dietary restriction as a potential intervention to retard age-associated brain function impairments in male rats." Age (Dordr) **34**(4): 917-933.
Singhal, N. K., G. Srivastava, et al. (2012). "Melatonin as a neuroprotective agent in the rodent models of Parkinson's disease: is it all set to irrefutable clinical translation?" Mol Neurobiol **45**(1): 186-199.
Sjovall, F., S. Morota, et al. (2010). "Temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis." Crit Care **14**(6): R214.
Smith, W. W., Z. Pei, et al. (2005). "Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration." Proc Natl Acad Sci U S A **102**(51): 18676-18681.
Snell, T. W., A. M. Fields, et al. (2012). "Antioxidants can extend lifespan of Brachionus manjavacas (Rotifera), but only in a few combinations." Biogerontology **13**(3): 261-275.
Sohal, R. S. and R. Weindruch (1996). "Oxidative stress, caloric restriction, and aging." Science **273**(5271): 59-63.
Someya, S., W. Yu, et al. (2010). "Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction." Cell **143**(5): 802-812.
Song, N., A. J. Kim, et al. (2012). "Melatonin suppresses doxorubicin-induced premature senescence of A549 lung cancer cells by ameliorating mitochondrial dysfunction." J Pineal Res **53**(4): 335-343.
Sorbi, S., E. D. Bird, et al. (1983). "Decreased pyruvate dehydrogenase complex activity in Huntington and Alzheimer brain." Ann Neurol **13**(1): 72-78.
Spangenburg, E. E., K. C. Jackson, et al. (2013). "AICAR inhibits oxygen consumption by intact skeletal muscle cells in culture." J Physiol Biochem **69**(4): 909-917.

Srinivasan, S. and N. G. Avadhani (2012). "Cytochrome c oxidase dysfunction in oxidative stress." Free Radic Biol Med **53**(6): 1252-1263.
Srinivasan, V., C. Kaur, et al. (2010). "Melatonin and its agonist ramelteon in Alzheimer's disease: possible therapeutic value." Int J Alzheimers Dis **2011**: 741974.
St-Pierre, J., S. Drori, et al. (2006). "Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators." Cell **127**(2): 397-408.
Stanimirovic, D. B. and A. Friedman (2012). "Pathophysiology of the neurovascular unit: disease cause or consequence?" J Cereb Blood Flow Metab **32**(7): 1207-1221.
Steinberg, G. R. and B. E. Kemp (2009). "AMPK in Health and Disease." Physiological Reviews **89**(3): 1025-1078.
Stichel, C. C., X. R. Zhu, et al. (2007). "Mono- and double-mutant mouse models of Parkinson's disease display severe mitochondrial damage." Hum Mol Genet **16**(20): 2377-2393.
Strazielle, C., C. Sturchler-Pierrat, et al. (2003). "Regional brain cytochrome oxidase activity in beta-amyloid precursor protein transgenic mice with the Swedish mutation." Neuroscience **118**(4): 1151-1163.
Strittmatter, W. J., A. M. Saunders, et al. (1993). "Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease." Proc Natl Acad Sci U S A **90**(5): 1977-1981.
Subramaniam, S. R., L. Vergnes, et al. (2014). "Region specific mitochondrial impairment in mice with widespread overexpression of alpha-synuclein." Neurobiol Dis **70**: 204-213.
Suwa, M., H. Nakano, et al. (2015). "A comparison of chronic AICAR treatment-induced metabolic adaptations in red and white muscles of rats." J Physiol Sci **65**(1): 121-130.
Swerdlow, R. H., J. M. Burns, et al. (2013). "The Alzheimer's disease mitochondrial cascade hypothesis: Progress and perspectives." Biochim Biophys Acta: doi: 10.1016/j.bbadis.2013.1009.1010.
Swerdlow, R. H., J. M. Burns, et al. (2014). "The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives." Biochim Biophys Acta **1842**(8): 1219-1231.
Swerdlow, R. H. and S. M. Khan (2004). "A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease." Med Hypotheses **63**(1): 8-20.
Swift, S., J. Lorens, et al. (2001). "Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems." Curr Protoc Immunol **Chapter 10**: Unit 10 17C.
Tajes, M., J. Gutierrez-Cuesta, et al. (2009). "Anti-aging properties of melatonin in an in vitro murine senescence model: involvement of the sirtuin 1 pathway." J Pineal Res **47**(3): 228-237.
Tanner, C. M., F. Kamel, et al. (2011). "Rotenone, paraquat, and Parkinson's disease." Environ Health Perspect **119**(6): 866-872.
Tao, R. R., J. Y. Huang, et al. (2013). "Ischemic injury promotes Keap1 nitration and disturbance of antioxidative responses in endothelial cells: a potential vasoprotective effect of melatonin." J Pineal Res **54**(3): 271-281.
Taylor, E. B. and J. Rutter (2011). "Mitochondrial quality control by the ubiquitin-proteasome system." Biochem Soc Trans **39**(5): 1509-1513.
Thakur, P. and B. Nehru (2015). "Inhibition of neuroinflammation and mitochondrial dysfunctions by carbenoxolone in the rotenone model of Parkinson's disease." Mol Neurobiol **51**(1): 209-219.

Thomas, C. and A. V. Tulin (2013). "Poly-ADP-ribose polymerase: machinery for nuclear processes." Mol Aspects Med **34**(6): 1124-1137.
Torres-Farfan, C., M. Seron-Ferre, et al. (2006). "Immunocytochemical demonstration of day/night changes of clock gene protein levels in the murine adrenal gland: differences between melatonin-proficient (C3H) and melatonin-deficient (C57BL) mice." J Pineal Res **40**(1): 64-70.
Travnickova-Bendova, Z., N. Cermakian, et al. (2002). "Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity." Proc Natl Acad Sci U S A **99**(11): 7728-7733.
Trifunovic, A., A. Wredenberg, et al. (2004). "Premature ageing in mice expressing defective mitochondrial DNA polymerase." Nature **429**(6990): 417-423.
Tsika, E., M. Moysidou, et al. (2010). "Distinct region-specific alpha-synuclein oligomers in A53T transgenic mice: implications for neurodegeneration." J Neurosci **30**(9): 3409-3418.
Turina, P., D. Samoray, et al. (2003). "H+/ATP ratio of proton transport-coupled ATP synthesis and hydrolysis catalysed by CF0F1-liposomes." EMBO J **22**(3): 418-426.
Valerio, A., G. D'Antona, et al. (2011). "Branched-chain amino acids, mitochondrial biogenesis, and healthspan: an evolutionary perspective." Aging (Albany NY) **3**(5): 464-478.
Valla, J., L. E. Schneider, et al. (2007). "Quantitative Cytochrome Oxidase Histochemistry: Applications in Human Alzheimer's Disease and Animal Models." Journal of Histotechnology **30**(4): 235-247.
Valsecchi, F., W. J. Koopman, et al. (2010). "Complex I disorders: causes, mechanisms, and development of treatment strategies at the cellular level." Dev Disabil Res Rev **16**(2): 175-182.
Varghese, M., W. Zhao, et al. (2011). "Mitochondrial bioenergetics is defective in presymptomatic Tg2576 AD Mice." Translational Neuroscience **2**(1): 1-5.
Varma, S. D. and K. Chandrasekaran (2015). "High sugar-induced repression of antioxidant and anti-apoptotic genes in lens: Reversal by pyruvate." Mol Cell Biochem **403**(1-2): 149-158.
Vassilopoulos, A., J. D. Pennington, et al. (2014). "SIRT3 Deacetylates ATP Synthase F Complex Proteins in Response to Nutrient- and Exercise-Induced Stress." Antioxid Redox Signal **21**(4): 551-564.
Ved, R., S. Saha, et al. (2005). "Similar patterns of mitochondrial vulnerability and rescue induced by genetic modification of alpha-synuclein, parkin, and DJ-1 in Caenorhabditis elegans." J Biol Chem **280**(52): 42655-42668.
Vezina, C., A. Kudelski, et al. (1975). "Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle." J Antibiot (Tokyo) **28**(10): 721-726.
Vielhaber, S., D. Kunz, et al. (2000). "Mitochondrial DNA abnormalities in skeletal muscle of patients with sporadic amyotrophic lateral sclerosis." Brain **123 (Pt 7)**: 1339-1348.
Vilar, A., L. de Lemos, et al. (2014). "Melatonin suppresses nitric oxide production in glial cultures by pro-inflammatory cytokines through p38 MAPK inhibition." Free Radic Res **48**: 119-128.
Villemagne, V. L., S. Ataka, et al. (2009). "High striatal amyloid beta-peptide deposition across different autosomal Alzheimer disease mutation types." Arch Neurol **66**(12): 1537-1544.

Vincent, D. A., Jr., M. A. Gratton, et al. (1995). "Effect of postmortem autolysis on Na,K-ATPase activity and antigenicity in the gerbil cochlea." Hear Res **89**(1-2): 14-20.
Vincent, E. E., P. P. Coelho, et al. (2014). "Differential effects of AMPK agonists on cell growth and metabolism." Oncogene.
Volles, M. J. and P. T. Lansbury, Jr. (2002). "Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism." Biochemistry **41**(14): 4595-4602.
von Gall, C. and D. R. Weaver (2008). "Loss of responsiveness to melatonin in the aging mouse suprachiasmatic nucleus." Neurobiol Aging **29**(3): 464-470.
Wahlestedt, M., A. Ameur, et al. (2014). "Somatic cells with a heavy mitochondrial DNA mutational load render induced pluripotent stem cells with distinct differentiation defects." Stem Cells **32**(5): 1173-1182.
Wakabayashi, T. (2002). "Megamitochondria formation - physiology and pathology." J Cell Mol Med **6**(4): 497-538.
Walker, J. E., R. Lutter, et al. (1991). "Identification of the subunits of F1F0-ATPase from bovine heart mitochondria." Biochemistry **30**(22): 5369-5378.
Wallace, D. C. and W. W. Fan (2009). "The pathophysiology of mitochondrial disease as modeled in the mouse." Genes & Development **23**(15): 1714-1736.
Wanagat, J., N. Ahmadieh, et al. (2015). "Skeletal muscle mitochondrial DNA deletions are not increased in CuZn-superoxide dismutase deficient mice." Exp Gerontol **61**: 15-19.
Wang, J. and T. Zhou (2010). "cAMP-regulated dynamics of the mammalian circadian clock." Biosystems **101**(2): 136-143.
Wang, L. M., N. A. Suthana, et al. (2005). "Melatonin inhibits hippocampal long-term potentiation." Eur J Neurosci **22**(9): 2231-2237.
Wang, X., A. Sirianni, et al. (2011). "The melatonin MT1 receptor axis modulates mutant Huntingtin-mediated toxicity." J Neurosci **31**(41): 14496-14507.
Wang, X., A. Sirianni, et al. (2011). "The Melatonin MT1 Receptor Axis Modulates Mutant Huntingtin-Mediated Toxicity." The Journal of Neuroscience **31**(41): 14496-14507.
Wang, Y., X. Li, et al. (2010). "alpha-Lipoic acid increases energy expenditure by enhancing adenosine monophosphate-activated protein kinase-peroxisome proliferator-activated receptor-gamma coactivator-1alpha signaling in the skeletal muscle of aged mice." Metabolism **59**(7): 967-976.
Wang, Z., D. Liu, et al. (2012). "Cytoprotective effects of melatonin on astroglial cells subjected to palmitic acid treatment in vitro." J Pineal Res **52**(2): 253-264.
Wang, Z., C. Ma, et al. (2012). "Melatonin activates the Nrf2-ARE pathway when it protects against early brain injury in a subarachnoid hemorrhage model." J Pineal Res **53**(2): 129-137.
Weindruch, R., R. L. Walford, et al. (1986). "The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake." J Nutr **116**(4): 641-654.
Weiss, H., T. Friedrich, et al. (1991). "The respiratory-chain NADH dehydrogenase (complex I) of mitochondria." Eur J Biochem **197**(3): 563-576.
Westlund, K. N., R. M. Denney, et al. (1988). "Localization of distinct monoamine oxidase A and monoamine oxidase B cell populations in human brainstem." Neuroscience **25**(2): 439-456.

Wiedemann, F. R., G. Manfredi, et al. (2002). "Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients." J Neurochem **80**(4): 616-625.
Wilkins, H. M., J. L. Harris, et al. (2014). "Oxaloacetate activates brain mitochondrial biogenesis, enhances the insulin pathway, reduces inflammation and stimulates neurogenesis." Hum Mol Genet **23**(24): 6528-6541.
Wilson, L., Q. Yang, et al. (2007). "Pyruvate induces mitochondrial biogenesis by a PGC-1 alpha-independent mechanism." Am J Physiol Cell Physiol **292**(5): C1599-1605.
Wisniewski, J. R., A. Zougman, et al. (2009). "Universal sample preparation method for proteome analysis." Nat Methods **6**(5): 359-362.
Wolf, A. B., B. B. Braden, et al. (2012). "Broad-based nutritional supplementation in 3xTg mice corrects mitochondrial function and indicates sex-specificity in response to Alzheimer's disease intervention." J Alzheimers Dis **32**(1): 217-232.
Wolfe, D. M., J. H. Lee, et al. (2013). "Autophagy failure in Alzheimer's disease and the role of defective lysosomal acidification." Eur J Neurosci **37**(12): 1949-1961.
Wu, S. B. and Y. H. Wei (2012). "AMPK-mediated increase of glycolysis as an adaptive response to oxidative stress in human cells: implication of the cell survival in mitochondrial diseases." Biochim Biophys Acta **1822**(2): 233-247.
Wu, Y. H., J. N. Zhou, et al. (2007). "Decreased MT1 melatonin receptor expression in the suprachiasmatic nucleus in aging and Alzheimer's disease." Neurobiol Aging **28**(8): 1239-1247.
Wu, Z., P. Puigserver, et al. (1999). "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1." Cell **98**(1): 115-124.
Xia, Q., L. Liao, et al. (2008). "Proteomic identification of novel proteins associated with Lewy bodies." Front Biosci **13**: 3850-3856.
Xie, H., J. Guan, et al. (2013). "Mitochondrial Alterations near Amyloid Plaques in an Alzheimer's Disease Mouse Model." J Neurosci **33**(43): 17042-17051.
Xie, L., H. Kang, et al. (2013). "Sleep drives metabolite clearance from the adult brain." Science **342**(6156): 373-377.
Xilouri, M., O. R. Brekk, et al. (2013). "alpha-Synuclein and protein degradation systems: a reciprocal relationship." Mol Neurobiol **47**(2): 537-551.
Xu, D., T. Nishimura, et al. (2014). "Fialuridine induces acute liver failure in chimeric TK-NOG mice: a model for detecting hepatic drug toxicity prior to human testing." PLoS Med **11**(4): e1001628.
Xu, X., S. Duan, et al. (2013). "Mitochondrial regulation in pluripotent stem cells." Cell Metab **18**(3): 325-332.
Yagi, T. and A. Matsuno-Yagi (2003). "The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: the secret unlocked." Biochemistry **42**(8): 2266-2274.
Yamamoto, H. A. and P. V. Mohanan (2002). "Melatonin attenuates brain mitochondria DNA damage induced by potassium cyanide in vivo and in vitro." Toxicology **179**(1-2): 29-36.
Ye, L., B. Varamini, et al. (2012). "Rapamycin has a biphasic effect on insulin sensitivity in C2C12 myotubes due to sequential disruption of mTORC1 and mTORC2." Front Genet **3**: 177.
Youle, R. J. and A. M. van der Bliek (2012). "Mitochondrial fission, fusion, and stress." Science **337**(6098): 1062-1065.
Yuan, H. and S. F. Pang (1991). "[125I]Iodomelatonin-binding sites in the pigeon brain: binding characteristics, regional distribution and diurnal variation." J Endocrinol **128**(3): 475-482.

Yuan, Y., J. Sun, et al. (2010). "Overexpression of alpha-synuclein down-regulates BDNF expression." Cell Mol Neurobiol **30**(6): 939-946.
Yui, R., Y. Ohno, et al. (2003). "Accumulation of deleted mitochondrial DNA in aging Drosophila melanogaster." Genes Genet Syst **78**(3): 245-251.
Zaleska, M. M. and R. A. Floyd (1985). "Regional lipid peroxidation in rat brain in vitro: possible role of endogenous iron." Neurochem Res **10**(3): 397-410.
Zaouali, M. A., E. Boncompagni, et al. (2013). "AMPK involvement in endoplasmic reticulum stress and autophagy modulation after fatty liver graft preservation: a role for melatonin and trimetazidine cocktail." J Pineal Res **55**: 65-78.
Zarranz, J. J., J. Alegre, et al. (2004). "The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia." Ann Neurol **55**(2): 164-173.
Zee, J. M. and D. M. Glerum (2006). "Defects in cytochrome oxidase assembly in humans: lessons from yeast." Biochem Cell Biol **84**(6): 859-869.
Zeman, M., K. Szantoova, et al. (2009). "Effect of rhythmic melatonin administration on clock gene expression in the suprachiasmatic nucleus and the heart of hypertensive TGR(mRen2)27 rats." J Hypertens Suppl **27**(6): S21-26.
Zhang, X. M., K. Xiong, et al. (2010). "Functional deprivation promotes amyloid plaque pathogenesis in Tg2576 mouse olfactory bulb and piriform cortex." Eur J Neurosci **31**(4): 710-721.
Zharikov, S. and S. Shiva (2013). "Platelet mitochondrial function: from regulation of thrombosis to biomarker of disease." Biochem Soc Trans **41**(1): 118-123.
Zhou, G., Y. Miura, et al. (2001). "Platelet monoamine oxidase B and plasma beta-phenylethylamine in Parkinson's disease." J Neurol Neurosurg Psychiatry **70**(2): 229-231.
Zhou, H., J. Chen, et al. (2012). "Melatonin protects against rotenone-induced cell injury via inhibition of Omi and Bax-mediated autophagy in Hela cells." J Pineal Res **52**(1): 120-127.
Zhou, J. N., R. Y. Liu, et al. (2003). "Early neuropathological Alzheimer's changes in aged individuals are accompanied by decreased cerebrospinal fluid melatonin levels." J Pineal Res **35**(2): 125-130.
Zhou, Q., C. Liu, et al. (2015). "Rotenone induction of hydrogen peroxide inhibits mTOR-mediated S6K1 and 4E-BP1/eIF4E pathways, leading to neuronal apoptosis." Toxicol Sci **143**(1): 81-96.
Zhu, H., K. Itoh, et al. (2005). "Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury." FEBS Lett **579**(14): 3029-3036.
Zhu, M., J. Li, et al. (2003). "The association of alpha-synuclein with membranes affects bilayer structure, stability, and fibril formation." J Biol Chem **278**(41): 40186-40197.
Zid, B. M., A. N. Rogers, et al. (2009). "4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila." Cell **139**(1): 149-160.
Zlokovic, B. V. (2005). "Neurovascular mechanisms of Alzheimer's neurodegeneration." Trends Neurosci **28**(4): 202-208.

APPENDIX A: MASS SPECTROMETRY DATA

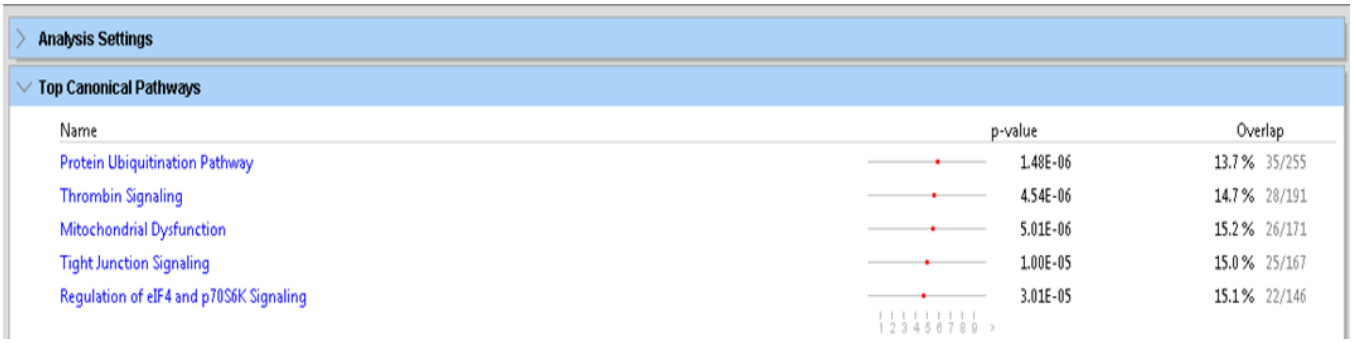


Figure 37 Top canonical pathways
Using ingenuity pathway analysis software (IPA), top canonical pathways were identified. Mitochondrial canonical pathways contributing to AD, and PD progression were among the top pathways identified in this data set.

Table 5 Statistically significant fold change differences in at least 2 biological replicates
Ratio of light (α-syn WT) to heavy isotope labeled (M17 control). The fold change for each ID was determined to be statistically significant using Perseus software, using Significance A setting (outlier test) to determine significance with a p value < 0.05. Only IDs that were significant in 2/3 or 3/3 biological replicates are reported.

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Cellular retinoic acid-binding protein 2	7.8	+	7.2	+	6.8	+	3	7.2
Keratin, type II cytoskeletal 1	2.2	+	7.5	+	9.3	+	3	7.5
Keratin, type I cytoskeletal 10	4.1	+	8.8	+	10.6	+	3	8.8
Dihydropyrimidinase-related protein 3	4.0	+	3.8	+	2.7	+	3	3.8
Zinc-binding alcohol dehydrogenase domain-containing protein 2	4.7	+	2.9	+	3.5	+	3	3.5
Alpha-aminoadipic semialdehyde synthase, mitochondrial;Lysine ketoglutarate reductase;Saccharopine dehydrogenase	2.4	+	2.3	+	3.0	+	3	2.4
Amine oxidase [flavin-containing] A	1.5	+	3.1	+	1.6	+	3	1.6
RNA-binding protein Musashi homolog 1	2.0	+	2.3	+	1.9	+	3	2.0
Galanin peptides;Galanin;Galanin message-associated peptide	2.3	+	2.2	+	1.9	+	3	2.2
Insulinoma-associated protein 2	1.8	+	2.4	+	2.2	+	3	2.2
Neurofilament light polypeptide	1.7	+	1.3	+	2.2	+	3	1.7
Protein NDRG4	1.9	+	2.4	+	1.8	+	3	1.9
Ancient ubiquitous protein 1	1.7	+	1.8	+	2.4	+	3	1.8

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Tissue-type plasminogen activator;Tissue-type plasminogen activator chain A;Tissue-type plasminogen activator chain B	2.7	+	1.7	+	1.6	+	3	1.7
Transgelin-3	1.8	+	1.5	+	1.6	+	3	1.6
Neurofilament medium polypeptide	1.6	+	1.6	+	1.7	+	3	1.6
Isoaspartyl peptidase/L-asparaginase;Isoaspartyl peptidase/L-asparaginase alpha chain;Isoaspartyl peptidase/L-asparaginase beta chain	1.7	+	1.5	+	1.5	+	3	1.5
Secretogranin-1;GAWK peptide;CCB peptide	1.5	+	1.6	+	1.9	+	3	1.6
Plastin-2	1.9	+	1.8	+	1.4	+	3	1.8
Bromodomain-containing protein 3	1.5	+	1.3	+	1.6	+	3	1.5
HIG1 domain family member 2A, mitochondrial	1.6	+	1.4	+	2.7	+	3	1.6
Synaptosomal-associated protein 25	1.7	+	1.4	+	1.5	+	3	1.5
Ganglioside-induced differentiation-associated protein 1	1.5	+	1.5	+	1.5	+	3	1.5

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Phosphatidylglycerophosphatase and protein-tyrosine phosphatase 1	1.4	+	1.3	+	1.5	+	3	1.4
Antigen KI-67	1.4	+	1.5	+	1.5	+	3	1.5
Nuclear factor NF-kappa-B p100 subunit;Nuclear factor NF-kappa-B p52 subunit	1.4	+	1.5	+	1.4	+	3	1.4
Vesicle-associated membrane protein 7	1.7	+	1.5	+	1.8	+	3	1.7
Cyclin-dependent kinase 6	1.4	+	1.6	+	1.5	+	3	1.5
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	1.7	+	1.5	+	1.8	+	3	1.7
Retinol-binding protein 1	1.5	+	1.4	+	1.4	+	3	1.4
Ornithine aminotransferase, mitochondrial;Ornithine aminotransferase, hepatic form;Ornithine aminotransferase, renal form	1.7	+	1.4	+	1.4	+	3	1.4
Retrotransposon-derived protein PEG10	1.9	+	1.4	+	1.5	+	3	1.5
Laminin subunit gamma-1	1.4	+	1.4	+	1.5	+	3	1.4

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2;Guanine nucleotide-binding protein subunit gamma	1.5	+	1.4	+	1.4	+	3	1.4
Creatine kinase U-type, mitochondrial	1.4	+	1.3	+	1.4	+	3	1.4
Nucleolysin TIAR	0.7	+	1.4	+	1.7	+	3	1.4
Syntaxin-binding protein 1	1.3	+	1.3	+	1.4	+	3	1.3
Threonine--tRNA ligase, mitochondrial	1.4	+	1.6	+	0.7	+	3	1.4
CB1 cannabinoid receptor-interacting protein 1	0.7	+	0.7	+	0.7	+	3	0.7
39S ribosomal protein L17, mitochondrial	0.3	+	0.3	+	1.5	+	3	0.3
BRCA1-associated protein	1.7	+	1.8	+	1.5	+	3	1.7
Cyclin-D1-binding protein 1	0.6	+	0.7	+	0.6	+	3	0.6
Talin-1	0.7	+	0.7	+	0.7	+	3	0.7
Argininosuccinate synthase	0.7	+	0.7	+	0.7	+	3	0.7
Charged multivesicular body protein 5	0.7	+	0.8	+	0.6	+	3	0.7
Tyrosine-protein kinase Lyn;Tyrosine-protein kinase HCK	0.7	+	0.7	+	0.7	+	3	0.7
Talin-2	0.7	+	0.7	+	0.7	+	3	0.7

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Isochorismatase domain-containing protein 1	0.7	+	0.7	+	0.7	+	3	0.7
Paxillin	0.7	+	0.8	+	0.7	+	3	0.7
Insulin-like growth factor II;Insulin-like growth factor II;Insulin-like growth factor II Ala-25 Del;Preptin	0.6	+	0.6	+	0.5	+	3	0.6
Aldo-keto reductase family 1 member C3	0.7	+	0.7	+	0.7	+	3	0.7
NAD-dependent protein deacetylase sirtuin-1;SirtT1 75 kDa fragment	0.7	+	0.7	+	0.6	+	3	0.7
A-kinase anchor protein 12	0.7	+	0.6	+	0.6	+	3	0.6
Lanosterol synthase	0.5	+	0.6	+	0.7	+	3	0.6
Phospholipase A-2-activating protein	0.7	+	0.7	+	0.6	+	3	0.7
	0.7	+	0.6	+	0.6	+	3	0.6
Cysteine and glycine-rich protein 2	0.6	+	0.7	+	0.6	+	3	0.6
Adenylate kinase isoenzyme 6	0.7	+	0.6	+	0.5	+	3	0.6
Isopentenyl-diphosphate Delta-isomerase 1	0.6	+	0.6	+	0.6	+	3	0.6
Integrin alpha-V;Integrin alpha-V heavy chain;Integrin alpha-V light chain	0.6	+	0.6	+	0.6	+	3	0.6

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Ubiquitin-associated protein 2	0.6	+	0.6	+	0.6	+	3	0.6
Chromogranin-A;Vasostatin-1;Vasostatin-2;EA-92;ES-43;Pancreastatin;SS-18;WA-8;WE-14;LF-19;AL-11;GV-19;GR-44;ER-37	0.6	+	0.7	+	0.6	+	3	0.6
Proto-oncogene tyrosine-protein kinase receptor Ret;Soluble RET kinase fragment;Extracellular cell-membrane anchored RET cadherin 120 kDa fragment	0.6	+	0.6	+	0.6	+	3	0.6
Aldo-keto reductase family 1 member C1;Aldo-keto reductase family 1 member C2;Aldo-keto reductase family 1 member C4	0.6	+	0.6	+	0.6	+	3	0.6
S-methyl-5-thioadenosine phosphorylase;Purine nucleoside phosphorylase	0.6	+	0.6	+	0.6	+	3	0.6
Phosphofurin acidic cluster sorting protein 1	0.6	+	0.5	+	0.6	+	3	0.6
Neuroblast differentiation-associated protein AHNAK	0.6	+	0.6	+	0.5	+	3	0.6

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Neural cell adhesion molecule 1	0.6	+	0.5	+	0.5	+	3	0.5
Perilipin-2	0.5	+	0.4	+	0.6	+	3	0.5
Synemin	0.5	+	0.5	+	0.5	+	3	0.5
BTB/POZ domain-containing protein KCTD12	0.5	+	0.5	+	0.5	+	3	0.5
Quinone oxidoreductase PIG3	0.4	+	0.5	+	0.5	+	3	0.5
Heterogeneous nuclear ribonucleoprotein K	0.7	+	0.3	+	0.4	+	3	0.4
Hydroxymethylglutaryl-CoA synthase, cytoplasmic	0.5	+	0.4	+	0.6	+	3	0.5
Tropomyosin alpha-1 chain	0.4	+	0.6	+	0.4	+	3	0.4
Cellular retinoic acid-binding protein 1	0.4	+	0.4	+	0.4	+	3	0.4
Protein-glutamine gamma-glutamyltransferase 2	0.4	+	0.3	+	0.3	+	3	0.3
Keratin, type I cytoskeletal 9	NaN		5.9	+	3.2	+	2	4.5
Lysocardiolipin acyltransferase 1	3.1	+	7.2	+	NaN		2	5.1
Neuronal migration protein doublecortin	2.7	+	3.1	+	NaN		2	2.9
Pleckstrin homology domain-containing family A member 6	2.8	+	2.3	+	NaN		2	2.6

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Protein asunder homolog	1.6	+	3.5	+	NaN		2	2.5
Dachshund homolog 1	NaN		1.4	+	2.5	+	2	2.0
Protein DEK	6.1	+	1.2		2.8	+	2	2.8
Tripartite motif-containing protein 67	1.7	+	2.1	+	NaN		2	1.9
UPF0444 transmembrane protein C12orf23	NaN		2.2	+	1.6	+	2	1.9
Periphilin-1	1.3		1.9	+	1.7	+	2	1.7
ProSAAS;KEP;Big SAAS;Little SAAS;Big PEN-LEN;PEN;Little LEN;Big LEN	NaN		1.7	+	1.7	+	2	1.7
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-8	1.6	+	1.6	+	NaN		2	1.6
Protein-tyrosine sulfotransferase 1	1.5	+	NaN		1.4	+	2	1.4
Ribose-phosphate pyrophosphokinase 2;Ribose-phosphate pyrophosphokinase	1.5	+	1.6	+	NaN		2	1.5
Kinesin-like protein KIF1A	1.5	+	1.6	+	1.2		2	1.5
Gamma-glutamylcyclotransferase	NaN		1.5	+	1.7	+	2	1.6
Aspartate--tRNA ligase, mitochondrial	1.5	+	0.9		1.5	+	2	1.5
Transmembrane protein 205	1.6	+	1.5	+	1.3		2	1.5

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Dehydrogenase/reductase SDR family member 7	1.5	+	1.4	+	1.3		2	1.4
Alpha-galactosidase A	1.6	+	NaN		1.4	+	2	1.5
Spartin	1.3		1.7	+	1.4	+	2	1.4
DNA topoisomerase 2;DNA topoisomerase 2-beta	1.6	+	1.1		1.4	+	2	1.4
Plastin-1	1.6	+	1.7	+	1.2		2	1.6
Zinc finger MYM-type protein 3	1.4	+	2.2	+	NaN		2	1.8
Midkine	NaN		1.4	+	1.5	+	2	1.4
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	1.4	+	1.3	+	1.2		2	1.3
Uncharacterized protein C11orf96	1.4	+	1.4	+	1.3		2	1.4
Calcium/calmodulin-dependent protein kinase type IV	1.4	+	1.3		1.4	+	2	1.4
Myc box-dependent-interacting protein 1	1.4	+	1.5	+	1.2		2	1.4
Leucine-rich repeat and calponin homology domain-containing protein 1	NaN		1.5	+	1.5	+	2	1.5
Ribosome production factor 1	1.7	+	1.1		1.5	+	2	1.5
RNA binding protein fox-1 homolog 2;RNA binding	1.2		1.4	+	1.4	+	2	1.4

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Melanoma-associated antigen G1	NaN		1.4	+	1.3	+	2	1.4
Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial	1.3		1.4	+	1.4	+	2	1.4
Ester hydrolase C11orf54	1.3	+	1.4	+	1.3		2	1.3
Heterogeneous nuclear ribonucleoprotein A3	1.8	+	1.4	+	1.3		2	1.4
Spectrin beta chain, non-erythrocytic 2	1.3		1.3	+	1.4	+	2	1.3
Aldehyde dehydrogenase;Fatty aldehyde dehydrogenase	1.3		1.3	+	1.5	+	2	1.3
Tudor and KH domain-containing protein	1.4	+	NaN		1.4	+	2	1.4
Metastasis-associated protein MTA1	1.4	+	1.4	+	1.3		2	1.4
Dedicator of cytokinesis protein 11	1.4	+	1.5	+	1.2		2	1.4
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	1.4	+	1.3	+	NaN		2	1.4
Zinc fingers and homeoboxes protein 1	1.4	+	1.3	+	NaN		2	1.4
Small nuclear ribonucleoprotein G;Small nuclear ribonucleoprotein G-like protein	NaN		1.4	+	1.4	+	2	1.4

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Glutaredoxin-related protein 5, mitochondrial	1.1		3.1	+	1.7	+	2	1.7
Probable global transcription activator SNF2L1	1.3	+	1.3		1.4	+	2	1.3
Equilibrative nucleoside transporter 1	1.9	+	1.3	+	0.8		2	1.3
Nesprin-2	1.2		1.5	+	1.6	+	2	1.5
Acyl-coenzyme A thioesterase 9, mitochondrial	1.4	+	1.3		1.4	+	2	1.4
Tripartite motif-containing protein 65	NaN		1.3	+	1.4	+	2	1.4
UBX domain-containing protein 4	1.4	+	1.3	+	NaN		2	1.4
REST corepressor 2	1.4	+	1.3	+	NaN		2	1.4
Apoptosis regulator BAX	1.4	+	1.3	+	1.3		2	1.3
Kinectin	1.3	+	1.3		1.4	+	2	1.3
Bromodomain adjacent to zinc finger domain protein 2A	1.7	+	1.3	+	NaN		2	1.5
Cdc42 effector protein 4	1.3		1.3	+	1.4	+	2	1.3
Phosphoinositide 3-kinase regulatory subunit 4	1.3		1.3	+	1.4	+	2	1.3
Golgi apparatus protein 1	1.4	+	1.1		1.3	+	2	1.3
Bromodomain-containing protein 2	1.5	+	1.3	+	1.2		2	1.3

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Annexin;Annexin A4	1.3	+	0.1	+	1.3		2	1.3
Probable asparagine--tRNA ligase, mitochondrial	1.4	+	1.6	+	1.2		2	1.4
3-hydroxyisobutyrate dehydrogenase, mitochondrial	1.4	+	1.2		1.3	+	2	1.3
Prostaglandin reductase 2	1.3	+	1.3	+	1.0		2	1.3
Bifunctional lysine-specific demethylase and histidyl-hydroxylase NO66	1.4	+	0.6	+	NaN		2	1.0
Helicase-like transcription factor	1.4	+	1.3	+	1.1		2	1.3
Surfeit locus protein 1	NaN		1.9	+	1.4	+	2	1.6
Mediator of RNA polymerase II transcription subunit 12	0.7	+	1.5	+	1.2		2	1.2
Abnormal spindle-like microcephaly-associated protein	1.0		1.4	+	1.4	+	2	1.4
Uncharacterized protein C19orf43	2.0	+	0.9		1.3	+	2	1.3
Apolipoprotein O	1.8	+	1.4	+	1.2		2	1.4
Cell division cycle-associated protein 2	1.6	+	1.3	+	NaN		2	1.5
Protein archease	1.1		1.7	+	1.7	+	2	1.7
Translocator protein	1.1		1.8	+	2.3	+	2	1.8
28S ribosomal protein S18b, mitochondrial	1.3	+	1.6	+	1.0		2	1.3

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Serine/threonine-protein kinase SMG1	1.1		0.7	+	1.5	+	2	1.1
D-beta-hydroxybutyrate dehydrogenase, mitochondrial	1.5	+	1.3	+	NaN		2	1.4
E3 ubiquitin-protein ligase HECTD1	1.0		1.3	+	0.7	+	2	1.0
TGF-beta-activated kinase 1 and MAP3K7-binding protein 1	0.6	+	1.0		0.7	+	2	0.7
WD repeat-containing protein 5	1.3	+	1.5	+	1.1		2	1.3
Focadhesin	0.7	+	0.5	+	NaN		2	0.6
Gamma-glutamyl hydrolase	0.7	+	0.6	+	1.0		2	0.7
Integrin alpha-2	NaN		0.7	+	0.6	+	2	0.6
Ubiquitin carboxyl-terminal hydrolase;Ubiquitin carboxyl-terminal hydrolase 19	1.8	+	1.3	+	1.2		2	1.3
Inositol-3-phosphate synthase 1	0.6	+	0.5	+	NaN		2	0.6
DNA polymerase epsilon subunit 4	0.6	+	0.6	+	1.0		2	0.6
Rab3 GTPase-activating protein catalytic subunit	0.6	+	0.7	+	0.9		2	0.7
Endophilin-B1	0.9		0.7	+	0.6	+	2	0.7
LanC-like protein 1	1.4	+	0.6	+	NaN		2	1.0
DNA-directed RNA polymerase II subunit RPB7	0.5	+	1.4	+	NaN		2	1.0

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1	0.8		0.6	+	0.7	+	2	0.7
Syndecan;Syndecan-2	0.8		0.7	+	0.7	+	2	0.7
Protein FAM219A	NaN		0.7	+	0.7	+	2	0.7
RNA exonuclease 4	0.8		0.6	+	0.7	+	2	0.7
Band 4.1-like protein 1	0.7	+	0.7	+	0.8		2	0.7
Phytanoyl-CoA hydroxylase-interacting protein-like	0.8		0.7	+	0.7	+	2	0.7
Early endosome antigen 1	0.7	+	NaN		0.6	+	2	0.7
Cleft lip and palate transmembrane protein 1-like protein	0.7	+	NaN		0.7	+	2	0.7
Protein S100-A13	0.8		0.7	+	0.7	+	2	0.7
Hexokinase-1	0.7	+	0.7	+	0.7		2	0.7
CD166 antigen	0.7	+	0.7	+	0.7		2	0.7
DnaJ homolog subfamily A member 1	0.7	+	0.7	+	0.8		2	0.7
Superoxide dismutase [Mn], mitochondrial;Superoxide dismutase	0.6	+	0.7	+	0.8		2	0.7
Myotubularin-related protein 5	0.5	+	1.1		0.5	+	2	0.5

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Actin-binding LIM protein 1	0.7	+	0.7	+	0.8		2	0.7
Glutaredoxin-1	0.5	+	0.8	+	NaN		2	0.6
Coiled-coil domain-containing protein 6	0.8		0.7	+	0.7	+	2	0.7
Synaptotagmin-1	0.8		0.6	+	0.7	+	2	0.7
GTP:AMP phosphotransferase AK3, mitochondrial	0.7	+	0.9		0.5	+	2	0.7
Glutathione peroxidase;Glutathione peroxidase 1	0.6	+	0.6	+	0.8		2	0.6
Translation factor GUF1, mitochondrial	0.6	+	0.7	+	NaN		2	0.6
Hippocalcin-like protein 1	0.7	+	0.7	+	0.9		2	0.7
Aprataxin	0.7	+	NaN		0.6	+	2	0.7
Selenoprotein O	0.7	+	0.7	+	NaN		2	0.7
Kelch-like protein 13;Kelch-like protein 9	0.1	+	NaN		0.1	+	2	0.1
Glutathione S-transferase theta-2B;Glutathione S-transferase theta-2	0.7	+	0.9		0.7	+	2	0.7
HAUS augmin-like complex subunit 7	0.7		0.6	+	0.6	+	2	0.6
Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha	0.6	+	NaN		0.7	+	2	0.6
Pyruvate kinase PKM;Pyruvate kinase	NaN		0.7	+	0.7	+	2	0.7

Table 5 continued

Protein names	Rep 1 (1/x)	Rep 1 Sig A	Rep 2 (1/x)	Rep 2 Sig A	Rep 3 (1/x)	Rep 3 Sig A	Count	Median Ratio
Angiotensin-converting enzyme;Angiotensin-converting enzyme, soluble form	0.6	+	0.5	+	0.8		2	0.6
Four and a half LIM domains protein 2	NaN		0.6	+	0.6	+	2	0.6
Palmdelphin	0.7		0.6	+	0.5	+	2	0.6
Zyxin	0.5	+	0.7	+	NaN		2	0.6
HAUS augmin-like complex subunit 6	0.7	+	0.8	+	NaN		2	0.7
N-alpha-acetyltransferase 38, NatC auxiliary subunit	0.5	+	0.6	+	NaN		2	0.6
NEDD4 family-interacting protein 1	NaN		0.5	+	0.5	+	2	0.5
Junctional adhesion molecule C	0.6	+	0.5	+	NaN		2	0.5
Triple functional domain protein	0.5	+	0.6	+	NaN		2	0.6
Armadillo repeat-containing protein 10	0.8		0.3	+	0.6	+	2	0.6
Vacuolar-sorting protein SNF8	NaN		0.5	+	0.5	+	2	0.5
Chondroitin sulfate proteoglycan 4	0.5	+	0.5	+	NaN		2	0.5
CD44 antigen	0.2	+	NaN		0.2	+	2	0.2
Annexin;Annexin A1	0.1	+	NaN		0.1	+	2	0.1
DNA-3-methyladenine glycosylase	0.7	+	NaN		0.3	+	2	0.5

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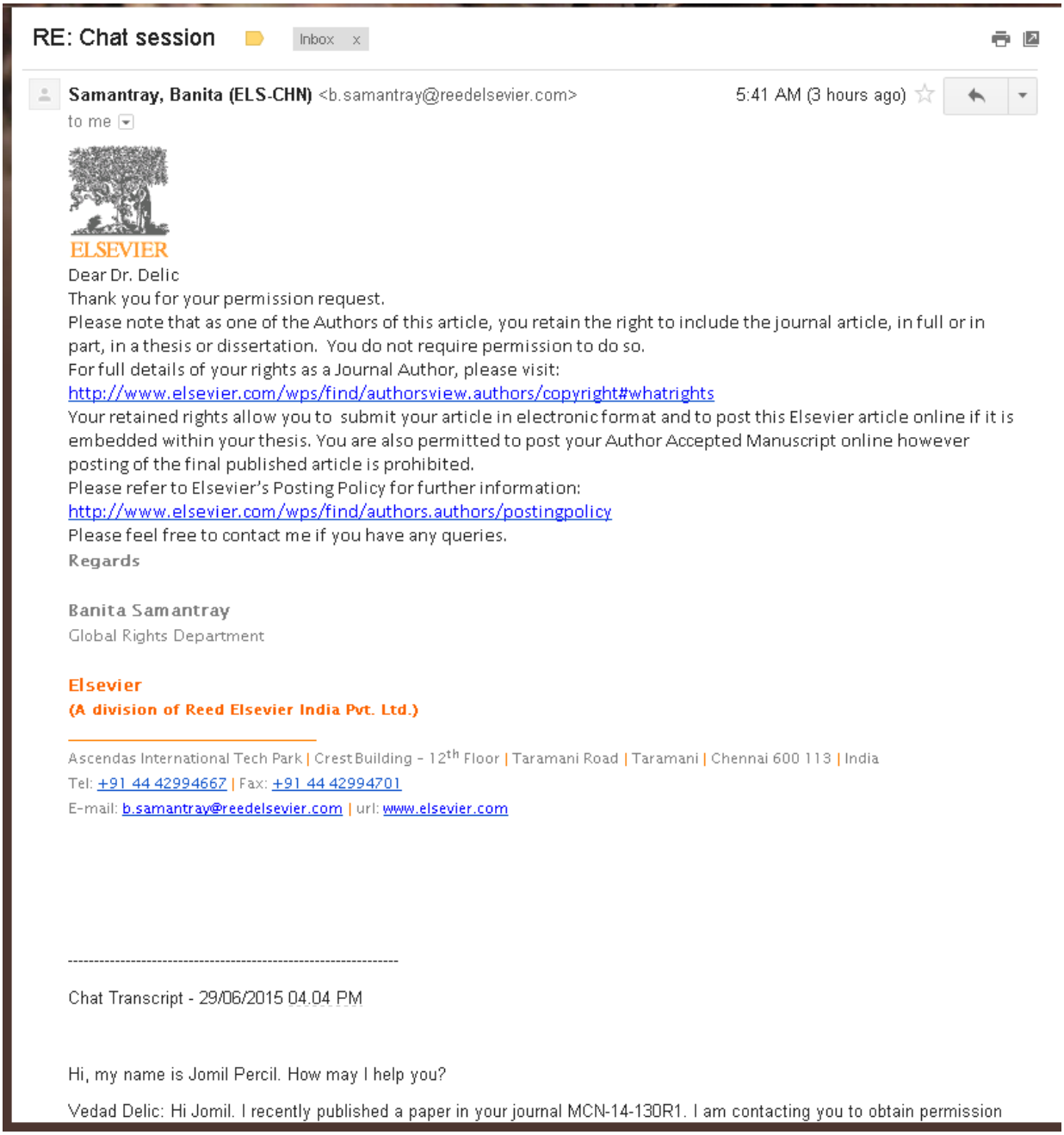

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