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NOVEL TARGETS FOR MITOCHONDRIAL DYSFUNCTION FOLLOWING TRAUMATIC BRAIN INJURY

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NOVEL TARGETS FOR MITOCHONDRIAL DYSFUNCTION FOLLOWING
TRAUMATIC BRAIN INJURY

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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2016

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ABSTRACT OF DISSERTATION

NOVEL TARGETS FOR MITOCHONDRIAL DYSFUNCTION FOLLOWING TRAUMATIC BRAIN INJURY

Mitochondrial dysfunction is a phenomenon observed in models of Traumatic Brain Injury (TBI). Loss of mitochondrial bioenergetics can result in diminished cellular homeostasis leading to cellular dysfunction and possible cellular death. Consequently, the resultant tissue damage can manifest as functional deficits and/or disease states. Therapeutic strategies to target this mitochondrial dysfunction have been investigated for models TBI and have shown promising effects.

For this project, we tested the hypothesis that mitoNEET, a novel mitochondrial membrane protein, is a target for pioglitazone mediated neuroprotection. To test this, we used a severe Controlled Cortical Impact (CCI) injury model in mitoNEET null and wild-type mice. We then dosed these animals with pioglitazone or NL-1, which is a compound that has a similar structure to pioglitazone allowing us to hone in on the importance of mitoNEET binding. Wild-type animals treated with the mitoNEET ligands, both pioglitazone and NL-1, had improved mitochondrial function, tissue sparing and functional recovery, compared to mitoNEET null animals.

In addition to this specific hypothesis tested, our experiments provided insight casting doubt on the central dogma that mitochondrial dysfunction following TBI is the result of vast oxidative damage and consequential irreversible mitochondrial loss. The data from these studies show that when mitoNEET is targeted with pioglitazone at 12 hours’ post-injury, mitochondrial dysfunction can be reversed. Additionally, when bypassing proteins upstream of Complex I with an alternative biofuel, such as beta-hydroxybutyrate (BHB), TBI related mitochondrial dysfunction is once again reversed. This leads to novel hypothesis for future work which posits mitoNEET as a redox sensitive switch; when mitoNEET senses changes in redox, as seen in TBI, it inhibits mitochondrial respiration. When targeted with an agonist/ligand or bypassed with a biofuel TBI mitochondrial dysfunction can be reversed.

These studies support the role of mitoNEET in the neuropathological sequelae of brain injury, supporting mitoNEET as a crucial target for pioglitazone mediated neuroprotection following TBI. Lastly, these studies propose a
mechanism of TBI related mitochondrial dysfunction which can reversed with pharmacological agents.

KEYWORDS: mitoNEET, NL-1, Pioglitazone, Traumatic Brain Injury, Mitochondrial Function

Heather M. Yonutas

December 2nd, 2015
NOVEL TARGETS FOR MITOCHONDRIAL DYSFUNCTION FOLLOWING TRAUMATIC BRAIN INJURY

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December 2nd, 2015
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The work presented here is the culmination of countless hours of work which I invested during my last five and a half years at the University of Kentucky, within the Spinal Cord and Brain Injury Research Center (SCoBIRC). My success within graduate school would not have been possible if it were not for my mentor, Dr. Patrick G. Sullivan. His belief in my abilities and his dedication to teaching me how to successfully “Pile it Higher and Deeper” made me the researcher, and person, I am today. Although I know I pushed the limit of his “open door policy”, he was always there to help me. Additionally, thanks to his lab, specifically Andrea Sebastian, Dr. Jignesh Pandya, Dr. Ryan Readnower and Dr. Hemendra Vekaria, as well as various members of the SCoBIRC such as Dr. Edward Hall, Dr. Indrapal Singh, Dr. Samir Patel and Dr. Rachel Hill, I was able to complete the project which I will be presenting. Without their help and
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- mitoNEET binding with a mitoNEET specific ligand, NL-1, increases cortical sparing and improves functional outcome following a TBI
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CHAPTER 1: THE POWERHOUSE OF THE CELL: MITOCHONDRIA

Mitochondria are key to cellular energetics and cellular homeostasis and have profound implications in the initiation of cell death. The study of these microscopic organelles has provided fundamental insights into the workings of a cell that covers a broad spectrum from genetics to biophysics to cell biology. Studies have demonstrated their importance in various neurodegenerative diseases and have been linked to the secondary injury cascade following a brain injury. For these reasons, mitochondria occupy a central position in the science of Cell Biology and Neurobiology.

Neuronal homeostasis and function has been found to be dependent on mitochondrial bioenergetics. As the “Power Plant” of the cell, mitochondria provide 90 to 95% of the energy needed for the cell to function properly [1-8]. As mitochondria become dysfunctional, cellular dysfunction soon follows. Therefore, research has focused on the discovery of pharmaceutical interventions that target mitochondrial dysfunction associated with various diseased states. Many therapeutic interventions that target mitochondrial function in these diseased states have shown positive results. One disease model that is characterized by vast mitochondrial dysfunction and has shown improved outcome when dysfunctional mitochondria are targeted is Traumatic Brain Injury, TBI. In this chapter there will be a historical overview of mitochondria in the scientific literature. Mitochondrial bioenergetics and their importance to proper brain function will also be discussed. The following chapters will explain cell specific and age related changes that occur within mitochondria with a detailed
explanation into the pathobiology of traumatic brain injury. Further details will also be provided on therapeutic interventions that target mitochondria leading to improved outcomes.

**History of Mitochondria**

Mitochondria have a very rich history in the scientific literature. They have been a subject of study for many Nobel Prizes, demonstrating their importance to overall cellular homeostasis. Although their history is rich, the time between initial observations of mitochondria and the understanding into their functions was long, initially being overshadowed by the discovery of the nucleus. The first official observations of mitochondria date back to the 1841 when cytologist Jakob Henle observed mitochondria in the muscles of insects [9-11]. In 1856, Von Kölliker, a Swiss Anatomist and Physiologist, described this “sarcomere”/organelle with more detail during his studies in spermatogenesis [12]. However, at this point, the function of these unknown intracellular structures was still ill defined, at the time hypothesized as a physical structure that had a role in the transmission of genetic information.

Remarkably, in the 1890s, Richard Altmann termed these intracellular granules “bioplasts” and proposed them to be autonomous, forming bacterial-like colonies within their host cells [13]. Based on our current knowledge and the evolutionary theory of a prokaryotic and eukaryotic symbiosis being formed billions of years ago, this was indeed a very astute observation. However their significance was still unappreciated [14-16]. Scientists in the field continued to described these intracellular structures by terms like “chondros”, “Korn” and
“grain’. Then, in 1898, with the development of improved staining techniques, the Greek term “mitos” was adopted by Carl Benda leading to the new term “mitochondria” [review by Chowdry 1918, quoted in [17] and [13].

Following the discovery of these organelles from a physical perspective, much time passed in the scientific literature before mitochondria were brought up by name again. However, during this down time, researchers were on track for some big findings due to continued progress in the field of cellular metabolism and cellular respiration. Dehydrogenase studies completed by Battelli and Sterns, which was later expanded upon by Heinrich Otto Wieland, and the discovery of insoluble iron containing “respiratory enzymes” (Atmungsferment, which are ironcolloid complexes) by Otto Heinrich Warburg lead to the initial findings which gave insoluble cellular enzymes credit for cellular respiration. However, these founding studies were also followed by great debate. Due to his work with iron containing molecules, Warburg hypothesized that cellular respiration was mediated through oxygen transfer between enzymes. Wieland, due to his investigations into dehydrogenases, believed respiration was mediated through hydrogen transfers. Then, in 1925, David Keilin, during his research involving cytochromes, provided deeper insight into the understanding of the electron transport chain that included dehydrogenases at one end and Atmungsferments (ironcolloids) are the other end, ending the current debate between Weiland and Warburg. Further research was completed by David Keilin, and his understudy E. F. Hartree, leading to the acknowledgement of an “unspecific colloidal structure” which localized these respiratory enzymes that
were the topic of such great debate. It wasn’t until work from Cleland and Slater in the 1950s that these “unspecific colloidal structures” were defined as phospholipids in the membrane of the mitochondria. Additionally, the dehydrogenases that were upstream to the ironcolloid structures were eventually defined by Hans Adolf Krebs as the Citric Acid Cycle, which is often called the Krebs Cycle.

When attempting to understand the importance of mitochondria a discussion about adenosine triphosphate, ATP, is crucial. ATP is a crucial macromolecule in the cell with an importance debated as second to DNA. It was first discovered in 1929 by a German Chemist name Karl Lohmann. However, it wasn’t until the work of Vladimir Engelhart, in 1935, and then Fritz Lipmann in the early 40s, that this compound was credited for many important functions in the cell. Work from Engelhart showed that ATP was necessary for proper muscle contraction. Building upon that, Lipmann was then credited for coining the term “energy-rich phosphate bonds” when he discovered that ATP is the main bearer of chemical energy for the cell. Now, it is well accepted that ATP is made up of the nucleoside adenosine that is bound to three phosphate groups.
Figure 1.1 Chemical Equation for Hydrolysis of ATP: Hydrolysis of ATP leads to the generation of 30.5 kJ/mol of energy plus an ADP molecule and a free inorganic phosphate. [18]
Upon the removal of the outermost, third, phosphate group, adenosine diphosphate (ADP) is formed. The cleavage of this phosphate group leads to the release of $\sim 30.5$ kJ/mol of energy. Conversely, generating an ATP molecule requires an ADP molecule with the addition of an inorganic phosphate group, which is more often than not completed in the mitochondria. Large quantities of ATP are formed and consumed in the human body daily. One report made the assumption that the average adult male has approximately 1 trillion metabolically active cells in the body. Each cell contains roughly 1 billion ATP molecules at any given time. With these values, one can calculate $10^{23}$ ATP molecules in the human body at any given time [19]. As mentioned previous, 90-95% of these ATP made in the body is carried out within the mitochondria by the enzyme ATP synthase, leading into further insight into the importance of these, once ill defined, organelles.

The time of the discovery of ATP, Warburg sought out to discover how these molecules were made. Keeping in mind that he was a proponent of oxidative phosphorylation, he attempted to describe the production of ATP through substrate level phosphorylation in which an ATP molecule was produced as a result of the enzymatic oxidation of a specific compound. As we currently understand cellular metabolism, his hypothesis was not wrong since we do observe substrate phosphorylation during glycolysis and the Krebs cycle. However, this process does not account for the majority of ATP made by the cell. Conversely, H.M. Kalckar proposed a separate method to explain ATP production. He hypothesized that the process of phosphorylating ADP to
generate ATP was linked respiration. Specifically, the energy generated in the process of oxidizing the nutrients within our bodies was harnessed to make ATP, which is a process now termed oxidative phosphorylation [20-25]. As we know, this method is favored in cells with active mitochondria.

About the time that progress was being made in the field of ATP and ATP production, Albert Claude pioneered a separation technique allowing researchers to isolate functioning mitochondria. Within this technique he defined the appropriate buffers and tissue fractionation method based on differential centrifugation [26-31]. These techniques were then applied by Albert L. Lehninger and his group to further validate his previous work concluding that fatty acid oxidation is primarily completed in the mitochondria and that the rate of reactions within the citric acid cycle was high enough to provide the majority of energy to liver cell [32-38]. This observation has never been disproven considering that mitochondria function within liver cells is known to be very high. During these studies, they also determined that fatty acid oxidation and citric acid cycle activity lead to ATP production in a manner that was dependent on oxidative phosphorylation, confirming Klackar’s previous work [35].

With the invention of electron microscopes in the earlier 50s, the structure of mitochondria could be better defined. It is now well accepted that mitochondria are intracellular organelles with a dual (inner and outer) membrane system, each responsible for specific functions.
Figure 1.2 An electron micrograph of mitochondria: This image shows the dual membrane nature and intact cristae observed within mitochondria [39]
The outer membrane (OM) contains many transporter proteins tasked with the import and export of ions and proteins necessary to maintain mitochondrial function [40]. This includes the Voltage Dependent Anion Channel (VDAC) as well as Translocase of the Outer Membrane (TOM) Channel. The inner membrane (IM) has many folds, termed cristae (Figure 1.2), and have areas enriched with inner mitochondrial membrane proteins such as Complex I, Complex III, Complex VI, ATP Synthase, the Adenosine Nucleotide Transporter (ANT), the mitochondrial Calcium Uniporter (mCU), and the Hydrogen Calcium and Sodium Calcium Exchangers (HCE and NaCE respectively), which will all be discussed later due to their importance in mitochondrial bioenergetics and dysfunction. It is these cristae which help to increase the surface area available for mitochondrial respiration and are the site of electron transport and oxidative phosphorylation (OXPHOS). The space between the outer and inner mitochondrial membrane is called the inner mitochondrial membrane space and the space enclosed by the inner membrane is termed the matrix. This matrix space contains the enzymes involved in the Krebs Cycle and electron rich compounds that feed into the electron transport chain.

When discussing mitochondrial bioenergetics and the history of these important organelles, it is impossible to skip the Nobel Prize winning work of Peter Mitchell. Keep in mind that within the late 50s, the idea of substrate level phosphorylation and oxidative phosphorylation were both proposed as mechanisms leading to ATP production. Work from Edward Charles (Bill) Slater, who was trained under Keilin (cytochrome work in the mid-20s), proposed a
chemical nature for oxidative phosphorylation. In this “chemical-coupling hypothesis”, redox reactions could occur between 2 molecules generating the energy necessary to produce ATP [41-43]. However, this theory was lacking information in that a high-energy intermediate necessary to produce the ATP molecule from ADP + Pi was unknown. Then, in 1960s, two theories were proposed, both of which proposed the flux of protons as the energy source necessary for ATP synthesis. The first theory was proposed by Robert Joseph Paton Williams who thought that the flux of protons occurred within the lipid phase of the membrane [44]. The energetics allowed for a dehydration reaction leading to condensation of ADP and Pi to ATP. Mitchell, on the other hand, proposed a more physics based approach in which the lipid membrane acted as a resistor and capacitor helping to separate charges in a manner similar to a battery. This revolutionary theory was called the Chemiosmotic Hypothesis of Oxidative Phosphorylation and will be discussed in further detail later in this chapter [45-49].

At the time that researchers were trying to understand cellular respiration and ATP production, Abraham E. Axelrod (1941) made an observation that Calcium uptake increased oxygen consumption [50]. Although he proposed a mechanism that was disproven, it is the first indication in the literature that hinted to mitochondrial mediated Calcium uptake. Once the proper mitochondrial isolation techniques were known, Siekewitz and Potter (1953) and then Lehninger (1970) both confirmed that mitochondria were able to buffer calcium in a manner that lead to increased oxygen consumption. To further
expand upon this work, Vasington and Murphy (1961) showed that calcium uptake was dependent on the respiratory chain and could be inhibited with the use of 2,4 DNP and FCCP, two well known membrane uncoupler [50]. As we now know, this calcium uptake is dependent on the mitochondrial Calcium Uniporters (mCU) that uses the membrane potential generated by the electron transport chain in buffer cytosolic calcium.

In addition to ATP production and calcium cycling, mitochondria also seem to have an important role in cell survival. Although early on the topic was heavily debated as an in vitro artifact that had no pathophysiological relevance, the mitochondrial permeability transition pore (mPTP) is now pretty well accepted as a potential mechanism of cell death. In the early 50s and 60s, when mitochondrial bioenergetics were being heavily studied, the ability for mitochondrial swelling which was dependent on calcium, inhibited by Mg2+ and ADP, was well documented [51-64]. Additionally, Mitchell even discussed this phenomenon as a detriment to energy conservation during his proposal of his Chemiosmotic Hypothesis. However, the mechanisms behind this swelling were not explained fully until the late 1970s by Haworth and Hunter coined term “permeability transition”. During their studies they described the reversible opening of a proteinaceous pore in the inner mitochondrial membrane leading to the possible flux of ions and solutes up to 1500kDa from the mitochondria to the cytosol [65-68]. This theory was not well accepted until approximately 10 years later when it was discovered that this pore could be inhibited by submicromolar concentrations of cyclosporin A [69-75]. To date, the mPTP is thought to play a
role in oncosis and apoptotic mediated cell death, all which will be discussed later in detail due to their importance in cell death and, therefore, TBI.

Since their initial discovery in the 1840s, mitochondria are now known to be an important and integral organelle in the cell. Their function, superficially, is to mediate cellular respiration and cellular metabolism, in which the mitochondria produce the energy rich compound, ATP, in order to provide an energetic environment within the cell. However, due to work in the 50s and 60s, it is also known that mitochondria have an important function in mediating calcium cycling, which is dependent upon the function of the mitochondrial calcium uniporter. This uniporter uses the potential in the inner membrane space to pump mitochondria from the cytosol into the matrix. Lastly, mitochondria seem to have a very important role in cell survival which can be mediated through the initiation of the mitochondrial permeability transition pore.

**Cellular Metabolism: Today’s Understanding**

Cellular energetics is directly linked to ATP production which occurs through cellular metabolism. Aerobic cellular metabolism can be broken down in 4 separate steps. The first step is the generation of compounds which can be chemically recognized by metabolic proteins within the mitochondria and transport into the mitochondria. Anaerobic metabolism does not include the use of mitochondria generating ATP without the use of mitochondrial mediated oxidative phosphorylation. The next step in aerobic phosphorylation is the Krebs Cycle which is the site of production of the electron rich compounds NADH and succinate. These electron rich compounds are then able to feed into the
Electron Transport System/Chain (ETS), which is considered the third step of cellular metabolism. Lastly, the fourth step which is the site of the ATP synthase, producing an overwhelming majority of the ATP used by the cell. Each of these steps and key players within these step will be discussed throughout the rest of this chapter.

**Glycolysis, Fatty Acid Oxidation and Urea Cycling**

When the cell takes up metabolic compounds such as glucose, lactate, fatty acids or various amino acids, it has to have a mechanism to break them down into substrates that can be transported into the mitochondria and then recognized by appropriate metabolic proteins. Three processes which are important in generating substrates for mitochondria are Glycolysis, Fatty Acid Oxidation and Urea Cycling. Glycolysis is a term generated from “glucose and lysis” referring to the metabolic process of breaking down glucose, a 6-carbon molecule, to pyruvate, a 3-carbon molecule. Pyruvate can be transported into the mitochondria through either diffusion or the mitochondrial pyruvate carrier (MPC). The existence of this transporter that has lead to some debate as to whether it is truly necessary for pyruvate transport, which is a topic that will be discussed some later in this chapter. However, whether through diffusion or MPC, pyruvate will then translocate into the mitochondrial matrix where it will be further broken down into acetyl co-A by pyruvate dehydrogenase (PDH). During the 10 step glycolytic reactions that eventually produce pyruvate from glucose, 2 NADH molecules are generated as well as 2 ATP molecules.
There is a small subset of cells who produce the majority of their energy through glycolysis. One of the most documented and heavily researcher topic related to this are those cells who have undergone mutations, transforming them into cancer cells. The scientific literature shows that cancer cells, which are primarily glycolytic, and have little to no mitochondrial contribution to their energetic profiles. These cells undergo a high rate of glycolysis followed by lactic acid fermentation, having very little contributions from mitochondria. This is one of many situations in which different cells types as well as different regions within the body within the same cell type experience different metabolic profiles which arise as variations in mitochondrial bioenergetics.

Another highly debated topic in terms of metabolic profiles of cells occur specifically within the brain tissue. It has been well accepted that the brain is considered immune privileged and is confined from the rest of the body by the blood brain barrier. The vasculature within the cranial vault is able to provide nutrients to the brain through either free diffusion of small lipid soluble molecules, like oxygen and carbon dioxide, or through active transport. Glucose is thought to reach cells within the brain through active transport by the GLUT1 or GLUT3 transporters. GLUT transporters are enriched in astrocytes allowing for glucose to be favorably taken up by astrocytes and then broken down to pyruvate and then potentially lactate. Although this topic is heavily debated, it is thought that lactate is then transported from astrocytes to the neurons through the Astrocyte-Neuron Lactate Shuttle, which was initially proposed by Pellerin and Magistretti in the late 90s [76-80]. Once lactate is shuttled into neurons, it can undergo a
dehydrogenation reaction with the assistance of lactate dehydrogenase (LDH) producing pyruvate. Pyruvate can then enter the mitochondria as mentioned previously. Differing opinions on this topic believe that neurons are able to take up glucose through the GLUT 3 transporter leading to the initiation of glycolysis in a manner similar to what was described previously.

Additionally, mitochondria can use byproducts of fatty acid oxidation, specifically fatty acetyl CoA, and urea cycle, specifically generating malate, as substrates for ATP production. These metabolic processes are often overlooked when energy to the brain is considered because traditionally glucose, and its derivative lactate, are considered the main energy source. However, it has been shown that up to 20% of the total brain’s energy is obtained through mitochondrial mediated fatty acid oxidation [81]. Additionally, in diseased states, such as following a traumatic brain injury, hypoglycemia is observed leaving cells within the brain to adapt to changes in glucose.

Within this process a fatty acid molecule is shortened by 2 carbons for every turn of the fatty oxidation cycle, producing 1 acetyl coA molecule, 1 NADH molecule and 2 FADH2 molecules [82]. Additionally, the urea cycle has been found to be an important mechanism in the brain for the removal of free nitrogen. This cycle uses free ammonia in the body to feed into mitochondria at malate. In addition, this urea cycle seems to be an important mechanism when considering the generation of \( \alpha \)-ketogluterate from glutamate, which is supported through GDH activity, since ammonia is given off a byproduct in this reaction. This
mechanism will be discussed later in this chapter, however is important to note when thinking about the entirety of mitochondrial related metabolism.

**Krebs Cycle**

Once the appropriate substrates have reached the mitochondrial matrix, the next important step in the metabolic process is initiation of the Krebs Cycle, also called the Citric Acid Cycle and the Tricarboxylic Acid Cycle. This is considered the central metabolic pathways for all aerobic organisms. The process begins with a 2 carbon compound, specifically acetate in acetyl coA, which is generated through the action of Pyruvate Dehydrogenase converting pyruvate. From acetyl coA, 8 chemical reactions completely oxidize the carbons to 2 carbon dioxide molecules, reducing NAD+ to NADH and generating succinate in the process. These two compounds can then be used as substrates within the electron transport chain, specifically at Complex I and Complex II respectively. Additionally, as mentioned previously, malate can be generated during amino acid oxidation feeding into the Krebs cycle during the 7th listed reaction and glutamate is fed into the Krebs Cycle through the activity of GDH producing α-ketoglurate (see below)
The multi-step Krebs cycle is the site where electron dense NADH and Succinate (FADH2) molecules are produced. These substrates can then donate their elections to either Complex I or Complex II, beginning a series of Redox which occur within the Electron Transport Chain.
Within the Krebs cycle, any of the intermediate steps can be substituted for molecules that are generated through processes outside of the Krebs Cycle. One important reaction which seems to have some importance when investigating the function of mitoNEET is the dehydrogenation reaction of Glutamate to α-ketogluterate by Glutamate Dehydrogenase.
Figure 1.4 Glutamate Dehydrogenase can produce α-ketoglutarate: Glutamate Dehydrogenase (GDH) is able to condense glutamate into α-ketoglutarate while simultaneously generating an NADH molecule.
Glutamate Dehydrogenase (GDH) is an enzyme found in all organisms, however is specifically located in the mitochondria of humans. It catalyzes the reversible oxidative deamination of glutamate and H2O producing α-ketogluterate and ammonia. α-Ketogluterate is then able to feed into the Krebs Cycle leading to more NADH for the electron transport chain and ammonia is then cleared from the mitochondria by the Urea Cycle. Interestingly, in lower order organisms, GDH is modulated through transcriptional regulation however in humans its modulation occurs through metabolites. Some important inhibitors are GTP, NADH, ATP and Zn2+ and some important activators include ADP and mitoNEET [83].

Traditionally speaking, PDH is thought to be the gatekeeper of mitochondrial respiration. However, with compelling evidence showing mitoNEET as a redox switch and showing mitoNEETs direct positive effects on GDH activity, it proposes a theory in that GDH mediates mitochondrial respiration through mitoNEET activity. This activity is inhibited in high oxidative states, such as during calcium cycling, leaded to decreased substrates feeding into the electron transport chain and therefore inhibiting state III mediated respiration.

**Electron Transport Chain**

The electron transport chain consists of a series of proteins that are located within the inner mitochondrial membrane, with the exception of complex II that is within the mitochondrial matrix. As mentioned previous, mitochondria have invaginations within their inner mitochondrial membrane which is called
cristae. Within these cristae there are the protein complexes that comprise the electron transport chain (ETC). The components of the ETC include complex I, II, III, IV as well as the mobile carriers, coenzyme Q10 and cytochrome C.
Figure 1.5 The Electron Transport System: The electron transport chain is the site for an overwhelming high production rate of ATP with respects to any other location in the cell. The ETC/ETS cycles through a series of redox reactions. This results in protons being pumped to the inner membrane space which generates a proton-motive force that is harnessed by Complex V to produce ATP.
As electrons are transferred through the electron transport chain, the redox potential of those electrons increase from -400mV, when they are associated with NADH, to 800mV which is where they terminate the process, associating with free oxygen. As the redox potential increases, the Gibbs Free energy decreases in the following manner:

\[-\Delta G = nF\Delta E\]

Where \(n\) is the number of electrons being transferred, \(F\) is defined by Faraday’s Constant and \(\Delta E\) is defined by:

\[\Delta E = \Delta E^\circ + \frac{RT}{nF} \ln \left( \frac{[A_{ox}]_i [B_{red}]_i}{[A_{red}]_i [B_{ox}]_i} \right)\]

For the following redox chemical reaction.

\[A_{red} + B_{ox} \rightarrow A_{ox} + B_{red}\]

Within this equation, \(\Delta E^\circ = \Delta E^\circ_{(cathode)} - \Delta E^\circ_{(anode)}\)

Within this process, the complexes act as an energy-conversion devices, using the free-energy to pump protons from the matrix into the inner membrane space.
Figure 1.6 ETS Energy Changes: This diagram provides the redox potential and the free energy changes that occur as electrons are cycled through the electron transport chain and protons are pumped into the inner membrane space.
Complex I

As mentioned previously, once NADH is produced within the mitochondria it is able to diffuse to the site of the Electron Transport Chain (ETC), specifically to Complex I. Complex I acts as a “gatekeeper” or “entry point” of the ETC therefore serving as a direct link between glycolysis, the Krebs Cycle, fatty acid oxidation, GDH activity and the electron transport chain. It is often called NADH-Ubiquinone Oxidoreductase, or NADH dehydrogenase, since it mediated the oxidation of NADH oxidation and the reduction of Ubiquinone. It is the largest catalytic complex within the electron transport chain having of mix of 45 nuclear and mtDNA generated subunits. Within this ~1000kDa structure are 7, out of the 45, subunits that are encoded by the mitochondrial DNA (mtDNA). Its molecular mass is little under 1000kDa and has a structure similar to a boot. The “foot” of this “boot” is hydrophobic and integrated into the inner mitochondrial membrane [84, 85]. This is the region which one would find the mtDNA encoded subunits. The “ankle” of this “boot” is hydrophilic and extends into the mitochondrial matrix.

The exact coupling mechanism and structure of this protein is unknown however has been proposed by many scientists including Efremov [86]. Within the hydrophilic “ankle” region is the primary electron acceptor flavin mononucleotide (FMN) which is the initial redox site for the oxidation of NADH to NAD+. The electrons are then transferred strategically through seven conserved iron-sulfur (Fe-S) clusters, reaching the N2 cluster which is where the electrons are thought to then be transferred to the ubquinone binding site (Q-site). The hydrophilic region is also called NADH-ferricyanide reductase due to this exact
process. This Q-site is located at the interface of the membranous domain. The atomic structure of the hydrophobic region of complex I is unknown however within this region there are 3 well defined subunits (NuoL/Nqo12, NuoM/Ngio13, NuoN/Nqo14) which are homologous to one another and the Na+ or K+/H+ antiporter. It is thought that this is the site where proton translocation occurs however this too is debated. A scheme is provided below to better explain the previous process.

The overall reaction that occurs at complex I is:

$$\text{NADH} + \text{CoQ} + 5\text{H}^+ \rightarrow \text{NAD}^+ \text{CoQH}_2 + 4\text{H}^+$$

In which NADH is converted to NAD+ by accepting an electron into the Fe-S center of the protein and then transferring them to coenzyme Q10 [87-91].

Historically, there are three well known inhibitors of Complex I that have been found to inhibit the reduction of ubiquinone. These inhibitors have been categorized into 2 to 3 different types depending on your source. This classification is dependent on kinetic studies including Class I/A-type, Class II/C-type and C-type. Class I/A-type is represented by an antibiotic called piercidin A, Class II/B-type is represented by a fish poison called rotenone, and C-type is represented capsaicin [92, 93]. These inhibitors all seem to bind within the same relative region of the complex however the exact mechanism of action are seemingly different. Work from Fato found that the Class I and Class II, categorized into Class A, inhibitors have been shown to increase reactive oxygen species where the Class B, referred to as Class III/C-Type in other locations, leads to decreased ROS production. The mechanism of actions of
these inhibitors are important to know in order to A) further understand the exact structure of Complex I and B) to understand how mutations and toxins which effect the structure and function can lead to specific diseases.

It is seemingly important to provide further clarification at this point. The use of mitochondrial inhibitors should not be confused with mitochondrial uncouplers. In the literature, the term uncoupler seems to be very freely. When using uncouplers, the redox reactions that occur within the electron transport chain persist, however the uncoupler in question depletes the membrane potential leading to no functionality at the ATP synthase, and therefore no ATP production. Specifically, uncouplers are able to unlink the redox reactions (electron flow) within the ETC from the phosphorylation of ADP molecules at the site of the ATP synthase. During the use of uncouplers, the redox reactions within the ETC are generally running at maximum capacity in order to regenerate the concentration of hydrogen molecules within the inner membrane space. However, as these protons are pumped into this inner membrane space, they are immediately translocated back into the matrix, leading to no overall change in the membrane potential and no protonmotive force to run allow ATP synthase to function. On the contrary, when using mitochondrial inhibitors, this is a term that is specific for compounds that actually interact with proteins to stop their function, leading to a halt in the redox reactions at the ETC. For instance, rotenone is a commonly used Complex I inhibitor which will be discussed later in this document. Rotenone interacts with Complex I, inhibiting the flow of electros
through the complex. This leads to no electrons entering the ETC and therefore, no functionality of the ETC.

ROS production is seemingly a hallmark of Complex I activity. It has been well documented that Complex I is one of the main sites of the production of reactive oxygen species for the ETC [94]. Within this complex, electrons are at their highest relative potential compared to any other site within the ETC. It is thought that this high potential leads to increased electron leak within the complex. As electrons leak out of the complex, they are able to bind to free O2 generating superoxide anions. These anions can be neutralized by antioxidant defense mechanisms such as the mnSOD (manganese superoxide dismutase) however at times they are able to evade this mechanism and damage nearby proteins. The exact site of this electron leak is unknown however strong evidence has been proposed by Fato that it occurs at the N2 cluster mentioned previously, which is the site immediately before the electron is transferred to ubiquinone. However, contradicting evidence has also been proposed implicating ubisemiquinone, FMN, and the iron-sulfur cluster N1a as contributor to ROS.

In addition to the damage that ROS can do to nearby proteins, mtDNA can also become damaged due to the havoc wrecked by ROS production. It is thought that throughout the life of an organism somatic mutations to mitochondrial DNA accumulate due to years of ROS, as well as RNS (reactive nitrogen species), mediated damage. This DNA damage leads to the production of damages proteins. Since Complex I has 7 subunits encoded by the
mitochondrial genome, it is thought that this protein takes the largest hit, leading to increased electron slippage. This theory is termed the “Mitochondrial Theory Of Aging” which will be discussed in more detail in Chapter 2.

**Complex II**

Complex II (Succinate:Ubiquinone Oxidoreductase), also referred to as Succinate Dehydrogenase, utilizes the conversion of succinate to fumarate to generate FADH2 from the FAD (flavin-adenine dinucleotide) coenzyme. This is the only Complex within the ETC that is also an integral part of the Krebs cycle, being utilized in the 6th chemical reaction where succinate is converted to fumarate [95].. This complex has a covalently linked FAD molecule within it. Once in contact with Succinate, electrons are donated to the FAD molecule, generating FADH2. From the production of FADH2, electrons are then transferred sequentially between 3 different Fe-S groups until reaching the membranous location of Complex II. At this point the electron is passed to heme b group (CybL-CybS) and then transferred to ubiquinone. This is the only complex within the ETC that does not contribute to the membrane potential discussed previously since there is no translocation of protons from the matrix to the IMS, making it a less energetically favored process compared to Complex I.

The overall reaction generated by this complex is

\[
\text{Succinate} + Q \leftrightarrow \text{Fumarate} + \text{QH}_2
\]

Where Q is ubiquinone and QH2 is ubiquinol, the reduced form of ubiquinone.
Complex III

Complex I and II transfer their electrons to Ubiquinone (Coenzyme Q10) located within the IM. Complex III is the next player in this process which oxidizes Ubiquinol producing Ubiquinone (Ubiquinol-Cytochrome-C Oxidoreductase) eventually reducing Cytochrome C via the protonmotive Q-cycle. This complex is much smaller than Complex I containing only 11 subunits, 3 of which are respiration subunits (cytochrome B, cytochrome C1 and Reiske protein which is an Fe-S cluster), 2 which are core proteins and 6 are low-molecular weight supporting proteins.

2 electrons are transferred to Complex III when Ubiquinol is oxidized to Ubiquinone. One of these electrons is transferred onto the 2Fe-2S group of the Rieske center. Rieske proteins are a specific iron-sulfur proteins found within cytochrome bc1 and cytochrome b6f complexes. From this center, electrons are able to transfer to the heme group of Cytochrome C1 and then picked up by cytochrome c and diffuses to Complex IV. The other of the 2 electrons is transferred onto a heme group in cytochrome b before being transferred to a ubiquinone molecule. This leaves ubiquinone in a partially reduced form generating a semi-ubiquinol radical. Then, a second ubiquinol molecule diffuses to complex III donating its electrons in a similar manner. Now, the semi-ubiquinol molecule can gain a second electron resulting in the production of another ubiquinol and the process repeats itself. During this process, 2 ubiquinol molecules are used to translocate 4 protons from the mitochondrial matrix to the inner membrane space.
The overall reaction that occurs at the site of complex III is

\[ \text{QH}_2 + 2\text{Cyt C}^3 + 2\text{H}^+ \rightleftharpoons \text{Q} + 2\text{Cyt C}^2 + 4\text{H}^+ \]

As mentioned previously, Complex I contributes to a large amount of the electron leak that occurs within ETC. However, Complex III also contributes to this electron leak. It is thought that approximately 1 to 2% of the O2 used within the mitochondria is due to this electron leak [2, 96]. It has been discovered that the within the center of Complex III is a high reduction potential chain called Q0 which contains the Rieske protein (Fe-S cluster). This is the site in which the majority of electron slippage is thought to occur.

**Complex IV**

As the last step in the ETC, Cytochrome C transports its electrons to Complex IV (Cytochrome-C Oxidase). Because of its position within the ETC, Complex IV is often considered a major regulator of Oxidative Phosphorylation. It is made up for 13 subunits which is contributed to by both nuclear (10 proteins) and mitochondrial (3 proteins) encoded proteins [97]. It is thought that the nuclear encoded proteins primarily regulate oxygen consumption and proton translocation and the mitochondrial encoded proteins are the catalytic sites.

There are 4 redox-active centered within Complex IV. Listed in the order of function, CuA, heme a (Fea), heme a3 (Fea3) and CuB. CuA is initially reduced by cytochrome c. From here the electron travels to Fea and then enters the “catalytic” site of the complex (where Fea3 and CuB are located) where free oxygen is reduced eventually leading to the production of H2O. During this
process 2 protons are pumped from the mitochondrial matrix to the inner membrane space.

The overall chemical reaction for Complex IV is

$$4 \text{Fe}^{2+}\text{-cytochrome c} + 8 \text{H}^+ + \text{O}_2 \rightarrow 4 \text{Fe}^{3+}\text{-cytochrome c} + 2 \text{H}_2\text{O} + 4 \text{H}^+$$

In which electrons are given off by a reduced form of cytochrome c, translocate through the complex until they reduce free oxygen and eventually produce $\text{H}_2\text{O}$. During this process, $\text{H}^+$ ions are pumped into the inner mitochondrial space helping to contribute to the membrane potential, which will be used by Complex V/ATP Synthase to generate ATP molecules.

**ATP Synthase**

Following the contribution of an NADH molecule and consequently 2 electrons being donated to the ETC, 10 protons will be translocated into the IMS. As we know, the ETS is continuously cycling and therefore protons are continuously being pumped out of the matrix. As the concentration of protons within the IMS increases, a multi-component proton-motive force is generated having both a differential pH and an electrochemical gradient leading to a membrane potential ($\Delta\Psi$) [98]. This high energy environment is utilized by Complex V (ATP Synthase) to facilitate the phosphorylation of ADP into ATP.

Complex V, like the other Oxidative Phosphorylation Complexes, is located, partially, within the inner mitochondrial membrane. It has multiple subunits which are contributed to by both nuclear and mitochondrial genes. In addition to having multiple subunits, this complex also has multiple domains including the Fo domain, the F1 domain and the stalk/accessory domain. The Fo
domain is the hydrophobic domain located within the membrane. This domain is often referred to as the “motor” of the complex. It has 1a, 2b and 12c (debated 10-15c) subunits [99]. The c subunits are aligned in a ring shape forming the proton channel that funnels the protons from the IMS to the F1 domain. These c subunits are thought to interact directly with the F1 domain to regulate proton flow and consequently ATP production [99].

As mentioned previously, the Fo domain is often thought to be the motor of the complex. When the c-ring rotates, being “pushed” by the high-energy proton-motive force mentioned previously, it causes movement of the γ subunit within the F1 domain, which is attached to the c-ring. The ε subunit causes a confirmation change in the α and β subunit within the F1 domain. As these confirmation changes occur, it opens the α and β subunit to the matrix allowing the diffusion of ADP and Pi into the catalytic region of these subunits. Then, the γ subunit rotates again causing the condensation reaction for ADP + Pi to ATP generation. The next rotation of the γ subunit causes a third confirmation change to the α and β subunit which opens the site to the matrix again. This allows ATP to flow out and ADP + Pi to diffuse in, repeating the process [100].

In addition to the Fo and F1 subunit, there is also an accessory/shaft domain which includes an Oligomycin Sensitivity Conferring Protein (OSCP) subunit. This is the site where a bacterial antibiotic called oligomycin interacts. When oligomycin binds to this OSCP subunit, it inhibits the rotation within the Fo domain and consequently blocks the catalytic site of the F1 subunit leading to a complete inhibition of the ATP Synthase [101].
Figure 1.7 ATP Synthase: A diagram of Complex V/ATP Synthase including the subunits where were specified previous within the text.
Chemiosmotic Coupling

Properly defined, the principal of Chemiosmotic Coupling is based upon the physical chemical property called Chemiosmosis. Chemiosmosis is the physical movement of ions from a highly concentrated region to a lower concentration region when these regions are separated by a selectively permeable membrane. This process requires the use of a transport protein since the ions in question are not permeable across the selectively permeable membrane. This principle is analogous to the principles of osmosis however as opposed to the flow of water, chemiosmosis concentrates on the flow of ions. Within the mitochondria, these ions are protons. In 1961, Dr. Peter Mitchell was awarded a Nobel Prize for defining this process which is harnessed to make ATP within mitochondria and provides a physical chemical understanding to the ETS.

Within mitochondria, Complex I, III, and IV use the energy rendered by local redox reaction to pump hydrogens into the IMS. These protons within the IMS are not permeable through the inner membrane, which is the selective permeable membrane in question. The ATP Synthase/Complex V then uses the principles of Chemiosmosis to provide the energetics necessary to generate the high energy ATP molecules.

ROS Production

Although OXPHOS is an amazing process, it does have the propensity to create toxic byproducts. Because of this, mitochondria are not only known as the cell’s power plant but they are also known as one of the cell’s major supplier
of Reactive Oxide Species (ROS) and Reactive Nitrogen Species (RNS). During OXPHOS, complex IV will release an electron to molecular oxygen (O$_2$) allowing for the eventual formation of H$_2$O. However, electrons “slippage” can also occur. This occurs as a result of inefficiencies within primarily Complex I and III, however slippage also occurs at Complex IV. Once an electron slips, or is released, from the ETC, it can reduce O$_2$ and/or react with proteins, nucleic acids and fatty acids within the mitochondria and mitochondrial membrane leading to damage. This process is often referred to as metabolic mediated oxidative damage.

Following slippage of an electron from the ETC, mitochondrial manganese superoxide dismutase (MnSOD, SOD2) which is a mitochondrial located metalloenzyme that is a crucial for the dismutation of this highly reactive O$_2$•$^-$ into H$_2$O$_2$, can neutralize any reactive O$_2$•$^-$ formed. This protein is located within the mitochondrial matrix, within the vicinity of the ETC. However, H$_2$O$_2$ is also a strong oxidizing agent and therefore needs to be further neutralized. Glutathione peroxidase, which is also located within the mitochondria, is then used to convert H$_2$O$_2$ into neutral H$_2$O [102, 103].

The mechanisms used to neutralize of these toxic byproducts are very efficient however even with them in place the cells within the brain still seem to be more vulnerable to oxidative stress compared to other tissues. This has been attributed to the fact that the brain is a high energy organ that consumes approximately 20% of the body’s total oxygen and has naturally lower levels of endogenous antioxidant activity relative to other tissue [104-106]. Neurons have
high energy demands compared to other cell types. The Na+/K+ ATPase pumps located within their cellular membrane function to maintain the membrane potential necessary for action potentials to be generated. It is estimated that 1/3 of the entire body’s ATP production in 1 day is used by these pumps [107]. Due to the fact that neurons primarily use mitochondrial oxidative phosphorylation to generate their ATP pools, they produce more metabolic mediated oxidative stress which can be detrimental to the cell over time.

**Calcium Cycling**

In addition to producing 95% of the cell’s energy currency, ATP, and being a major source of oxidative stress, mitochondria also play an integral role in the buffering of cytosolic calcium. This is important since calcium is a potent intracellular signaling molecule which has beneficial and detrimental roles in neurons. In the brain during normal cellular interactions, neurons become activated following the binding of various neurotransmitters to their respective metabotropic and/or inotropic receptors. One interesting neurotransmitter that has been implicated in the calcium signaling pathway and has an important role in the secondary injury cascade of TBI is glutamate. Glutamate binds many receptors but one of importance when discussing calcium cycling is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, also known as the AMPA receptor. Voltage dependent Na+ channels within these same synaptic densities can also activated by glutamate. Once glutamate is bound, these ionotropic receptors move to an open confirmation leading to the influx of Na+ causing a local depolarization. This local depolarization leads to the displacement of the
Mg$^{2+}$ blockage in the N-methyl-D-aspartate receptor, NMDA receptor. Once removed, the NMDA receptor has a higher probability of being in an “open” confirmation allowing Calcium to enter the neuron.

As the NMDA receptors remain in an open confirmation, Calcium will continue to enter the neuron. At lower concentrations, this Calcium has been shown to regulate neurotransmitter release within the synaptic cleft. Also, as transient increases in cytosolic Calcium level occur, mitochondria sink Calcium in an electrogenic fashion, removing the ions from the cytosol. These increased Calcium levels within the mitochondria have been shown to directly regulate the Krebs cycle through activation of pyruvate dehydrogenase, PDH, NAD+-dependent isocitrate dehydrogenase, ICDH and α-ketoglutarate dehydrogenase complex, KGDHC. Activation of these various metabolic proteins all leads to increased NADH production, which can, in turn, generate increased ATP production by the ETC. Calcium mediated activation has also been shown to increase Complex V (ATP Synthase) activity resulting in increased ATP synthesis as well [108, 109].

In addition to the direct effects of Calcium within the mitochondria, these ions also have indirect effects which is equally as important to mitochondrial function and therefore cellular metabolism. As mentioned previously, the ETC generates a negative membrane potential, $\Delta\Psi$, within the mitochondria. Calcium uniporters, located in the inner mitochondrial membrane, use this $\Delta\Psi$ generated by the ETC to pump calcium ions down their electrochemical gradient into the mitochondrial matrix. As the calcium uniporters continue to translocate calcium
into the matrix, the $\Delta \Psi$ diminishes leading to further activation of the ETC in an attempt to restore the depleted potential. This process provides an intimate relationship between mitochondrial bioenergetics, calcium homeostasis and ATP production. This same mechanism can eventually lead to mitochondrial dysfunction when calcium levels become too high. In many diseased state, such as following an injury to the CNS, calcium dysregulation is often observed. This can lead to electron slippage at the site of the ETC and pathogenic decreases in mitochondrial membrane potential. In the process, oxidative stress increases which can initiate the formation of the mitochondrial permeability transition pore, mPTP.

**Mitochondrial Permeability Transition Pore**

Healthy mitochondria generally maintain a Nernstian membrane potential of around 180mV. As mitochondrial matrix Calcium concentrations, oxidative stress and damage to ETC proteins increases, this $\Delta \Psi$ can become depleted. Once around ~100-120mV, the ETC will begin to slow down, stop and potentially start running backwards. Once this occurs, ATP production will decrease and if the ETC begins to run backwards, then ATP will be consumed by the ATP synthase\Complex V. Not only will this lead to decreased ATP stores but generates ROS and RNS [110]. The increase in ROS and RNS can then lead to increased oxidative damage within the mitochondria. Mitochondrial dysfunction is further exacerbated as the damage to lipid, protein and nucleic acid modifications continues. Eventually, the redox potential within the mitochondria changes and crucial mitochondrial proteins like adenine nucleotide translocator (ANT) undergo
oxidative modifications. This occurs specifically as a thiol oxidation on ANT resulting in a conformational change that promotes cyclophilin D (cypD) binding. Once ANT, an inner membrane mitochondrial protein, is bound to cypD, an appropriate conformational change occurs in order to promote ANT, cypD, and many other proteins within the complex to bind with voltage-dependent anion channel, VDAC [111-113]. VDAC is an outer membrane mitochondrial protein that once bound to this inner mitochondrial membrane protein complex, including Cyclophilin D, ANT, and many other proteins, forms the mitochondrial permeability transition pore, mPTP. The formation of the mPTP is a heavily debated topic with many models proposed. This specific mPTP description is the classic model which provides a good foundation for some of the therapeutic approaches which will be discussed further in Chapter 2.

Whatever model you prefer for the mPTP formation, once formed the pore exposes the inner mitochondrial matrix to the cytosol, allowing the mitochondria to release apoptotic initiating factors like Cytochrome C, SMAC/diablo and AIF which all promote cell death. Additionally, mitochondria can also initiate another route of cell death called necrosis. This occurs as a result of ion concentration difference, like chlorine, across the mitochondria membrane. These ionic concentration differences allow water to freely diffuse across the mitochondria membrane as a result of osmosis. As this perpetuates, swelling of the inner membrane occurs due to osmotic pressure, ATP production decreases due to dysfunction within the ETC, the mitochondrial membrane potential decreases and oxidative stress to increases. This causes a complete destruction of the
mitochondria as a result of the swelling of the inner mitochondrial membrane. Eventually these mitochondria will burst leading to the initiation of cell death.
Figure 1.8 The mechanisms of mitochondrial mediated cell death:
Mitochondrial mediated methods to cellular death. This figure was adapted from [108]
Redox Sensitive Switch in Mitochondria

In terms of history, to date, research related to mitochondrial homeostasis or dysfunction in various disease states is flourishing. Following the generation of a specific group of PPAR ligands called thiazolidinediones, TZDs or glitazone, which will be discussed at the end of chapter 3 and within chapter 4, a novel mitochondrial membrane protein was discovered (2004). This protein was found to bind to a specific TZD which is FDA approved for type 2 diabetes called pioglitazone. This novel mitochondrial membrane protein was termed mitoNEET due to its CDGSH-type zinc finger domain which actually binds iron, as opposed to zinc. mitoNEET has been labeled as an outer mitochondrial membrane protein however from the same report that published the work, it was found to have a localization within the inner mitochondrial membrane as well.
Figure 1.9 Location of mitoNEET is not just the outer mitochondrial membrane: Work from Wiley showing the mitoNEET is located within the outer membrane however can also be found in the inner mitochondrial membrane [114]. This figure was edited from [76] showing the presence of mitoNEET within inner membrane fractions.
Further supporting the notion that mitoNEET can also be localized within the inner mitochondrial membrane is work from Roberts et. al. at Eastern Illinois University. They found that mitoNEET is able to bind to Glutamate Dehydrogenase I (GDHI), which is a protein located in the mitochondrial matrix, and that this interaction leads to increased GDHI activity [83]. Additionally, within the work, mitoNEET was found to form homodimers proposing a mechanism is that mitoNEET binding GDHI leads to increased GDHI activity however in oxidized states mitoNEET can homodimerize leading to decreased GDHI activity [83]. Recent work from Landry also found that once oxidized, glutathione reductase is able to reduce mitoNEET back to its original structure, in theory rendering it active again [115].

As mentioned previously, mitoNEET will be discussed further in the following chapters, however this protein has become of great interest to researchers who investigate redox shift within mitochondria. Due to its structure, which contained Fe-S clusters, this protein is structured perfectly to react to changes in redox state. Additionally, mitoNEET has also been shown to mediate mitochondrial respiration, allowing for the hypothesis that this protein would be able to sense possible harmful changes in redox and inhibit respiration to avoid damage to inter-mitochondrial proteins. This is a novel idea which will be expanded upon later in this document.
Methods Used to Measure Mitochondrial Respiration

Prior to 1944, when Albert Claude published the first recipe for the appropriate buffers to isolate mitochondria, the field of mitochondrial studies were heavily based on microscopic studies. Upon the isolation of healthy, viable, respiring, well coupled mitochondria, many more studies could be done. Some of these initial methodologies are still used till this day.

As mentioned previously, mitochondrial oxidative phosphorylation couples the use of oxygen, which is reduced to water at Complex IV, to the phosphorylation of ADP, which occurs at Complex V. Therefore, the rate at which oxygen is consumed by mitochondria will give insight into the bioenergetics, which is specific for the substrate given to allow the mitochondria to respire.

Clark Electrode/Oxytherm

In order to measure the amount of oxygen used by mitochondria, a method for measuring small changes in oxygen within a liquid had to be discovered. This was done by Leland Clark in 1953. Known as the “Father of Biosensors”, Dr. Clark used a Ag/AgCl electrode and a Pt cathode separated by a KCl electrolyte to measure oxygen within a sample. The principles of this design were adapted in 1972 by Hansatech Limited, an electronic company, located out of King’s Lynn in Northern Europe. Within this device, a potentiating voltage is applied across two electrodes. This causes the platinum to become negative and the silver to become positive, generating a cathode and anode respectively. These surfaces are then covered with a thin layer of KCl solution.
and an oxygen permeable membrane. As oxygen diffuses through the membrane, it is reduced by the platinum allowing for a current to flow through the KCl solution. The silver is then oxidized and silver chloride is deposited on the anode. **Figure 1.10** shows images of an oxytherm electrode being prepared and a diagram to indicate the importance of each component within the electrode.
Figure 1.10 Clark Electrode: Building the electrode is an important step when using the oxytherm. The electrode has a platinum cathode and silver anode within the device. A drop of KCl buffer is added to the top of the cathode and anode and then a piece of cigarette paper is added to the top of the electrode, which should have contact with the cathode and anode, in order to build the appropriate KCl bridge. A piece of Oxygen Permeable Membrane is then added to the top of the electrode and secured in place with a rubber o-ring.
The chemical reaction that occurs are each cell is as follows:

At the platinum cathode:

\[ \text{O}_2 + \text{H}_2\text{O} + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2 + 2\text{OH}^- \]

\[ \text{H}_2\text{O}_2 + 2\text{e}^- \rightarrow 2\text{H}^- \]

At the silver anode:

\[ 4\text{Ag} \rightarrow 4\text{Ag}^+ + 4\text{e}^- \]

\[ 4\text{Ag}^+ + 4\text{Cl}^- \rightarrow 4\text{AgCl} \]

As one may notice, this leave 4AgCl as an end product. This does occur and leads to accumulation on the anode which has to be removed overtime. [116]

Once the electrode is “built”, it is attached to a cord and the corded electrode is secured within the back of the Oxytherm Electrode Control Unit. The signal from the electrode then travels through the cord to the control unit where it is converted into a digital readout that is then read by the computer.
Figure 1.11 The Hansatech Oxytherm: The Oxytherm which is where the electrode from figure 1.10 is secured. Mitochondria with respiration buffer are placed within the chamber and the electrode is placed on the underside of the chamber. This allows the electrode to measure the change in oxygen content within the mitochondrial slurry located within the chamber.
Seahorse

Another novel device that has gained increasing popularity over the last few years as a tool for measuring mitochondrial bioenergetics is the Seahorse Bioscience Flux Analyzer. This device uses an 8, 24 or 96 well microplate allowing the researcher the ability to measure multiple sample at one time. Similar to the Oxytherm, this device measures oxygen within a slurry however the measurement techniques are different and therefore allow for the measurement of the proton concentration as well. The Seahorse disposable assay kit has two parts. The first part is the sample cell culture microplate which is where your 5-10ug of mitochondrial protein are placed in approximately 500uL of respiration buffer (recipe provided in the methods section). The second portion of the cartridge is called the solid state biosensor cartridge. This cartridge has four injection ports and two biosensor sleeves per well. The four injection ports allow for the injection of 4 different mitochondrial substrates or drugs per well for your study. The biosensor sleeves are strategically placed in the center of the four ports and are the site at which oxygen, and proton, levels within the mitochondrial sample are measured.

At the base of each sleeve are patented polymer embedded fluorophores. The oxygen biosensor quenches in the presence of oxygen and the other fluorophore is sensitive to protons. As the substrates or drugs from the ports are injected, the mitochondria within the sample begin to respire respectively. This leads to a signal which is read by optic fibers within the actual seahorse machine.
Figure 1.12 Seahorse Diagram: Representative figures of the Seahorse Bioscience Flux Analyzer with the Biosensor Cartridge, microplate, polymer fluorophores and fiber optics (within the Seahorse) where the signal is read.
Pros and Cons of Both Methods

Although both methods are efficient at measuring mitochondrial bioenergetics, depending on the experiment that one is running, one method may be superior to the other.

Oxytherm

Pros

1. Allows for intricate studies where drug or substrate concentrations can easily be adjusted throughout the experiment.
2. No Limit on the number of substrates added.

Cons

1. Requires 50-100ug of mitochondrial protein.
2. Can only run one sample per Oxytherm device. *Multiple Oxytherm devices can be run in serial order
3. Takes approximately 15 minutes per sample

Seahorse:

Pros

1. Can run 8, 24 or 96 samples at one time.
2. Only requires 5-10ug of mitochondrial protein.
3. Takes approximately 37 minutes to run each plate, see table in methods section for specific details.

Cons

1. Only has 4 injection ports
2. Cannot vary any of the experiment once the plate is placed in the Seahorse

3. If too much mitochondria or substrate is added during the loading process of the plate, the reliability of the OCRs are lost. This requires further calculations after the experiment is run.
CHAPTER 2: MITOCHONDRIAL FUNCTION VARIES WITH AGE AND TISSUE TYPE

As discussed in Chapter 1, mitochondria are extremely important for normal cellular metabolism, ATP production, calcium cycling and the mediation of cell death. These functions lead to overall cellular homeostasis and, therefore, proper cellular function. When observing mitochondrial function however, it is important to consider the cellular environment of those specific mitochondria. It has been shown that mitochondria found in one tissue type may not act the same as mitochondria in another tissue type, such as spinal cord mitochondria compared to cortical tissue mitochondria or liver mitochondria. Additionally, even mitochondria within the same cell type, such as synaptic versus non-synaptic regions of a neuron, seem to exhibit different bioenergetics. To further confound this topic, in many diseased states mitochondrial dysfunction has been observed. Aging is one culprit which can lead to increased cellular damage resulting in cellular dysfunction. Throughout this chapter, the effects of aging within brain derived mitochondria and how these changes become more apparent within specific tissue types will be discussed. Additionally, from the research it seems as if TBI mediated mitochondrial dysfunction may mimic accelerated forms of the mitochondrial theory of aging. This is important because it proposes a therapeutic use of the pharmacological agents used to target the cellular and mitochondrial dysfunction related to pathological aging states to assist with TBI related dysfunction as well.
Age Related Changes in the Brain

Throughout a person’s life, their brain is constantly undergoing structural changes. Imaging studies in humans have repeatedly shown that aged brains have decreased gray and white matter and enlarged ventricles [117-119]. However, surprisingly, these age related changes in brain volume do not seem to be due to a loss of neurons but are seemingly the result of shrinking neurons, reduced synaptic spines, decreases in synapses and changes in myelination [119-128]. As these structural changes in the brain occur, functional changes also ensue. With age related structural changes, the functional changes observed are generally thought to result in a deficit. For instance, reduced cognitive abilities, such as processing speed, attention, executive functions, and episodic memory, are all commonly seen in healthy aging [129-134]. In a large population these age related functional deficits can progress into a neurodegenerative disease states like Alzheimer’s Disease (AD).

With age related structural and functional changes, increasing evidence has provided strong support that specific cellular changes occur in brain as well [135-138]. The cellular changes observed are thought to be linked to the anatomical and functional changes that occur in the aging brain. Some of the most common cellular changes observed with age are the oxidative damage of lipids, proteins, RNA and DNA. In the 1950’s, a researcher at the University of Berkley, Denham Harman, proposed a compelling mechanistic link between the functional and cellular changes observed with aging. At the time, Dr. Harman had conducted years of radiation experiments, which is known to induce free radicals
and therefore cause damage. During these studies he also observed metabolic changes that were similar to what was seen in aging. With these findings he hypothesized that with aging free radical damage accumulated, similar to what was seen with radiation treatment. The years of accumulating free radicals eventually resulted in oxidative damage to intracellular components leading to cellular dysfunction and changes in metabolism. This theory became the foundation for the Free-Radical Theory of Aging (FRTA) [139] and provides a mechanistic link between oxidative damage, cellular dysfunction, changes in metabolism and age. To further support this theory, studies of human autopsy tissue show higher levels of oxidative damage to nucleic acids [140-142], proteins [141, 143-146] and lipids [142, 146, 147] in aged brain as compared to young brain and changes in brain metabolism has been linked to various age related disease [148-150].

As mechanistic studies into the sources of oxidative stress progressed, Chance et al showed that the mitochondrial electron transport chain was a source for the production of reactive H$_2$O$_2$ which is a strong oxidizing agent, which was mentioned earlier [151]. Further expanding upon these findings, it was also found that the H$_2$O$_2$ produced was a product of MnSOD converting O$_2$\textsuperscript- into H$_2$O$_2$ [2, 152-154]. As mentioned previously, SOD2 is now knows as a mitochondrial located metalloenzyme that is a crucial for the dismutation of highly reactive O$_2$\textsuperscript- into H$_2$O$_2$ and O$_2$ [155]. This was an important finding for two reasons: One: It proved mitochondria were an important site for ROS production. Two: It proved, for the first time, that oxidative stress was linked directly to
metabolism. As mentioned previously, in normal metabolic processes, ROS and RNS are produced. [156-159]. In a healthy system, after production, these reactive species are immediately neutralized, avoiding deleterious consequences. However, if not neutralized, they can diffuse away from the site of production and damage proteins, lipids, DNA or RNA [156].

With the production of ROS and RNS being understood as a byproduct of OXPHOS, Harman expanded upon FRTA to acknowledge mitochondria as the major source of oxidative stress within a cell. He modified FRTA to the mitochondrial free radical theory of aging (MFRTA) [139, 160]. Linking age related functional deficits to the function of an organelle became an important turning point in the study of aging. As the “powerhouse” of the cell, mitochondria are extremely important organelles for normal cellular homeostasis [7]. Interestingly, within anaerobic cells and tissues, 90% of oxygen consumed is used by mitochondria. Mitochondria primarily use this oxygen for the production of ATP during oxidative phosphorylation within the electron transport chain. In most aerobic healthy cells, mitochondria are the main source of the reactive oxygen species (ROS) produced [160-163]. However, due to the high production of ROS, mitochondria are extremely vulnerable to oxidative damage [164]. Well aligned with MFRTA is the understanding that one of the primary hallmarks of aging is mitochondrial dysfunction [161, 165]. The literature suggests that mitochondrial function is compromised with age in the human brain [158, 159, 166, 167]. In normal aging, mitochondrial respiratory chain activity declines [168], mitochondrial metabolism-associated enzymatic activity such as aconitate
decreases [169] and the rate of somatic mitochondrial DNA (mtDNA) mutations increases [14, 170].

MFRTA was the first theory of its kind to acknowledge a possible link between mitochondrial oxidative phosphorylation, ROS production, cellular damage and functional deficits related to aging [171]. In this theory, it is proposed that mitochondrial produced ROS will diffuse away from the site of production to damage proteins and mtDNA. This leads to increased damage to proteins within the ETC, resulting in continually increased ROS production as electron slippage occurs by the damaged proteins [15, 172]. Therefore, mitochondrial dysfunction and the production of ROS, combined with lower endogenous antioxidant activity, may lead to increasing oxidative damage to molecules critically important to neuronal function. The combination of mitochondrial dysfunction and production of ROS may be a key contributor to the deleterious effects of aging on the brain [14, 167, 173-179]. This mitochondrial dysfunction can lead to increased oxidative stress, changes in protein processing and changes in metabolism. Although there is evidence arising that contradicts the Dr. Harman’s MFRTA, there are still many supporters [171]. This will be the mechanistic foundation used in this chapter to explain the deficits seen related to aging and the therapeutic rational for antioxidants and mitochondrial co-factors to improve aging and decrease the onset of cognitive disorders.

Age Related Cellular Dysfunction as a causation for Neurodegenerative Diseases

Many diseased states are thought to be precipitated by increased oxidative stress within the cell causing damage and consequently dysfunction.
This is congruent with the MFRTA which was discussed earlier. Further elaboration on this theory helps to explain the pathology seen in Alzheimer's Disease [180] since oxidative damage has specifically been linked to age-associated neurodegenerative diseases such as Alzheimer's disease (AD) [181-183]. AD is a progressive neurodegenerative disease that causes dementia in the elderly, affecting an estimated 5.5 million people in the United States [184]. It is characterized by the accumulation of beta-amyloid (Aβ) in extracellular senile plaques and intracellular hyperphosphorylated tau protein in neurofibrillary tangles [185]. Consequently, extensive neuron loss is observed in the AD brain in the cortex and particularly within the hippocampus, a region of the brain involved with memory. In an AD affected brain, further increases in oxidative damage to protein [141, 144, 145, 186-193], lipid [142, 147, 194-197], DNA [198-200] and RNA [175, 201, 202] are seen relative to elderly controls. In addition, endogenous antioxidant activity in the AD brain is reduced relative to age-matched controls [141, 146, 203]. Proteins particularly vulnerable to oxidative damage have been identified by proteomics, with a subset of these proteins putatively involved directly or indirectly in the production and accumulation of AD neuropathology [204].

Mitochondrial dysfunction also occurs in AD, with decreased respiratory chain activity [177, 205, 206] and increased mitochondrial DNA mutations [207] observed at higher rates when compared to age-matched controls. Further, decreased cytochrome oxidase activity in the posterior cingulate cortex of AD patients is correlated with hypometabolism seen by positron emission studies.
A gene array study in the cingulate cortex shows that energy-metabolism related genes decrease in AD, with a 65% reduction in expression of mitochondrial electron transport chain genes [148].

The mitochondrial dysfunction related to AD is further explained in the literature by a theory called the “Calcium hypothesis of AD”. As mentioned earlier, mitochondria are bioenergetically active due to the maintenance of a membrane potential that is primarily generated by the electron transport chain. As a result of this membrane potential, mitochondria are the cell’s best Calcium buffering mechanisms. As Calcium enters the cell, mitochondria are able to quickly uptake the Calcium in an electrogenic fashion. In the Calcium hypothesis of AD, mitochondria are inherently dysfunctional, therefore hindering their ability to generate the membrane potential necessary to properly buffer intracellular calcium. As cytosolic Calcium levels remain high, signaling cascades that affect synaptic stability and function are initiated, such as the activation of calcineurins and calpains, leading to detrimental neuronal health [208]. Based on the MFRTA, the initial mitochondrial dysfunction that inhibits proper Calcium buffering is the result of mitochondrial oxidative stress within the electron transport chain leading to diminished membrane potential. Without the generation of an appropriate membrane potential, mitochondria are unable to buffer calcium.

Based on these mechanistic theories that eloquently link mitochondrial function, oxidative stress and metabolism to aging and AD, diet seems to be an important factor in healthy aging. It is thought that proper dietary supplementation may hinder the onset of many age-related neurodegenerative diseases such as
AD. For instance, antioxidants are a critical component of the proper cellular homeostasis. These compounds are necessary for keeping levels of oxidative stress low and therefore decreasing damage to lipid, proteins, DNA and RNA [209]. Based on correlative human neuropathology studies, antioxidants are predictive of healthy aging, may reduce the risk of developing AD and may improve cognitive function in AD patients. However, studies in humans have shown either a positive effect of antioxidant use on cognition and risk reduction for developing AD [210-212] or no significant effects [213-216]. Few systematic and controlled clinical trials have evaluated the effects of antioxidants on cognition in aged individuals or patients with AD.

**Dietary Supplementation that target mitochondrial function to improve age related deficits**

As mentioned previously, mitochondria dysfunction, oxidative stress and changes in metabolism are important factors in natural aging. Based on the MFRTA, it is thought that appropriate dietary supplementation can help to avoid many of the age related deficits that arise, such as reduced cognition. Two major supplementations that have shown some promise are the use of antioxidants as well as mitochondrial co-factors.

One specific antioxidant which has been well studied in aging and various age related neurodegenerative states is vitamin E. Vitamin E is an important lipid-soluble antioxidant that is obtained through the diet [217]. Commonly referred to as tocopherol, these vitamin E isomers are found in high concentrations in almonds, roasted sunflower seeds, various plant oils, such as olive oil, and can also be taken as a supplement. It is thought that proper
supplementation with Vitamin E can protect cells against deteriorating effects of oxidative damage and the progression of degenerative diseases and aging however findings in the literature has shown mixed results [209]. As a compound, vitamin E is an important antioxidant shown to protect against lipid peroxidation in cell culture models [218]. However in single-cell organisms, rotifers, Caenorhabditis elegans, Drosophila melanogaster and rodent models, vitamin E has shown the full gamut of results, being increased lifespan with supplementation, no observed changes in lifespan and detrimental effects with supplementation [219-231]. Similar to these previous finding, human clinical trials with vitamin E has also produced mixed results. In one study, intake of vitamin E delayed institutionalization of AD patients [232], suggesting some beneficial effects. However, vitamin E alone did not improve cognition in patients with mild cognitive impairment, which is thought to precede AD [233]. Further, in non-demented elderly women, vitamin E treatment was associated with little improvement in cognition [215].

Vitamin C is another antioxidant that has been studied for years but has shown mixed results in the treatment of various age related diseases. Since vitamin C is a water-soluble vitamin, it is not stored in the body and therefore is needed to be consumed regularly through diet. Not only has it shown to be important in the maintenance of connective tissues, it can also assist in the conversion of the amino acid, tryptophan, to the neurotransmitter, serotonin and is an antioxidant that protects body from free radical damage [234]. Foods rich in vitamin C are guava, kale and kiwi. To date, research using vitamin C as
an antioxidant are mixed and seemingly are improved when combined with other antioxidants such as vitamin E [235]. Research has found that in low concentrations, it has antioxidant properties, however at high concentrations is a potent pro-oxidant, with intra- and extracellular mechanisms that generate hydrogen peroxide [236]. Treatment with aspirin plus ascorbic acid/vitamin C in aged rats was also shown to enhance cognitive performance and increase the expressions of several receptors related to learning and memory process [237]. Another recent rodent student also saw that high supplementation of vitamin C was able to decrease the amyloid plaques found in the cortex and hippocampus of an AD transgenic mouse model that normally has increased amyloid plaques in the brain compared to wild-type mice [238]. However, even with the positive results found in animal testing, the results pertaining to human clinical trials are not as positive [239]. As study performed in Germany found that vitamin C and β-carotene concentrations were significantly lower in the blood of demented than in control persons [240].

Coenzyme Q10, which is also known as CoQ10, is another important naturally occurring compound that has been hypothesized as an integral component of various neurodegenerative diseases and cognitive disorders related to age [241]. As an important cofactor needed in the mitochondrial electron transport chain, CoQ10 is necessary for proper aerobic cellular respiration and ATP production, both of which have been found to decline with age. It is thought that when CoQ10 is deficient, mitochondrial function decreases, ROS production increases and inflammation ensues [184, 242]. Also, in its
Reduced form, CoQ10H2 is a fat-soluble antioxidant that is generally found in cell membranes. This important lipid soluble antioxidant is the only antioxidant that animal cells synthesize de novo [243]. Although it has an integral role in proper cellular function related to both metabolism and oxidative stress, its importance in aging and proper supplementation in many diseased states is highly debated. In laboratory studies where CoQ10 was supplemented in cell-culture and slice-culture studies, it has been found to be neuroprotective through the reduction of oxidative stress leading to decreased cell death [244]. Rodent studies have also shown improvements in cognition with later life supplementation of CoQ10 [245]. Additionally, a study measuring cognition in aged beagles following statin usage found lower levels of serum CoQ10 in the parietal cortex was correlated with decreased cognition, suggesting the need for proper CoQ10 level in order to retain proper cognition [184]. Decreases in plasma CoQ10 levels seen in aged canines seemingly translate well to humans. A correlative study recently completed in humans found that plasma levels of CoQ10 are significantly reduced older patients suggesting decreased antioxidant capacity [246]. Since both cognition and CoQ10 levels tend to decline with age, it is hypothesized that decreases in CoQ10 may lead to the mitochondrial dysfunction seen with AD [247]. However, another interpretation of the data suggests that age related impaired mitochondrial function which may make aged cells more vulnerable to naturally occurring decreased levels of CoQ10 [248].

Taken together, studies of dietary or supplemental antioxidant intake in humans reveal variable results and appear far less robustly associated with
positive functional outcomes than those reported in the rodent aging literature [164, 179, 249-256]. Variability in the outcomes of human antioxidant clinical trials outcomes may reflect inconsistencies in the amount of supplements provided, their form and source (e.g. lower AD brain neuropathology is associated with cerebrospinal fluid levels of alpha-tocopherol and not gamma-tocopherol [257]), their duration and regularity of use and challenges in determining the exact background of dietary antioxidants [258]. Interestingly, combinations of antioxidants may be superior to single compound supplementation [259] and dietary intake of antioxidants is superior to supplements in human studies on cognition and risk of developing AD [260, 261]. Further, supplementation of elderly women with a combination of vitamins E and C can lead to improved memory [262]. Thus, antioxidants may prove to be more efficacious if administered in combination with other antioxidants (e.g. vitamin C, which helps to recycle Vitamin E) and through diet, rather than as a supplement.

In addition to investigating the effects of cellular antioxidants on cognition and risk of AD, several studies examined the effects of targeted co-factors that improve mitochondrial function, including acetylcarnitine (ALCAR) and lipoic acid (LA). ALCAR and LA may improve mitochondrial function and reduce the production of ROS, thus also reducing oxidative damage to proteins, lipids and DNA/RNA [263]. In studies where ALCAR was administered to patients with moderate to severe AD, either improved cognition and/or slower deterioration was observed [264-267]. In early-onset AD patients (less than 65 years of age), only small cognitive improvements were noted [268], although younger patients
with AD (less than 61 years) may also have experienced slowed disease progression [269, 270]. When the results of all these studies are combined in a meta-analysis, ALCAR administration in patients with AD was clearly beneficial, particularly with respect to slowing cognitive decline [271]. Further, combining ALCAR with acetylcholinesterase therapy in AD may provide additional benefits [272]. Similar evidence of maintenance of function was observed in an open label study of 9 patients with AD or related dementias receiving 600 mg/day of LA for an average of 337 days [273]. In a larger follow up study of 48 patients for a 48 month treatment period, maintenance of function was also observed [274].

A panel of experts for the Duke Evidence-based Practice Center for the US Department of Health and Human Services recently reviewed the literature and, not surprisingly, reported no consistent or robust evidence to suggest that single or dual antioxidant use is protective against AD [275]. In terms of preventing cognitive decline with aging, vegetable intake was only weakly associated with decreased risk of developing AD, whereas cognitive training was strongly associated with decreased risk. Thus, the role of either dietary or supplemental antioxidants and level of protection against cognitive decline or AD has yet to be clearly established. Additional reasons for the small or negative effects of antioxidants on cognition in the elderly and for treatment of AD [249, 276] include the limitations of animal models (primarily rodent) in terms of ability to predict human response. Therefore, it is useful to consider other animal models of human aging and AD, and also to test the potential for combinations of antioxidants/ mitochondrial co-factors to improve cognition and reduce Aβ.
Specifically, dogs are frequently used to evaluate safety of drugs and in food metabolism studies given their substantial similarities to humans. Therefore, preclinical studies of mitochondrial function and interventions should be completed in an aging canine model of human brain aging and AD to give results that can better be translated to humans.

**Targeting Oxidative Stress in a Canine Model of AD**

Dogs may be particularly useful in studying human brain aging because they naturally develop cognitive decline with age, accumulate oxidative damage and Aβ protein [277]. In dog brain, oxidative damage to proteins increases with age [278, 279] and is associated with reduced endogenous antioxidant enzyme activity or protein levels [278, 280-282]. In several studies, a relation between age and increased oxidative damage has been inferred by measuring the amount of end products of lipid peroxidation to predict oxidative damage to lipids. These end products including 4-hydroxynonenal [282-285] and malondialdehyde [278]. Additionally, we and others have reported evidence of increased oxidative damage to DNA or RNA (8OHdG) in aged dog brain [277, 285].

Oxidative damage may also be associated with behavioral decline in dogs. Rofina and collaborators examined oxidative end products (lipofuscin-like pigment and protein carbonyls) in aged companion dog brain and found a correlation between increased oxidative end products and severity of behavior changes due to cognitive dysfunction [279, 284, 285]. Similarly, in work completed by Opii and Head et. al. aging beagles, higher protein oxidative damage (3-nitrotyrosine) and lower endogenous antioxidant capacity (superoxide
dismutase and glutathione-S-transferase) are associated with poorer prefrontal-dependent and spatial learning [281]. These correlative studies suggest a link between cognition and progressive oxidative damage in the dog, suggesting their utility in testing antioxidant treatment strategies.

To test the hypothesis that reduced oxidative stress leads to cognitive benefits, longitudinal studies in aged dogs were completed. In this study, a combination of antioxidants and mitochondrial co-factors was provided in food [286-290]. 48 aged beagles (between ~8-12 years) were divided into four groups that were balanced with respect to baseline cognitive ability, sex and age: (1) no behavioral enrichment/control diet group; (2) behavioral enrichment/control diet; (3) no behavioral enrichment/antioxidant diet; and (4) combined behavioral enrichment and antioxidant diet. In a subset of experiments, an additional 17 young beagles (<5 years of age) were included for comparison to aged dogs. Young dogs were all placed in the behavioral enrichment condition, with half provided with the antioxidant diet (i.e. similar to groups 2 and 4).

Three unique features of the experiment included: 1) a combination of antioxidants and mitochondrial co-factors; 2) incorporation of all antioxidants and mitochondrial co-factors into food and; 3) evaluation of dietary treatments in combination with behavioral enrichment. An antioxidant-enriched dog diet was formulated to include a broad spectrum of antioxidants and two mitochondrial co-factors [289]. Based on an average weight of 10 kg per animal, the daily doses for each compound were 800 IU or 210 mg/day (21 mg/kg/day) of vitamin E, 16 mg/day (1.6 mg/kg/day) of vitamin C, 52 mg/day (5.2 mg/kg/day) of carnitine and
26 mg/day (2.6 mg/kg/day) of lipoic acid. Fruits and vegetables were also incorporated at a 1 to 1 exchange ratio for corn, resulting in 1% inclusions (dehydrated) of each of the following: spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp. This was equivalent to raising fruits and vegetable intake from 3 servings per day to 5-6 servings per day based upon ORAC values [291]. Additionally, vitamin E was increased by ~75% in dogs treated with the antioxidant diet [292]. The behavioral enrichment condition consisted of additional cognitive experience (20-30 min/day, 5 days/week), an enriched sensory environment (housing with a kennel-mate, weekly rotation of play toys in kennel) and physical exercise (two 20 min outdoor walks/week) [289].

Dogs were evaluated over a 2.8 year period to evaluate short term and chronic treatment effects. Treatment with the antioxidant diet lead to cognitive improvements in learning within two weeks, with aged animals showing significant improvements in spatial attention (landmark task) [292]. Subsequent testing of animals with a more difficult complex learning task (oddlity discrimination) also revealed benefits of the diet [286]. With antioxidant treatment, visual discrimination improved and reversal (frontal function) learning ability was maintained over time while untreated animals showed a progressive decline [289]. This was despite the fact that for each time point where discrimination learning was re-administered, the task was made more difficult (harder to distinguish objects) to prevent a practice effect. Thus the progressive increase in error scores over time in untreated dogs reflects both increased task
difficulty and possibly, longitudinal aging effects. Interestingly, the dogs fed an antioxidant diet benefited from behavioral enrichment, in that cognitive scores of aged dogs receiving both treatments were superior to either treatment alone [288, 289]. For example, in singly treated animals spatial memory showed a trend toward improvement, reaching statistical significance only after long-term treatment (>2 years) with a combination of both the antioxidant diet and behavioral enrichment [290]. The antioxidant diet selectively repaired an aging deficit, in that cognitive scores from young dogs treated with the antioxidant diet did not differ from those of young dogs fed control diet [293].

Neurobiological studies showed reduced oxidative damage and increased endogenous antioxidant activity in antioxidant-fed dogs, particularly among animals receiving the combination of antioxidants and behavioral enrichment [281]. Interestingly, the antioxidant diet increased the levels of glutathione suggesting a possible involvement of a possible vitagene network that might account for the increased expression of antioxidant molecules and growth proteins [294, 295]. Given that the diet provided to the dogs also included acetylcarnitine, resulting in increased levels of heme oxygenase (HO-1) also support the possibility that vitagene networks are engaged [294].

Mitochondrial function was significantly improved in the antioxidant fed dogs and not in behaviorally enriched dogs [248]. Interestingly, behavioral enrichment but not the antioxidant diet protected against neuron loss in the hilus of the dog hippocampus [296]. Further, brain derived neurotrophic factor mRNA increased in aged dogs provided with the combination treatment [297]. These
results suggest that cognitive benefits of antioxidants can be further enhanced with the addition of behavioral enrichment, perhaps due to different yet synergistic mechanisms of action in the brain, including reduced oxidative damage and maintenance of neuron health. In addition to brain, however, peripheral benefits were also seen, including less cellular degeneration in the inner ear [298].

Interestingly, in a recent study of aged dogs, the formulation of the diet was modified to compare only the mitochondrial co-factors used in this previous study and effects on cognition [299]. Aged dogs were treated with lipoic acid, ALCAR or the combination and tested with spatial learning and discrimination/reversal tasks. When these compounds were included with a broader spectrum of antioxidants described above, no cognitive benefits were observed when evaluated singly or in combination. Additionally, protein carbonyl accumulation in the plasma of treated dogs was increased. Increased oxidative damage may reflect either higher doses of the mitochondrial co-factors used in this study or increased oxidative stress resulting from not counterbalancing mitochondrial cofactors with cellular antioxidants.

**Changes in mitochondrial bioenergetics that occur with age**

Through this Calcium mediated mitochondrial cell death mechanism and others, mitochondria have been implicated in various age related neurodegenerative diseases such as Amyotrophic Lateral Sclerosis, ALS [300]. Therefore, scientists have turned their interests to finding novel therapeutic approaches in order to target mitochondrial function with the hopes of avoiding
the onset and/or progression of diseases where mitochondrial dysfunction is present and amendable. However, as scientists discovered approaches that were able to target mitochondrial function, a debate ensued as to whether mitochondria are a homogenous population across various tissue types.

With the investigation of mitochondrial targeting therapeutic approaches for various disease states, unexpected insights into the nature of mitochondria have surfaced. Mitochondrial function does not seem to be consistent across all tissue types or even within different regions of a specific cell type, specifically synaptic versus non-synaptic mitochondria [301]. Additionally, it is also thought that aging further increases these differences [300]. Therefore, to date, the field is beginning to probe the idea that mitochondria may not all be alike and may react to insults, such as aging or toxins, differentially. Within this article, we will review these differences and highlight new data supporting this hypothesis from our laboratory.

Based on the information provided previously, mitochondrial bioenergetics and age related changes to these bioenergetics is an important area of research. Denham Harman’s work on the Mitochondrial Free Radical Theory of Aging (MFRTA) [139, 150, 158-160, 302] is heavily debated, however there are specifics that have yet to be disproven. Within MFRTA, Harman acknowledged that through the normal function of the electron transport chain, reactive electrons are released. These electrons are then able to react with free oxygen, generating reactive oxygen species, which can thereby react with nearby proteins, nucleic acids, etc. leadings to metabolic mediated oxidative stress. Over
time, the oxidative insult perpetuates and mitochondrial and cellular damage can occur. After many years, this damage begins to accumulate leading to decreased bioenergetics efficiency, increased electron slippage/release and decreased ATP production. In cell types with low bioenergetics profiles, this change in bioenergetics is not a large concern. However, in cells that have a high energy demand such as neurons, decreased ATP production can lead to serious detrimental effects which can result in severe functional changes due to disruption of cellular homeostasis.

Age-associated impairments in mitochondrial oxidative phosphorylation (OXPHOS) due to oxidative damage has been shown in multiple tissues including the brain, muscle, liver and heart [205, 303-306]. However, the proteins damaged seem to be very specific and not necessarily the result of a complete destruction to all mitochondrial structures. Therefore, specific mitochondrial proteins seem to be more vulnerable to age-related metabolic mediated oxidative stress. For instance, mitochondria isolated from various rodent tissues have shown decreased mitochondrial electron transport chain enzymatic activity with age, specifically within complexes I and IV, however complexes II and III seemingly remain unaffected [307, 308]. Based on data obtained from our laboratory, it has also become apparent that age related mitochondrial dysfunction may be the result of a regional specific dysregulation in Calcium buffering by the mitochondria [300]. In this specific study, mitochondria isolated from aged rat cortex and hippocampus showed increased ROS production and decreased Calcium buffering capacity compared to young rats
however this was not observed in mitochondria isolated from the cerebellum of these animals. This study also found that aged brain mitochondria were able to undergo mitochondrial permeability at a lower Calcium threshold than young animals with the exception of cerebellar mitochondria where no difference was measured.

It is thought that as oxidative damage and the resulting mitochondrial dysfunction increases with age, mitochondria will become more vulnerable to insults by environmental toxins, etc. leading to an increase in mitochondrial mediated cell death. This rational is supported by the increased prevalence of neurodegenerative diseases in the aged population [102]. Additionally, these age-related changes in mitochondrial bioenergetics do not seem to occur equally in all regions of the central nervous system. Certain regions within the central nervous system experience increased mitochondrial dysfunction, increased ROS concentrations and decreased Calcium buffering capacity making these tissue types more vulnerable to the initiation of mitochondrial mediated cell death pathways.

**Mitochondrial differences in the brain versus spinal cord**

Back in the early 2000s, our laboratory and others were investigating Cyclosporin A for the treatment of injuries to the central nervous system, CNS. At the time, Cyclosporin A was a known immunosuppressant however it also had a secondary target within the mitochondria. Specifically, it was found to bind a protein within the mitochondria called Cyclophilin D, thereby inhibiting the mitochondrial permeability transition. In the model of brain injury, this drug
provided a means of limiting part of the excitotoxic cell death cascade that occurs following injury. However, within these studies it was noticed that the therapeutic effect of Cyclosporin A was lost when it was moved from brain injury to a model of spinal cord injury. This work provided evidence that mitochondria in one tissue type differs from mitochondria within another tissue [300]. Upon further research, it became apparent that these differences could also occur in tissues that were histologically similar. For instance, the brain and the spinal cord are one continuous structure, thereby sharing similar cell types. However, it has been shown that mitochondrial from the brain have different bioenergetics than those in the spinal cord. Later it became apparent that even mitochondria within one area of a cell can have a different energetic profiles compared to mitochondria within another area of cell. Specifically, our work has shown that neuronal mitochondria located in the synapse of neurons have different bioenergetics, calcium buffering capacities and respond differently to mitochondrial targeting drugs compared to mitochondria found within non-synaptic pools [39, 301, 309-314]. This data further supports the theory that mitochondrial bioenergetics may be location specific.

From our current understanding, it seems as if there is convincing evidence supporting the theory that mitochondria within the spinal cord may have significant differences in metabolic profiles than mitochondria within the brain. Work from our laboratory and others have found that mitochondria within the spinal cord produce more reactive oxygen species and have increased oxidative damage compared to age-matched brain mitochondria. Additionally, in normal,
non-aged mitochondria obtained from 3 month old rats, spinal cord mitochondria have decreased maximum NADH linked mitochondrial respiration. However, in this study no difference in FADH2/succinate mediated respiration was demonstrated [313]. Additionally, spinal cord mitochondria have a lower calcium mediated threshold for the formation of the mitochondrial permeability transition pore, which is likely related to increased expression of cyclophilin D compared to brain mitochondria. Because of these differences, drugs that inhibit the formation of the mitochondrial permeability transition pore such as Cyclosporin A require significantly higher concentrations in order to provide the same degree of neuroprotection in the spinal cord that is seen in the brain. Although these findings were interesting, they lead to more questions. Specifically, does age influence the differences seen between mitochondria from the brain versus the spinal cord?

**Age related differences between brain and spinal cord mitochondria**

We hypothesized that since age and location of mitochondria both have individual effects on mitochondrial bioenergetics, then when combined together an even further deviation in mitochondrial function will occur. To test this hypothesis we compared mitochondrial bioenergetics within the brain to those within the spinal cord in rats at different ages, specifically 3 month compared to 12-15 month old male Sprague-Dawley rats (9 per age group, tissue from 3 animals pooled for mitochondrial assessments). Within this study we looked at differences in the total mitochondrial population within the cortex of the brain and cervical, thoracic and lumbar regions of the spinal cord using techniques as
described in [39, 300, 301, 313]. Analysis of the NADH-linked maximum respiration data using a 2way ANOVA demonstrated a significant age X region interaction (F=4.955, p = 0.012) as well as significant effects of age (F=72.69, p < 0.0001) and region (F=39.46, p < 0.0001). FADH2-linked maximum respiration demonstrated significant effects of age (F=31.30, p < 0.0001) and region (F=29.68, p < 0.0001) with no significant age X region interaction (F=3.238, p = 0.050). To further establish age dependent differences, unpaired t-tests were performed for each region in young vs aged animals. We determined aged rats had a 20 to 50% decrease in NADH mediated respiration within all regions of the spinal cord compared to the young cohort (p < 0.05). Aged animals also showed a significant decrease in succinate/FADH2 mediated respiration within the various regions of the spinal cord (p < 0.05), which was not demonstrated in the brain for either NADH or FADH2 mediated respiration (FIGURE 2-1A and 2-1B and TABLE 2-1). These findings are very interesting because significant NADH mediated inhibition of cortical mitochondria has been documented with age in the past however this study did not find the same conclusions [300]. The differences observed however may be due to inherent strain differences and/or the ages of animals examined in these two studies. We did, however, find a significant decrease in both NADH and FADH2 mediated respiration in all the tested areas of the spinal cord in the aged animals compared to young. With the results found in young animals, mitochondria within the spinal cord seem to have decreased bioenergetics, compared to brain mitochondria, which continues to decrease with age. This decrease in bioenergetics has the potential of making these areas
more vulnerable to insults due to decreased energetic reserves, deceased ATP production and decreased capacity for ROS, which would increase the probability of a transition to cell death and therefore, increase the possibility of neurodegeneration.

Mitochondrial mediated oxidative stress and mitochondrial dysfunction generally occur together however the causation between the two is debated, however MFRTA supports the theory that oxidative stress causes mitochondrial dysfunction. To further support this hypothesis that the deviations in mitochondrial respiration are the result of oxidative stress, we assessed mitochondrial ROS production as a function of age across CNS regions using methods previously described in [39, 301]. A 2way ANOVA of basal mitochondrial ROS production demonstrated a significant age X region interaction (F=12.81, p = 0.0002) as well as significant effects of age (F=183.6, p < 0.0001) and region (F=16.10, p < 0.0001). Unpaired t-tests revealed that mitochondria isolated from aged rats compared to young rats (12 months compared to 3 month old Sprague-Dawley rats) had a significant (p < 0.01) increase in reactive oxygen species (ROS) production within all regions of the CNS, specifically the cortex and the cervical, thoracic and lumbar regions of the spinal cord (FIGURE 2-2 and TABLE 2-2). One factor that is ambiguous within this study is whether the high ROS production leads to decreased mitochondrial bioenergetics or the decreased bioenergetics leads to increased ROS production. However, what is known is that this age related increase in ROS can eventually lead to cellular dysfunction making aged animals more vulnerable to
cellular insults. This increased vulnerability makes the animal more likely to experience cellular dysfunction and possibly cellular death within this region.

With increased production of ROS and decreased mitochondrial respiration, mitochondrial and cellular structures become more vulnerable to oxidative damage. As previously reported by our group, mitochondrial DNA, mtDNA, damage is significantly higher within the spinal cords compared to the brain [313]. This increased damage to mtDNA can further perpetuate the mitochondrial dysfunction observed since protein structures made within the mitochondria have an increased chance of being mutated which can render mitochondrial proteins encoded by the mtDNA dysfunctional.

Summary

Well supported by the literature and the studies conducted in our lab, NADH and FADH2 mediated mitochondrial respiration decreases in the cortex and within the cervical, thoracic and lumbar regions of the spinal cord as age increases. In addition to changes in respiration, we have also observed increased mitochondrial ROS production with age in these same regions. Lastly, within the spinal cord of aged animals, it seems as in mtDNA damage is increased which could be a direct result of the increased mitochondrial ROS production. Although more studies may be needed to determine the exact age when the mitochondria become less bioenergetically efficient and to determine whether ROS or mitochondrial bioenergetics increase first with age leading to the other, MFRTA and the previously mentioned studies do give great insight into a potential therapeutic target that scientists could exploit in order to improve age
related functional decline and thereby improving the quality of life for the older population.
TABLE 2-1: Mitochondrial Mediated Respiration
Mean (nmol oxygen/min/mg) ± SD

<table>
<thead>
<tr>
<th>Region</th>
<th>State III</th>
<th>State IV</th>
<th>State V</th>
<th>State Vsuc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young Cortex</td>
<td>94.64 ± 4.61</td>
<td>16.09 ± 1.42</td>
<td>114.09 ± 5.50</td>
<td>125.46 ± 1.77</td>
</tr>
<tr>
<td>Young Cervical</td>
<td>70.67 ± 7.72</td>
<td>10.73 ± 1.88</td>
<td>94.44 ± 13.19</td>
<td>100.16 ± 14.02</td>
</tr>
<tr>
<td>Young Thoracic</td>
<td>42.32 ± 3.99</td>
<td>7.75 ± 1.06</td>
<td>81.97 ± 2.80</td>
<td>85.60 ± 4.36</td>
</tr>
<tr>
<td>Young Lumbar</td>
<td>48.52 ± 11.80</td>
<td>7.96 ± 0.70</td>
<td>72.24 ± 11.29</td>
<td>77.79 ± 5.74</td>
</tr>
<tr>
<td>Aged Cortex</td>
<td>85.29 ± 14.47</td>
<td>17.87 ± 5.67</td>
<td>102.98 ± 4.72</td>
<td>118.01 ± 11.43</td>
</tr>
<tr>
<td>Aged Cervical</td>
<td>38.06 ± 8.52</td>
<td>9.34 ± 2.49</td>
<td>45.73 ± 9.69</td>
<td>52.44 ± 19.15</td>
</tr>
<tr>
<td>Aged Thoracic</td>
<td>26.38 ± 8.57</td>
<td>5.58 ± 1.82</td>
<td>45.40 ± 10.51</td>
<td>58.70 ± 13.67</td>
</tr>
<tr>
<td>Aged Lumbar</td>
<td>31.31 ± 4.32</td>
<td>5.44 ± 1.34</td>
<td>46.29 ± 7.20</td>
<td>56.10 ± 9.79</td>
</tr>
<tr>
<td>Tissue Type</td>
<td>Mean (DCF Fluorescence AU/µg) + SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young Cortex</td>
<td>95.88 ± 9.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young Cervical</td>
<td>121.95 ± 32.61</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young Thoracic</td>
<td>145.28 ± 39.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young Lumbar</td>
<td>144.95 ± 61.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aged Cortex</td>
<td>235.30 ± 45.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aged Cervical</td>
<td>625.79 ± 63.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aged Thoracic</td>
<td>293.04 ± 78.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aged Lumbar</td>
<td>485.65 ± 52.79</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 2-1

A  Max NADH-linked Respiration
12-15 Month Old Naive Rats
nmol/min/mg

B  Max FADH2-linked Respiration
12-15 Month Old Naive Rats
nmol/min/mg
FIGURE 2-1 Increased age in rats lead to decreases in (a) NADH and (b) FADH2 mediated respiration.

Total mitochondria were isolated from young (3 month) and aged (12-15 months) male Sprague-Dawley rats (9 per age group, tissue from 3 animals pooled for final n=3 per group) and oxygen consumption assessed in a sealed, stirred, thermostated chamber equipped with a Clark-type electrode and expressed as nmol of oxygen consumed per min per mg of mitochondrial protein. Maximum NADH-linked (A) and FADH2-linked (B) respiration data were then analyzed using 2-way ANOVAs following by unpaired t-tests when warranted to assess age effects on mitochondrial bioenergetics. Data are expressed as % of respiration measured in young animals, *p < 0.05.
FIGURE 2-2: Mitochondrial ROS production increases with age.

Aged rats had a 200% to 600% increase in ROS production mitochondria isolated from the cortex and spinal cord compared to young rats. Total mitochondria were isolated from young (3 month) and aged (12-15 months) male Sprague-Dawley rats (9 per age group, tissue from 3 animals pooled for final n=3 per group) and ROS production assessed using the indicator DCF in the presence of respiratory substrates. Control wells included FCCP to induce minimum ROS production and oligomycin to induce maximum ROS production. Wells in which mitochondria were omitted were used for background subtraction. Raw data (DCF fluorescence AU/ug) were then analyzed using a 2way ANOVA following by unpaired t-tests when warranted to assess age effects on
mitochondrial basal ROS production. Data are expressed as % of respiration demonstrated in young animals, *p < 0.05.

Portions of this chapter have been previously published in:
CHAPTER 3: TRAUMATIC BRAIN INJURY

In the United States, the CDC estimates that approximately 1.7 million Americans experience a traumatic brain injury (TBI) every year [315]. Expanding this scope, it has been further estimated that 57 million people have experienced a brain injury worldwide [315, 316]. Even more astounding is that these numbers do not account for the population who sustain a TBI, likely more mild in nature, and do not seek medical care. With such a large affected population, it is estimated that approximately 77 billion dollars are spent annually on direct and indirect health related costs in the US alone [316]. Therefore, it becomes obvious that there is a great need for pro-regenerative and/or neuroprotective therapeutic agents for the treatment of this disease however to date there is still no FDA approved drug for its treatment. Although ample manpower has gone into the discovery of drugs to treat TBI, many tribulations have been encountered along the way due to the multifaceted complicated set of pathways that are initiated following the primary the injury.

The pathophysiology of TBI

The pathophysiological nature of TBI is complicated and is thought to occur in a biphasic manner. The first phase is termed the primary injury and the second phase is termed the secondary injury.

Primary Injury

initial impact is where kinetic energy is transferred into a mechanical disruption within the brain causing immediate tissue damage. The extent of damage, and therefore the severity of the injury, depends on the nature of the
injury as well as the degree of mechanical force transferred to the brain tissue when the injury ensues. One complication that physician and researchers encounter when attempting to treat this disease is that injuries are generally heterogeneous in nature, providing a “significant barrier” for therapeutic interventions [317]. According the the Mayo Clinic, injuries may include one or more of the following factors:

1) Tissue damage limited to an acute area directly below the point of impact on the skull. This type of injury is often referred to as a contusion injury.

2) Tissue damage at multiple points in the brain due to movement of the cerebrum, brain stem, and cerebellum within the cranial vault, which is defined as a coup contrecoup injury.

3) Tearing of cellular structures due to severe rotational movement or spinning motion. This type of injury can often include a coup contra-coup injury as well as damage to deeper cerebral structures. This is often called a rotational injury.

4) Widespread tissue damage due to a blast from an explosive device. This is defined as a blast injury.

5) Tissue damage can occur as the result of an object penetrating the skull such as a bullet or some other rigid structure with enough kinetic energy to break the skull. This type of injury can often be referred to as a ballistic injury, when referring to a bullet, or produce a contusion type of injury as well.
6) Changes in vasculature such as bleeding in and/or around the brain, swelling of the brain and blood clots which disrupt the oxygen supply also causing tissue damage.

7) Lastly, which was not noted by the Mayo Clinic, brain damage can occur as the result of drowning or any other environment where oxygen supply to the brain is diminished. This is defined as an anoxic or hypoxic type of TBI.

These types of primary injuries are most commonly produced by either falls, vehicle related-collisions, assault, sports injuries or explosive blast or other combat related injuries.

To date, there are a few known risk factors that increase a person's chances of sustaining a TBI. One of the first risk factors is age including children from newborn to 4 years old, young male adults between the ages of 15 to 24, and adults ages 75 and older [318]. The rate of TBIs per 100,000 in the US population is also greater in males, between 25 to 28 over the past decade compared to females which ranges more between 9 and 10.
Figure 3.1 Gender is a risk factor for TBI: Based on CDC reports, males have double the risk of sustaining a TBI compared to females. [319]
Drug usage, nutrition and psychosocial status also are included as risk factors for TBI [320]. Additionally, athletes involved in high impact sports, such as boxing, football or soccer, and solders involved in military combat have also been defined as two groups who are more susceptible to sustaining a TBI. Since this phase of the injury is cannot be therapeutically influenced, the best practice to avoid the primary injury is through prevention techniques [321]. The CDC proposed prevention techniques including:

1) Using proper restraints for you and your children while operating or riding in a motor motor vehicle.

2) Never driving while under the influence of alcohol or drugs.

3) Wear a helmet, or have your children wear helmets, while

   a. Riding a bike, motorcycle, snowmobile, scooter, or all-terrain vehicle;

   b. Playing a contact sport such as football, ice hockey or boxing;

   c. Using in-line skates or riding a skateboard;

   d. Riding a horse; or

   e. Skiing or snowboarding

4) Making living areas safer for seniors by:

   a. Removing tripping hazards;

   b. Using nonslip mats on surfaces that become slippery when wet such as bathroom floors, bathtubs, showers, etc.;
c. Installing handrails in stairways;
d. Proper lighting within the home
e. Maintaining a physical activity regiment to provide increased body strength and balance.

5) Making living areas safer for children by:
a. Using window guards on open windows to avoid them falling out of one
b. Using safety gates at the top and bottom of stairwells.

6) Having safe shock-absorbent floor surfaces around play areas [321].

In addition to using proper precautions to help avoid the occurrence of a TBI, researchers have also continued to improve upon current helmet designs. Researchers have found that a hard shell design alone for helmets will not help protect the brain from injury. Not surprisingly, the cushion (foam) within the helmet seems to be of the upmost importance in order to decrease the head impact acceleration and therefore the mechanical force that the brain encounters [322]. However, as researchers at the Lawrence Livermore National Laboratory discovered, there does not seem to be one type of foam that protects against all injuries. For instance, dense foams within the helmet seem to protect the brain to a greater extent when high-speed interactions are observed. On the contrary, when low speed interactions are encountered, less dense foam seems to outperform the higher density foam. Additionally, the thickness of the foam within the helmet is also an important factor in that the thicker the foam, the less force
the brain will experience during any impact [323]. However, these researchers also determined that at high enough speeds, the kinetic energy is too high and no thickness or density of the foam could protect against damage to the brain [324]. Therefore, although prevention techniques are currently being improved upon, mechanical insults to the brain tissue are still able to occur leading to necessary progress being made once the injury occurs.

Even with proper prevention techniques in place, traumatic brain injuries are still occurring within the US as an alarming rate. When this injury occurs, as mentioned previously, it starts the injury cascade. This cascade is initiated by the primary injury leading to tearing of blood vessels, sheering of axons and immediate cell death which have all been documented and are dependent on the severity of the impact [325]. The initial insult causes cellular membranes around the injury to become permeable to various proteins, ions and other molecules which is thought to be one factor leading to the initiation the secondary phase of the injury [326].

Following the primary injury, within the first seconds, to minutes, to days and now seemingly expanding to months and possibly years later is the second phase of this bi-phasic injury cascade. This phase is often called the secondary injury cascade and causes delayed non-mechanical tissue damage [327]. This tissue occurs due to a complex set of events including alterations of endogenous neurochemical mechanisms, ischemic insult, massive edema, increases in oxidative stress and mitochondrial dysfunction which all lead to progressive damage around the site of the primary injury [109]. It is the tissue that is
undergoing this progressive damage that researcher and physicians have the greatest interest in when investigating therapeutic interventions following injury. It is thought that with proper intervention, improved functional outcomes can be obtained due to decreased tissue dysfunction and neurodegeneration. This secondary injury cascade will be discussed in more detail later in this chapter.

**Experimental Models of TBI**

The heterogeneous nature of clinical TBIs has sparked the invention of many experimental injury models [317]. Contusion injury, coup contra-coup, rotational injuries, blast injuries, penetrating injuries, anoxic or hypoxic injury, plus others, as well as combination injuries, have all been documented in clinical setting. When experimentally investigating these injuries, a single model has not been shown to be superior so a few of the more commonly used models will be discussed. However, a concentration will be placed on the contusion injury modeled by the controlled cortical impact since this was the model used for the studies in this thesis.

The experimental injury models can be either static or dynamic. A static injury model in one which produces a compression injury and the force applied is never removed. Dynamic injury models are the most commonly studied forms of injury [328, 329]. These injuries can be the result of an indirect force being applied to the brain, an example of this would be an explosion which is modeled by the blast wave injury model, or the direct injury model, where there is an object which interacts with the head to produce the injury. From here, direct injury models can be further classified as penetrating or non-penetrating models of
injury. Two well-studied models of dynamic, direct, penetrating injuries are the fluid percussion model and the controlled cortical impact model [328].

**Blast Traumatic Brain Injury, mTBI**

The first model discussed will be the blast wave injury model which would be considered a dynamic non-penetrating injury. This model has gained more interest due to the marked functional changes observed by the men and women returning home from the war after encountering a blast or explosion. In this experimental design, a stabilized animal is attached to the end of cylindrical metal tube that has a closed on the other side. This close end is where a plastic explosive or compressed air device is located which can be detonated to produce the blast wave. The pathology of this form of injury is still highly debated and therefore is being continually investigated. Following the primary insult, shock waves from the explosion can pass through the brain tissue leading to minute neuroanatomical changes [328]. This blast overpressure causes the transfer of kinetic energy into a force on the brain which is thought to induce afferent hyperexcitability leading to increase synthesis and release of various neurotransmitters [330-332]. Although no apparent mitochondrial dysfunction has been documented in this model, neuron swelling, astroglial response and myelin debris in the hippocampus have all been observed. Damage to neuronal cytoskeletal structures in layers II-IV of the temporal cortex, the cingulate gyrus, the piriform cortex, the dentate gyrus and CA1 regions of the hippocampus has also been seen over 7 days following injury [333]. Increases in oxidative stress, alterations of antioxidant defense systems, increased nitric oxide metabolism and
cognitive deficits have also been documented in patients who have sustained a blast TBI, bTBI. Additionally, compression of blood vessels can also occur leading to changes in blood flow to the brain, possibly inducing a hypoxic state.

As the blast wave encounters the brain tissue, a coup-contracoup injury can also occur in which the brain is pushed in the direction of the shock wave compressing itself along the backside of the cranial cavity. Based on the law of conservation of energy, once the brain impacts the cranial cavity at the site opposite to the point of impact, the brain will then move back towards the site of injury within the cranial cavity. Therefore, this injury causes a coup and contracoup injury which are 180° from one another.

Fluid Percussion Injury, FPI

The next model of brain injury discussed will be the fluid percussion injury model, FPI, which is a direct penetrating injury model. In this model, water confined in a tube is impinged by a weight with a specific kinetic energy. This generates a pressure pulse in the water. The water is then rapidly injected into the cranial cavity, through the Luer cap placed in the skull, providing a mechanical force to the brain that is diffuse in nature \[334\]. Following injury, animals experience apnea and the severity of the injury is measured by the animals “righting time”. Depending on the location of this injury, either midline or lateral, and the severity, this injury does not produce any overt histopathology or contusion at the site of injury, however with the lateral fluid percussion, apparent cavitation and cell death is markedly observed within the ventricular region out to one year post-injury. As a model, FPI produces an injury that is thought to mimic
a rotational injury on the brain which can be the result of a clinically traumatic event such as a car accident or sports related injury.

*Controlled Cortical Impact, CCI*

The last model discussed, and the most important model related to this thesis project, produces a much more focal contusion injury compared to the diffuse injury seen with FPI. This is the controlled cortical impact, CCI, which uses a rigid impactor which impinges exposed, intact dura producing cortical tissue loss, acute subdural hematoma, axonal injury, concussion, a breakdown of blood brain barrier and coma [329, 335-340]. This experimental models dates back to the late 1980s where Dr. James Lighthall produced a novel contusion injury model in ferrets. This open-skull midline injury was generated by a pneumatic impact device that was attached to a cylinder shaft. The cylinder shaft had a 1.25cm diameter aluminum rod at the end, which was the tip that interacted with the brain. Dr. Lighthall then drilled a 1.5cm craniotomy in on the midline of the skull, between bregma and lambda, to expose the brain tissue. Great care was taken to remove the drilled bone flap in order to not damage the dura mater. The brain was then impacted at various velocities producing a graded injury which became lethal at higher velocities [340].

Then, in 1991, Dr. Edward Dixon, under the advisement of Dr. Ronald Hayes, expanded upon the work from Dr. Lighthall, generating an experimental CCI injury model which is most commonly used to study contusion brain injuries today. This work from Dr. Dixon was completed in rats and provided a very thorough investigation into the neurological, cardiovascular and histopathological
changes that occur following a midline contusion injury. The neurological deficits seen were consistent with changes to somatomotor system, showing decreased beam balance latencies and increase beam walking latencies which was dependent on the depth of the impact. These changes in motor function were also further supported with observations of axonal injury in the cerebellar peduncles and pontomedullary junction. Changes in mean arterial blood pressure were also observed leading to hypotension following a moderate injury (2mm cortical deformation) where a severe injury (3mm cortical deformation) produced immediate hypertension followed by hypotension. Most importantly noted however is that this type of injury paradigm could be graded, which was dependent on the extent of cortical deformation during the impact, leading to either no overt macroscopic or microscopic changes to the brain when the brain was only impacted at a depth of 1mm compared to a brain that received a large cortical contusion and intraparenchymal hemorrhage with a 3mm cortical defomation [336].

The work from Dr. Lighthall and Dr. Dixon was further expanded upon when Dr. Douglas Smith, in 1995, used this model to injure the left parietotemporal cortex in mice. In this study, a 3mm beveled flat-tip metal impactor tip was used to impinge the brain at a velocity of 5.7-6m/s. With this injury paradigm, decreased cognition was measured at 2 days post-injury with the use of the Morris Water Maze. With this cognitive tests, animals are placed in a tub of water and tasked to remember visual cues around the room to find a platform hidden under the surface of the water. In support of the changes in
memory/cognition that was observed, postmortem histological studies from 48 hour post-injured mice revealed substantial cortical loss at the site of the impact and cell loss in the CA2, CA3 and CA3a regions of the hippocampus. Bilateral degeneration in the gray matter-white matter interface of the corpus callosum and reactive gliosis was observed bilaterally in the cortex, hippocampus and thalami at 48 hours post-injury. Additionally, a breakdown of the blood brain barrier was also documented in the cortex, hippocampus and thalamus [339].
Figure 3.2 Injury diagram for the CCI model: The controlled cortical impact model of TBI produces a contusion injury that leads to cell death and dysfunction acute to the injury. This injured tissue can be found in the cortex, hippocampus and corpus callosum on the ipsilateral injured hemisphere as well as the corpus callosum on the contralateral hemisphere [339].
The controlled cortical impact uses a pneumatic piston with a specific velocity, depth and dwell time to induce an injury. The severity of the injury can be adjusted based on these parameters. This injury provides a non-penetrating deformation to the cortex leading to a hematoma that has a similar histopathology to a clinical contusion injury, analogous to a lesion due a fall or a sports related injury. Immediately following the injury, a bruise occurs which begins to undergo progressive neurodegeneration that can continue to extend based on the secondary injury cascades for up to years [341]. At this time, differences in damage to the blood brain barrier, BBB, and cytoskeletal in the cortex and hippocampal formation have all been seen and well documented within the secondary injury cascade [342]. Glutamate excitotoxicity, changes in calcium homeostasis, increased free radical production and lipid peroxidation, mitochondrial dysfunction, inflammation and apoptosis are all hallmarks of this injury paradigm and used as potential therapeutic targets following injury [110, 311, 312, 343-354].

**Secondary Injury Cascade**

Following the primary injury is a vast and encompassing secondary injury cascade that is initiated at various time point post-injury. This secondary injury cascade arises due to alterations in excitatory amino acids (EAA) such as glutamate [309, 355], increases in oxidative stress (ROS) [356-358], the disruption of Calcium homeostasis [359, 360], and mitochondrial dysfunction [108, 311, 312, 343, 346, 347, 350, 353, 354, 361-364], which can all contribute to increased tissue damage at the site of the injury. Scientists believe that by
targeting these secondary injury cascades, improved histological and function outcomes will follow.

Although a complete understanding of the secondary injury cascade injury is still uncertain, the implications of abnormally high excitatory amino acids, EAA, levels around the site of the injury are generally accepted. The most common of these EAAs is glutamate. High glutamate levels are thought to be a result of the breakdown of the blood-brain barrier, BBB, excessive synaptic release from neurons, nearby hyperexcited astrocytes and excitatory amino acids forming micropores within the cell membrane [365]. Once in the synaptic cleft, these EAAs are able to activate multiple receptors and channels including, AMPA and NMDA. Activation of these receptors leads to Calcium influx into the cell. As mentioned previously, at low to moderate levels, this intracellular Calcium, $[\text{Ca}^{2+}]_i$, is used as a potent signaling molecule; important in many pathways such as increased metabolism, neurotransmitter release and alterations in cytoskeleton. However, following an injury, EAA concentrations around the injury site increase to pathogenic levels, leading to drastic increases in Calcium influx which has the potential of leading to excitotoxic mediated cell death. The most important organelles involved in the rapid buffering of this Calcium, in order to avoid neuronal cell damage or death, are the mitochondria, which was discussed previously in detail.

Mitochondria have low-affinity, high capacity Calcium pumps in their inner membrane that allow for rapid sequestration of $[\text{Ca}^{2+}]_i$. These pumps use electrochemical gradients generated by the electron transport chain’s (ETC)
translocation of protons across the inner membrane to build a membrane potential, \( \Delta \Psi \). Although the primary usage of \( \Delta \Psi \) is to drive ATP synthesis, it also allows for electrogenic movement of \( [\text{Ca}^{2+}] \), into the mitochondria. As the mitochondria buffer Calcium, a reduction in \( \Delta \Psi \) occurs. The heightened intracellular Calcium levels place excessive demand on the mitochondria sequestering ability eventually resulting in mitochondrial dysfunction that has been documented for up to 48 hours post-injury [110]. It has been shown that cortical mitochondria Calcium levels in sham-operated rats range around \(~25\text{nmol Calcium/mg}\). However, after traumatic brain injury, mitochondrial Calcium levels increase nearly 60% to levels hovering \(~40\text{nmol Calcium/mg}\) [363]. This increase leads to a drastic change to the mitochondrial membrane potential eventually causing a detrimental disruption to mitochondrial homeostasis. Since \( [\text{Ca}^{2+}] \), reach pathogenic levels following injury, extreme \( \Delta \Psi \) reduction occurs and consequently ETC mediated ATP synthesis is hindered leading to increased ROS production. The dogma is that this ROS can then oxidize mitochondrial proteins leading to further mitochondrial dysfunction and the eventual initiation of cell death pathways. However, work Opii et al. found that even though important proteins, such as PDH, are oxidized, their function is not loss [343].

As pointed out above, the cascade of posttraumatic mitochondrial ROS generation and oxygen radical reactions begins in response to rapid elevations in intracellular Ca2+ immediately following the primary mechanical injury to the brain or spinal cord with the single electron (e-) reduction of an oxygen molecule
(O2) to produce superoxide radical (O2•-) which is considered to be a modestly reactive primordial radical that can potentially react with other molecules to give rise to much more reactive, and thus more potentially damaging radical species. The reason that O2•- is only modestly reactive is that it can act as either an oxidant by stealing an electron from another oxidizable molecule or it can act as a reductant by which it donates its unpaired electron to another radical species (i.e. an electron-donating antioxidant).

Although O2•- itself is less reactive than •OH radical, its reaction with nitric oxide (•NO) radical forms the highly reactive oxidizing agent, peroxynitrite (PN, ONOO-). This reaction (O2•- + •NO → ONOO-) occurs with a very high, diffusion-limited rate constant. Subsequently, at physiological pH, ONOO- can either undergo protonation to form peroxynitrous acid (ONOOH) or it can react with carbon dioxide (CO2) to form nitrosoperoxocarbonate (ONOOCO2). The ONOOH can break down to form highly reactive nitrogen dioxide(●NO2) and •OH (ONOOH → ●NO2 + •OH). Alternatively, the ONOOCO2 can decompose into •NO2 and carbonate radical (●CO3) (ONOOCO2 → ●NO2 + ●CO3).

Increased production of reactive free radicals (i.e. “oxidative stress”) in the injured brain has been shown to cause “oxidative damage” to cellular (including mitochondrial) lipids and proteins leading to functional compromise and possibly cell death in both the microvascular and brain parenchymal cells. The major form of radical-induced oxidative damage involves oxidative attack on cell membrane polyunsaturated fatty acids triggering the process of lipid peroxidation (LP) which has three distinct chemical phases: initiation, propagation and termination. The
initiation of LP is triggered when a highly reactive (i.e. electron-seeking) oxygen radical (e.g. \( \cdot \text{OH}, \cdot \text{NO}_2, \cdot \text{CO}_3 \)) reacts with membrane polyunsaturated fatty acids such as arachidonic acid, linoleic acid, eicosapentaenoic acid or docosahexaenoic acid resulting in disruptions in membrane integrity. Specifically, initiation of LP begins when a highly “electrophilic” radical steals the hydrogen electron from an allylic carbon of the peroxidizable polyunsaturated fatty acid. The allylic carbon is susceptible to free radical attack because it is surrounded by two relatively electronegative double bonds which tend to pull one the carbon electron away from the hydrogen electron it is paired with. Consequently, a reactive free radical has an easy time pulling the hydrogen electron off of the carbon because the commitment of the carbon electron to staying paired with it has been weakened by the surrounding double bonds. This reaction results in the original radical being quenched while the polyunsaturated fatty acid (L), becomes a lipid radical (L•) due to its having lost an electron.

In the subsequent propagation step, the unstable L• reacts with O2 to form a lipid peroxyl radical (LOO•). The LOO• in turn abstracts a hydrogen atom from an adjacent polyunsaturated fatty acid yielding a lipid hydroperoxide (LOOH) and a second L•, which sets off a series of propagation “chain” reactions. These propagation reactions are terminated in the third step when the peroxidizable substrate becomes depleted and/or a lipid radical reacts with another radical or radical scavenger to yield relatively stable non-radical end products. One of those end products that is often used to measure LP is the 3 carbon-containing malondialdehyde (MDA) which is mainly a stable non-toxic compound that when
measured represents an LP “tombstone”. In contrast, two highly toxic aldehydic products of LP are 4-hydroxynonenal (4-HNE) or 2-propanal (acrolein) both of which have been well characterized in experimental brain or spinal cord injury models [366-368]. These latter two aldehydic LP end products can covalently bind to basic amino acids (e.g. lysine, histidine, arginine, cysteine) in cellular proteins, altering their structure and functional properties.

In addition to LP, free radicals can cause various forms of oxidative protein damage. A major mechanism involves carbonylation by reaction of various free radicals with susceptible amino acids. Secondly, •NO2 can nitrate the 3 position of tyrosine residues in proteins forming 3-nitrotyrosine (3-NT) which is a specific footprint of PN-induced cellular damage. Similarly, lipid peroxyl radicals (LOO●) can promote tyrosine nitration by producing initial oxidation (loss of an electron) which would enhance the ability of •NO2 to nitrate the phenyl ring [369]. Multiple commercially available polyclonal and monoclonal antibodies are available for immunoblot of immunohistochemical measurement of proteins that have been nitrated by PN. Thus, in summary, LP can cause cellular damage by destroying the integrity of the phospholipid bilayer as well as membrane-localized proteins by modification by carbonylation, nitration and/or binding of aldehydic LP end products such as 4-HNE or acrolein.

Mitochondrial oxidative stress and resulting dysfunction plays an especially critical role in the post-traumatic cell death cascade in the injured brain [359, 360, 370, 371] or spinal cord [372, 373]. The initiator of this mitochondrial homeostatic dysfunction involves a rapidly evolving post-injury intracellular
accumulation of Ca++ ions which causes the mitochondria to try to buffer (sequester) the excess Ca++ which in turn causes mitochondrial respiratory dysfunction and lessened oxidative phosphorylation[354, 359, 370, 371] and Ca++ buffering capacity [360, 370] and mitochondrial failure due to mitochondrial permeability transition (MPT) [108, 364]. In addition to the potential activation of mitochondrial caspase-dependent and caspase-independent cell death cascades, this mitochondrial failure causes a disruption of synaptic function, and indeed synaptic mitochondria are more susceptible than non-synaptic mitochondria [354].

The disruption of respiratory function is preceded by, or at least coincident with, an increase in mitochondrial free radical production and/or free radical-triggered LP-mediated oxidative damage [371-373]. Evidence has accumulated which shows that a particularly important oxidant that is being formed by injured brain of spinal cord mitochondria is PN [373-378]. The relationship of PN generation to mitochondrial dysfunction in the injured spinal cord has been recently documented by studies that have shown that the timing of post-SCI mitochondrial dysfunction (i.e. respiratory and Ca++ buffering impairment) is preceded by an increase in PN-induced 4-HNE (i.e. LP), 3-NT (i.e. protein nitration) and 4-HNE and protein carbonyl content in mitochondrial proteins. Nitric oxide and a mitochondrial nitric oxide synthase (NOS) isoform (mtNOS) have been shown to be present in mitochondria [379, 380]. Exposure of mitochondria to Ca++, which is known to cause them to become dysfunctional, leads to PN generation which in turn triggers mitochondrial Ca++ release (i.e. limits their
Ca++ uptake or buffering capacity) [381]. In vitro application of each of the three PN forms, ONOO-, ONOOH and ONOOCO2, have been found to deplete mitochondrial antioxidant stores and to cause protein nitration, which is the hallmark of PN oxidative damage [382]. The loss of mitochondrial function and the increase in oxidative damage markers, including 3-NT, is antagonized by early in vivo post-TBI treatment with the PN radical scavenger tempol [376]. Furthermore, exposure of non-injured mitochondria to PN quickly leads to respiratory dysfunction that is antagonized by tempol pretreatment along with a reduction in nitration of mitochondrial proteins [383].

Additionally, the neurotoxic LP end products 4-HNE or acrolein are also able to produce a concentration-related attenuation of the respiratory function of normal brain or spinal cord mitochondria [384]. In those experiments, acrolein was observed to be 10x more potent than 4-HNE. Furthermore, spinal cord mitochondria were found to be significantly more susceptible that brain mitochondria. However, scavenging of these carbonyl-containing compounds with the carbonyl scavenging compound phenelzine has been shown to attenuate mitochondrial respiratory depression along with a reduction in the levels of aldehyde-modified mitochondrial proteins [385].

As mitochondria remain in this dysfunctional state due to dysregulation of Calcium the ETC will reach a point where it can no longer maintain the necessary $\Delta \Psi$ needed to allow Complex V to function properly. As the mitochondria remain in a dysfunctional state with a decreased $\Delta \Psi$ hovering around 100-120mV, which is a drastic decrease from the normal membrane potential ranging around
180mV, the electron transport systems will begin to slow down or even halt. As discussed in Chapter 1, this change in potential allows for an increase in ROS production [110]. ANT can then be oxidized resulting in a conformational change that promotes cypD binding, along with other accessory proteins. VDAC is then able to bind this protein complex forming the mitochondrial permeability transition pore, MPTP, which was discussed previously. The MPTP formation exposes the inner mitochondrial matrix to the cytosol, allowing for the release of apoptotic initiating factors like Cytochrome C, SMAC/diablo and AIF, which all promote cell death. Neuroprotective agents that target mitochondrial dynamics and the membrane permeability pore formation can improve neuronal function in many disease states and therefore is important to consider when studying Traumatic Brain Injury.

**Pharmacological Treatments for TBI**

TBI can lead to drastic cognitive and functional deficits for the injured person. These deficits can decrease the quality of life for the injured person and their family as well as place extreme financial burdens on the individuals involved. To date there is still no FDA approved treatment for this disease; however, researchers are optimistic that a pharmaceutical intervention can be found. Scientists interested in finding a cure for TBI are most interested in the tissue where the the secondary injury cascade occurs following the initial injury. This is the tissue that is adjacent to the impacted tissue and can undergo a non-mechanical cell death. Due to the neuroinflammation, the generation of free radicals, the accumulation of other reactive species and sustained mitochondrial
dysfunction, it is this tissue that is thought to undergo drastic tissue dysfunction and cell death through various apoptotic and necrotic cell death pathways. By minimizing this injury cascade it is believed that the injured party will experience decreased functional deficits and increased tissue sparing providing a neural substrate for rehabilitation.

**Targeting mitochondrial dysfunction**

Some of the most promising neuroprotective agents following TBI are drugs that target mitochondrial dysfunction. Within the category of mitochondrial targeting drugs, there are multiple classifications. These include uncouplers, alternative fuels, antioxidants, oxidative phosphorylation modulators, mPTP inhibitors and O2 enhancers. Each of these drugs have specific pathways that are independent of one another and some show synergy when paired together. When using mitochondrial targeted approached, the time of delivery seems to be a critical factor. Mitochondrial targeting drugs seem to work best when delivered within the first hour or two of injury, however some seem to show positive effects when the therapeutic window is extended even further [110].

One way to avoid mitochondrial dysfunction and the resulting cell death is by decreasing mitochondrial calcium buffering. Researchers have found that weak lipophilic acids that act like mitochondrial uncouplers, such as carbonyl cyanide 4-trifluromethoxy phenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP), can produce positive therapeutic effects in animal studies of TBI. Acting as uncouplers are weak lipophilic acids that are able to deplete the membrane potential and thereby decrease the electromotive force by which calcium enter
the mitochondria. With decreased calcium uptake, markers of oxidative stress decreased as well leading to improved cortical sparing of up to 12% and enhanced cognition measured with the Morris Water Maze test when compared to vehicle treated animals [350, 363]. Although this technique has shown much promise, the dose response is steep providing an extra burden if it were to be used clinically.

TBI induced oxidative stress occurs leading to oxidative modifications on various proteins rendering them dysfunctional. Oxidative stress with Pyruvate Dehydrogenase (PDH) has been documented following injury however the function of PDH does not seem to decrease with this increased oxidation [343]. This leads to the question as to where the mitochondrial dysfunction is originating. Additionally, glucose mediated depression has been seen in both animal and humans following TBI which is dependent on the injury severity [386, 387]. Therefore, with metabolic enzymatic dysfunction and decreased metabolic substrates, a state of acute metabolic crisis has been shown to occur [388].

Alternative biofuels like ketones and beta-Hydroxybutyric acid (BHB), which are substrates that easily feed into the ETC without the contribution PDH or GDH, become a promising area of research [389]. For this reason, the use of ketone bodies in various animal models have been shown to decrease the production of free radicals through increases in glutathione production as well as increase the expression of various endogenous mitochondrial uncoupling proteins [361, 389, 390]. A study from Davis et. al. showed that with a moderate contusion injury in rats, treatment with D-beta-hydroxybuterate can decrease
markers of oxidative stress and increase mitochondrial oxidative phosphorylation following injury [361]. With these marked improved mitochondrial bioenergetics, cortical sparing and functional recovery has been seen as well. [391, 392].

Another important secondary injury mechanism that seems to show therapeutic effects when targeted following a TBI are those which up-regulate endogenous antioxidant activities. As mentioned previously, GSH/Glutathione is an endogenous antioxidant which is located in the mitochondria and converts the $\text{H}_2\text{O}_2$ generated by mnSOD to $\text{H}_2\text{O}$. Present at mM levels, it is known to be the most abundant antioxidant found within the cell. Previous reports have shown that targeting GSH using a modified molecule of gamma-glutamylcysteine reduces markers of oxidative damage following TBI [393, 394]. Similarly, studies have also shown that n-acetyl cysteine, NAC, administration, the non-amide form of NACA, can increase brain GSH levels, improve mitochondrial function, reduce BBB permeability, and decrease brain edema following TBI [395-397]. NAC has also been shown to be effective in reducing free radical dependent cerebrovascular responsiveness in fluid percussion model of TBI which are likely the result of NAC’s ability to improve free radical scavenging mechanisms [398-400]. Additionally, NAC has also been shown to be an effective compound in CCI by inhibiting cerebral inflammatory responses [401] and, when used in combination with minocycline, it has been shown to improve cognition and memory function following TBI [402, 403]. Most recently, NAC treatment has been evaluated in U.S. service members deployed to Iraq who had been exposed to a blast induced mild traumatic brain injury (mTBI). The initial report
indicates significantly improved behavioral outcome measures when patients were treated with NAC for 7 days compared to the placebo treatment [398, 404]. The Authors reported that if NAC treatment were received within 24 hrs of blast injury, members had significantly better chances of symptom resolution which included dizziness, hearing loss, headache, memory loss, sleep disturbances, and neurocognitive dysfunction. However, given the limited CNS bioavailability of NAC due to its limited BBB, cellular and, most importantly, mitochondrial permeability, the potential benefit of GSH as a therapeutic target has most likely been underestimated and has not reached its full potential. This justifies the foundation for a clinical trial that is currently underway to utilize the membrane transporter inhibitor probenecid to improve bioavailability of NAC following TBI in pediatric patients (Pro-Nac clinical trial, ClinicalTrials.gov ID NCT1322009).

Building upon this, researchers have now started to investigate the use of NACA, the novel amide form of NAC which has increased bioavailability and ability to cross cellular and mitochondrial membranes. NAC is negatively charged at physiological pH, and mostly lipid insoluble in vivo, whereas the chemical derivative NACA is neutral and has high lipid solubility under physiological conditions [405, 406]. Although the literature on NACA is still somewhat limited it has been shown to increase tissue sparing and functional recovery following a CNS injury when compared to NAC and vehicle treated animals. These results were seen in both models of TBI and Spinal Cord Injury and have been attributed to the increased mitochondrial function and decreased oxidative stress which were both observed [344, 407-409].
Interventions with multiple targets

Lastly, it is thought that due to the complicated nature of the secondary injury cascade, the best pharmaceutical interventions will target multiple steps in this cascade. Cyclosporin A, CSA, is a drug that has been investigated for the use for a use in TBI and has shown great promise. CSA had multiple indications, including immune suppression, inhibition of mPTP formation and indirect antioxidant properties. In its action of targeting mPTP, CSA binds Cyclophilin D, CypD. Once CSA binds CypD, it is not able to contribute to the structure of the megapore leading to non-functionality thus preventing Calcium overload induced MPT. As an indirect antioxidant, CSA is not able to directly scavenger reactive radicals or aldehydes. Rather, CSA inhibition of MPT in TBI models secondarily lessens mitochondrial free radical formation and decreases the further accumulation of 4-HNE and 3-NT together with a histological neuroprotection in terms of decreased lesion volume with a therapeutic window as long as 8 hours [110, 360, 410-413]. This effect is totally unrelated to immunosuppression since the non-immunosuppressive cyclosporin A analog, NIM811, is able to duplicate the posttraumatic mitochondrial and neuroprotective effects [410, 414]. A phase 2 dose escalation and safety study in 40 severe TBI patients has suggested that CSA treatment initiated within the first 8 hours and continued for 3 days may improve their functional recovery [415]. However, additional clinical trials are needed to determine the reliability of this effect.

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and

CHAPTER 4: PIOGLITAZONE AS A TARGET FOR TBI INDUCED MITOCHONDRIAL DYSFUNCTION

Traumatic brain injury (TBI) is a serious healthcare problem resulting in health, emotional, behavioral and financial complications [315]. The discovery of effective pharmacological agents is necessary to help this ailing population. Alas, there are no approved pharmacological treatments for TBI so progress in this area is vital. Appropriately aligned, this study will investigate the mechanism of neuroprotection offered by pioglitazone, an FDA approved pharmacological agent, and the importance of mitoNEET within this mechanism. This study will also help advance the field of traumatic brain injury by investigating the importance of mitochondrial bioenergetics to neuronal loss following TBI, while possibly uncovering a novel neuroprotective therapeutic target, mitoNEET.

Complications that arise from TBI are multifaceted and originate from a mechanical insult. This insult leads to tearing of blood vessels, axonal shearing and cellular death, resulting in increased membrane permeability and the initiation of multiple signaling cascades [325]. Since the initial insult cannot be reversed, it is this secondary injury cascade where drug interventions can be targeted and advantageous. Some well accepted occurrences in the secondary injury cascade are alterations in excitatory amino acids (EAA) [309, 355], disruption of Calcium homeostasis [350, 359, 416], and mitochondrial dysfunction [108, 110, 313, 346, 347, 354, 359-361, 364, 416-420]. The implications of abnormally high excitatory amino acids, EAA, levels around the site of the injury are generally accepted. These EAAs activate multiple receptors and channels,
such as the NMDA receptor [421, 422]. Activation of these receptors leads to Calcium influx into neurons. At low to moderate levels, this intracellular Calcium concentration, \([\text{Ca}^{2+}]_i\), is used as a potent signaling molecule; however, following an injury, EAA concentrations drastically elevate Calcium levels leading to an overwhelming influx of Calcium and consequential neuronal cell death [423]. One of the most important organelles involved in the rapid buffering of this Calcium is the mitochondria.

Mitochondria and TBI Link: Mitochondria have low-affinity, high capacity Calcium uniporters in their inner membrane that allow for rapid sequestration of \([\text{Ca}^{2+}]_i\). These uniporters “piggyback” off the membrane potential, \(\Delta \Psi\), generated by the electron transport chain’s (ETC) translocation of protons across the inner membrane. As the mitochondria buffer Calcium, a reduction in \(\Delta \Psi\) occurs. Following injury, \([\text{Ca}^{2+}]_i\) rapidly increases and mitochondria work feverishly to buffer it leading to decreased \(\Delta \Psi\) and decreased ETC mediated ATP synthesis. This mechanism continues until \(\Delta \Psi\) is depleted. At this point, mitochondria become dysfunctional and cell death pathways are initiated. Mitochondrial dysfunction related to excitotoxicity may be a therapeutic target for future drug interventions. Following a CNS (central nervous system) injury excitotoxic event, mitochondrial uncouplers have been studied to inhibit mitochondrial Calcium uptake through the reduction of \(\Delta \Psi\). These uncouplers have been shown to be neuroprotective due to their ability to modulate mitochondrial dysfunction [350, 363, 420, 424]. Recent studies have also shown that post-injury treatment of pioglitazone is able to attenuate mitochondrial dysfunction and increase cortical
sparing, however the exact target of pioglitazone within this mechanism is still unknown.

PPAR, PPAR agonists and TBI: Peroxisome Proliferator Activated Receptors (PPARs) are Nuclear Receptor proteins that can heterodimerize in a ligand-dependent and -independent manner to regulate gene expression of multiple molecular processes. There has been 3 PPARs identified including α, δ/β or and γ [425-429]. PPAR-γ the most widely studied subtype in the area of CNS injury due to its well-established role in the regulation of adipogenesis, glucose homeostasis, cellular differentiation, apoptosis and modulation of inflammation resulting from chronic and acute neurological insult. One class of drugs which have shown much promise as neuroprotective agents are thiazolidinedione which are anti-diabetic drugs used for the treatment of type 2 diabetes. This class of drugs were generated for their ability to activate various Peroxisome Proliferating Activating Receptor isoforms [427, 428]. One of the isoforms in this class of drugs that has drawn much attention due to its ability to provide neuroprotection in models of CNS injury is pioglitazone [347, 430]. Originally, the mechanism of neuroprotection for this drug was thought to be through the modulation of PPARγ, however recent data analyzing PPARγ activation, as well as pioglitazone binding affinities for a specific mitochondrial membrane protein, may prove that this initial hypothesis needs reconsideration. Data has suggested that the therapeutic effects of pioglitazone may be more complex than just activation of PPAR. Not only has pioglitazone been shown to decrease inflammation it has also been shown to decrease oxidative damage,
attenuate mitochondrial dysfunction and reduce cell death following CNS injury, pathways which seem to be slightly more encompassing and on a quicker timeline than what could be attributed solely as PPARγ activation [311, 347, 430, 431]. To further support this, a study by Thai et al. used PPAR antagonists to show that the neuroprotective effects seen with pioglitazone were independent of PPARγ. Additionally, a study by Bieganski et al. showed that pioglitazone bound to mitoNEET, a mitochondrial membrane protein with an unknown role in the cell [432]. Lasly, a recent study by Zuris et al. showed that pioglitazone binding to mitoNEET was able to inhibit [2Fe2S] cluster transfer upon binding however the role of such a mechanism in providing therapy in CNS injury is unknown [433]. The combination of this data has stemmed ample research from our lab showing that pioglitazone is unable to increase mitochondrial bioenergetics in transgenic mice who lack mitoNEET and that pioglitazone loses some of its neuroprotection following injury in these animals as well.

mitoNEET: mitoNEET was discovered in 2004 by Colca et al. in the brain, liver and skeletal muscles of rodents [434]. This discovery was a result of a pioglitazone pull-down assay that bound to the novel m-17 kDa mitochondrial membrane protein, which was later termed mitoNEET [434]. The exact role of mitoNEET still remains unclear. However, it is known that mitoNEET knockout mice experience reduced oxidative capacity hinting to an importance in mitochondrial mediated respiration [435, 436]. A recent publication also found that mitoNEET binds Glutamate Dehydrogenase I allowing for direct control over the Krebs cycle and therefore control over mitochondrial respiration [83].
Therefore, I propose mitoNEET to be important for mitochondrial homeostasis and therefore a crucial therapeutic target for pioglitazone mediated neuroprotection.

Results

Pioglitazone is able to increase mitochondrial respiration in a biphasic manner following calcium insult.

The mitochondrial effects of pioglitazone have been debated in the literature [347, 430, 437]. Pioglitazone has been shown to interact with multiple proteins at the cytosolic, nuclear and mitochondrial level [431, 432, 434, 437, 438]. In order to study the importance of mitochondria bioenergetics in pioglitazone mediated neuroprotection, we isolated mitochondria, removing any interactions due to cytosolic or nuclear proteins such as the PPARs. This allows for insight into what exactly is occurring in the mitochondrial level. Previous results from Murphy et. al. have shown that micromolar concentrations of pioglitazone are able to decrease mitochondrial respiration [437]. However, results from Sauerbeck et al show that lower concentrations, nanomolar to micromolar range, of pioglitazone can increase mitochondrial respiration in isolated mitochondria [347]. In order to determine where this discrepancy arose, cortical mitochondria were isolated from naive CD57/BL6 mice, insulted with Calcium (150nm/mg) and then treated with various concentrations of pioglitazone. Mitochondrial bioenergetics were analyzed (Figure 4.1). This calcium insult provides a similar effect to that seen within the excitotoxicity mechanism following a TBI. 50nM pioglitazone was able to increase
mitochondrial respiration 12.7% compared to naïve non-insulted mitochondria and 32.8% compared to vehicle treated calcium insulted mitochondria. Also, a biphasic effect was seen where lower dosages of pioglitazone (0.05uM) provided increased mitochondrial bioenergetics and higher concentrations provided decreased mitochondrial bioenergetics. This data supports what has been published in the literature where lower concentrations of pioglitazone increased mitochondrial respiration and higher concentrations decreased mitochondrial respiration.
Figure 4.1 Pioglitazone Dose Response: Pioglitazone is able to increase state V mediated maximal mitochondrial respiration in isolated from wild-type C57BL/6 mice cortex insulted with 150nm/mg of calcium. One-way ANOVA, Compared Drug Treated to Ca + Vehicle, Bonferroni Post-Hoc – protects from a type I error (Detecting an effect that is not present), N=3, SEM
Pioglitazone loses its neuroprotective effects in mitoNEET KO animals.

As mentioned previously, mitoNEET is a novel mitochondrial membrane protein which has been shown to modulate mitochondrial respiration. Pioglitazone has also been shown to bind mitoNEET however the importance of this interaction in pioglitazone’s neuroprotective effects remains unknown. Therefore, we wanted to perform a similar test to what was performed in mitochondria isolated from wild-type mice however use mitoNEET null mice instead. The mitoNEET null mice were generated by Dr. Stewart Ross at the university of Kentucky and used previously Western blot was performed in WT versus mitoNEET null cortical homogenate to confirm the presence, or lack, of mitoNEET [435] (Figure 4.2). As shown in figure 1, calcium is able to decrease mitochondrial respiration in wild-type mitochondria and pioglitazone is able to reverse these inhibitory effect. However, in mitochondria isolated from the cortical tissue of mitoNEET null mice, calcium (150nm/mg of protein) had little effect on mitochondrial respiration and the use of pioglitazone provided no increased respiration (Figure 4.3). In order to further understand this phenomenon, we evaluated the membrane potential using TMRE and Ca5 indicators and there was no differences observed, data not shown. Combined, these results show that mitoNEET is important in calcium mediated decreases in mitochondrial bioenergetics and that mitoNEET is necessary for pioglitazone mediated increased respiration following an insult from Calcium.
Figure 4.2 WT versus mitoNEET null protein expression: Western blot analysis of mitoNEET expression in WT versus mitoNEET null animals.
Figure 4.3 Pioglitazone dose response in mitoNEET null mice: In mitochondria isolated from the cortical tissue of mitoNEET null mice, calcium insult (150nm/mg protein) has no significant effect and pioglitazone is unable to increase state V mediated maximal mitochondrial respiration. One-way ANOVA, Compared Drug Treated to Ca + Vehicle, Bonferroni Post-Hoc, N=3, SEM
Work from Sullivan et. al. and many others have shown that mitochondrial dysfunction is progressive over the first 24 to 48hrs following a control cortical impact and that amelioration of this mitochondrial dysfunction can improve neurological function and cortical sparing. This mitochondrial dysfunction can be targeted with various therapeutic interventions in order to improve tissue and functional outcomes following an injury. Pioglitazone is one drug that has been shown to both target mitochondrial dysfunction and improve tissue and functional outcome. However, the question that arises when studying pioglitazone mediated neuroprotection following a traumatic brain injury is ‘what is its mechanism?’.

This is a difficult question to answer because pioglitazone has been shown to interact with multiple proteins within the cell. In the past, much interest has been placed on pioglitazone interaction with various isoforms of PPAR however one specific protein that is starting to gain some interest in the literature is mitoNEET. This protein is found within the mitochondria giving it direct localization to improve mitochondrial bioenergetics. In order to test the importance of mitoNEET in pioglitazone’s ability to increase mitochondrial bioenergetics following injury, mitoNEET null and wild-type mice were injured and received one IP injection of pioglitazone at 12 hours post-injury. The animals were sacrificed and cortical mitochondria were isolated at 13 hours post-injury. Mitochondria isolated from mice treated with pioglitazone had a 54% increase in mitochondrial bioenergetics compared to vehicle treated wild-type mice. On the contrary, mitoNEET knockout mice experienced no increase in mitochondrial bioenergetics compared to vehicle treated animals of the same genotype. This data provides support that
the ability of pioglitazone to improve mitochondrial dysfunction following traumatic brain injury is directly dependent on the presence of mitoNEET.
Figure 4.4 Pioglitazone response in WT versus mitoNEET null mice: FCCP mediated, maximal mitochondrial oxygen consumption rate, in wild-type and mitoNEET null mice (n=3) who received pioglitazone (1 IP injection, 10mg/kg) at 12 hours post-injury with isolations at 13 hours post-injury. Mitochondria from wild-type mice treated with pioglitazone had significantly increased bioenergetics compared to mitoNEET null mice. Data represents means + SEM, *p=0.0322 wild-type compared to mitoNEET null mice.
Pioglitazone treatment following TBI is able to increase cortical sparing however the effect is diminished when mitoNEET is removed.

As mentioned previously, research has found that pioglitazone is able to alter mitochondrial bioenergetics and improve neuroprotection [347, 430]. Many pharmacological treatments that target mitochondrial dysfunction have also shown promising neuroprotective effects following an injury to the CNS [361, 439-444]. To test whether the change in mitochondrial bioenergetics lead to increased cortical sparing following a brain injury, adult wild-type and mitoNEET null mice (n=6) received a moderate contusion injury and then received either vehicle (DMSO) or 10mg/kg of pioglitazone through IP injection at 15min and then daily for the first 7 days post-injury. Cortical tissue sparing was accessed at 14 days post-injury and pioglitazone was found to be neuroprotective following TBI in wild-type (+/+) but not mitoNEET null (-/-) mice (n=6, p=0.0049). As shown in Figure 3, pioglitazone treatment significantly increases tissue sparing in wild-type (+/+ ) mice but this effect was lost in mitoNEET null (-/-) mice. The effects of pioglitazone that are seen in the mitoNEET mice are likely due to other neuroprotective effects mediated at the cytosolic and/or nuclear level. It is also important to note that no significant differences were measured between vehicle-treated animals of either genotype, showing that the removal of mitoNEET from the mitochondrial membrane did not increase the animals’ susceptibility to injury.
Figure 4.5: Pioglitazone is neuroprotective following TBI in wild-type (+/+)
but not mitoNEET null (-/-).
In this study, mice (n=6 per group) were injured (1.0 mm, severe) followed by
pioglitazone injections (10mg/kg) at 15min, and 24hours post injury with cortical
tissue sparing was assessed at 7 days. Pioglitazone treatment significantly
increases tissue sparing in wild-type (+/+)
but not mitoNEET null (-/-) mice. Additionally, no significant differences were measured between vehicle treated animals of any genotype. One-way ANOVA, Compared Drug Treated to
Vehicle within same genotype, Bonferroni Post-Hoc, n=6, p=0.0049, mean
+/- SEM
mitoNEET binding with a mitoNEET specific ligand, NL-1, increases cortical sparing and improves functional outcome following a TBI.

Due to the slight trend of neuroprotection seen in mitoNEET null mice who were treated with pioglitazone, we decided to probe the importance of targeting mitoNEET specifically, with a mitoNEET ligand. In regards to this non-significant, off-target effect seen with pioglitazone, it is likely due to interactions at the cytosolic level, quite possibly with PPAR. We know that this effect is due to cytosolic contributions because we do not see increases in isolated mitochondrial respiration in mitoNEET KO mitochondria however do see an effect in wild-type mitochondria. Therefore, we moved our studies forward hypothesizing that NL-1 would provide the same degree of neuroprotection which was seen with pioglitazone, however render no trends towards neuroprotection in the mitoNEET null mitochondria. Therefore, collaborators synthesized a compound that binds to mitoNEET called NL-1. Pharmacodynamic studies have shown that this drug is very similar to pioglitazone, retaining the traditional glitazones structure, binding to mitoNEET with equal affinity as pioglitazone, however lacks the PPAR binding region. Following a severe injury, either WT or mitoNEET null mice received either an IP injection of 10mg/kg NL-1 or vehicle (DMSO) at 15 minutes and then daily for the first 7 days post-injury. At 7 days post-injury, animals were sacrificed and tissue sparing was calculated. As seen in Figure 4.6, NL-1 administration following injury significantly increases (p=0.033, n=16) cortical sparing by 37% compared to vehicle treated animals.
Figure 4.6: NL-1 administration increases cortical tissue sparing after TBI. CD57bl6 WT or mitoNEET null mice received the mitoNEET ligand, NL-1, (n=16, IP injection, 10mg/kg, at 15min and then daily for the first 7 days post-injury) following a moderate injury (1.0mm) had a significant increase in cortical sparing at 37% compared to vehicle treated mice (n =16) at 7 days, an effect that was lost in the mitoNEET null animals. One-way ANOVA, Compared Drug Treated to Vehicle within same genotype, Bonferroni Post-Hoc, n=16, p=0.0222, mean +/- SEM
Figure 4.7 Qualitative Images of NL-1 Cortical Sparing: Representative figures of the cortical sparing seen in WT animals treated with NL-1 compared to either vehicle treated or drug treated without mitoNEET's presence.
The neuroprotective effects seen with NL-1 lead us to believe that treatment with NL-1 could also increase functional recovery following injury. With the model of injury used in this study, animals experience damage to regions of the motor cortex leading to motor deficits. To test these deficits, animals are trained before their TBI surgery to traverse various sized beams. Following injury, the animals are then placed on the various sized beams and foot faults are measured as an indication of deficits to the corticospinal tracts. Therefore, the animals were trained to traverse the beams and then provided a severe injury to both wild-type and mitoNEET null mice. We treated them with either vehicle or 10mg/kg of NL-1 at 15 minutes' post-injury and then daily for the first 7 days. Foot faults on the beam were tested at 1 hour, 1 day, 3 days and 5 days post-injury. Injections on behavioral test days were provided following the behavioral tests in order to avoid any confounding stresses. The wild-type mice treated with NL-1 had a significant increase in functional recovery at day 5 compared to vehicle treated, which is an effect that was not seen in the mitoNEET null mice.
Figure 4.8: Wild-type mice treated with NL-1 had a significant increase in functional recovery, an effect lost in the mitoNEET null mice. Wild-type and mitoNEET null mice were trained on the beam walk test and then received a severe CCI injury 24 hours later. They were given 8 IP injections of 10mg/kg of NL-1 at 15 minutes and then every 24 hours thereafter for the first 7 days post-injury. At 1 hours, 1 day, 3 days and 5 days post-injury, the beam walk test was performed and foot faults were measured. Wild-type mice treated with NL-1 had improved performance on this test compared to vehicle treated mice. This effect was lost in mitoNEET null mice. **Two-way ANOVA, Compared Drug Treated to Vehicle within same genotype, Bonferroni Post-Hoc, n=16, p=0.0168, mean +/- SEM**
mitoNEET is able to interact with GDH providing insight to a possible neuroprotective mechanism.

Following a brain injury, a dysregulation of excitatory amino acids occurs, specifically related to Glutamate. This eventually leads to the initiation of excitotoxicity through pathogenic influxes of calcium. Mitochondria are the first line of defense in order to rapidly buffer high intracellular calcium levels. As they continue to buffer Calcium in a process mediated by the mitochondrial calcium uniporter, the membrane potential, generated by the electron transport chain, becomes depleted. The current theory is that as Calcium levels reach high enough level, reactive oxygen species and reactive nitrogen species (ROS and RNS specifically) production increases leading to the damage of proteins within the mitochondria. However, work published by Pandya, show that with increased calcium insult in isolated mitochondria did not lead to increased oxidative stress. Additionally, calcium insult leads to decreased state III respiration, which hints to an inhibition that is mediated either at Complex I or upstream. According to this 2013 paper however, neither Complex I nor PDH activity were inhibited by calcium load hinting at another possible mechanism [362]. After observing decreased calcium inhibited respiration in the mitoNEET null mice, we became interested in a possible mechanism. Work from Roberts observed that mitoNEET was able to interact with GDH, which was observed during a pull-down assay [83]. Additionally, work from Cole found that GDH expression increases following TBI, which was proposed to be due to a lack of BCAA [445]. However, I hypothesize that decreased mitoNEET activity leads to inhibition of GDH
following TBI, which leads to compensatory increased expression. I propose that this effect would be reversed following treatment with either NL-1 or pioglitazone.

Discussion

The mechanism of pioglitazone mediated neuroprotection has been highly debated for years. Historically, the effect of this glitazones has been thought to be through its interactions with PPAR-γ. However, many research groups have found pioglitazone retains its therapeutic effects in the presence of PPAR inhibitors. Additionally, researchers have found that pioglitazone is able to target mitochondrial bioenergetics and we have found that it can increase mitochondrial bioenergetics in pure isolated mitochondria, removing any effect from the cytosol or nucleus. Therefore, with this study, we attempted to understand the importance of mitoNEET within the mechanism of neuroprotection provided by pioglitazone following a control cortical impact injury.

One topic that has been highly debated regarding pioglitazone is whether it increases or decreased mitochondrial bioenergetics. In a previous study completed my Murphy et. al. mitochondrial bioenergetics were decreased with permeabilized cells were incubated with micromolar concentrations of pioglitazone [437]. However, research from Sauerbeck et. al. found that pioglitazone is able to increase mitochondrial bioenergetics when isolated mitochondria were incubated with nanomolar concentrations of pioglitazone [347]. Results from this study provide possible insight into where this discrepancy may be arising in that pioglitazone has a biphasic effect which increased
mitochondrial bioenergetics at low concentrations but can become toxic to mitochondria when concentrations become too high.

Building upon the importance of dosage when working with pioglitazone, the next important topic to discuss is the mechanism of neuroprotection. In the literature, pioglitazone is described as a PPAR agonist that provides neuroprotection through changes in gene expression of a plethora of important proteins such as cytokines and various endogenous antioxidants. These changes are extremely important when treating traumatic brain injury and should not be disregarded. However, interestingly enough, the improvements of mitochondrial dysfunction that have been seen following an injury to the brain occur on a timescale much more rapid than the effects seen with changes in gene expression. As seen with the previous data, animals treated with pioglitazone at 12 hours post-injury and sacrificed 1 hour later had increased mitochondrial function compared to vehicle treated animals. Since the drug was only introduced one hour before the mitochondria were isolated, the effects are thought to be based on direct interactions and not through changes in gene expression.

This observation raises the question, if pioglitazone is not providing neuroprotection through changes in gene expression, then what is pioglitazone interacting with directly? From this study it seems as if the answer to this question lies within mitoNEET. From the data we see that pioglitazone is able to increase mitochondrial bioenergetics by over 54% in wild-type mice who express mitoNEET in their mitochondria however lose its ability to increase mitochondrial bioenergetics in mitoNEET null (-/-) mice. To further support this, wild-type mice
that were injured with a moderate CCI injury had a 64% increase in tissue sparing compared to mitoNEET null (−/−) mice. This provides evidence that mitoNEET is crucial for pioglitazone mediated neuroprotection.

In order to further test that hypothesis that binding mitoNEET with a mitoNEET ligand is indeed able to increase mitochondrial bioenergetics, NL-1 was synthesized as a truncated glitazones, retaining a structure similar to pioglitazone which allows it to bind mitoNEET but not interact with the PPAR isoforms. This drug was not only found to provide a 37% increase in cortical sparing but was able to improved motor function in wild-type mice, which was not seen in mitoNEET null (−/−) mice.

The data provided from this study provides strong evidence that pioglitazone mediated neuroprotection following a contusion injury in rodent models is able to provide increase tissue sparing and improved functional outcome is dependent on interactions with mitoNEET. This data also supports mitoNEET as a potential therapeutic target for brain injury as well as other neurodegenerative diseases where mitochondrial dysfunction is prevalent.
CHAPTER 5: METHODS USED FOR THESIS WORK

**Animals:** All of the studies performed were approved by the University of Kentucky Institutional Animal Care and Usage Committee. All experiments were conducted using either adult (male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300-350g (chapter 2) or adult (~8-10 week old) male and females adult wild-type (C57BL/6) or mitoNEET null mice with an average mass of 30 grams. Studies with wild-type (WT) and mitoNEET -/- (KO) mice were the same mice described in [435] which are bred and housed in the SCoBIRC transgenic animal core. The rats were housed 3 per cage and the mice were house 5 per cage. Both sets of animals were maintained in a 12-hr light/12hr dark cycle and fed a balanced diet ad libitum.

**mitoNEET Null Colony:** Genotyping of these animals has been done by our laboratory and wild-type littermates will be used as controls. These animals have been backcrossed into the C57BL/6 strain of mice (minimum of 30 generations) to account for differential strain sensitivity to neuronal insult and injury. C57BL/6 mice are an inbred strain, maintained in a barrier-reared environment, widely used in TBI research and as genetic background strains in transgenic models.

**Controlled Cortical Impact Brain Injury:** All surgical procedures were performed as previously described [110, 346, 350, 354, 361, 363, 364, 416, 418, 444] and were classified as severe (1.0 mm (mice) deep contusion at 3.5
meters/second for 500ms). Prior to injury, animals were anesthetized using 2% isofluorane, weighed and their head was shaved to remove hair at the site of the surgery. Animals were then placed in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA) for proper positioning under a pneumatic head impactor (Precision Science Instruments) and body temperature was maintained at 37°C with the use of a isothermal pad. A 5mm craniotomy was drilled lateral to the central fissure on the left side of the skull centered between lambda and bregma, with much care taken to not disrupt the dura. Injury groups then received a unilateral injury directly to the surface of the brain. Sham animals received a 5.0mm craniotomy but did not receive an impact to the brain. Following the injury, a hardened circular piece of dental acrylic made the night before, and secured with sterile surgical adhesive over the craniotomy. The incision was then closed with medical grade sutures.

After the surgery sites were closed on all animals, the isofluorane was shut off and the animals were removed from the stereotaxic frame. They were then placed in a clean cage that was temperature controlled at 37°C with a heating pad until the animals were mobile and fully responsive.

To investigate the neuroprotective effects of either pioglitazone or NL-1 after TBI, animals were given a CCI in the manner described above and then given an IP injection of either pioglitazone or NL-1. Both drugs were solubilized in vehicle (100% Ethanol, 1uL 38% HCl, and 25uL of 0.9% saline for pioglitazone and 100% DMSO for NL-1) before injection. All drugs were made at an
appropriate concentration in order to inject ~100uL per animals and exact adjustments for weight were made before the injection was given.

**TABLE 5.1 Dosage Paradigm for Animals**

<table>
<thead>
<tr>
<th>Desired Dosage For Animal</th>
<th>Concentration of Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mg/kg</td>
<td>0.3mg/ml</td>
</tr>
<tr>
<td>10mg/kg</td>
<td>3mg/ml</td>
</tr>
<tr>
<td>20mg/kg</td>
<td>6mg/ml</td>
</tr>
<tr>
<td>40mg/kg</td>
<td>12mg/ml</td>
</tr>
</tbody>
</table>
**Mitochondrial isolation:** Mitochondria were isolated using previously employed differential mitochondrial isolation methods [347, 362, 446]. Animals were sacrificed by asphyxiation with CO$_2$ and then rapidly decapitated. This CO$_2$ asphyxiation method is the standard euthanasia method used. The animal’s consciousness is quickly removed allowing for the rapid removal of the brain without any confounding factors that other methods would pose, such as the use of FatalPlus. The brain is then washed with ice-cold (around 4°C) mitochondrial isolation buffer with EGTA (MIB+EGTA) made-up of 215 mM Mannitol, 75 mM Sucrose, 0.1% BSA, 1 mM EGTA, and 20 mM HEPES at pH 7.2. This step removes excess blood and debris, while chilling the brain rapidly. The used of MIB+ETGA at this point is a crucial step as EGTA is used to chelate free calcium released during the homogenization process. Cortical tissue, either total or a 5mm punch if the brains were injured, were then homogenized in a Potter-Elvejhem manual homogenizer with 3mL of MIB+EGTA. Once the tissue was fully homogenized, the samples were spun via centrifugation at 1300 × G for 3 min.

Following the last spin, the supernatant is placed in a fresh tube and the pellet is re-suspended in MIB+EGTA. The samples are then spun again at 1300 × G for 3 min. This step allows for the separation of heavy cellular debris such as the plasma membrane, from smaller intercellular organelles, etc. The supernatant from the first and second spins were collected in separate tubes and spun at 13,000 × G for 10 min. This spin allows for the removal of light-weight objects such as proteins, RNA, etc. The pellets from both tubes are then
combined, resuspended in 500 μl MIB+EGTA and placed in a nitrogen bomb at 1,200 psi for 10 min. The pressure in the nitrogen bomb is rapidly released after 10 min in order to pop the synaptosomes and release synaptic mitochondria.

The samples are then placed as the top layer on a Ficoll separation column which consisted of a 10% Ficoll layer (bottom) and a 7.5% Ficoll layer (top). The Ficoll column with sample carefully placed on top is centrifuged at 32,000 × G for 30 min at 4 °C. Following the Ficoll purification, the mitochondrial pellet, bottom layer, is re-suspended in mitochondrial isolation buffer without EGTA (MIB-EGTA) and centrifuged at 10,000 × G for 10 min at 4 °C in order to remove residual Ficoll and EGTA from the purified mitochondrial sample. The final mitochondrial pellet is re-suspended in MIB-EGTA to yield a final concentration of approximately 10 mg/ml. These samples are stored immediately on ice. Protein concentrations for each sample is determined with all the samples on the same plate using the BCA protein assay kit and measuring absorbance at 560 nm with a Biotek Synergy HT plate reader (Winooski, VT).

**Measurement of mitochondrial bioenergetics: Oxytherm**

As mentioned in Chapter 1, and seen in Figures 1.10 and 1.11, the Oxytherm which uses a Clark Electrode, is historically used for mitochondrial bioenergetics assays. Before the mitochondrial samples are evaluated, the electrode is built in the manner described in Chapter 1. The Oxytherm is then calibrated at 37°C, measuring 100% oxygen, 0% oxygen in the presence of Sodium Borohydride (Sigma 213462-25G) and then 100% oxygen again. Once calibrated, 250uL of Respiration Buffer (RB) composed of 215 mM mannitol,
75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KH$_2$PO$_4$ at pH 7.2, is added to the chamber along with 50ug of mitochondrial protein and the Oxytherm program, Oxy32, is started to read oxygen changes.

Substrates and inhibitors are then added allowing us insight into the functionality of the proteins within the Electron Transport System.
<table>
<thead>
<tr>
<th>Targeted State of Respiration</th>
<th>Substrate or Inhibitor Added</th>
<th>Amount Added</th>
<th>Concentration</th>
<th>Rational of Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>State I</td>
<td>Mitochondria</td>
<td>~5ul</td>
<td>10ug/ul</td>
<td>No Substrate added</td>
</tr>
<tr>
<td>State II</td>
<td>Pyruvate/Malate</td>
<td>2.5ul</td>
<td>5mM/2.5mM</td>
<td>Added to “charge” the membrane. Pyr and Mal will start the Krebs Cycle allowing for the production of NADH. These are then fed into the ETS, building the proton-motive force however this force cannot be converted into energy (ATP) due to a lack of ADP.</td>
</tr>
<tr>
<td>State III_{ADP}</td>
<td>ADP</td>
<td>1.25ul x 2</td>
<td>150uM</td>
<td>The presence of ADP allows ATP Synthase to convert the proton-motive force (Membrane Potential) built by the addition of Pyr/Mal into ATP. With differential mitochondria, ATPases will use the ATP as it is generated leading to a depletion leading to a depletion of Oxygen. In isolated mitochondria, the ATP synthase will convert all the ADP to ATP and the oxygen consumption will plateau. A second amount of ADP is</td>
</tr>
<tr>
<td>State</td>
<td>Additive</td>
<td>Concentration</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>---------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Oligomycin</td>
<td>0.5ul, 1uM</td>
<td>As mentioned in Chapter 1, Oligomycin is an ATP Synthase inhibitor. It binds the Oligomycin Sensitive Conferring Protein (OSCP), consequently inhibiting State III respiration. This allows the proton-motive force to build back up and any oxygen consumption measured here is a result of proton leak across the inner membrane.</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>FCCP /Uncoupler</td>
<td>0.5ul, 1uM</td>
<td>FCCP is a weak lipophilic acid which acts as a membrane uncoupler. It binds protons in the IMS and translocates them to the matrix. The rate of oxygen consumed here provides the maximal mitochondrial respiration.</td>
<td></td>
</tr>
</tbody>
</table>
| V<sub>Succinate</sub> | Rotenone + Succinate | 0.1ul + 5ul, 1mM + 10mM | Rotenone is added to inhibit Complex I, as discussed in Chapter 1. Once Complex I is
inhibited, succinate is able to feed electrons into the ETC through Complex II. The rate of oxygen consumption here provides information about State III Complex II mediated respiration.
A representative figure of an Oxytherm plot is as follows:

Figure 5.1 A representative figure of healthy mitochondria respiring during a coupling experiment: Oxygen consumption within healthy mitochondria.
Measurement of mitochondrial bioenergetics: Seahorse

Measurements of mitochondrial bioenergetics in isolated mitochondrial were completed using a Seahorse XF24 Flux Analyzer as published previously using slight modifications \[447\]. Stock mitochondrial substrates of 500 mM pyruvate, 250 mM malate, 30 mM ADP, 1 mg/ml oligomycin-A, 1 mM FCCP, 1 mM rotenone and 1 M succinate were prepared and the pH was adjusted to pH 7.2. The day before the planned experiment, a 24 well dual-analyte solid state bio-sensor cartridge was hydrated with Seahorse XF Calibrant (pH 7.4) and incubated in a non-CO\(_2\) incubator at 37 °C.

On the experiment day, the bio-sensor cartridge ports A through D were loaded with 75ul of the appropriate mitochondrial substrates or inhibitors (Port A = 8 x, Port B = 9 x, Port C = 10 x, and Port D = 11 x concentration made from stocks and RB), and injected into the assay plate according to protocol procedure to reach the final concentration of the compound (1 x) in each well. The amount of substrates/inhibitors loaded for each port is based upon the initial 525 µl RB volume in the mitochondrial plate as follows: Port A – 75 µl (mixture of pyruvate, malate and ADP), Port B – 75 µl (Oligomycin A), Port C – 75 µl (FCCP), and Port D – 75 µl (rotenone and succinate). Once the bio-sensor cartridge was loaded with all of the experimental reagents it was placed into the Seahorse XF24 Flux Analyzer for automated calibration.

During the calibration phase, the Seahorse Standard XF24e flux assay plates were utilized for mitochondrial analysis. Isolated mitochondria (7µg) from every experimental group were analyzed together on a single plate.
Mitochondrial samples were re-suspended in 50 μl RB and added in experimental wells whereas background control wells contained 50 μl of RB without mitochondria. Once every well was loaded, it was centrifuged for 4 minutes at 3,000 rpm room temperature. Following the centrifugation of the plates, 475 μl (37 °C) of pre-warmed RB was gently added to each well for a final volume of 525 μl per well. Plates were then placed into the calibrated Seahorse XF24 flux analyzer for mitochondrial bioenergetics analysis after the sensor cartridge calibration was concluded.

An optimized protocol was utilized for the analysis of bioenergetics function in purified mitochondria using the Seahorse Biosciences XF24 Flux Analyzer. The protocol contains sequential and/or cyclic steps of a cartridge probe calibration, mixing substrates in the assay system, a delay for some time, injections of substrates/inhibitors and then measurement of the oxygen consumption rates (OCR) as elaborated upon previously [447].
<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Elapsed Time</th>
<th>Procedure</th>
<th>Reason</th>
<th>Potential Issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration</td>
<td>-30 minutes</td>
<td></td>
<td>Calibrate Biosensors</td>
<td>Calibration of the biosensors, measuring standard Oxygen and pH</td>
<td></td>
</tr>
<tr>
<td>Insert microplate</td>
<td>0 minutes</td>
<td>0 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equilibration</td>
<td>12 minutes</td>
<td>12 minutes</td>
<td>2 minutes Mix x 3</td>
<td>Allows samples in microplate to adjust to temperature changes. Consists of 2 minute mix and a 2 minute wait which is repeated 3 times.</td>
<td></td>
</tr>
<tr>
<td>Basal Respiration</td>
<td>1 minutes</td>
<td>13 minutes</td>
<td>Mix</td>
<td>Measures the State I respiration. No substrates, only mitochondria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:30 minutes</td>
<td>14:30 minutes</td>
<td>Wait</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:00 minutes</td>
<td>16:30 minutes</td>
<td>Measure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5mM Pyruvate 2.5mM Malate 1.0mM ADP</td>
<td>~30 seconds</td>
<td>17 minutes</td>
<td>Inject Port A</td>
<td>This step measures State IIIp/M mitochondrial respiration. It will produce the electron rich NADH molecules leading to a charged inner mitochondrial</td>
<td>When running this step, it is crucial to go back to the raw O2 levels to assure that you have not depleted the oxygen stores. This</td>
</tr>
<tr>
<td></td>
<td>25 seconds</td>
<td>17:25 minutes</td>
<td>Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 seconds</td>
<td>17:25 minutes</td>
<td>Wait</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:00 minutes</td>
<td>19:25 minutes</td>
<td>Measure</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
membrane through Complex I while providing the ADP necessary to allow ATP Synthase to make ATP will lead to errors in your calculated OCR values.

<table>
<thead>
<tr>
<th>Membrane through Complex I while providing the ADP necessary to allow ATP Synthase to make ATP</th>
<th>membrane through Complex I while providing the ADP necessary to allow ATP Synthase to make ATP will lead to errors in your calculated OCR values</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Mix 1</th>
<th>1 minute</th>
<th>20:25 minutes</th>
<th>Mix</th>
<th>Helps to re-equilibrate the system to prepare for the next substrate</th>
<th>Sensors needs to re-equilibrate to ambient O2 in order to read effectively. If this step is skipped, sensor can drop below its threshold of reading O2</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>1uM Oligomycin</th>
<th>~30 seconds</th>
<th>20:55 minutes</th>
<th>Inject Port B</th>
<th>This step measures the State IV respiration. Any oxygen consumption here is the result of proton leak across the membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 seconds</td>
<td>21:20 minutes</td>
<td>Mix</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0 seconds</td>
<td>21:20 minutes</td>
<td>Wait</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4:00 minutes</td>
<td>25:20 minutes</td>
<td>Measure</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mix 2</th>
<th>1 minute</th>
<th>26:20 minutes</th>
<th>Mix</th>
<th>Same as Mix 1</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>4uM FCCP</th>
<th>~30 seconds</th>
<th>26:50 minutes</th>
<th>Inject Port C</th>
<th>This step measures the State V respiration. This is the measurement of O2 consumption when it is a</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 seconds</td>
<td>27:00 minutes</td>
<td>Mix</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0 seconds</td>
<td>27:00 minutes</td>
<td>Wait</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4:00 minutes</td>
<td>31:00 minutes</td>
<td>Measure</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

| Similar issue seen here as seen with the injection of Port A. Make sure you have not depleted oxygen | Similar issue seen here as seen with the injection of Port A. Make sure you have not depleted oxygen |

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uncoupled from ATP production and is defined as the Maximal Mitochondrial Respiration levels when your OCR is calculated.

<table>
<thead>
<tr>
<th>Mix 3</th>
<th>1 minute</th>
<th>32:00 minutes</th>
<th>Mix</th>
<th>Same as Mix 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1uM Rotenone 10mM Succinate</td>
<td>~30 seconds</td>
<td>32:30 minutes</td>
<td>Inject Port D</td>
<td>This step measures State $V_{succ}$ mitochondrial respiration. It will produce the electron rich $\text{FADH}_2$ molecules leading to activation of the ETS through Complex II while inhibiting Complex I with Rotenone</td>
</tr>
<tr>
<td></td>
<td>25 seconds</td>
<td>32:55 minutes</td>
<td>Mix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 seconds</td>
<td>32:55 minutes</td>
<td>Wait</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4:00 minutes</td>
<td>36:55 minutes</td>
<td>Measure</td>
<td></td>
</tr>
</tbody>
</table>
Mitochondrial oxygen consumption rates (OCR) rates were recorded in the absence or the presence of various mitochondrial substrates/inhibitors which were added from port A to port D. The data files collected from each experiment were analyzed and reported as percent OCR (% OCR) using GraphPad Prism 6 software package (GraphPad Software, Inc. La Jolla, CA).

**Reactive Oxygen Species:**

Mitochondrial ROS production was measured using 25uM 2'-7'-dichlorodihydro-fluorescein diacetate (DCF) purchased from Molecular Probes (Eugene, OR) (excitation 485nm, emission 528nm) in the Biotek Synergy HT plate reader as described by [300, 301, 313, 418]. 25ug of isolated total mitochondria were added to the a 96 well plate with 100uL of a mixture of RB, 5mM pyruvate, 2.5mM malate. Plate was warmed to 37°C within the plate reader. DCF was added and the plate was incubated for 15 minutes. ROS production was then calculated as the maximum DCF fluorescence expressed in arbitrary fluorescence units. ROS production in the presence of oligomycin, which will produce the maximal ROS production, and FCCP, which will produce the least amount of ROS production, was also determined to assure that the measurements were within the range of the ROS indicator.

**Histological Analysis:**

Animals were anesthetized using FatalPlus and immediately perfused with saline followed by 4% paraformaldehyde. The brains were removed and placed in 4% paraformaldehyde and 30% sucrose solution in PBS for 24hrs. After 24hrs, the brains were transferred to a 30% sucrose PBS buffer solution without
paraformaldehyde. 35uM coronal sections were cut with a freezing microtome throughout the rostral caudal extent of the damaged cortex. Sections were stained with cresyl violet and subjected to image analysis for lesion volume assessment. Quantitative assessment of cortical damage employed a blinded unbiased stereological protocol using the cavalieri method as previously described. All slides were assessed blindly with respect to treatment group using Image J for simple ROI analysis of cortical sparing.

**Adjusted Neurological Severity Score:**

Injured wild-type or mitoNEET null mice had functional recovery tested using the adjusted neurological severity score (aNSS). This 14-point test measures coordinated motor function and balance as a measurement of motor function/recovery following injury. One day prior to injury, all animals were trained to traverse elevated beams of 3, 2, 1, and 0.5 cm width and a 0.5 cm diameter rod. At 1 hour, 24 hours, 3 day and 5 days post-injury, mice were allowed to traverse the beams and points were deducted for footfalls, hanging upside-down, an unwillingness to traverse the beam or falling off the beam.
CHAPTER 6: SUMMARY AND CONCLUSIONS

With over 150 years of research into mitochondria, it is now well-accepted that these once ill-defined bioplasts are crucial for cellular homeostasis. Their role within the cell transcends mere ATP production. As more than the proclaimed cellular “Power Plants”, these organelles are also important in calcium cycling, the generation of reactive oxygen and nitrogen species and play an integral role in apoptotic and necrotic cell mediated death pathways. Because of their importance in overall cellular homeostasis, mitochondrial dysfunction has been linked to many diseased states including Diabetes, Parkinson’s Disease, Alzheimer’s Disease, Amyotrophic Lateral Sclerosis and, most important to this dissertation, Traumatic Brain Injury. Mitochondria function has also been found to be altered in models of aging, hinting to a possible mechanism as to neurodegenerative diseases which generally appear in aged population.

Mitochondria are essential for appropriate metabolism and energy flow within neurons but they have also been found to contribute to metabolic mediated oxidative stress and for the initiation of cell death. Because of this, mitochondria have been proposed as a therapeutic target in models of aging and Alzheimer’s Disease, AD. With mitochondria being a key player in metabolism, dietary interventions and supplementations with various antioxidants and mitochondrial co-factors have been proposed to target mitochondrial dysfunction in order to avoid age and AD related deficits, however the results from these studies have been controversial.
Oxidative damage seems to be a consistent feature seen in models of aging [156-159]. The brain is particularly vulnerable to oxidative damage as a result of it consuming approximately 20% of the body’s total oxygen, having a high content of polyunsaturated fatty acids and lower levels of endogenous antioxidant activity relative to other tissue [104-106]. Normal metabolic processes result in the release of reactive oxygen species (ROS), which in turn can lead to oxidative damage to proteins, lipids, DNA and RNA [156]. Since mitochondrial are also a primary production site for ROS [163], these organelles become particularly vulnerable to oxidative damage [164]. It has been hypothesized that the combination of mitochondrial dysfunction and production of ROS may be a key contributor to the deleterious effects of aging on the brain [14, 167, 173-179]. In addition to playing a role to the deficits seen with aging, mitochondria dysfunction is also thought to be hallmark feature with this secondary injury cascade in TBI, in which a similar mechanism has been proposed.

Similar to aging models, traumatic brain injury is another state that seems to be plagued with mitochondrial dysfunction. Due to the heterogeneous nature of this disease, experimental modeling has been challenging leading to the invention of numerous injury paradigms in order to encompass a full range of what is seen clinically. The rodent model of TBI that shows the most apparent mitochondrial dysfunction is the controlled cortical impact. This is likely a result of the focal nature of the injury which allows scientists to isolate tissue only undergoing the secondary injury cascade. It is this tissue, which surrounds the impact site and is defined as the penumbra and core tissue, that has been
highly investigated for therapeutic interventions in an attempt to avoid the non-mechanical tissue damage that occurs following the primary injury.

The current dogma within the TBI literature is that following injury, excitatory amino acids are released into the extracellular space in a dysregulated manner. This leads to excitation of nearby neurons causing pathogenic influxes of calcium. Mitochondria buffer this calcium leading to intramitochondrial changes in the redox potential and consequently the initiation of oxidative stress. This oxidative stress is thought to lead to lipid peroxidation and protein damage, similar to what has been proposed in aging models. This oxidative stress was traditionally thought to be irreversible however work within this dissertation project proposes a mechanism of mitochondrial dysfunction that is enzymatically regulated and not due to vast oxidative damage, as originally hypothesized [327].

After over 15 years, mitochondrial studies in the field of TBI have concluded a well-accepted time course of mitochondrial dysfunction. Following a focal injury significant dysfunction is seen at 1 hour post-injury and persists out to 48 hours, peaking around 12 to 24 hours [311, 348, 350, 363]. Still till today, the mechanism behind this dysfunction is debated. As mentioned previously, the current dogma proposes a mechanism which leads to irreversible oxidative damage. However, as seen in Figure 4.4, pioglitazone, a drug which has been shown to improve mitochondrial dysfunction, is able to improve mitochondrial function when given at 12 hours post-injury, a time-point linked to maximal mitochondrial dysfunction. In addition to pioglitazone, the use of an alternative
biofuel called beta-hydroxybuterate, which is able to feed directly into Complex I, effectively skipping any metabolic processes upstream up of Complex I, is also able to increase mitochondrial function when given ex vivo.
Figure 6.1 BHB intervention following injury can improve mitochondrial respiration: In this experiment, BHB was given at 24 hours ex vivo in mitochondria. BHB was able to increase mitochondrial respiration showing that mitochondria are not completely damaged, rather inhibited at a point upstream of Complex I.
In healthy, uninjured, cortical tissue, Pyruvate Dehydrogenase, PDH, is traditionally thought to be the gatekeeper of mitochondrial respiration [448]. Therefore, it was only reasonable to hypothesize that the limiting step that occurs upstream of Complex I leading to inhibited respiration following injury would be PDH. However, as seen by Opii et. al., PDH activity does not significantly decrease following injury [343].

If not PDH, then what? Well, insight into this question may have surfaced in 2004 when Colca et al discovered a novel mitochondrial protein which is now hypothesized to be a Redox sensitive switch which is able to modulate mitochondrial respiration [434, 449-452]. Work from Roberts et. al. has found that mitoNEET is able to increase Glutamate Dehydrogenase activity [83].

With compelling evidence showing mitoNEET as a redox switch and showing mitoNEET’s direct positive effects on GDH activity, it proposes a theory in that GDH mediates mitochondrial respiration through mitoNEET activity. This activity is then inhibited during high oxidative states, such as during calcium cycling and following injury to the cortical tissue, leading to decreased substrates feeding into the electron transport chain and therefore inhibiting state III mediated respiration as seen with this research. When using a mitoNEET ligand, such as NL-1 or pioglitazone, mitoNEET activity is increased during this inhibitory state leading to increase mitochondrial respiration resulting in increased cortical sparing and functional recovery.

From an evolutionary perspective, GDH is a highly modulated enzyme that has been previously linked to mitochondrial function through the production
of NADH and α-ketoglutarate from glutamate and NAD+. It is thought that this protein is an evolutionary byproduct from protozoa, which used this enzyme as a sensor in its antenna in order to sense its environment and avoid encounters to dangerous environments [453-456]. This information leads to a model as seen below:
Figure 6.2 Thesis Overview: This diagram proposes the mechanism which is hypothesized for mitoNEET. mitoNEET targeting pharmacological treatments such as pioglitazone or NL-1 are able to activate mitoNEET, which leads to Glutamate Dehydrogenase (GDH) activity. Under normal, homeostatic conditions, mitoNEET activates GDH. Following a redox change within mitochondria, mitoNEET loses its activity leading to an inhibition on GDH and therefore decreased mitochondrial respiration.
Future work within this project is ample. At this point, an in depth analysis into GDH activity following injury needs to be completed, since the current research related to GDH activity following TBI is limited and possibly nonexistent. One will need to the hypothesis that pioglitazone’s ability to improve functional recovery and increase tissue sparing following moderate CCI is dependent upon GDH activity, which is modulated through mitoNEET. To further expand this study, one will need to test whether NL-1, the mitoNEET specific ligand, can reproduce the same effects as pioglitazone. The rationale behind this is that our data supports the hypothesis that pioglitazone can significantly increase mitochondrial bioenergetics following TBI resulting in increased tissue sparing but does not provide the same degree of neuroprotection in mitoNEET -/- mice. The literature also shows that pioglitazone binds mitoNEET, that pioglitazone confers neuroprotection in mice after TBI at 1.0 and 10mg/kg and that GDH is activated by interactions with mitoNEET. I have also demonstrated that the mitoNEET ligand (NL-1) can confer neuroprotection following TBI. These data drive the novel hypothesis that pioglitazone neuroprotection following TBI is dependent upon its interactions with mitoNEET.

The experimental designs for these studies should assess GDH activity following injury in addition to neuroprotection, through tissue sparing and behavioral outcomes, following moderate CCI in mice administered the optimal dose of a known GDH activator and/or inhibitor. Adult wild-type (+/+ ) and mitoNEET null (-/-) male mice, should be subjected to either a sham surgery or moderate TBI and either sacrificed at 24 hours post-injury for GDH activity
studies or allowed to survive for 30 days, with naïve mice included for behavioral outcome measures, depending on the cohort. Animals should all receive intraperitoneal (i.p.) injections of vehicle or the 10mg/kg pioglitazone or NL-1 or the ideal dosage of GDH activator and inhibitor at 15 minutes post-injury, in accordance with their grouping. Changes in cortical and hippocampal morphology should be assessed longitudinally (days 7, 14 and 28 post-injury) using parametric T2 weighted imaging and apparent diffusion weighted coefficients from DTI in addition to employing unbiased stereology at 30 days post-injury. The extent of cortical sparing should be measured in a blinded fashion using the variations in signal intensities at the region of interest, ROI. These ROI measurements will be hand drawn and calculated with ImageJ. Behavioral measures should include motor and cognitive function to assess the degree of functional recovery as a result of treatment, employing both the Adjusted Neurological Severity Score (aNSS) and Morris Water Maze (MWM). Recovery of motor function will be assessed using the NSS at 1 hr, 24 hr, 72 hr, and 120hrs after CCI or sham injury.

Additionally, since GDH is tightly linked to insulin production, which has not been investigated within this paradigm. However, in a study completed by Davis, it was found that insulin injections at 3 hours post-injury lead to rapid death in a rodent model of CCI injury and did not increase cortical sparing or functional recovery following injury. It is well accepted that metabolic dysfunction occurs following injury and therefore further research into important metabolic signaling molecules, like insulin, should be further investigated as well.
Lastly, and seemingly most important to this study, is that further studies need to completed in order to investigate the hypothesis that mitoNEET is a redox sensitive mitochondrial protein that is able to modulate mitochondrial function (“switching” them on and off) during times that redox potential has shifted to a potentially pathogenic state. We currently understand that following TBI, calcium dysregulation occurs leading to changes in the redox potential of mitochondria. We also know that mitoNEET is able to inhibit mitochondrial respiration. Research from this study showed the mitoNEET null mitochondrial did not experience the same extent of mitochondrial respiration depression when calcium was added ex vivo, compared to mitoNEET containing mitochondrial. Therefore, mechanistic studies using mitochondria need to be further investigated to determine if mitoNEET is indeed the “switch” that we think it is.
REFERENCES


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VITA

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Education

PhD in Anatomy and Neurobiology
University of Kentucky, Lexington, KY
Investigated mitochondrial function and the role of mitoNEET in pioglitazone mediated neuroprotection following traumatic brain injury in rodent models.

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PhD in Physical Chemistry, program not completed
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B.S. Degree in Biology, Minor in Chemistry
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Academic and Professional Honors

• Funded F31 through NINDS

• Neurotrauma Student Travel Award, 32nd Annual Symposium of the National Neurotrauma Society, San Francisco, CA, June 2014

• Voted Graduate Student Representative for the Bluegrass Society for Neuroscience
  o 2014-2015 School Year
  o 2012-2013 School Year

• Voted President of Graduate Student Congress, University of Kentucky
  o 2014-2015 School Year
  o 2013-2014 School Year

• Poster winner, Bluegrass Society for Neuroscience Research Day, Lexington, Kentucky
  o March 2015
  o April 2014
  o April 2013
• Voted Committee Member for See Tomorrow: The University of Kentucky Strategic Plan 2014-2020 Committee (Voted by Provost Office), Fall 2013 to Present

• Issued the Medical Neuroanatomy Teaching Certificate, Anatomy and Neurobiology Department, University of Kentucky, June 2013

• Finalist in the 2013 Three Minute Thesis (3MT) Competition, University of Kentucky, Lexington, KY Spring 2013, http://www.youtube.com/watch?v=z1MegzEKtTk

• Top Research Student Award in the Physical Science Department, Spring 2005

• Award to Graduate with Honors due to the completion of my Honors Thesis and other prerequisites, Spring 2004

• Outstanding Progress in the Physical Sciences, at Mount St. Mary’s College, April 2004, April 2003

• American Chemical Society Travel Grant for the 225th ACS National Meeting, New Orleans, LA, March 23-27, 2003

• St. Catherine Medal, National Achievement Award, Kappa Gamma Pi, April 26th 2003

• Outstanding Freshman in Biology Student, 2002 at Mount St. Mary’s College

Peer-Reviewed Publications and Abstracts

• **Yonutas, HM,** Vekaria, H., Pandya, JD, Sebastian, A, Sullivan; Pioglitazone neuroprotection following CNS injury is mediated via interaction with mitoNEET; Cell Metabolism; submitted

• **Yonutas, HM,** Vekaria, H., Sullivan, PG; Mitochondrial Specific Therapeutic Targets Following Brain Injury; Brain Research special issue on therapies for TBI; In Press

• **Yonutas, H.M;** Sullivan, P.G., Mechanism of Action of PPAR Agonists in CNS Injury, Current Drug Targets, 14, 7 (2013) PMID: 23627890

• **Yonutas HM,** Hall ED, Sullivan PG; The Functions, Disease-Related Dysfunctions, and Therapeutic Targeting of Neuronal Mitochondria.


• Yonutas H.M., Pandya, J.D., Sebastian, A.H., Geldenhuys, W.J., Carroll, R.T, Sullivan, P.G., A “NEET” mitochondrial target: The Importance of mitoNEET in Pioglitazone Mediated Neuroprotection following TBI; Society of Neuroscience 2014, Washington, DC, November 2014 – Poster Presentation and asked to present a 3MT of my research to Francis Brooke, Legislative Assistant for U.S. Representative Andy Barr, KY-6

• Yonutas H.M., Pandya, J.D., Sebastian, A.H., Geldenhuys, W.J., Carroll, R.T, Sullivan, P.G., Interactions between Pioglitazone and mitoNEET Ameliorate Mitochondrial Dysfunction following Traumatic Brain Injury; Neurotrauma 2014 Symposium; San Francisco, CA; July 2014 – Poster Presentation - Student Travel Grant Winner


• Yonutas H.M., Geldenhuys, W.J., Carroll, R.T, Sullivan, P.G., A mechanism of neuroprotection provided by pioglitazone following traumatic brain injury, Society of Neuroscience 2013, San Diego, CA, November 2013 – Poster Presentation


**Teaching Experiences**

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<td>University of Kentucky, College of Medicine</td>
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Lecture Experiences

- Principles of Human Anatomy – ANA 209 – 1 Hour Lecture on Peripheral and Central Nervous System, February 2015

- Neuroanatomy for PT students – ANA 802 – 2 Hour Lecture on the Peripheral Nervous System, September 2014

- Neuroanatomy for PT students – ANA 802 – 2 Hour Lecture on the Peripheral Nervous System, September 2013

- Medical Neuroanatomy – MD 816 – 2 Hour Lecture on the Sensory Receptors in the Peripheral Nervous System, March 2013

Memberships & Affiliations

- Student Member, National Neurotrauma Society

- Student Member, Society for Neuroscience

- Student Member, Bluegrass Society for Neuroscience
  2012-2013 Graduate Student Representative
  2014-2015 Graduate Student Representative

- Member, Graduate Student Congress, University of Kentucky
  2013-2014 President
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- Member, Delta Epsilon Iota Honor Society, University of Kentucky