2008

THE UNDERLYING MECHANISM(S) OF FASTING INDUCED NEUROPROTECTION AFTER MODERATE TRAUMATIC BRAIN INJURY

Laurie Michelle Helene Davis
University of Kentucky, ldavi4@uky.edu

ABSTRACT OF DISSERTATION

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ABSTRACT OF 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
Laurie Michelle Helene Davis

Lexington Ky

Director: Dr. Patrick G. Sullivan,
Associate Professor of Anatomy and Neurobiology

Lexington, Ky

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ABSTRACT OF DISSERTATION

THE UNDERLYING MECHANISM(S) OF FASTING INDUCED NEUROPROTECTION AFTER MODERATE TRAUMATIC BRAIN INJURY.

Traumatic brain injury (TBI) is becoming a national epidemic, as it accounts for 1.5 million cases each year. This disorder affects primarily the young population and elderly. Currently, there is no treatment for TBI, which means that ~2% of the U.S. population is currently living with prolonged neurological damage and dysfunction. Recently, there have been many studies showing that TBI negatively impacts mitochondrial function. It has been proposed that in order to save the cell from destruction mitochondrial function must be preserved. The ketogenic diet, originally designed to mimic fasting physiology, is effective in treating epilepsy. Therefore, we have used fasting as a post injury treatment and attempted to elucidate its underlying mechanism. 24 hours of fasting after a moderate TBI increased tissue sparing, cognitive recovery, improved mitochondrial function, and decreased mitochondrial biomarkers of injury. Fasting results in hypoglycemia, the production of ketones, and the upregulation of free fatty acids (FFA). As such, we investigated the neuroprotective effect of hypoglycemia in the absence of fasting through insulin administration. Insulin administration was not neuroprotective and increased mortality in some treatment groups. However, ketone administration resulted in increased tissue sparing. Also, reduced reactive oxygen species (ROS) production, increased the efficiency of NADH utilization, and increased respiratory function. FFAs and uncoupling proteins (UCP) have been implicated in an endogenously regulated anti-ROS mechanism. FFAs of various chain lengths and saturation were screened for their ability to activate UCP mediated mitochondrial respiration and attenuate ROS production. We also measured FFA levels in serum, brain, and CSF after a 24 hour fast. We also used UCP2 transgenic overexpressing and knockout mice in our CCI injury model, which showed UCP2 overexpression increased tissue sparing, however UCP2 deficient mice did not show a decrease in tissue sparing, compared with their wild type littermates. Together our results indicate that post injury initiated fasting is neuroprotective and that this treatment is able to preserve mitochondrial function. Our work also indicates ketones and UCPs may be working together to preserve mitochondrial and cellular function in a concerted mechanism, and that this cooperative system is the underlying mechanism of fasting induced neuroprotection.

KEYWORDS: Traumatic Brain Injury, Mitochondria,
Ketone, Uncoupling Protein, HIF-1

Laurie M. Davis

12/18/08

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By

Laurie Michelle Helene Davis

Dr. Patrick Sullivan
Director of Dissertation

Dr. Jane Joseph
Director of Graduate Studies

12/18/08

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ACKNOWLEDGEMENTS

It is impossible to adequately thank all of the people who have helped me both personally and professionally throughout my graduate career. This work would not have been possible without my dissertation chair, Dr Patrick G. Sullivan, Ph.D., who has given me the opportunity to ask fascinating questions and allowed me to grow as an independent scientist. I would also like to thank the members of my dissertation committee, Dr. Edward D. Hall Ph.D.; Dr. James W. Geddes Ph.D.; Dr. Sheldon M Steiner Ph.D.; Dr. Stephen W Scheff Ph.D. and my outside examiner Dr. Francisco H Andrade for the dedication of their time and attention to my doctoral work. I would like to thank Dr. Maile R. Brown Ph.D. and Dr. Vidya Nag Nukala Ph.D. for their unwavering patience as my first laboratory mentors on my journey through mitochondrial research. I also could not have accomplished this work without the help of the members of the Sullivan lab, without whom science would not have been as enjoyable. Special thanks to Dr. Jeff Boskin Ph.D., who was instrumental in development and troubleshooting the GC/MS work. Also, as SCoBIRC is an open lab environment, I would like to thank the department as a whole for their continual support. This doctoral work was supported by a National Institutes of Health, U.S. Public Health Service grants (NS48191) and National Institutes of Health Training Grant (1T32DA022728) which endeavors to move the field of neuroscience from the bench to the bedside.

Finally, I would like to express my appreciation for the love and dedication of my mother, Debbie Davis. She has been my ardent supporter through thick and thin and a consistent source of strength and friendship throughout my life; and it is to her that I attribute my development into the person I am today. I am also deeply indebted to my

extensive network of friends, who have celebrated with me during good times and supported me in times of tribulation. This process has proven to be a very collaborative and scientifically rewarding experience, and I am truly grateful for all of the people I have had the privilege to work with throughout the completion of this graduate work.

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Traumatic Brain Injury, Mitochondria, and Fasting

Traumatic Brain Injury

With over 1.5 million injuries every year, Traumatic brain injury (TBI) has become a widespread phenomenon in our country. As this disorder can present without any outward sign of physical damage and because the effects often render the patient unable to communicate effectively, it has become a largely 'silent epidemic' (Jennett 1998; Thurman et al. 1999; Jager et al. 2000; CDC 2003; Langlois et al. 2006). Although advancements in medicine over the past century have increased the survival rates of TBI patients, there is unfortunately a growing population of individuals who sustain a mild to moderate TBI who do not seek immediate medical treatment (~25%), and often develop prolonged and chronic neurological symptoms (CDC 2003). The growing population of injured patients has presented our society with an enormous economic and social burden, as these patients are frequently unable to properly reintegrate in to their previous professional and social networks. They become exceedingly dependent on family and social outreach programs to maintain daily quality of life; which can cause their health care to cost the nation tens of billions dollars per year (Thurman et al. 1999; Langlois et al. 2006). While there are treatment options designed to allow the person to survive their injuries, consisting of minimizing acute brain edema, decreasing intracranial pressure, and the prevention of peripheral complications, there is no current treatment to attenuate or recover the loss of neural tissue (Hatton 2001).

Perhaps the most insidious aspect of TBI is that it can occur without obvious signs of injury to the patient's body. There have been recorded medical incidences of

mysterious neurological disorders dating back to World War I. Physicians in the British armed forces had then given it the somewhat enigmatic label of "shell shock" (SS) (Jones et al. 2007). Although some cases could be attributed to psychosis, by 1917 SS was responsible for 14% of all discharges from the armed forces, and accounted for 33% of all discharges of non-wounded soldiers (Jones et al. 2007). It had become so prevalent throughout the armed forces and had such a wide array of presenting symptoms that it was highly debated whether or not it was a real condition, and the etiology and management was highly disputed for much of the early 20th century. By the end of WWI the prevalence of SS began to incur a large financial burden upon the British armed forces, primarily due to the 32,000 pensions that had been awarded to "neurasthenic" soldiers suffering from SS with no obvious cerebral injury (Jones et al. 2007). The controversial definition of the disorder and its method of treatment, in addition to the development of public controversy and stigma over diagnosis, delayed the development of a treatment protocol and even caused the British army to ban the use of the term "shell shock" from medical reports (Jones et al. 2007).

During WWII the British army banned the SS terminology in hopes of avoiding another epidemic of these cases, which they may or may not have viewed as physical disorders. However, with the start of the war it became readily apparent that disavowing the existence of this disorder did not prevent another epidemic. In response to the army regulations regarding this disorder, alternative terminology arose in its place, such as postconcussional syndrome (PS) or posttrauma concussion state (coined by (Schaller 1939). Eventually, physicians began to realize that many of the soldiers that suffered from this concussed state had been in close proximity to an explosion during battle. This

led them to speculate that some force, that had no perceptible outward affect on the body, had a substantial effect on fragile neural tissue. In an attempt to, once again, clarify the etiology of this disorder, Denny-Brown suggested that it was the timeline of symptom presentation within the individual patient instead of the symptom type that was the key factor between severe head injury and PS. His etiological account indicated that severe head injury would present with immediate neurologic symptoms that would trend toward recovery; whereas PS would have delayed onset of neurologic symptoms with a trend toward worsening symptoms (Jones et al. 2007). It has been estimated that 50% of patients with a mild TBI can develop post concussive syndrome consisting of dizziness, headaches, cognitive dysfunction, sleep disorders, and depression (Alexander 1995; Bazarian et al. 1999; CDC 2003; Langlois et al. 2006; Rapoport et al. 2006). This delayed development of symptoms in the mild to moderate patient populations is perhaps the most unfortunate aspect of this condition, as soldiers and civilians can often suffer immense psychiatric morbidity without realizing that they require medical treatment for a physical injury (Setnik et al. 2007). A recent online polling study indicated that 42% of their respondents who suffered a TBI failed to seek medical care, which is considerably higher than the CDC estimate of 25%. It has been observed clinically that even mild or moderate TBI can require neurosurgical intervention, and any delay in treatment could prove to be costly in terms of cognitive and functional recovery (Setnik et al. 2007).

Of the more than 1.5 million military personnel deployed since 2001 to the Middle East, approximately 25% of the injured service members have reported brain injury (Hoge et al. 2008). Unpublished data from the department of defense indicates that blast injuries are the leading cause of TBI in war zones; and has been labeled as a

signature injury of the current Middle Eastern conflicts (Hoge et al. 2008). Recently the ravages of TBI has been documented through the unfortunate injury of network news anchor Bob Woodruff, which showed the grim reality of the recovery process of this disorder and its effects on patients lives in terms of cognitive dysfunction and its impact on family dynamics (Woodruff et al. 2007). Although the TBI sustained by Woodruff was severe, mild and moderate injury have also become a long term problem coupled with prolonged cognitive dysfunction within the armed forces population, with an approximately 18% prevalence in various reports (Hoge et al. 2008). In addition to prolonged cognitive deficits, this injury population also has an increased predisposition to the development of post traumatic stress disorder (PTSD). There is also a problem of failure to report due to a perceived stigma concerning psychological problems within the armed forces population; which could contribute to the development of chronic neurologic dysfunctions within this population of injured patients (Hoge et al. 2004).

Within the civilian population of the United States ~2% of the population (5.3 million) is currently living with long-term disabilities resulting from TBI (Langlois et al. 2006). The leading causes of TBI (Figure 1.1) are falls and motor vehicle accidents, followed closely by assault and incidents in which the head is "struck by/against" an object (Langlois et al. 2006). There has also been an increasing population of pediatric (5-18 years) TBI cases resulting from sports related injuries, which can often be misdiagnosed as the symptoms manifest as lethargy, irritability or fatigue (CDC 2003; Yang et al. 2008). TBI has a biphasic age-related incidence; occurring in young (< 25)

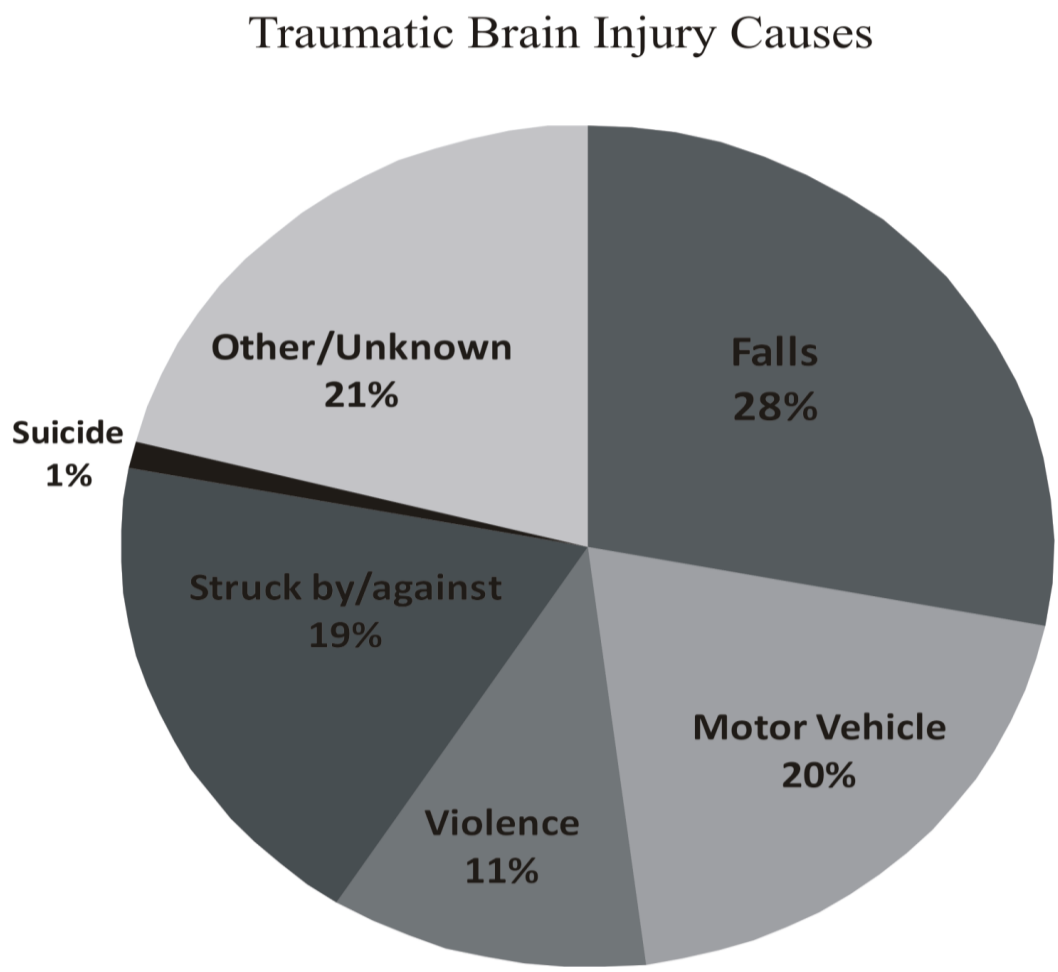


Figure 1.1: <u>Traumatic brain injury causes.</u>
The leading cause of traumatic brain injury is falls at 28%. This is closely followed by motor vehicle accidents (20%), struck by/against events (19%), and violence (11%). Suicide and other/unknown injury mechanisms account for the remaining 22% of traumatic brain injuries. This figure was compiled based on data from (CDC 2003; Langlois et al. 2006)

and elderly (>75) populations (Langlois et al. 2006; Rutland-Brown et al. 2006). Over 50,000 deaths are attributed to TBI each year, as well as 235,000 hospitalizations and 1,111,000 emergency department visits (Figure 1.2). With such a high incidence and great propensity for the development of chronic symptoms, the total medical costs incurred by individuals currently living with TBI within the U.S. can reach

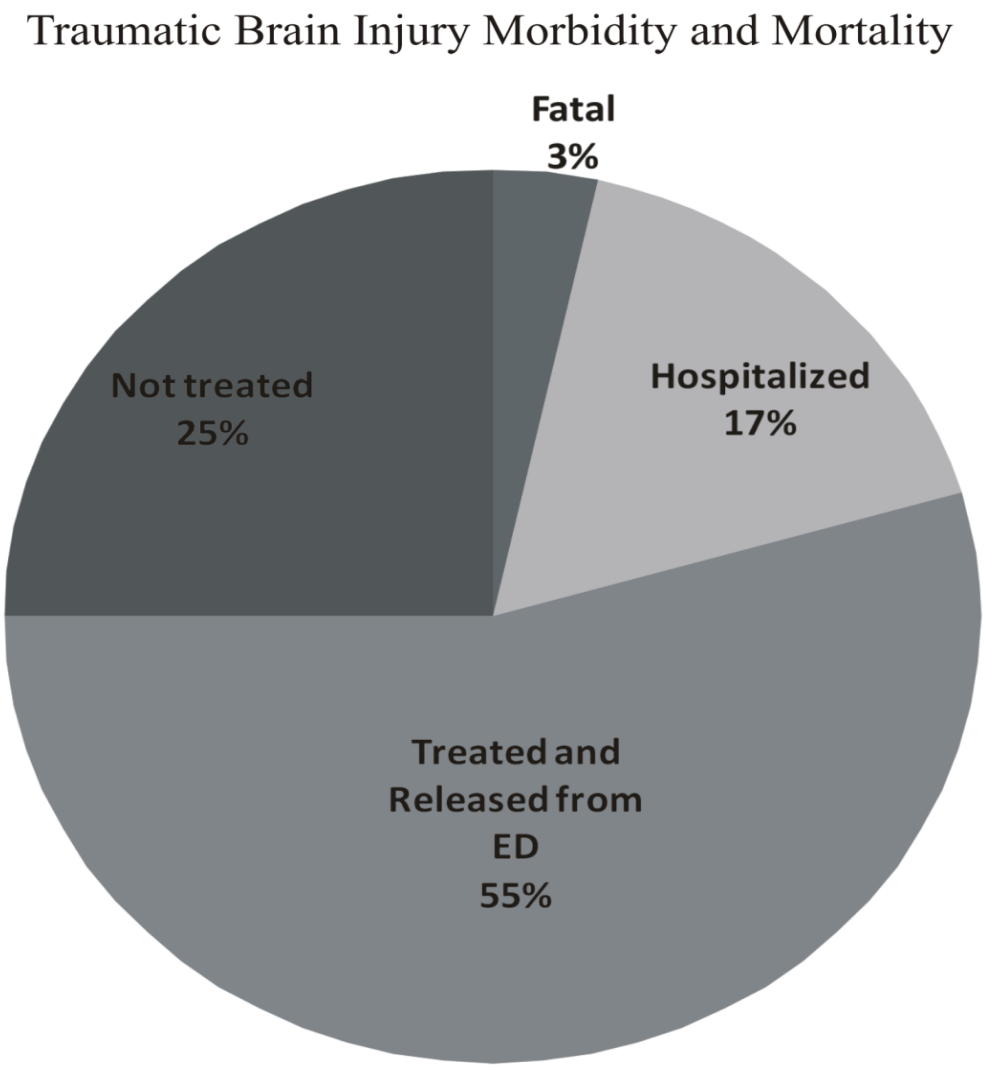


Figure 1.2: <u>Traumatic brain injury morbidity and mortality.</u>
Of the 1.5 million annual cases of traumatic brain injury in the United States, 3% result in fatalities, whereas 55% are treated and released from emergency departments (ED). Hospitalization accounts for 17% of the cases seen annually, and 25% of total cases do not seek medical treatment. This figure was compiled from data contained in (CDC 2003; Langlois et al. 2006)

$50 billion dollars per year. This figure increases to $60 billion when lost productivity of these individuals is factored in; however, these figures do not account for how this disorder impacts social and family dynamics (Langlois et al. 2006; Rutland-Brown et al. 2006). As such, there is a clear need for the development of neuroprotective therapies and effective protocols for the treatment of TBI.

Mitochondrial Structure, Function, and Posttraumatic Dysfunction

The development of mitochondrial function was the basis for the progression toward multi-cellular organisms. It was at this evolutionary crossroads that the cell was able to produce enough energy, in the form of ATP, to form highly complex interconnected networks that developed into the organ systems we see in the human body as well as all other organisms (Lane 2006). Underscoring the dependence on mitochondrial ATP production is the evolutionary development of all multi-cellular organisms upon this planet to require oxygen utilization through some sort of respiration. It is essential that mitochondria are provided with adequate oxygen in order for the cell to maintain homeostatic regulation of its intracellular processes (Lane 2006). The importance of oxygen consumption is highly evident when we examine any pathological disease in which tissues become oxygen deprived (ischemic) for even the shortest time period. These regions undergo massive cellular loss as a result of mitochondrial damage and dysfunction, leading to the initiation of cell death pathways, such as necrosis and apoptosis (Obrenovitch 2008).

Although we have only been studying mitochondria since the turn of the 20th century, these organelles have proven to be one of the most important discoveries in the history of cellular research. Ever since Kolliker (1850), Altman (1890), and Benda (1898) described their presence in cells we have been fascinated with their function. The first Nobel Prize for mitochondrial research was awarded to Meyerhof in 1922 for the discovery of the connection between substrate oxidation and oxygen consumption in relation to glycolysis. Next to be awarded in 1931 was the work done by Warburg on the nature and mode of action of the "respiratory enzyme", indicating that ATP production

was coupled with enzymatic oxidation of glyceraldehyde phosphate. Szent-Gyorgyi was awarded the Nobel Prize in 1937 for the discovery of the connection with biological combustion process of dicarboxylic acids within mitochondrial respiration. The most recent Nobel was awarded in 1997 to Boyer and Walker for their discovery of the enzymatic mechanism underlying the synthesis of ATP. Although we have learned much over the past century about mitochondrial bioenergetics, there remains a great deal to be discovered.

Indeed, mitochondrial function plays an integral role in all cellular function, therefore in order to discuss mitochondrial dysfunction we must first discuss normal mitochondrial structure and function (Figure 1.3). Mitochondria are intracellular organelles with a dual (inner and outer) membrane system, each of which is responsible for specific functions. The outer membrane (OM) contains many transporter proteins and the inner membrane (IM) exhibits many folds, termed cristae, which increase the surface area available for mitochondrial respiration (Nicholls et al. 2002). The space enclosed by the IM is called the matrix and contains enzymes involved in cellular metabolism and calcium regulation. Within the IM lipid bilayer there are a series of five protein complexes that comprise the electron transport chain (ETC), which functions as the primary source of ATP production within the cell. Complex I (NADH-Ubiquinone Oxidoreductase), which is embedded within the IM, converts NADH to NAD^+ by accepting an electron into the Fe-S center of the protein (Nicholls et al. 2000). As a byproduct of this electron donation, a proton is translocated from the matrix to the intermembrane space (IMS), which is located between the inner and outer membranes.

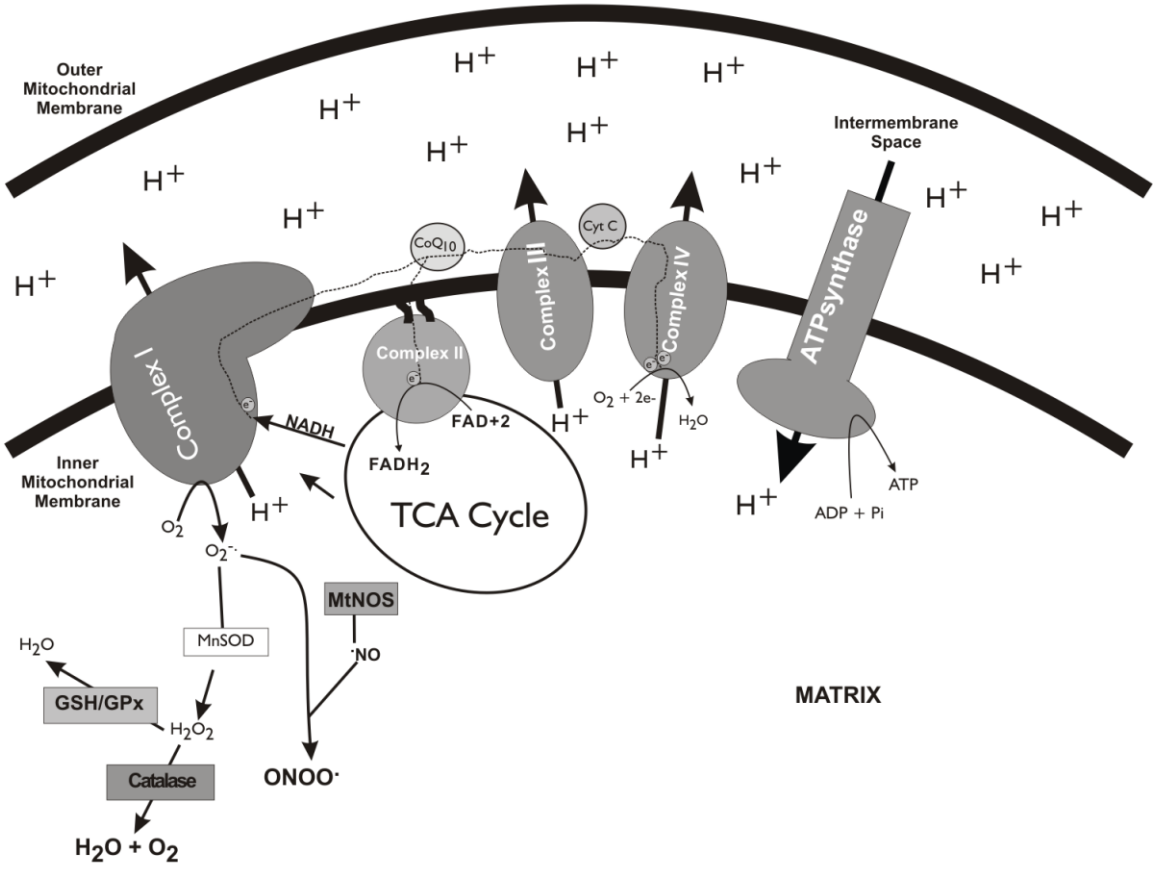


Figure 1.3: <u>Overview of the electron transport chain.</u>
The electron transport chain (ETC) is made up of 5 inner membrane proteins within the mitochondria. The first protein in this chain is complex I, which accepts electrons from N**A**DH, facilitating proton (H^+) translocation into the intermembrane space from the mitochondrial matrix. Complex II, which is only attached to the inner side of the inner membrane, accepts electrons from FADH2, however does not translocate protons (H^+). Complex II is also a component of the Krebs cycle, which is responsible for the production of ETC substrate production. The electrons from complex I & II are transported to complex III via ubiquinone (Co enzyme Q10), which also causes a translocation of protons (H^+) into the intermembrane space. The Q-cycle of complex III transfers the electrons to Cytochrome c, which then transfers them to complex IV, resulting in another proton (H^+) translocation. Complex IV is responsible for the production of water by using oxygen as the final electron acceptor. Complex V (ATPsynthase) uses the proton (H^+) gradient ($\Delta\Psi$) created by complex I-IV to catalyze the phosphorylation of ADP to create energy in the form of ATP. Superoxide ($O_2^{-\cdot}$) is produced by the escape of electrons from complex I or III. Normally it is converted by Manganese Superoxide Dismutase (MnSOD) to hydrogen peroxide, and then to H_2O by catalase or glutathione systems. However, in the presence of excess superoxide production, $O_2^{-\cdot}$ can be converted into peroxynitrite ($ONOO^-$) by nitric oxide ($^{\cdot}NO$).

Complex II (Succinate Dehydrogenase), in addition to its function as an ETC protein, is also a key component of the Krebs Cycle; which through the conversion of the glycolytic product pyruvate produces substrates for the ETC. This complex utilizes the conversion of succinate to accept electrons from $FADH_2$ into the ETC. As it is anchored to the inner half of the IM, there is no translocation of protons from the matrix to the IMS. Complex I and II transfer their electrons to ubiquinone (CoEnzyme Q_{10}) located within the IM (Nicholls et al. 2000). These electrons are then passed to Complex III (Ubiquinone-Cytochrome-C Oxidoreductase) via the Q-cycle, resulting in proton is translocation into the IMS. Another electron transfer protein, Cytochrome c, accepts this electron and transports it to Complex IV (Cytochrome-C Oxidase); again translocating a proton into the IMS via complex IV. It is at Complex IV that oxygen plays its vital role as the final electron acceptor for the ETC, where it is reduced to form H_20. All of the protons that have been pumped into the IMS create a proton concentration gradient ($\Delta\Psi$) which is utilized by Complex V (ATPsynthase) to facilitate phosphorylation of ADP into ATP for use as an energy source for cellular processes (Nicholls et al. 2000).

Mitochondrial Dysfunction after TBI

It has become increasingly evident that mitochondrial dysfunction is intimately involved in the pathology of TBI, as well as the development of other neurological disorders (Hovda et al. 1992; Verweij et al. 1997; Xiong et al. 1997; Sullivan et al. 1998; Nicholls et al. 2000; Verweij et al. 2000; Hatton 2001; Pellock et al. 2001; Schurr 2002; Sullivan et al. 2002; Tieu et al. 2003; Lifshitz et al. 2004; Sullivan 2005; Sullivan et al. 2005). Unfortunately, there is very little we can do to prevent the initial blunt force

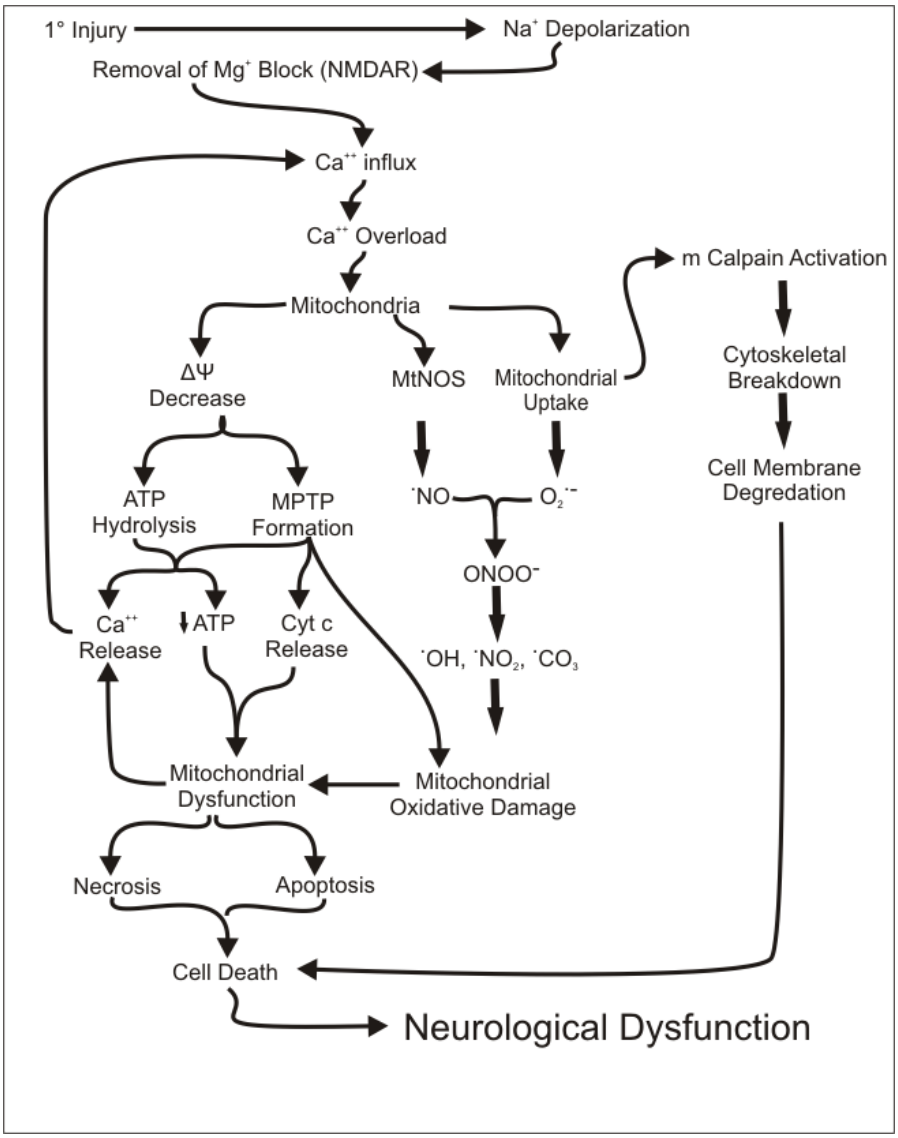


Figure 1.4: <u>Overview of the signaling cascade that follows traumatic brain injury.</u>
Traumatic brain injury has been characterized as a biphasic injury mechanism. Primary injury is defined as the initial blunt force trauma to the brain tissue. Secondary injury induces a cascade that begins with the depolarization of the cell membrane by Na+ channel opening, and the subsequent removal of the Mg+ block from NMDA channels. The activation of these channels results in a large influx of Calcium (Ca++), which is sequestered into the mitochondria by mitochondrial (membrane dependent) calcium channels. However, the Ca++ storage capacity is finite, and when it reaches its set point the mitochondria can be damaged. Mitochondrial damage can result in the production of reactive oxygen and reactive nitrogen species, which can damage mitochondrial and cellular components. Large amounts of Ca++ can also activate the formation of the mitochondrial permeability transition pore (MPTP), which causes the massive efflux of intramitochondrial proteins and the dissipation of membrane potential (ΔΨ). This cascade culminates in mitochondrial dysfunction and the initiation of cell death pathways (apoptosis or necrosis), and ultimately in cell death and neurological dysfunction.

trauma that is caused by TBI; however we may be able to intervene within the massive secondary signalling cascade that can last for hours to weeks following the primary insult (Figure 1.4). Secondary injury is initiated by a massive depolarization of the plasma membrane by voltage dependent Na^+ channels. Along with glutamate release, this depolarization causes the removal of the Mg+ block within N-methyl-D-aspartate (NMDA) channels, causing a massive calcium influx into the cell (Nicholls et al. 1999; Nicholls et al. 2000; Gunter et al. 2004). This calcium can activate many damaging cellular enzymes within the cytosol, and as such must be sequestered by the endoplasmic reticulum and mitochondria (Nicholls et al. 2000; Gunter et al. 2004).

Calcium is transported into mitochondria through a membrane potential-driven uniporter and is believed to be stored as a calcium phosphate compound within the matrix (Nicholls et al. 2000). However, the calcium buffering capacity of mitochondria is finite and eventually the calcium influx becomes too great, resulting in mitochondrial dysfunction and subsequent initiation of cell death pathways (Brookes et al. 2004; Lifshitz et al. 2004; Sullivan et al. 2004; Sullivan et al. 2005). Calcium seems to primarily effect complex I driven respiration, and damage to this major site of electron acceptance can significantly hinder the ability of the mitochondria to produce ATP (Tieu et al. 2003; Gunter et al. 2004; Sleven et al. 2006; Maalouf et al. 2007). Importantly, the loss of adequate membrane potential will cause the ATPsynthase to run in reverse, thereby dephosphorylating ATP and pumping protons into the IMS in an attempt to restore membrane potential and preserve mitochondrial homeostasis (Nicholls et al. 2000). However, by depleting ATP stores, energy dependent membrane channels required to maintain ionic balances will be unable to sustain their function. This causes

the mitochondria to swell and eventually burst, which are characteristic signs of necrotic cell death (Nicholls et al. 2000; Sullivan et al. 2005). Calcium overload can also activate intra-mitochondrial proteins, such as μcalpain, that may contribute to the formation of the mitochondrial permeability transition pore (mPTP) and release of IM proteins (Scheff et al. 1999; Nicholls et al. 2000; Garcia et al. 2005; Sullivan et al. 2005).

The formation of the mPTP results in mitochondrial dysfunction and has been shown to occur after acute TBI (Nicholls et al. 2000; Sullivan et al. 2000; Sullivan et al. 2005). This structure spans both inner and outer membrane, and causes a massive efflux of calcium into the cytosol and the release of apoptotic inducing proteins, ultimately leading to cellular loss and cognitive dysfunction (Springer et al. 1997; Nicholls et al. 2000; Sullivan et al. 2000; Nasr et al. 2003; Sullivan et al. 2005; Yu et al. 2007). The mPTP is a pore comprised of multiple mitochondrial proteins within the inner and outer membranes, including the adenine nucleotide translocase (ANT), inner and outer protein transporters (Tim/Tom), voltage-dependent anion channel (VDAC), and Cyclophilin D (Gunter et al. 2004). It allows nonspecific conductance of matrix and intermembrane space components to the cytosol, where they can activate detrimental signaling cascades leading to cell death. One such protein highly involved in both normal mitochondrial respiration and cell death cascades is cytochrome C. It is normally found in the IMS electrostatically associated with the P-side of the inner membrane where it shuttles electrons from complex III to complex IV. However, in the presence of increased calcium levels it is released from the inner membrane space (Sullivan et al. 2002). After mPTP opening, it is released into the cytosol where it binds to apoptosis activation factor-1 (Apaf-1), which is also bound to pro-caspase 9. This complex, known as the apoptosome,

initiates the activation of caspase 3 and subsequent cleavage of apoptotic substrates ultimately resulting in cellular loss. The opening of the mPTP also releases apoptosis inducing factor (AIF) and endonuclase G (Endo G), both of which are responsible for nuclear DNA degradation (Sullivan et al. 1999; Nicholls et al. 2000; Sullivan et al. 2000; Brookes et al. 2004; Sullivan et al. 2004; Sullivan et al. 2005).

A common byproduct of normal mitochondrial function is the production of reactive oxygen species (ROS), primarily produced by complexes I and III (Nicholls et al. 1999; Nicholls et al. 2000; Sullivan et al. 2003; Pandya et al. 2007). Oxygen is the final electron acceptor of the ECT; however if an electron is accepted by oxygen outside of the controlled reaction within complex IV, it can result in the production of potentially damaging radicals that target mitochondrial and cellular structures (Nicholls et al. 2000; Nicholls et al. 2002; Brookes et al. 2004; Brookes 2005). The Q-cycle of ubiquinone facilitates the transfer of electrons between complexes, and as such is a highly sensitive link within the ETC. Under the conditions of high membrane potential electrons become backed up within the chain and the probability of their escape from the Q-cycle increases (Nicholls 2002). Rogue electrons first combine with oxygen to form the superoxide radical ($O_2^{\cdot -}$). As the production of these molecules is a common occurrence, mitochondria are equipped with endogenous anti-ROS enzymes designed to neutralize these harmful molecules before they wreak havoc on mitochondrial and cellular systems.

Normally, within the mitochondria, superoxide is converted into hydrogen peroxide via Manganese Superoxide Dismutase (MnSOD) (Figure 1.3), and subsequently into water via catalase or glutathione driven antioxidant systems (Patel et al. 2003; Lambert et al. 2004; Liang et al. 2004; Xiong et al. 2005). However, when the amount of

superoxide being produced is greater than what endogenous anti-oxidant systems can manage, they can react with iron or nitrogen to form highly reactive molecules that can perpetuate damage to mitochondrial and cellular structures (Lambert et al. 2004). Homozygous MnSOD -/- knockout mice are embryonic lethal and heterozygous MnSOD +/- knockouts have increased susceptibility to neurological insult, which indicates the vital function of this gene in physiological function, developmental processes and neurologic pathology (Patel 2002). Also, overexpression of MnSOD produces lower amounts of inactive aconitase and 8-hydroxy-2-deoxyguanosine (8-OHdG), measures of oxidative protein and DNA (most likely mtDNA) damage, indicating a role in the preservation of mitochondrial function (Patel 2002; Gonzalez et al. 2005; Sleven et al. 2006). The affinity of MnSOD for superoxide is outmatched by its affinity for nitric oxide ($^{\cdot}NO$), which combine to form peroxynitrite anion ($ONOO^{\cdot}$) and then peroxinitrous (ONOOH); the latter molecule subsequently breaks down into nitrogen dioxide ($^{\cdot}NO_2$) and hydroxyl ($^{\cdot}OH$) radicals (Halliwell et al. 2007). Peroxynitrite anion can also combine with CO_2 to form nitrosoperoxocarbanate ($ONOOCO_2$), which then decomposes into carboxyl radicals ($CO_3^{\cdot-}$) and $^{\cdot}NO_2$. Additionally, superoxide that does manage to be converted to hydrogen peroxide by MnSOD, can then be converted by iron ($Fe_2^{+)}$, present due to the hemorrhage of blood vessels associated with acute injury, to ferric iron (Fe_3^{+}) and $^{\cdot}OH$ (Fenton reaction, Figure 1.4) (Halliwell et al. 2007). It is the breakdown products of these reactive oxygen species that have recently been shown to be a major source of trauma induced oxidative damage to mitochondrial lipids, proteins and DNA (Sullivan et al. 1999; Sullivan et al. 1999; Hall et al. 2004).

Recently, it has become more and more apparent that the key to maintaining cellular and mitochondrial function is to decrease the levels of oxidative stress induced damage after this excitotoxic calcium influx (Hatton 2001; Sullivan et al. 2004; Singh et al. 2006). However, because the production of oxidative stress molecules is a normal byproduct of mitochondrial function and mitochondrial function is required for proper cellular function, there must be a balance between preserving mitochondrial function and reducing oxidative damage. In order to properly fuel mitochondrial energy production it may be necessary to bypass damaged components of the ETC. Therefore, the metabolic pathways by which you supply the mitochondria become a critical component of the treatment of TBI.

Fasting & the Ketogenic Diet

Used for centuries as an unproven method for controlling seizure disorders, fasting causes the body to use stored fats as the primary energy source through the production of ketone bodies (Thiele 2003; Ziegler et al. 2003). The ketogenic diet (KD) was developed as a way to get a similar shift of metabolic utilization and increase ketone bodies without depriving patients of essential nutrients and energy (Stafstrom 1999). While the underlying mechanism(s) of action remain unclear, the ketogenic diet has been successfully used in the treatment of medically intractable epilepsy for over eight decades (Bough et al. 2003; Greene et al. 2003; Tieu et al. 2003; Sullivan et al. 2004; Yamada et al. 2005; Bough et al. 2006; Bough et al. 2007; Kim et al. 2007; Maalouf et al. 2007). The regime requires a change in the ratio of fat:carbohydrate consumption from roughly 1:2 to 4:1 (Thiele 2003; Rho et al. 2004). Many versions of the ketogenic diet have been

examined for efficacy in attenuating seizures, and the method that seems to be the most effective is a reduced calorie regime coupled with the increased fat:carbohydrate ratio (Rho et al. 2004). The administration of this diet has been shown to increase levels of antioxidant enzymes, such as glutathione peroxidase (GPx), and proteins with the ability to uncouple oxidative phosphorylation from proton transport (UCPs) in order to transiently decrease membrane potential and ROS production (Sullivan et al. 2003; Ziegler et al. 2003; Sullivan et al. 2004; Sullivan 2005). It has also been shown to increase mitochondrial biogenesis in various cell types via fatty acid upregulation (Totland et al. 2000; Bough et al. 2006).The reduction of ROS and oxidative damage coupled with preferential utilization of efficient ketone bodies in the brain causes this treatment to be a very effective therapy for epileptic seizures (Rho et al. 2004). Although the KD has been highly investigated in terms of epilepsy, the precise mechanism has yet to be elucidated. However, because the KD and fasting share similar metabolic pathways it is logical to speculate that they may share similar neurological benefits, and the elucidation of this fasting mechanism may lead to the development of efficacious therapies for the treatment of TBI.

Ketones

The primary modulatory target for fasting and the KD is ketosis, or the upregulation of ketone bodies. Ketones (Figure 1.5), made in the matrix of liver

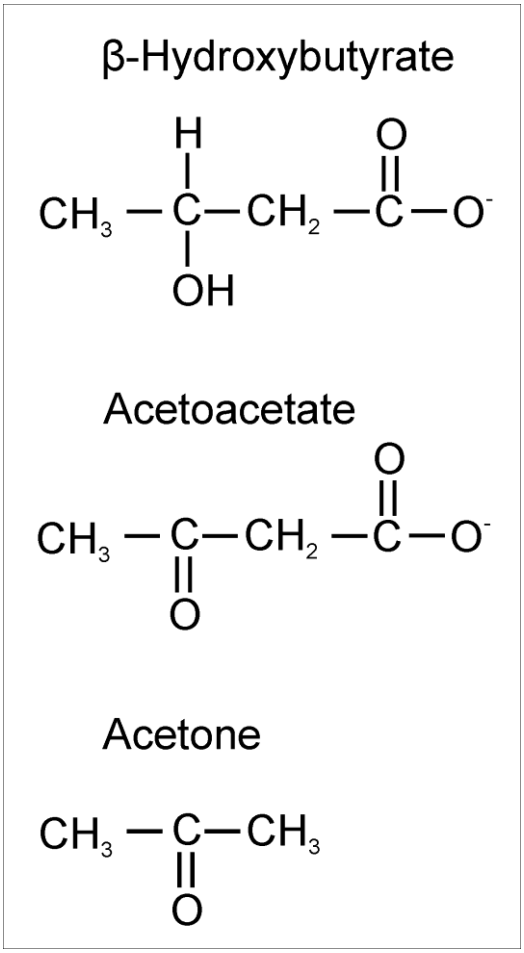


Figure 1.5: <u>Structure of β-Hydroxybutyrate, Acetoacetate, and Acetone.</u>
Ketones are produced by the liver in response to low glycolitic stores. The three major ketones produced are Acetoacetic acid, β-Hydroxybutyrate, and Acetone. β-Hydroxybutyrate is exported into the blood to the rest of the body. Acetone is mainly excreted and does not participate in energy production.

mitochondria, normally function to supplement glycolytic substrates for use in cellular function. Hepatic mitochondria do not have β-oxoacid-CoA transferase; therefore the liver cannot use ketones for energy production (McKee et al. 2003). This allows ketones to be exported to the rest of the body for use as fuel. Triacylglycerol (Tg) is a three-carbon alcohol with three fatty acids attached as esters. When intracellular lipases are activated in response to low glycolitic substrates, Tgs are converted into glycerol and 3

free fatty acids (FFA) (McKee et al. 2003). The liver then can use these components to produce ketones from the enzymatic combination of Acetyl-CoA (Figure 1.6). The primary product of this process is β-hydroxybutyrate (βHB), which is exported to the rest

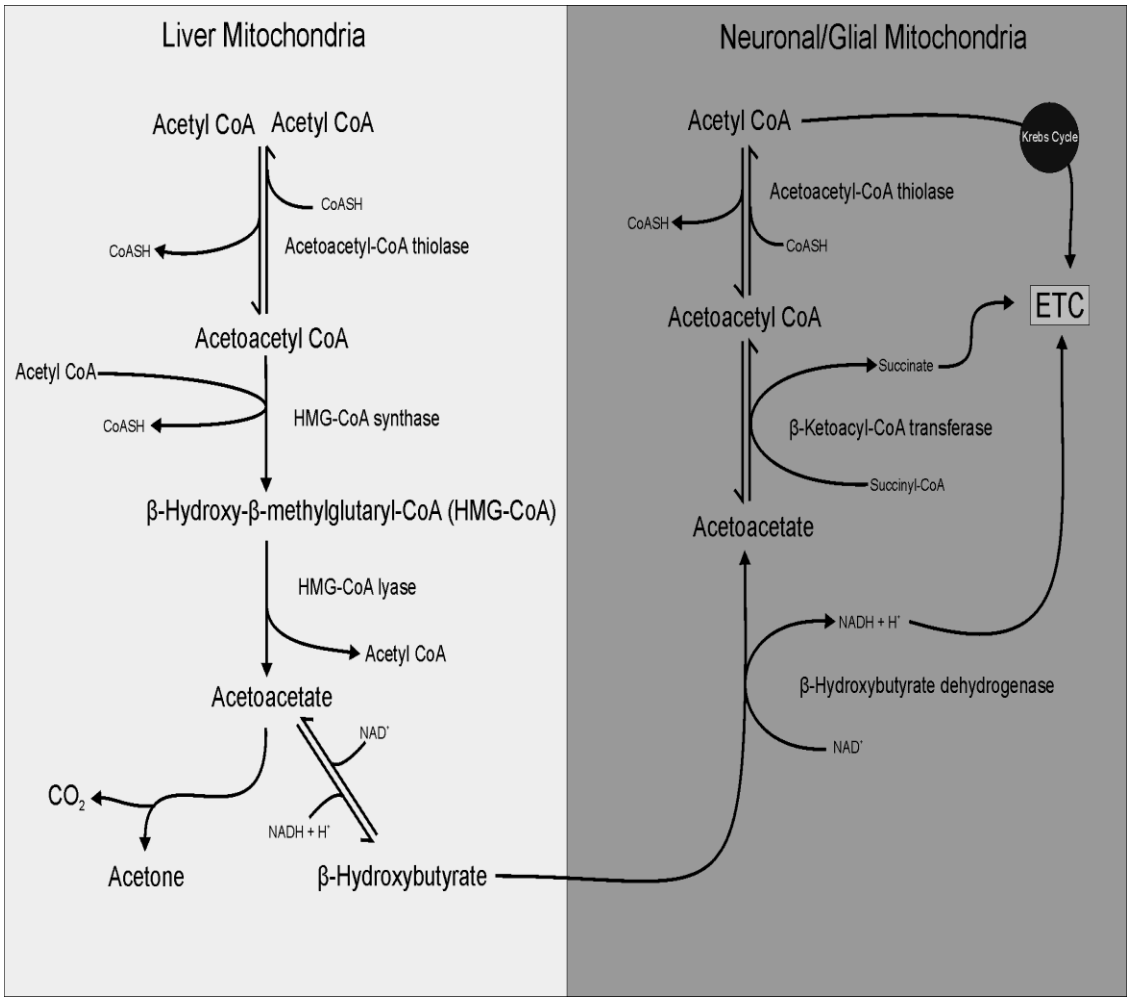


Figure 1.6: <u>Overview of ketone synthesis and metabolism.</u>
Ketone bodies are produced in the liver in response to low glycolitic substrate levels. Adipocytes mobilize their triglyceride stores to send glycerol and fatty acids to the liver. Then the liver produces acetyl Co-A, which it then combines to form acetoacetyl Co-A using acetoacetyl-CoA thiolase. This is combined with another acetyl-CoA to from HMG-CoA using HMG-CoA synthase. Acetoacetate is formed by HMG-CoA lyase, and an acetyl CoA is recycled back into the system. Acetoacetate can then be converted to β-hydroxybutyrate through the addition of NADH, and is a reversible reaction. Acetate and CO_2 is also formed. β-hydroxybutyrate is then transported to the brain where it is taken up by neurons and glia. It is then broken down into acetyl CoA, which is then fed into the Krebs Cycle to produce substrates for the electron transport chain (ETC). The conversion of acetoacetate to acetoacetyl CoA by β-Ketoacyl-CoA transferase produces succinate, which can be fed directly into the ETC.

of the body for fuel. Upon their uptake into the cell and the mitochondria, βHB is converted by βHB dehydrogenase into Acetoacetate, which is ultimately converted into Acetyl-CoA and utilized by the Krebs Cycle (Figure 1.6). However, as a byproduct of conversion succinate is also produced, which can be directly fed into the ETC (McKee et al. 2003). Ketone utilization is much more efficient in terms of enzymatic steps and ATP requirements. Glycolysis uses 11 enzymatic steps and 4 ATP per one molecule of glucose verses 3 enzymatic steps and 0 ATP per one molecule of βHB (McKee et al. 2003; Prins 2008).

Uncoupling Proteins

The basis of mitochondrial function is that the translocation of protons from the IMS to the matrix (down their concentration gradient) is coupled to the phosphorylation of ADP to ATP. Mitochondria are able to create the concentration gradient due to the function of the electron transport chain (ETC), which creates a potential difference in proton concentration across the inner membrane via the flow of electrons through a series of proteins within the ETC. The maintenance of this membrane potential ($\Delta\Psi$) is critical to the proper function of mitochondria, and subsequently cellular function and homeostasis. However, there is a narrow window in which the mitochondria must keep the $\Delta\Psi$, and deviation from this range can have serious dysfunctional effects on mitochondrial function and may lead to the initiation of cell death pathways. Increased $\Delta\Psi$ will cause a backup of electrons within the ETC, causing an increase in ROS production, which can damage mitochondrial proteins, lipids, and DNA. Decreased $\Delta\Psi$ will cause the reversal of the ATPsynthase, causing it to consume ATP in an attempt to

recover ΔΨ. This will cause decreased ATP levels within the cell, thereby preventing the critical function of ion translocators to maintain ionic balance within the cell, which is especially critical in neuronal function (Nicholls et al. 2000).

Endogenous mitochondrial uncoupling is mediated by members of the uncoupling protein (UCP) family that dissociate ATP production from the proton translocation in mitochondria of muscle and fat tissues, leading to heat generation (Nicholls et al. 2000). UCPs translocate protons across the inner mitochondrial membrane bypassing the ATP synthase and dissipating mitochondrial ΔΨ (Richard et al. 2001; Nicholls David et al. 2002; Nicholls et al. 2002). This UCP mediated loss of ΔΨ "uncouples" proton pumping by the ETC from the production of ATP and may lead to subsequent decreases in ATP, reactive oxygen species (ROS) production, and effect mitochondrial calcium cycling (Richard et al. 2001; Nicholls David et al. 2002; Sullivan et al. 2003; Sullivan et al. 2004). Their expression and function is believed to be upregulated by fasting and enhanced by the free fatty acids produced via beta oxidation within the mitochondrial matrix (Dulloo et al. 2001; Sullivan et al. 2004).

Five mitochondrial UCPs have been identified in the human genome (Dulloo et al. 2001; Nicholls David et al. 2002; Sullivan et al. 2003). Among these characterized UCPs, UCP2, UCP4 and UCP5/BMCP1 have recently been shown to be significantly expressed in the CNS (Horvath et al. 1999; Arsenijevic et al. 2000; Diano et al. 2000; Kim-Han et al. 2001; Sullivan et al. 2003). However, unlike UCP1 function in brown adipose tissue (BAT) is to generate heat in cold environments (i.e. thermogenesis), their physiological role(s) are unclear (Horvath et al. 2003).

UCP1 was first discovered in the 1960's when researchers focused their attention on the thermogenic capacity of BAT (Nicholls 2001). They were looking, more specifically, at the mitochondria in BAT to determine the mechanism of fat storage and mobilization in response to both dietary restrictions and temperature (Nicholls 2001). Experiments done during this time recognized that BAT stores of lipid substrates could be mobilized by lipases activated in response to the sympathetic nervous system (SNS) (Nicholls 2001). It has been proposed that there is a SNS-BAT-UCP axis, which is modulated by both diet and temperature (Dulloo et al. 2001).

It wasn't until the late 1990's that researchers found additional UCP1 homologs. UCP2 was found to be localized ubiquitously throughout the body, and a substantial amount was found to be localized in various parts of the brain; including the hypothalamus (suprachiasmatic, paraventricular, dorsomedial, ventromedial nucleus and arcuate nuclei), brainstem, and limbic system; which suggests that UCP2 plays a role in neuroedocrine, behavioral, and autonomic functions (Horvath et al. 1999; Richard et al. 2001). This expression had been found to be mainly neuronal; although the identity of the type of neuron that is participating has been somewhat unclear (Sullivan et al. 2003). It has recently been accepted that neurons involved in the upregulation of UCP2 possess an atypical β_3 adrenergic receptor that releases noradrenalin in response to SNS signaling, which activates BAT thermogenesis and liberation of fatty acids (Dulloo et al. 2001; Nicholls 2001). Highlighting this pathway were studies showing animals treated with a β_3 agonist experiencing weight loss associated with UCP expression (Dulloo et al. 2001). UCP2 knockout animals have an increased ability to secrete insulin, which may suggest a

role for UCP2 in energy metabolism by functioning as a negative regulator of insulin secretion (Richard et al. 2001; Erlanson-Albertsson 2002).

UCP3 is primarily found in muscle tissue and BAT, where it plays a role in the transport of fatty acids across the mitochondrial membrane during fasting or high-fat feeding when fatty acid oxidation predominates (Dulloo et al. 2001; Richard et al. 2001). UCP3 knockout animals have been shown to increase the efficiency of ATP production, decrease in baseline proton leak, increase ROS production, and show an impaired ability to shift its lipid partitioning between oxidation and storage in response to starvation (Dulloo et al. 2001). It has also been suggested that both UCP2 and UCP3 achieve their uncoupling function through the translocation of anions transported across the inner mitochondrial membrane bound to free fatty acids (Dulloo et al. 2001; Garlid et al. 2001). Still some others speculate that they transport protons through a pore-like structure (Garlid et al. 2001). UCP4 and Brain Mitochondrial Carrier Protien-1(BMCP-1 also called UCP5) have been localized to the brain; however whether they are even UCPs is still under debate (Dulloo et al. 2001). With the exception of the role of UCP1 in thermogenesis; all of the UCPs have a sequence homology, although their physiological role(s) are unclear particular with regard to the CNS.

Mitochondrial UCPs and Ca^{2+} Buffering

Although the complex mechanisms of secondary neuronal injury are poorly understood, it is clear that excitatory amino acid (EAA) neurotoxicity plays an important role (Rothman et al. 1995). Elevated EAAs increase the levels of intracellular Ca^{2+} ($[Ca^{2+}]i$) by activation of N-methyl-d-asparate (NMDA) receptor/ion channels, α-amino-

3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors and voltage-gated Ca^{2+} channels. This results in excessive entry of Ca^{2+}, leading to a loss of cellular homeostasis and subsequent neuronal Ca^{2+} overload. Ca^{2+} is the most common signal transduction element in cells, but unlike other second-messenger molecules, it is critical for normal cellular and mitochondrial function. Paradoxically, prolonged high levels of $[Ca^{2+}]_i$ lead to cell death (Choi 1992), however it is required for the activation of specific glycolytic and Krebs Cycle enzymes (Gunter et al. 2004). Excessive $[Ca^{2+}]_i$ can damage the structure of nucleic acids and proteins and interfere with kinase activity as well as activating proteases or phosholipases causing cellular damage. Therefore, maintenance of low $[Ca^{2+}]_i$ is necessary for proper cellular function and the initiation of second-messenger pathway in order to facilitate intracellular communication. Since Ca^{2+} cannot be metabolized like other second-messenger molecules, it must be tightly regulated by cells. Numerous intracellular proteins and some organelles have adapted to bind or sequester Ca^{2+} to ensure that homeostasis is maintained. Mitochondria are one such organelle (Ichas et al. 1998; Rizzuto et al. 1999; Rizzuto et al. 2000).

The mitochondrial membrane potential ($\Delta\Psi$), generated by the translocation of protons across the inner mitochondria membrane via the ETC, culminates in the reduction of O_2 to H_2O. This store of potential energy (the electrochemical gradient) can then be coupled to ATP production as protons flow back through the ATP synthase and complete the proton circuit. The potential can also be used to drive Ca^{2+} into the mitochondrial matrix via the electrogenic uniporter when cytosolic levels increase; and in turn when cytosolic levels of decrease, Ca^{2+} is pumped out of the matrix in order to precisely regulate cytosolic Ca^{2+} homeostasis (Gunter et al. 1994). During excitotoxic

insult, Ca^{2+} uptake into mitochondria has been shown to increase ROS production, inhibit ATP synthesis and induce mitochondrial permeability transitions (Dugan et al. 1995; Reynolds et al. 1995; White et al. 1996; Sengpiel et al. 1998; Brustovetsky et al. 2002). UCP2 has also been shown to play a role in determining the calcium buffering capacity of the mitochondria, which is a critical factor in maintaining cellular homeostasis (Richard et al. 2001). It is also important to note that inhibition of mitochondrial Ca^{2+} uptake by reducing $\Delta\Psi$ (chemical uncoupling) following excitotoxic insults is neuroprotective, emphasizing the pivotal role of mitochondrial Ca^{2+} uptake in EAA mediated neuronal cell death (Nicholls et al. 1998; Nicholls et al. 1998; Stout et al. 1998).

Role of Mitochondrial Uncoupling Proteins in ROS Reduction

As noted earlier, free radical production is a byproduct of ATP generation in mitochondria via the electron transport chain. Electrons escape from the chain and reduce O_2 to $O_2^{-\cdot}$. Normally cells convert $O_2^{-\cdot}$ to H_2O_2 utilizing both manganese superoxide dismutase, which is localized to the mitochondria, and copper-zinc superoxide dismutase found in the cytosol. H_2O_2 is rapidly converted to H_2O via catalase and glutathione peroxidase, but has the potential to be converted to the highly reactive $^{\cdot}OH$ via the Fenton reaction, underlying ROS neurotoxicity. The $^{\cdot}OH$ rapidly attacks unsaturated fatty acids in membranes causing lipid peroxidation and the production of 4-hydroxynonenal (HNE) that conjugates to membrane proteins, impairing their function (Keller et al. 1997; Keller et al. 1997; Mark et al. 1997; Sullivan et al. 1998). Such oxidative injury results in significant alterations in cellular function. In particular, ROS induction of lipid peroxidation and protein oxidation products may be particularly

important in neurodegeneration (for review see (Mattson 1998)) and TBI (Braughler et al. 1985; Braughler et al. 1989; Braughler et al. 1992; Sullivan et al. 1998).

Mitochondrial ROS production is intimately linked to $\Delta\Psi$ such that hyperpolarization (high $\Delta\Psi$) increases and promotes ROS production (Skulachev 1996; Skulachev 1998; Votyakova et al. 2001). The underlying mechanism is the altered redox potential of electron transport chain carriers (reduced) and an increase in semiquinone anion half-life time (high $\Delta\Psi$ prevents b_h oxidation of cytochrome b_1 in the Q cycle). In other words, at a high $\Delta\Psi$ protons can no longer be pumped out of the matrix (against the electrochemical proton gradient) by the chain so electron transport slows/stalls resulting in intermediates staying reduced longer and increasing the chance that the electrons escape from these intermediates, reducing O_2 and increasing ROS production. Since the magnitude of ROS production is largely dependent on--and correlates with--$\Delta\Psi$, even a modest reduction via increased proton conductance across the mitochondrial inner membrane (uncoupling) can reduce ROS formation (Skulachev 1996; Kim-Han et al. 2001; Votyakova et al. 2001). Endogenous mitochondrial uncoupling mediated by members of the UCP family could participate in the reduction of ROS production via this increased proton conductance. UCPs are activated by FFAs and $O_2^{-\cdot}$, and inhibited by purine nucleotides, indicating that they are sensitive to both ROS and ATP levels (Echtay et al. 2002) (also see (Harper et al. 2001; Argiles et al. 2002; Zackova et al. 2002) for review).

Several hypotheses have been put forth concerning possible physiological roles of the UCPs including energy partitioning, energy balance and control of metabolism which may be pivotal in obesity and diabetes (Argiles et al. 2002; Jezek 2002). Skulachev was

the first to hypothesize that mild uncoupling could be beneficial since it causes a decrease in ROS production (Skulachev 1996). Indeed, several studies have now demonstrated roles for UCPs in modulating ROS production. UCP2 (Arsenijevic et al. 2000) and UCP3 (Vidal-Puig et al. 2000) knockout mice exhibit increased ROS in macrophages and muscle, respectively. Leptin-deficient mice have decreased levels of UCP2 and also show increased ROS production in macrophages (Lee et al. 1999). *In vitro* overexpression of UCP2 (Li et al. 2001) or UCP5/BMCP1 (Kim-Han et al. 2001) decrease cell death following H_2O_2 exposure and ROS production respectively. Finally our lab has also reported a neuroprotective role for UCP2 in excitotoxic cell death *in vivo* (Sullivan et al. 2003). Our findings demonstrate that reducing UCP2 expression and activity, increases kainic acid induced mitochondrial ROS production and neuronal cell loss in p12 rats pups, which are classically resistant to excitotoxic insult (Sullivan et al. 2003). UCP2 overexpression has also been demonstrated to reduce ROS production and increase tissue sparing *in vivo* following ischemia or TBI (Mattiasson et al. 2003). Together these studies have implicated uncoupling (possibly via increased UCPs) in a neuroprotective role in protecting the mitochondria from both increased calcium uptake and increased oxidative stress (Sullivan et al. 2003).

Peroxisome Proliferator Activating Receptor

Although the exact mechanism remains unknown, UCPs have been suggested to be upregulated through the activation of the Peroxisome Proliferator Activating Receptor (PPAR). PPARs are a part of the hormone receptor super family and are mainly involved in the regulation of lipid metabolism. When activated, these receptors form a heterodimer

with the retinoid X receptor (RXR) then translocate into the nucleus where they bind to peroxisome proliferator response elements (PPRE); thereby initiating the transcription of specific enzymes involved in lipid homeostasis (Staels et al. 1997; Pineda Torra et al. 1999; Debril et al. 2001; Kiec-Wilk et al. 2005). As of now, three isoforms have been identified which seem to be involved in opposing pathways of lipid homeostasis. PPARγ, which is highly expressed in adipose tissue, is responsible for the differentiation of adipocytes and its expression is acutely induced by insulin (Debril et al. 2001). It functions primarily in lipogenesis and storage of lipids in adipocytes and other tissues. Its expression is upregulated by caloric restriction and is activated by certain naturally occurring FFA derivatives and a synthetic agonist thiazolidinediones (DZT), which is mainly used as a therapy for insulin resistance in diabetes (Debril et al. 2001; Shi et al. 2005). Its activation has been shown to increase high density lipoprotein (HDL) while reducing triglycerides; however this can come at a great risk due to the chance of increased levels of low density lipoprotein (LDL) created during the conversion of very low density lipoprotein (VLDL) to HDL, so it is likely that long term use may not be beneficial (Staels et al. 1997; Staels et al. 1998; Debril et al. 2001; Staels et al. 2005).

PPARα, which was the first of the isoforms to be identified and cloned, is mainly found in tissues which have a high metabolic rate for fatty acids (FA) such as liver, BAT, kidney, heart, brain, and skeletal muscle and is involved in a divergent pathway in lipid metabolism responsible for initiating the transcription of genes that are involved in the transport of FA into cells/mitochondria and in the subsequent oxidation of those FAs (Staels et al. 1997; Cullingford et al. 2002; Feinstein 2003; Shi et al. 2005). This isoform of PPAR is activated by fibrates (such as Ciprofibrate and Wy-14643) due to their dialkyl

fibrate head group which makes them highly selective for binding of the α isoform (Cullingford et al. 2002). Fibrates are used as an effective therapy for the reduction of high LDL cholesterol and triglyceride levels and the subsequent increase in HDL cholesterol levels due to the upregulation of apoA-I/II (Staels et al. 1997; Staels et al. 1998; Pineda Torra et al. 1999; Staels et al. 2005). These PPARα specific ligands also reduce apoC-III and apoB expression which inhibit the removal of LDL and VLDL (Staels et al. 1997; Staels et al. 1998; Shi et al. 2005). The PPARα isoform can also be activated by certain long chain FFA (i.e. α-linoleic acid and oleic acid) which have been described as natural ligands (Pineda Torra et al. 1999; Westin et al. 2004). PPARα has been shown to decrease pro-inflammatory FFA (i.e. lipid peroxides) as well as increasing mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHS) expression (Pineda Torra et al. 1999; Cullingford et al. 2002). This hepatic enzyme (mHS) is the key initiator of the conversion of acetyl-CoA, derived from FA, into ketone bodies (βHB and ACA). PPARα activation induces the transcription of many enzymes involved in the import and β-oxidation of triglycerides and FA (Staels et al. 1998; Kashiwaya et al. 2000). It has been implicated in an anti-inflammatory role since PPARα can directly interfere with NFκB transactivation by binding co-activator protein p65; however this sequestration does not inhibit all of NFκB-driven transcription products (Tan et al. 2005). This may be important in light of the evidence that TNFα, which is an activator of NFκB and ultimately MnSOD, knockout mice have decreased tissue sparing after a focal CCI injury (Sullivan et al. 1999). Activation of PPARα may lead to discriminatory inhibition of NFκB-driven transcription of pro-inflammatory cytokines, while leaving the pro anti-oxidant mechanism in place and inducible in response to TBI.

Both PPARs and UCPs are sensitive to FFA modulation and induction of function. FFAs act as natural PPAR ligands to activate target genes, and also play a role in modulating the activation of those target genes at a transcriptional level (Staels et al. 1997; Pineda Torra et al. 1999; Kashiwaya et al. 2000; Westin et al. 2004; Kiec-Wilk et al. 2005; Shi et al. 2005). In addition to inducing the upregulation of UCPs via PPAR activation, FFA activate UCPs; however it is still undetermined whether they induce a proton pore or if they utilize the membrane protein to translocate (flip-flop) proton ions across the inner membranes (Figure1.7) (Klingenberg 1999; Garlid et al. 2001). Our preliminary data have found that UCPs are upregulated during fasting, which could implicate PPARs as players in metabolic homeostasis (i.e. ketogenesis) as well as a neuroprotective mechanism due to their involvement in UCP upregulation. The elucidation of this mechanism could result in novel therapeutic treatment applications for TBI.

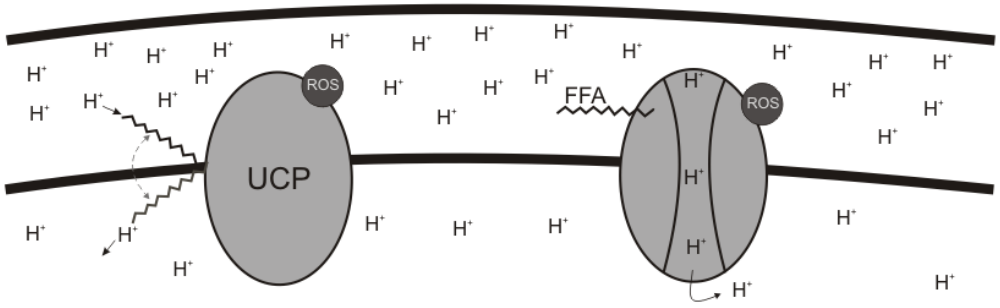


Figure 1.7: <u>Proposed mechanisms of UCP mediated proton translocation.</u>
There are two proposed mechanisms of UCP mediated proton translocation, which can uncouple the electron transport chain from ATP production. The first proposed mechanism states that free fatty acids (FFA) use UCPs as a platform so that they can become protonated and flip-flop across the inner membrane (left side of figure). The second is that they activate UCPs, which then form a pore through which protons can then flow (right side of figure). Both of these mechanisms require the presence of reactive oxygen species, and as such have been described as an endogenous anti-ROS mechanism.

Chapter 2

The Neuroprotective Effect of Fasting after TBI: Possible Mechanism(s) and Clinical Implications

Introduction

Mitochondria have recently been implicated as a critical component of cellular regulation and homeostasis. Ordinarily, mitochondria maintain cellular homeostasis through their vital role in Ca^{2+} sequestration and the production of ATP (Sullivan et al. 1998; Nicholls et al. 2000; Sullivan et al. 2002; Brookes et al. 2004). Mitochondria are also the communal mediator in the activation of cell death pathways; in effect acting as the "death switch" of the cell (Sullivan et al. 2005). As such, it seems reasonable to speculate that the preservation of normal mitochondrial function after TBI would alleviate the cellular stresses of secondary injury, and may result in improved post-injury cognitive outcome. This concept has gained much credibility in recent years due to a number of experimental studies showing that such treatment strategies aimed at maintaining normal mitochondrial function are neuroprotective (Sullivan et al. 1998; Scheff et al. 1999; Lifshitz et al. 2004; Sullivan et al. 2004; Sullivan 2005).

Extrapolating from such findings one might ask how improved mitochondrial function might be achieved clinically? Previous studies have shown that fatty acids increase mitochondrial biogenesis in skeletal muscle, heart, liver (Totland et al. 2000) and in brain (Bough et al. 2006). These observations suggest that a high-fat diet may ameliorate mitochondrial dysfunction in humans. One such diet designed to achieve a high fat to carbohydrate ratio paradigm is the anti-epileptogenic ketogenic diet. This dietary therapy was originally designed to mimic the physiological effects of fasting, and most notably produces mild hypoglycemia and systemic ketosis. While the underlying

mechanism(s) of action remain unclear, the ketogenic diet has been successfully used in the treatment of medically refractory epilepsy for over eight decades (Bough et al. 2003; Greene et al. 2003; Tieu et al. 2003; Sullivan et al. 2004; Yamada et al. 2005; Bough et al. 2006; Bough et al. 2007; Kim et al. 2007; Maalouf et al. 2007). Interestingly, ketone bodies appear to exert direct neuroprotective effects in a variety of *in vivo* and *in vitro* models (Bough et al. 2003; Tieu et al. 2003; Sullivan et al. 2004; Yamada et al. 2005; Kim et al. 2007; Maalouf et al. 2007). For example, when administered after MPTP, a mitochondrial toxin used in an experimental models of Parkinson's disease, beta-hydroxybutyrate (βHB) enhances mitochondrial respiration and prevents neuronal injury (Tieu et al. 2003). Also, we have previously shown that ketone bodies raise the threshold for induction of the mitochondrial permeability transition (Kim et al. 2007). Given these findings, we hypothesized that fasting will produce a neuroprotective effect in a controlled cortical impact (CCI) model of TBI, possibly through ketone body production.

In the present study, we found that fasting improved tissue sparing, cognitive function, and limited mitochondrial dysfunction when implemented post injury. To elucidate the underlying mechanism of fasting-induced neuroprotection, we modulated metabolic effects of fasting independently. To this end we found that after moderate TBI, ketone administration, but not acute insulin mediated hypoglycemia, exerted a significant neuroprotective effect. We also found that ketones alleviated mitochondrial dysfunction after excitotoxic calcium insult. Collectively, our study highlights a possible clinical implication for treatment, and underscores the importance of metabolic regulation after TBI.

Methods

Animals

All experimental animal procedures were approved by the Animal Care and Use Committee at the University of Kentucky. All experiments were conducted using adult male Sprague-Dawley rats (250-300g), which were housed 3 per cage and maintained in a 12-hour light/12-hour dark cycle. All animals were fed a balanced diet *ad libitum* unless otherwise specified.

Surgical Procedures and Experimental Paradigms

All surgical procedures were performed as previously described under 2% Isoflurane (Sullivan et al. 1998; Sullivan et al. 1999; Sullivan et al. 2000) Injuries were classified as moderate (1.5mm) or severe (2.0mm), based on the specified depth of mechanical cortical depression. To investigate the neuroprotective effect of fasting after TBI, animals were given a controlled cortical impact (CCI) of either a moderate or severe injury and were either fed *ad libitum* or fasted for 24 or 48 hours (3 groups n=5/group). Animals were allowed free access to water during the fasting time, and after fasting were fed *ad libitum*. Body temperature was maintained at 37°C throughout the experiment until the animals were awake and moving freely about their cage. Behavioral testing (see below) was performed beginning 10 days post injury and tissue harvesting for tissue sparing assessment was done at 15 days post injury. As a 24hr fast after moderate CCI injury was the only paradigm that was found to be neuroprotective, this was the only injury level and fasting duration examined in the remainder of our studies. In a separate set of animals, blood glucose and ketone levels were monitored by first anesthetizing

animals with 2.0% Isoflurane followed by tail prick blood extraction. Ketone levels were measured using a STAT-Site® analyzer (Stanbio Laboratory, Boeme, Texas). Glucose levels were measured using Therasense™ (Freestyle) glucose meter. To determine if TBI +/- fasting had an effect on ketone or glucose levels a separate set of animals received a 1.5mm injury, however only one of the groups was fasted for 24 hours; glucose and ketone levels were taken at 0, 3, 6, and 24hrs (Table 2.2, 2 groups 3/group).

An alternate group of animals were fasted or injected with either saline, 5U, 10U insulin (Humulin®R regular Eli Lilly and Co. Indianapolis, IN 46285), after which glucose and ketone levels were taken at -5min, 1hr, 3hr, 6hr, and 24hr (Table 2.1, 4 groups, 3/group). After determining the proper insulin dose, animals received a 1.5mm CCI injury and were injected with a 10U dose of insulin at 3, 6, 9, 12, 15, and 21 hrs post injury (7 groups 4/group). Animals were sacrificed at 7 days post injury at which time tissue preparation and tissue sparing assessments were performed.

To investigate the neuroprotective effect of exogenous ketone administration, a separate set of animals were given a 1.5mm CCI injury and administered D-βHB via mini-osmotic pumps at a constant dose of either of 1.66 mMoles/kg/day (n= 6), 0.83 mMoles/kg/day of D-βHB (n=10) (Sigma-Aldrich Co St. Louis, MO 63178) or saline (n=10). The pumps were subcutaneously inserted immediately after injury and removed after 3 days. Animals were sacrificed 10 days post injury at which time tissue preparation and tissue sparing assessments were performed.

Tissue Preparation, Histology and Tissue Sparing Assessments

All animals were sacrificed and their tissue prepared as previously described (Sullivan et al. 2000). Briefly, all animals were anesthetized by administering a 95 mg/kg dose of sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL) and subsequently transcardially perfused using phosphate-buffered saline (PBS) followed by PBS (pH 7.4) containing 4% paraformaldehyde (PFA). Brains were extracted and stored in a PBS solution containing 4% PFA and 15% sucrose at 4ºC for at least 24hrs until sectioning could be performed. Brains were coronally sliced on a freezing microtome at a thickness of 50µm. Sections were mounted on gelatinized slides and stained with Cresyl Violet. Slides were viewed using an Olympus AX70 microscope at 4X power and images were taken using an Olympus MagnaFire (model S99800) U-TVO .5XC camera. Tissue sparing was measured with Image J (NIH freeware) software (Michel et al. 1988; Sullivan et al. 1999; Sullivan et al. 2000; Pandya et al. 2007) . The cortical volume of each brain was measured by separately tracing the outline of the ipsilateral and contralateral cortex in 12 sections from each brain. The sections were taken 250um apart from anterior to posterior. The volume of ipsilateral tissue spared was compared to the volume of tissue contralateral to the injury and results were expressed in % tissue spared (ipsilateral/contralateral*100) (Figure 2.1).

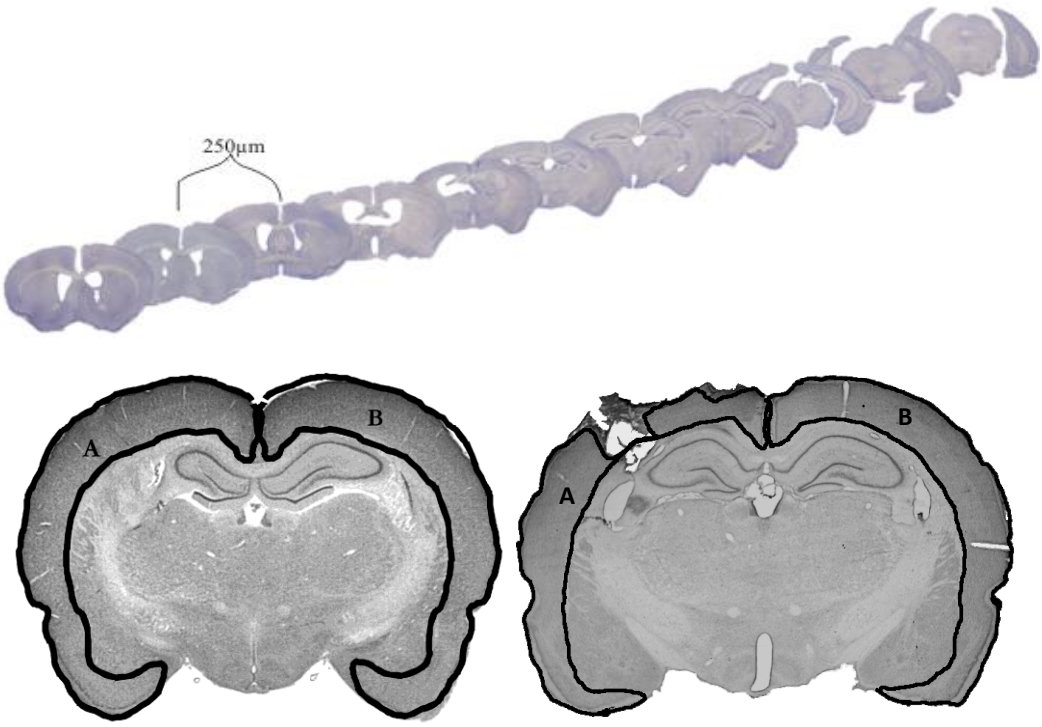


Figure 2.1: <u>Cavelleri method of tissue sparing assessment.</u>
Tissue sparing is calculated tracing the volume of the cortex of 12 sections from each animal. Then we determined the percent tissue spared by dividing the ipsilateral area (A) by the contralateral area (B) and multiplying by 100 to get the percentage of spared tissue. (A/B*100). Figure shows both and injured and naïve section from similar anatomical locations.

Behavioral Testing

Sham operated or animals which underwent a moderate TBI and either were fasted for 24 hrs, fed *ad libitum* were behaviorally assessed using a well characterized adaptation of the Morris Water Maze (MWM) to assess cognitive function at 10 days post TBI (Scheff et al. 1997; Verbois et al. 2003; Pandya et al. 2007). During these assessments the animals were tested for goal latency, target quadrant entries, target search time, swim speed and target annulus crossings as a measure of spatial learning and memory. The maze consisted of a darkened (nontoxic black powder pain) circular pool of

water (121cm across, 56cm high, 30cm deep) at 27 C. Just beneath the water (2cm), a Plexiglas platform was placed and used as the goal platform for targeting 40 cm from the side of the pool. The entire pool was in a 4m x 4m room with consistent extra-maze cues, and placed directly beneath a video camera that recorded swim performance. These video recordings were analyzed by a video motion analyzer (Videomex V, Columbus Instruments, Columbus, OH). Testing began 10 days post injury and continued for a total of 5 consecutive days. Early motor deficits that are occasionally seen within the first few days after injury will not confound our results because we are delaying behavioral assessment for 10 days post injury. Each day animals were separately placed in a randomized quadrant (north, south, east, west) of the pool facing the wall and each animal was allotted 120 seconds to locate the hidden platform. If the animals were unable to locate the platform they would be manually placed on the platform from 30s and subsequently placed in a heated incubator for 4 minutes between trials. In order to rule out nonspecific visual deficits animals were tested using a visible platform (2.0 cm above water) following transfer testing. Animals that failed to reach the platform during this testing paradigm were removed from the behavioral pool of data as this indicates that they are unable to see properly and therefore cannot use the designated visual cues. To asses swim speed used the mean of the total path length and latency to reach the platform during the four trials/day. On the last day of testing after the latency trials were completed, a probe trial was performed in which the platform was removed from the pool and the animal was allowed to search for 60 seconds. In order to assess how well the animal learned where the target platform (annulus) was located, target annulus crossings,

target search time, and target quadrant entries (expressed as a percentage of total quadrant entries) were used as endpoint measurements. (3groups n=5/group)

Mitochondrial Isolation

These procedures are based on, and contain, modifications of previously described protocols (Brown et al. 2004). A separate set of animals received a moderate TBI injury and were either fasted for 24hrs or fed *ad libitum.* At 24hrs post injury the brains of adult male Sprague-Dawley rats (~250g) were quickly removed and a 5mm punch of the injured cortex was dissected and removed. Cortical tissue was placed in cold isolation buffer with 1mM ethylene glycol tetraacetic acid (EGTA) (75mM sucrose, 215mM mannitol, 0.1% BSA, 20 Mm HEPES with a pH of adjusted to 7.2 using KOH). Tissue was homogenized and spun at 1,300 x g for 5 minutes at 4ºC. The supernatant was taken off and saved in separate tubes. The pellet was resuspended and spun at 1,300 x g for 5 minutes at 4ºC. The supernatant was again taken off and saved in separate tubes. The saved supernatant was spun at 13,000 x g for 10 minutes at 4ºC. The pellets were resuspended and a nitrogen bomb was used to rupture the cell membrane (Brown et al. 2004). After bombing, the sample was separated on a Percoll gradient via centrifugation in a high speed Sorval centrifuge for 10 minutes at 30,400 x g. The third fraction containing the purified mitochondria was removed and spun at 16,700 x g for 15 minutes. The pellet was resuspended and spun at 13,000 x g for 10 minutes. The pellet was resuspended in 500µL isolation buffer without EGTA and transferred to a microcentrifuge tube, which was then spun at 10,000 x g for 10 minutes. The supernatant was removed and the pellet was resuspended in enough isolation buffer without EGTA to obtain a concentration of 10-15ug/µl. BCA protein assay kit was used determine protein

concentration by measuring absorbance at 560 nm with a BioTek Synergy HT plate reader (Winooskin, Vermont).

Mitochondrial Respirations

Mitochondria isolated from punches of injured cortex were suspended in KCl respiration buffer (125mM KCl, 2mM MgCl, 2.5mM KH_2PO_4, 20mM HEPES, 0.1% BSA) inside a sealed constantly stirred thermo-regulated Oxytherm chamber containing a Clark-type oxygen electrode as previously described (Brown et al. 2004). Substrates were added (Pyruvate (5.0mM)/Malate (2.5mM), ADP (150µM) oligomycin (2µm), FCCP (1µM), Rotenone (0.8µM), succinate (150µM)) to determine the functionality of the mitochondria at various states (Brown et al. 2004). (n=4/group)

For respirations done in the presence of Ca^{2+}, mitochondria were isolated from whole cortex (from naïve animals), rather than a punch. KCl stock respiration buffer (as described above) also containing 5mM Pyruvate and 2.5mM Malate was used to for respiration studies. A final concentration of 0.5mM calcium was added to one aliquot of stock buffer. Ketones (final concentration of 1mM D-βHB and 1mM ACA) were added to the stock buffer with and without 0.5mM calcium. 250uL of buffer was added to the oxytherm chamber along with mitochondria from naïve unfasted animals. Respirations were conducted as described above. (n=4/group)

Oxidative Biomarkers

A separate set of animals were used to measure oxidative biomarkers. In these studies animals were given a moderate CCI injury and either fasted for 24hrs or fed *ad libitum.* At 24hrs post injury, the mitochondria from the injured site were isolated and assessed for oxidative damage markers. (n=6/group)

Protein Damage Assessment

Mitochondrial protein oxidation was assessed by measuring protein carbonyls using the OxyBlot Protein Oxidation Detection Kit (OxyBlot, Intergen Company, Purchase, New York) as previously described (Pandya et al. 2007). Breifly, mitochondrial samples taken from a 5mm punch of the injury site of fasted and unfasted injured animals isolated at 24 hours post injury were derivatized with 2,4-dinitrophenylhydrazine (DNP) to achieve the formation of 2,4-dinitrophynylhydrazone. Samples were then neurtralized and separated by gel electrophoresis. After transfer, the membrane was incubated with primary antibody to detect protein carbonyl groups through the binding to a specific DNP moiety. This incubation was done overnight at 4°C, after which the membrane was sufficiently rinsed with wash buffer (phosphate buffered saline, 0.2% Tween-20). The membrane was then incubated with a goat-anti-rabbit HRP-conjugated secondary antibody, and subsequently developed using enhanced chemiluminescent (ECL) detection solutions (Amersham-Pharmacia, Piscataway, NJ). Bands were quantified using densitometry using a Molecular Dynamics Storm 860 phosphoimager and NIH Image software. (n=6/group)

Lipid Peroxidation Assessment

The mitochondrial lipid peroxidation assessment was performed using a slot blot technique as previously described (Pandya et al. 2007). Breifly, mitochondrial samples taken from a 5mm punch of the injury site of fasted and unfasted injured animals were isolated using a protease inhibitor cocktail (Sigma) isolation buffer and subsequently flash frozen using liquid nitrogen. Using a Pierce BCA protein concentration kit, 250ng of solubilized mitochondrial protein was loaded to each slot blot well. Under a vacuum

the samples were transferred to a nitrocellulose membrane. This membrane was incubated with primary anti-4-hydroxynonenole (HNE) antibody (monoclonal 1:2500, Chemicon). After washing, the membrane was incubated with rabbit-anti-mouse antibody HRP-conjugated secondary antibody, and subsequently developed using ECL solutions (Amersham-Pharmacia, Piscataway, NJ). Band densities were assessed using afore mentioned imager and software. (n=6/group)

Mitochondrial In Situ Ca^{2+} Load Assessment

In order to assess mitochondrial Ca^{2+} cycling/loading *in situ*, we used a novel technique developed by our lab as previously reported (Pandya et al. 2007). Breifly, animals were sacrificed and perfused with cold buffer containing Ca^{2+} uniporter inhibitors (0.6µM ruthenium red), Ca^{2+} antiporter inhibitors (10µM CGP-37157 and 0.6µM ruthenium red), and mPTP inhibitors (5µM cyclosporin A). This "locking" buffer is designed to prevent the loss of Ca^{2+} during the mitochondrial isolation process. Brains were removed and a 5mm punch of the ipsilateral cortical injury site was dissected from injured fasted and injured unfasted animals, which was placed in "locking" isolation buffer containing the above described additions. One important difference between the locking isolation buffer and isolation buffer described previously is the lack of EGTA in the buffer throughout the isolation procedure. Ca^{2+} levels were assessed using Calcium Green 5N, which is a non membrane permeable indicator with excitation at 485nm and emission at 528nm. Mitochondrial samples were measured for fluorescence before and after the addition of 10% DMSO, which causes the release of intra-mitochondrial Ca^{2+}. A standard curve of Ca^{2+} fluorescence was used in order to extrapolate our values to molar Ca^{2+}/mg protein. Values were then expressed as a percentage of unfasted injured calcium

loading levels from fasted injured mitochondria. All assays and standards were performed using identical total volumes. (n=6/group)

ROS Assay

Mitochondrial samples were assessed for ROS production using the permeative indicator 2′-7′-dichlorodihydro-fluorescein (DCF) as described previously (Sullivan et al. 2003). Briefly, isolated mitochondria were incubated in respiration buffer at 37˚C in the presence of horseradish peroxidase (1μg/ml) and DCF (10μM) and measured using a fluorometric plate reader (excitation 485 nm, emission 528 nm). Fasted mitochondria were compared to mitochondria isolated from unfasted (control) animals and the fluorescence was expressed as a percentage of the control fluorescence (fasted value/control value *100). (n=6/group)

Calcium ROS/NADH Assay

ROS and NADH were measured using a Synergy HTTR com1 plate reader and analyzed using KC4 software program (Bio-Tek Instruments Winooski, VT). A KCl stock respiration buffer (as described above) which also contained 5mM Pyruvate, 2.5mM malate, 10μM DCF, and 100μM HRP was used to measure fluorescence. A final concentration of 0.5mM calcium was added to one aliquot of stock buffer. Ketones (final concentration of 1mM D-βHB and 1mM ACA) were added to the stock buffer with and without 0.5mM calcium. 25μg of protein was added to each well containing 50μL total volume of one of the previously described buffers. ROS production was measured at 37˚C at 485nm/528nm and NADH auto-fluorescence was measured at 360nm/460nm for 15 minutes at intervals of 1:24 minutes. Values from blank wells containing only buffer were subtracted from values obtained from sample wells. Groups were Ketones, Calcium,

and Ketones + Calcium (n=8/group). Values were expressed as % control (mitochondria with calcium free and ketone free buffer).

Statistical Analysis

For all statistical comparisons, significance was set at $p < 0.05$. Data were evaluated using analysis of variance (ANOVA) or unpaired t-tests when appropriate. When warranted by the ANOVA, post hoc comparisons employed the Student-Neuman-Keuls and Bonferroni post hoc analyses, respectively.

Results

Fasting is Neuroprotective after Traumatic Brain Injury

In this study, we found that the amount of cortical tissue sparing increased when animals were fasted for 24 hrs after a moderate (1.5mm) injury compared with unfasted injured animals (Figure 2.2). In contrast, 48 hrs of fasting post injury did not result in a significant increase in tissue sparing following a moderate injury, suggesting no additive effect for a prolonged fasting period (Figure 2.2). The neuroprotective effect of fasting was also lost after a severe (2.0mm) injury in both fasting paradigms (Figure 2.3). Cognitive function was significantly lower in unfasted injured animals, indicated by increased goal latency, decreased search time, annulus crossings, and target search time (Figure 2.4 B). However, fasted injured control animals had significantly improved scores on all of the aforementioned measures of cognitive function; which also were not significantly different from sham operated animals (Figure 2.4 B). Interestingly, fasted injured animals were not significantly different than sham animals in terms of goal

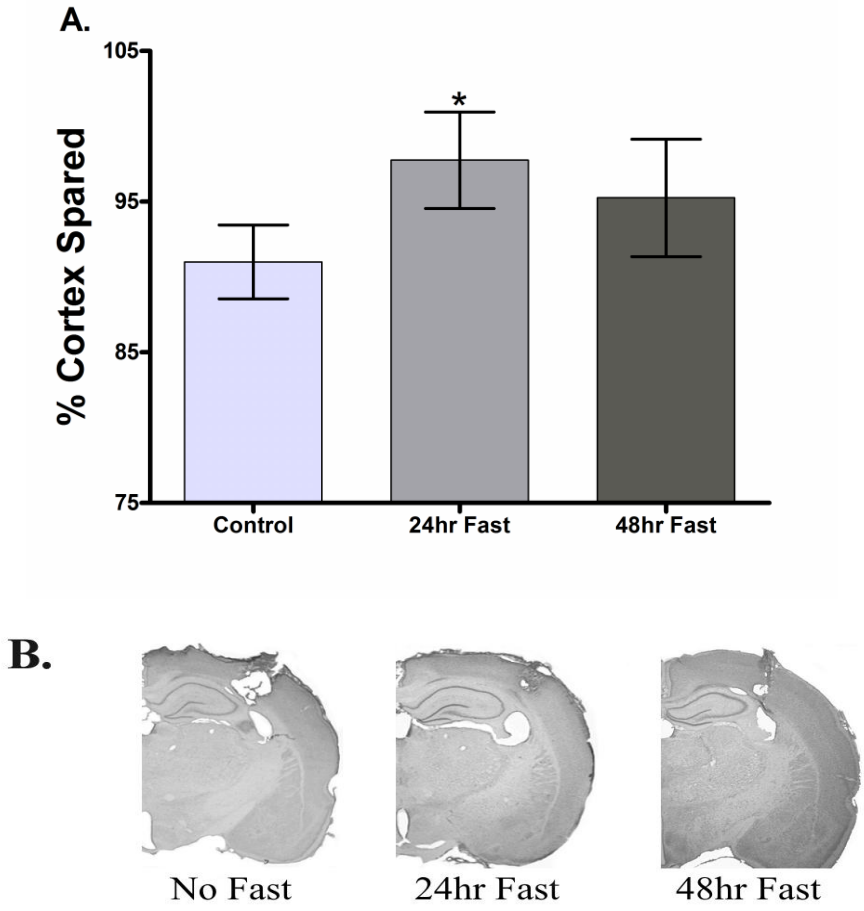


Figure 2.2 <u>Fasting increases tissue sparing after moderate (1.5mm) CCI injury</u>.
(A) Animals fasted for 24hrs after a moderate (1.5mm) CCI injury showed a significant increase in tissue sparing compared with control animals, which were continually fed ad libitum after TBI. Fasting animals for 48hrs did not show a significant amount of tissue sparing compared with control injured animals. Values are shown in percent contralateral volume. (n=5/group; ANOVA $p<0.05$, $F_{2, 14}=5.6$, $R^2=0.48$ *$p<0.05$ ± SD). (B) Shows a representative section from each treatment group a similar anatomical location within serial sectioning..

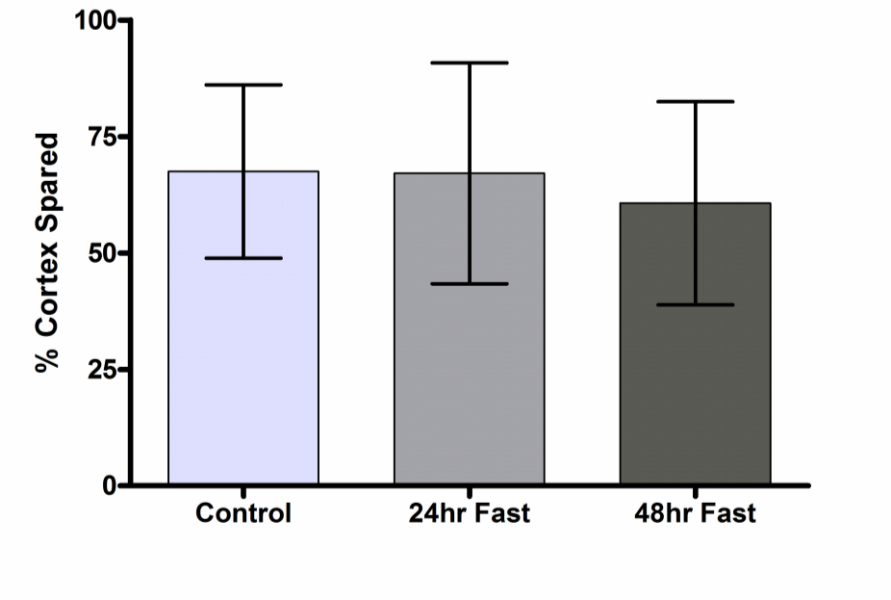


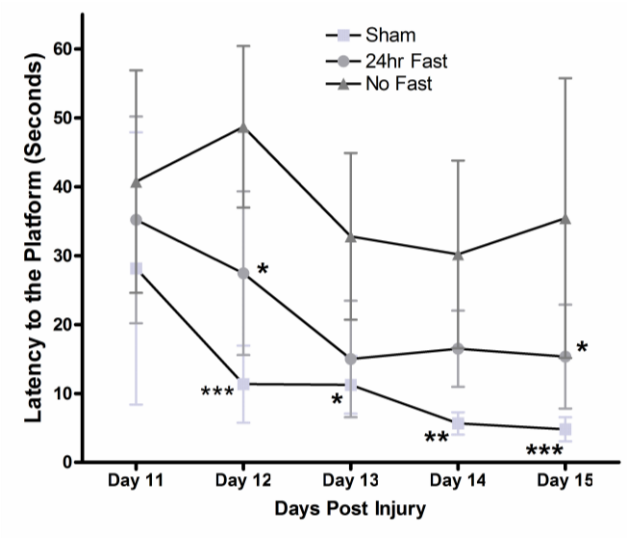
Figure 2.3 <u>Fasting is unable to increase tissue sparing when administered after severe (2.0mm) CCI injury.</u> When animals received a 2.0mm injur, fasting did not significantly increase tissue sparing when administered for either 24hr or 48hrs after injury. Values expressed as percent of contralateral cortex (n=5/group; ANOVA $p>0.05$ $F_{2, 14}=0.16$, $R^2=0.026$, ± SD).

latency; however they did show a significant difference compared to unfasted injured animals a 12 and 15 days post injury (Figure 2.4 A). Swim speed was not significantly different between any of the groups, indicating that physical limitations induced by the injury did not influence behavioral outcome (Figure 2.4B).

Fasting Reduces Mitochondrial Damage and Improves Function after TBI

Our studies also demonstrate that fasting after injury reduces the levels of several biomarkers of mitochondrial dysfunction and cellular damage including mitochondrial ROS production and Ca^{2+} loading, and to a lesser extent, protein carbonyls and lipid peroxidation (Figure 2.5). Mitochondria isolated from the injury site of fasted animals showed significantly higher ADP utilization (i.e., state III) rates when compared to mitochondria isolated from unfasted injured animals (Figure 2.6). Collectively, these

A.



B.

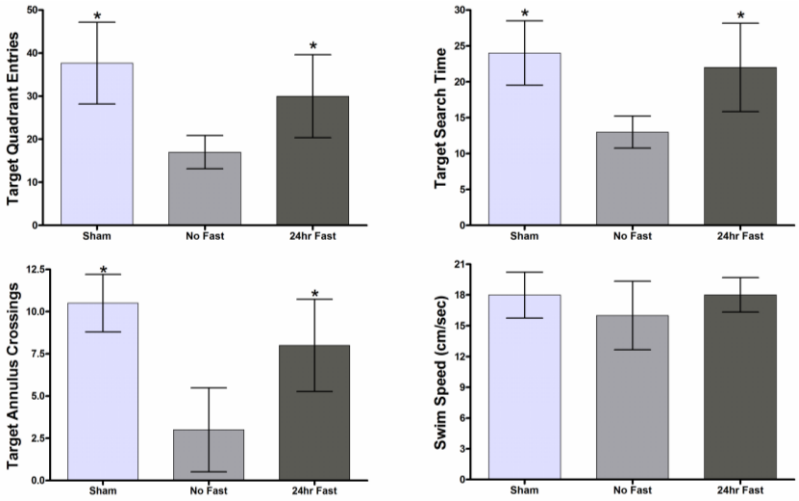


Figure 2.4 <u>Fasting improves cognitive recovery after moderate (1.5mm) CCI injury.</u> (A) Behavioral testing, using an adaptation of the Morris Water Maze, showed that injured unfasted animals showed significantly increased latency compared with sham animals. Fasted injured animals did not show a significant difference in goal latency compared with sham animals. (n=5/group; Two-Way Repeated Measures ANOVA $p<0.05$ $F_{2,48}=12$, *$p<0.05$ **$p<0.01$ ***$p<0.001$ compared to No Fast (unfasted injured) animals, ± SD). (B) Fasted injured animals did not perform significantly differently from sham (unfasted) animals. However, injured unfasted animals did show significant differences in target quadrant entries (% of all quadrant entries) ($F_{2,\ 14}=8.3$, $R^2=0.58$), target search time (seconds) ($F_{2,\ 14}=8.2$, $R^2=0.58$), and target annulus crossings ($F_{2,\ 14}=13$, $R^2=0.69$) when compared with sham unfasted and fasted injured animals. There was no significant difference between the treatment groups for swim speed ($F_{2,\ 14}=1.0$, $R^2=0.15$), indicating that the behavioral results were not influenced by physical limitations due to injury (n=5/group; ANOVA * $p<0.05$ compared with injured unfasted animals, ± SD).

data indicate that 24hrs of fasting post injury induces a beneficial mechanism for preservation of mitochondrial and therefore cellular function after TBI and, unlike other neuroprotective treatments such as creatine or some antioxidants (Sullivan et al. 2000), has the advantage of post injury administration.

As our data indicate, the optimal time point and injury level for intervention would be a 24hr post injury suspension of caloric intake following a moderate (1.5mm) TBI. Therefore, this was the paradigm we employed to elucidate a mechanism underlying the neuroprotective effects of post-injury fasting.

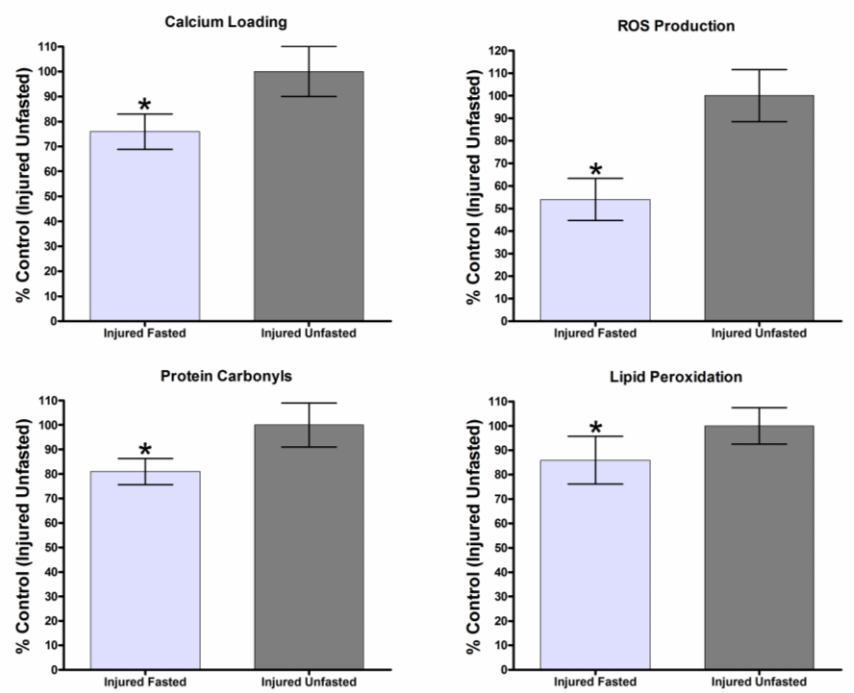


Figure 2.5: <u>Post Injury fasting reduces biomarkers of injury.</u>
Mitochondria from fasted injured animals showed a significant decrease in the biomarkers of damage compared with mitochondria from unfasted injured animals. Total calcium load, measured using locking buffer to block the efflux/influx of calcium during preparation, was decreased in fasted animals indicating a decrease in the TBI induced dysfunction of calcium cycling. Decreased ROS levels, indicative of decreased oxidative stress, combined with decreased markers of protein and lipid damage indicate that fasting decreases both oxidative stress and subsequent oxidative damage resulting from TBI (n=6/group; unpaired t-test, t_{10}= 4.8 (Ca^{2+} Loading), t_{10}= 7.6 (ROS Production) , t_{10}= 4.4 (Protein Carbonyls), t_{10}= 2.8 (Lipid Peroxidation) *$p<0.05 \pm$ SD).

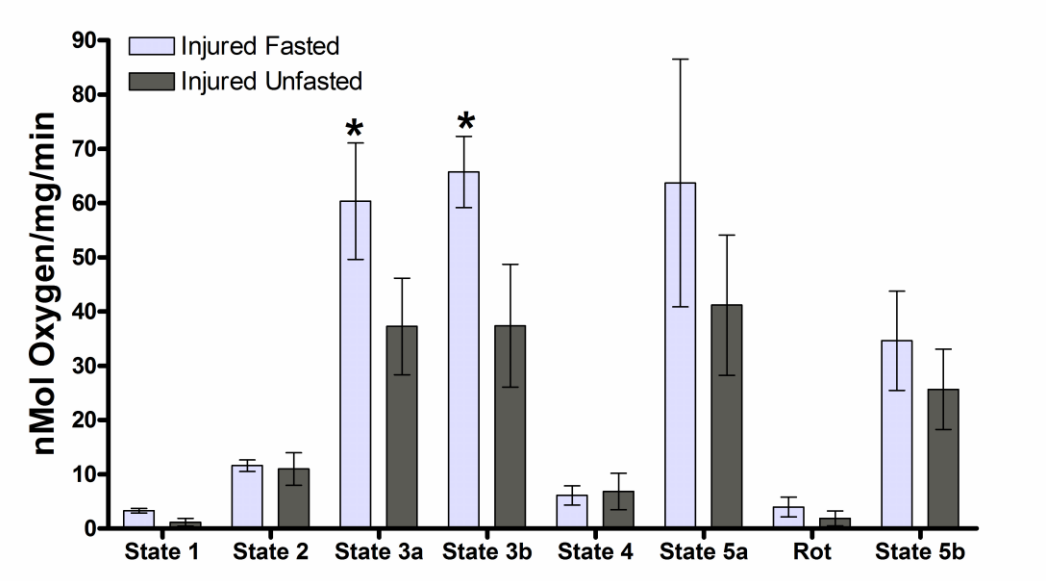


Figure 2.6: <u>Fasting increases mitochondrial function after TBI.</u>
Injured unfasted animals had no dietary modulations after CCI injury, whereas animals in the injured fasted treatment group were fasted for 24hrs post CCI. Mitochondria isolated from these punches at 24hrs post injury were monitored for oxygen consumption in response to mitochondrial substrates. The mitochondria from fasted injured animals showed significant increases in their ability to utilize ADP for ATP production (State III). The designations for state 3a (t_4=4.4) and 3b (t_4=6.3) are due to the order they were performed in. State 3b should be slightly higher than state 3a, due to the priming of the ETC and Krebs cycle state 3a. (Unpaired t-test; * $p < 0.05$ n=3/group ± SD, one animal from each group was dropped because they were more than 2 SD from group mean.)

Insulin and Ketone Administration after Injury

It is widely known that fasting induces many metabolic changes, including modulation of blood glucose and ketone levels (Burge et al. 1993). Naïve fasted animals monitored for glucose and ketone (βHB) levels showed simultaneous, significant decreases in glucose and increases in ketone levels after 24hrs compared with naïve unfasted animals (Table 2.1). Additionally, fasted injured animals show a significant decrease in glucose levels and an increase in ketone levels when compared with unfasted injured animals, indicating that fasting is modulating metabolism in ways that injury alone does not, and that unfasted animals return to normal eating habits after injury

(Table 2.2). These data present two possible underlying mechanisms of fasting-induced neuroprotection, namely, hypoglycemia and ketosis. To evaluate each of these possibilities, we independently modulated ketone and glucose levels in unfasted animals and assessed their effects on tissue sparing following a moderate TBI. Hypoglycemia was

A)

Glucose (% -5 min)	-5 min	1hr	3hr	6hr	24hr
0 U	100.00±41.97	88.16±36.16	103.72±28.51	82.61±18.21	89.41±17.63
5 U	100.00±57.01	50.18±16.05	35.59±8.89**,##	93.80±34.43	109.23±44.56##
10 U	100.00±28.51	49.59±15.45	47.02±19.34**	78.53±13.82	83.20±5.56
Fasted	100.00±21.71	91.03±11.06	84.78±11.62	89.06±13.56	47.22±9.87*

* p<0.05 compared with 0U; ** p< 0.01 compared with 0U
p<0.01 compared with fasted

B)

β-HB (mM)	-5 min	1hr	3hr	6hr	24hr
0 U	0.068±0.06	0.130±0.15	0.200±0.10	0.100±0.10	0.000±.0.00
5 U	0.033±0.06	0.230±0.12	0.067±0.16	0.067±0.06	0.000±0.00
10 U	0.200±0.17	0.170±0.06	0.100±0.10	0.000±0.00	0.000±0.00
Fasted	0.100±0.00	0.000±0.00	0.033±0.06	0.100±0.10	1.030±0.06$

$ p<0.001 compared with 0U, 5U, and 10U

Table 2.1: <u>Insulin Administration Decreases Glucose Levels without Affecting Ketone Levels in Naïve Animals.</u>
(A) Insulin artificially decreases glucose levels to 24hr fasting levels at 3hrs post injection (n=3/group; 2-way ANOVA $p<0.05$, $F_{3, 32}=2.5$; Bonferroni post test; * $p<0.05$ compared with 0U; ** $p< 0.01$ compared with 0U; ## $p<0.01$ compared with fasted, ± SD). (B) Ketones (βHB) were not increased in response to insulin administration. Fasting significantly increased ketone levels after 24hrs compared with 0U, 5U, and 10U. (n=3/group; 2-way ANOVA $p<0.05$, $F_{3, 32}=21$, Bonferroni post test; $ $p<0.001$ compared with 0U, 5U, and 10U; ± SD).

A)

Glucose % 0hr (Control)	0hr	3hr	6hr	24hr
Fasted Injured	100.0±10.94	110.2±6.78	119.9±27.87	86.7±8.67*
Unfasted Injured	100.0±30.45	96.3±4.67	131.4±33.04	124.58±7.24

* $p<0.05$ compared with unfasted injured animals.

B)

β-HB (mM)	0hr	3hr	6hr	24hr
Fasted Injured	0.23±0.15	0.4±0.19	0.33±0.12	0.57±0.06**
Unfasted Injured	0.27±0.12	0.07±0.06	0.13±0.06	0.27±0.06

** $p< 0.01$ compared with unfasted injured animals.

Table 2.2: <u>Fasting induced modulation of glucose and ketone levels after moderate (1.5mm) controlled cortical impact (CCI) injury.</u>
(A) Fasted animals showed a significantly decreased level of serum glucose at 24hrs post injury compared with injured unfasted animals (Paired t-test, $t_{2,\ 11}=31.27$ * $p<0.05$ compared with unfasted injured animals). (B) Fasting animals after injury induced significantly higher levels of ketones at 24hrs post injury compared with unfasted injured animals; (Paired Student's t-test, $t_{2,\ 11}=5.29$ ** $p< 0.01$ compared with unfasted injured animals 5). (n=3/group ± SD).

induced using insulin administration, due to its ability to artificially decrease blood glucose levels without altering ketone production (Table 2.1). Based on our preliminary dosing studies (Table 2.1), insulin was administered at different time points after a moderate injury, throughout which animals were fed *ad libitum*. Assessment of cortical tissue sparing showed no significant sparing of tissue with any of the insulin injection time points. In fact, insulin administration at 3, 15, and 21hrs resulted in an increased mortality rate (Figure 2.7). These treatment groups were excluded from the ANOVA analysis due to the high mortality. Although we have used an acute insulin administration

paradigm, we believe that a prolonged titration of insulin administration would produce similar results, as this would also cause glucose uptake without supplemental energy

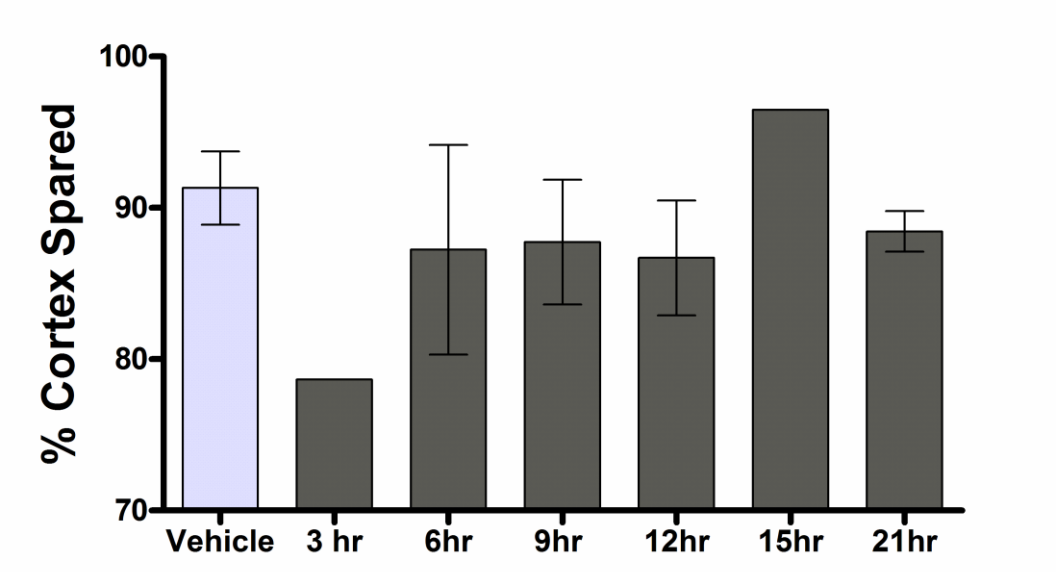


Figure 2.7: <u>Insulin is not neuroprotective after moderate CCI TBI</u>. Animals given a moderate (1.5mm) controlled cortical impact (CCI) injury were injected (i.p.) with either saline (3hrs post injury) or 10 Units (U) of insulin at either 3, 6, 9 12, 15, or 21hrs after injury. None of the injection time points showed a significant change in the amount of tissue spared. (ANOVA, $F_{3,13}$=0.6, R^2=0.15, p>0.05 ± SD; the 3hr and15hr time points were not included in the statistical analysis due to the high mortality in each group leaving only an n of 1 in each group. Also, the 21hr time group was excluded from the statistical analysis as it had only an n of 2. All other groups contained 4 animals). This lack of neuroprotection may be due to the sequestration of glucose away from the brain and into adipocytic stores, resulting in energetic failure and neuronal loss.

substrate production through ketosis. Our data suggest that hypoglycemia, independent of its downstream effects, is not the underlying mechanism of the neuroprotective effect afforded by fasting.

When monitoring ketone levels after a moderate injury, we found that fasted injured animals showed a significant increase in the levels of βHB after 24hrs compared with unfasted injured animals (Table 2.2). However, the level of ketones in fasted injured animals did not seem to reach the same levels as the fasted naïve animals (although these

groups could not be directly compared), which may indicate the uptake of ketones in the injured brain.

To examine the neuroprotective effects of ketones, we subcutaneously implanted osmotic mini-pumps filled with vehicle (saline), 0.8mmoles D-βHB/kg/day, or 1.6mmoles D-βHB/kg/day immediately after administering a moderate injury, doses which have been shown to maintain the serum concentration of D-βHB at similar levels seen with fasting (Tieu et al. 2003). Interestingly, the lower dose of D-βHB significantly increased cortical tissue sparing compared with vehicle, whereas the higher dose of D-βHB did not, indicating that there may be a dosage effect for the efficacy of ketone administration (Fig.2.8). These data clearly demonstrated that this treatment could have a beneficial effect on tissue sparing when administered post injury.

Ketones Attenuate Mitochondrial Dysfunction In The Presence Of Calcium

When mitochondria from naïve unfasted animals were incubated with excitotoxic levels of Ca^{2+}, we found a significant increase in ROS production, as well as increased NADH levels (Figure 2.9). This would indicate that the calcium is causing mitochondrial function to become aberrant. Interestingly, when we added ketones (1mM D-βHB and 1mM ACA) to the calcium buffer, ROS levels and NADH levels were significantly decreased from the calcium buffer levels, and were not significantly different from control levels (Figure 2.9). Somehow the mitochondria are able to utilize ketones as substrates to overcome calcium induced dysfunction. When mitochondrial respiratory capacity through oxygen utilization was measured in the presence of calcium, the

mitochondria with access to ketones showed significant improvements in state III and state V respiration rates (Figure 2.10), which are measures of ADP phosphorylation

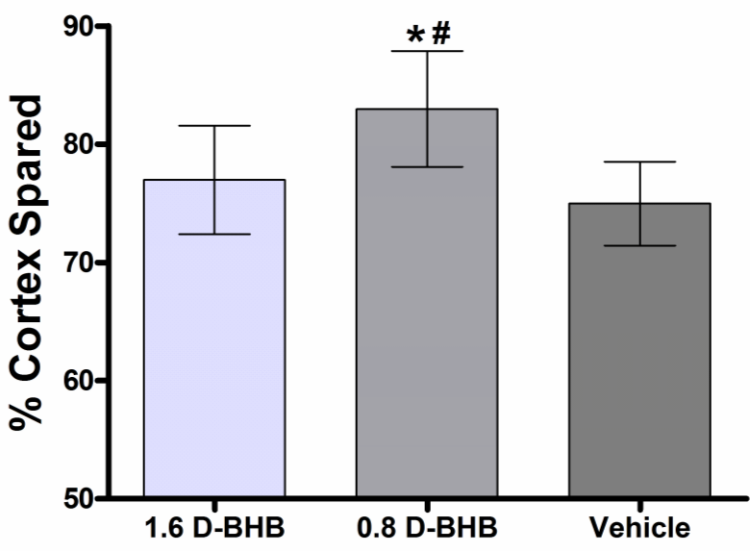
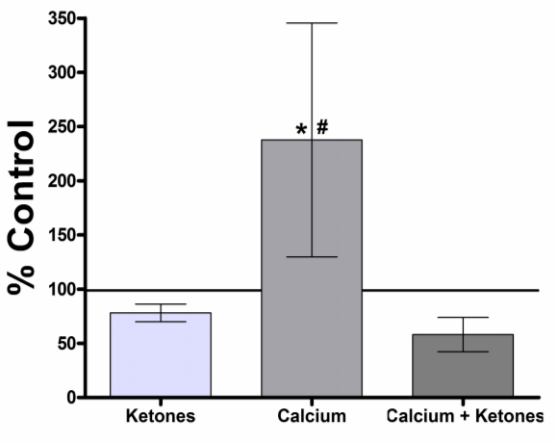


Figure 2.8: <u>Exogenous ketone administration increases tissue sparing when administered after moderate (1.5mm) controlled cortical impact (CCI) injury.</u> β-hydroxybutyrate was administered via subcutaneously implanted osmotic mini-pumps for 3 days post injury. The lower dose (0.8mMoles/kg/day; n=10) of βHB induced neuroprotection as measured by increased tissue sparing compared with saline (n=10) treated animals. There was also a significant difference between the high dose βHB (1.6mMoles/kg/day; n=6) and the low dose, indicating a dose response to ketone administration. However, there was no significant difference between vehicle and the high dose of βHB. (ANOVA, $F_{2, 25}$=9.0, R^2=0.44, p<0.001 SNK; * p<0.001 High βHB vs. Saline; #p<0.01 High βHB vs. Low βHB ± SD).

capacity and maximum respiratory capacity, respectively. These results are similar to what we have seen in mitochondria from injured fasted animals compared to uninjured fasted animals. These data collectively indicate that the presence of ketones is capable of maintaining mitochondrial function and this may explain how they are able to confer neuroprotection after injury.

A. **ROS**



B. **NADH**

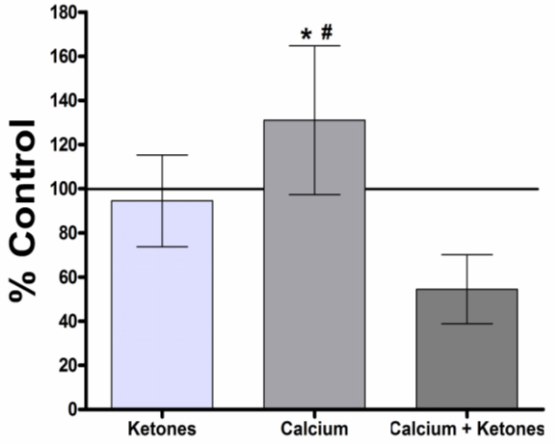
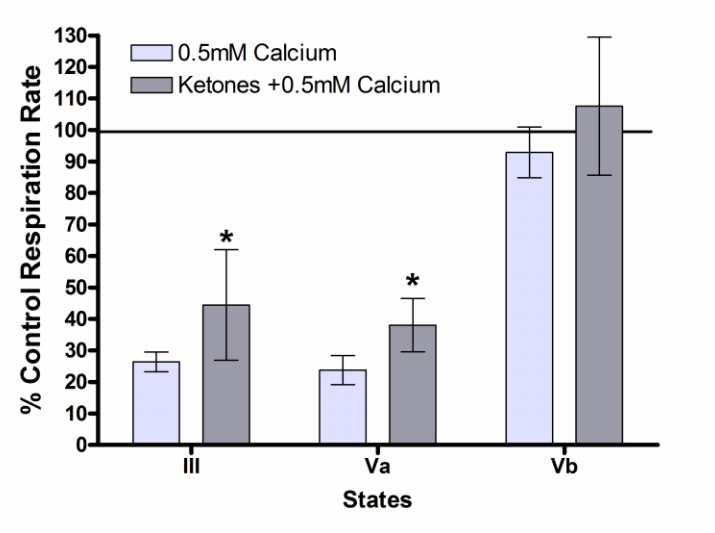


Figure 2.9: <u>Ketones attenuate Ca^{2+}induced ROS formation and NADH backup.</u> Exogenous Ca^{2+} causes changes in mitochondrial bioenergetics in terms of ROS production and NAD^{+}/NADH cycling. (A) When mitochondria from unfasted naïve animals are incubated with exogenous Ca^{2+} (0.5mM), the production of ROS significantly increases. This is attenuated when ketones (1 mM D-βHB and 1mM ACA) are added to the buffer, which may be due to their ability to affect the oxidation state of the Q-cycle within the ETC. (ANOVA, $F_{2,\ 23}$=19.420, R^2=0.649, p<0.0001 SNK; * p<.001 compared to Ketones, # p<0.001 compared to Ketones + Calcium, n=8, SD). (B) Exogenous Ca^{2+} (0.5mM) also increased NADH when incubated with mitochondria. Ketone (1mM D-βHB and 1mM ACA) addition significantly decreases these levels, which indicates that ketones can preserve complex I driven respiration in the presence of excitotoxicity. (ANOVA, $F_{2,\ 23}$=19.41, R^2=0.649, p<0.0001 SNK; * p<.001 compared to Ketones, # p<0.001 compared to Ketones + Calcium, n=8, SD).

A.



B.

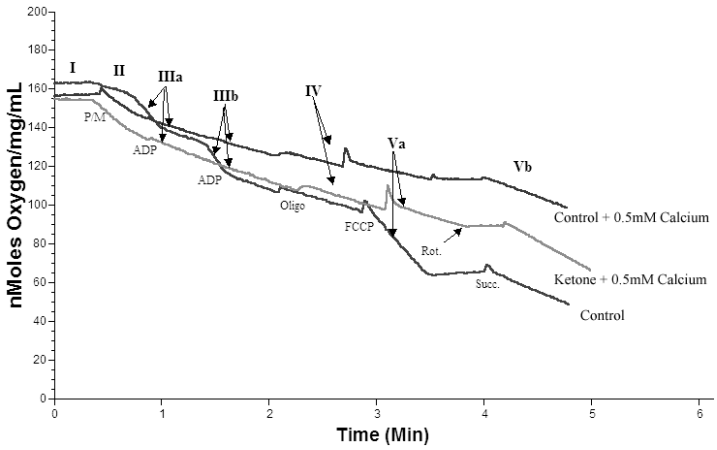


Figure 2.10: <u>Ketones increase mitochondrial function in the presence of exogenous calcium.</u>

(A) Mitochondria from unfasted naïve animals were isolated and incubated with calcium, which caused a decrease in mitochondrial function as measured by oxygen consumption. The addition of ketones (1mM D-βHB and 1mM ACA) significantly increased state III respiration (t_8=3.2) and state Va (t_8=3.0) respiration, which are measures of ADP phosphorylation and maximum ETC function (respectively). Complex II driven respiration (state Vb) was not significantly different between treatments (t_8=2.5), indicating that calcium mainly affects complex I function. Values are expressed as % control (mitochondria in buffer without calcium or ketones). Mito + Calcium; n= 5/group, Mito + Ketones and Calcium n=5/group; Unpaired Student's t-test, * $p<0.05$. (B) Graph shows representative oxygen consumption trace with states and corresponding substrate additions. Rate is measured in nMoles of oxygen/mg/mL over time. The presence of calcium causes a flattening of state III respiration as well as state Va, which is then attenuated by the addition of ketones (1mM D-βHB and 1mM ACA).

Discussion

Traumatic brain injury occurs in a biphasic manner, beginning with primary mechanical damage, followed by a more insidious secondary injury cascade. Several lines of evidence have implicated that mitochondria are key players in the events constituting secondary injury, which includes increased release of excitatory amino acids (EAAs), increased lactate and free fatty acid levels (FFA), impaired Ca^{2+} cycling, increased ROS production, mitochondrial dysfunction, and induction of cell death pathways (Faden et al. 1989; Hayes et al. 1992; Sullivan et al. 1998; Sullivan et al. 2000; Scheff et al. 2004).

Recently there have been growing concerns regarding the timing of nutritional support for TBI patients and what influence this could have on neurological outcome. After TBI, there is general unregulated increase in metabolic activity within the first 24 hours after injury, characterized by an increased demand for bioenergetic substrates and protein catabolism (Caron et al. 1991; Hovda et al. 1992; Hovda et al. 1995; Vespa et al. 2005). Previous clinical reports suggested that "early" dietary intervention may improve outcome in TBI patients (Pepe et al. 1999; Krakau et al. 2006; Perel et al. 2006), supporting the general notion that proper nutritional support is vital to sustain increased metabolic activity. Thus, it may appear counter-intuitive that an acute reduction in overall caloric intake would be neuroprotective after TBI. However, “early intervention”, as defined clinically and which has been shown to confer better outcome, is understood to be 24-72 hours after injury (Krakau et al. 2006; Perel et al. 2006). It is also important to note that although TBI patients are not getting nutritional support, they are getting intra-venous glycolytic compound (e.g. glucose) administration and glucose uptake regulation

via insulin, which could be shutting down the potentially protective ketogenic pathway (Robertson et al. 1991; Vespa et al. 2006). In the present study, animals fasted for 48 hours did not show a significant increase in tissue sparing, which may be due to the exhaustion of metabolic stores, and the collapse of cellular repair mechanisms. Thus, our experimental results are in accordance with clinical findings, as fasting beyond the initial 24-hour window was not beneficial to outcome.

We have previously shown that TBI induces mitochondrial damage and dysfunction (Sullivan et al. 1998; Sullivan et al. 2002). The present study clearly demonstrates that mitochondrial function can be improved by fasting. Specifically, fasting led to an attenuation of mitochondrial ROS production, oxidative damage, and enhanced Ca^{2+} buffering. Isolated mitochondria from fasted and injured animals also showed enhanced utilization of ADP, indicative of efficient ATP production. Moreover, our behavioral data indicated that the cognitive abilities of fasted injured animals were not significantly altered as compared to sham animals, highlighting the potential safety of appropriately timed fasting following TBI.

However, fasting induces many evolving biochemical changes at a systemic level, including ketogenesis, lipogenesis, and hypoglycemia (Burge et al. 1993; Pan et al. 2000). During extended fasting (more than 9-12 hours), decreasing insulin levels initiate the mobilization of stored energy deposits contained in hepatic and adipose tissues (Fromenty et al. 2004). Once glucose levels are depleted, the body shifts to the oxidation of fatty acids to maintain ATP levels. This results in increased hepatic synthesis of ketone bodies (principally, βHB and acetoacetic acid), and the release of free fatty acids (FFA) from the breakdown of triacylglycerols and their mobilization from adipose stores

(Fromenty et al. 2004; Nehlig 2004). Ideally, identification of the key mediator(s) of fasting-induced neuroprotection might allow us to obviate systemic complications associated with fasting and ensure a more uniform effect. As such, to ascertain potential underlying mechanism(s), we used a reductionist approach to independently target two well-known physiological adaptations resulting from fasting, namely, hypoglycemia and ketosis.

Hypoglycemia is an early response to fasting, and we have seen its induction within the first 9 hours. However, in our effort to mimic this condition with acute insulin administration in the absence of calorie restriction, we failed to see a neuroprotective effect after moderate TBI at any injection time-point examined in our study. Moreover, we found that acute hypoglycemia proved fatal in some treatment groups, which may have important clinical implications; however, we recognize that insulin boluses are not administered to TBI patients with normal glycemic levels. Our experimental paradigm involved abrupt increases in insulin levels, which may have caused glucose sequestration through enhanced selective insulin-sensitive glucose transporter activity, which is not present on neurons (El Messari et al. 1998; Alquier et al. 2001). As a result, there would be a deficiency in both glucose and alternative energy substrates (i.e., ketone bodies) to provide sufficient fuel for damaged neurons to facilitate repair mechanisms, which may account for the lack of protection seen with insulin administration after TBI. In the present study, we did not examine whether a more gradual elevation of blood insulin levels might yield a neuroprotective effect. However, we do not believe that sustained hypoglycemia through insulin administration would be protective due to observations in clinical patients that sustained insulin administration indicated poorer outcome after

injury (Vespa et al. 2006). Also, supporting our findings are previous reports that acute glucose administration (which also induces spikes in insulin levels) post injury decreases neuroprotection in both human and animal studies (Robertson et al. 1991; Cherian et al. 1998). However, we could have included an injured fasted group that was administered insulin after injury as a way to mimic the clinical treatment of a TBI patient, and as such is an acknowledged limitation of our current studies.

Given that acute insulin-induced hypoglycemia did not explain the neuroprotective effect of fasting against moderate TBI, we next examined whether compounds classically referred to as ketone bodies (i.e., D-βHB) could be underlying the neuroprotective effect of fasting. Ketone bodies, which are preferentially utilized by the brain after TBI, have been shown to increase cortical levels of ATP after injury and improve mitochondrial function, possibly through similar mechanisms (Prins et al. 2004). This upregulation of ketones occurs shortly after hypoglycemia has been induced, within 15hrs (data not shown) due to lack of caloric intake. Infusion of the principal ketone body, D-βHB, alone replicated our findings with a 24-hour fast after TBI in terms of tissue sparing. Interestingly, only the lower dose of D-βHB was neuroprotective after moderate injury, suggesting a preliminary dose-response relationship.

The neuroprotective activity of ketone bodies has been demonstrated in various models of neurological injury and disease, both *in vivo* and *in vitro* (Yager et al. 1992; Kashiwaya et al. 2000; Massieu et al. 2003; Tieu et al. 2003; Smith et al. 2005; Yamada et al. 2005; Noh et al. 2006; Bough et al. 2007; Maalouf et al. 2007). We have also previously demonstrated that ketone bodies attenuate ROS formation in isolated mitochondria exposed to oligomycin, the maximum ROS producing inhibitor of complex

V (ATP synthase) (Kim et al. 2007). As a part of its metabolic utilization, D-βHB is converted to acetoacetate (ACA) by β-hydroxybutyrate dehydrogenase, thereby reducing NAD^+ to $NADH+H^+$ (Dardzinski et al. 2000; Rho et al. 2002; Nehlig 2004). This limits the available pool of NAD^+ and alters the redox potential of ubiquinone (Co-enzyme Q_{10}) from a reduced to an oxidized state, and hence diminishes the number of electrons available to spin off and form superoxide ($O_2^{\cdot-}$) radicals (Nicholls et al. 2002; Nehlig 2004). In addition, the conversion of D-βHB to acetyl-CoA produces endogenous succinate, a complex II (succinate dehydrogenase) substrate independently of the TCA cycle; and acetyl-Co also enters the TCA cycle producing additional complex II substrates (McKee et al. 2003). Thus, administration of ketone bodies may yield two independent sources of substrates for Complex II, which coupled with the alteration of the redox state of ubiquinone, ultimately helps to reduce ROS and maintain normal mitochondrial function.

It is well known that TBI results in excessive influx of Ca^{2+} into the cell, resulting in increased Ca^{2+} loading and mitochondrial stress, and eventual dysfunction (Nicholls et al. 2003; Brookes et al. 2004; Gunter et al. 2004; Sullivan et al. 2004). In our studies we have shown co-incubation of isolated mitochondria with ketone bodies in the presence of excitotoxic levels of Ca^{2+} resulted in increased ADP utilization (state III) and maximum electron transport chain function (state V) (Figure 2.10), both indicative of an overall improvement in mitochondrial function. These data showing functional recovery are similar to our results from mitochondria from fasted injured animals. Together with our data showing that ketone bodies also decrease ROS production and increase the efficiency of NADH utilization in the presence of exogenous Ca^{2+} (Figure2.9), it suggests

that ketones increase the ability of the cell to withstand excitotoxic injury by glutamate mediated Ca^{2+} influx after traumatic injury. Our current data indicates that ketones could be working to maintain mitochondrial homeostasis through ETC maintenance as well as cellular Ca^{2+} regulation.

Studies investigating the metabolic fate of glucose after TBI have shown post injury glucose utilization shifting from energy production to cellular repair mechanisms via the pentose phosphate pathway. Therefore, the addition of exogenous ketone bodies may ameliorate systemic complications resulting from TBI, by allowing pyruvate and glucose to be utilized for support and repair mechanisms in other tissues while providing metabolic support for neuronal function (Bartnik et al. 2005; Bartnik et al. 2007; Dusick et al. 2007). Additionally, monocarboxylate transporters (the major ketone transporters) have been shown to be upregulated by TBI as well as the ketogenic diet; which would indicate that ketones can be effectively taken up and utilized preferentially after injury in our experimental paradigm (Morris 2005; Prins et al. 2006; Prins 2008). Indeed the uptake of ketones by these transporters seems to be substrate concentration dependent manner, thereby allowing for rapid uptake in the event of increased ketone availability (Prins 2008). Interestingly, there are also studies indicating that the human brain possesses a greater capacity for ketone utilization compared to the rodent brain, which may indicate that ketone administration may have greater neuroprotective effects in clinical TBI patients (Prins 2008). Together, the utilization of these endogenous repair systems, along with energetic substrate supplementation, could account for the protection seen with ketone body administration post injury. These previous studies, along with our

current data demonstrate strong support for a protective role for ketone bodies in the phenomenon of fasting-induced neuroprotection following moderate TBI.

Although significant, the neuroprotective effect seen with ketone administration was not as robust as that seen with fasting after injury. It is possible that co-administration of ACA along with D-βHB could prove more efficacious due to dual insertion sites in the metabolic system. Although, in human subjects there was a 13 fold increase in cerebral uptake of βHB after 3.5 days of fasting, whereas there was not increase in uptake of ACA (Hasselbalch et al. 1995). Also, other systems upregulated by the complex network of fasting may also be contributing to the neuroprotective effect seen with fasting post injury. Regardless of other possible neuroprotective mechanisms, our studies have shown that ketones play an integral role in the attenuation of tissue loss and mitochondrial dysfunction in the mechanism of fasting induced neuroprotection.

Hypoxia inducible factor- α (HIF-1α) is responsible for the upregulation of glycolytic enzymes, glucose transporters, angiogenesis genes, and monocarboxylate transporters (MCT), which are responsible for ketone uptake into the brain (Zhang et al. 2005). Its expression is upregulated during times of decreased oxygen levels, therefore HIF-1α expression could be transiently increased in a hypoxic region of the injury site (Acker et al. 2004; Prins 2008); however the duration of hypoxia may not be sufficient to preserve cellular function. Indeed, the upregulation of a key enzyme of the (PPP) pentose phosphate pathway (glucose-6-phosphate dehydrogenase (G6PD)) is less sensitive to HIF-1 mediated induction and as such has a slower induction rate (Gao et al. 2004). This transient ischemia may prevent the upregulation of G6PD and inhibit the supplementation

of the damaged endogenous antioxidant glutathione system after TBI (Ansari et al. 2008; Ansari et al. 2008).

Although HIF-1α expression has not been directly measured after fasting, it has been measured after ketogenic diet administration and direct intra-ventricular ketone infusion (Puchowicz et al. 2008). Interestingly, ketotic animals show an upregulation in the expression of HIF1α, and this upregulation can be achieved via diet or direct infusion of βHB into the brain (Puchowicz et al. 2008). Also, these animals have increased expression of Bcl-2 proteins, which could indicate that ketones are involved in promoting an anti-apoptotic mechanism (Puchowicz et al. 2008). It is postulated that the increased production of succinate via ketone utilization may be responsible for HIF1α stabilization as it can directly inhibit HIF1α- prolyl-dydroxylases, the protein responsible for HIF1α breakdown (Schofield et al. 2004; Puchowicz et al. 2008). Indeed, strategies that either provide additional succinate (propionate) or cause the inhibition of complex II by 3NP (3-nitropropionic acid), and therefore the increase of succinate, induce the stabilization of HIF-1α (Goldberg et al. 1966; Koivunen et al. 2007). Moreover, brain tissue of ketotic animals, be it from dietary supplementation or infusion of ketones, contains significantly higher succinate concentration (Goldberg et al. 1966; Puchowicz et al. 2008). As ketones are able to increase the pool of available succinate, this could also contribute to the upregulation of beneficial enzymes as well as the increased uptake of ketones for mitochondrial utilization. This also could explain why MCT upregulation coincides with ketone availability (Prins et al. 2004). It should be noted that ketones can also be utilized more readily than glucose by the cell to create proteins and lipids (cholesterol in myelin), which could further enhance the ability of the cell to repair itself (Morris 2005).

Furthermore, as glycolytic products are in a state of enzymatic equilibrium (McKee et al. 2003), the preferential uptake of ketones over pyruvate into the mitochondria could shift glycolytic intermediates toward utilization by the pentose phosphate pathway, which increases NADPH levels thereby supporting the endogenous glutathione system. Ketones would therefore increase mitochondrial substrate production, mitochondrial function, and increase HIF-1 mediated upregulation of proteins/transporters that could enhance cellular repair mechanisms and ketone uptake/distribution (Figure 2.11). It is also important to mention that HIF-1 is also responsible for the upregulation of pro-apoptotic proteins, however it has been suggested that these proteins are upregulated with prolonged HIF-1 activation (Acker et al. 2004), which could explain why our high dose of ketone administration did not show as robust neuroprotection as fasting and why a 48hr fast did not increase tissue sparing. This indicates that investigation into duration of treatment is needed in order to determine the maximum effectiveness of ketone administration.

In summary, we have shown that fasting after moderate TBI exerts a significant neuroprotective and positive cognitive effect compared with unfasted injured animals. In attempting to elucidate the underlying mechanism(s) of fasting-induced neuroprotection, we found that acute administration of insulin alone, which mimics fasting-induced hypoglycemia, was not neuroprotective and, in fact, was fatal in some treatment groups. More importantly, we demonstrated that exogenous ketone body administration in the absence of fasting resulted in neuroprotection after moderate injury as measured by cortical tissue sparing. Similar to observations seen in mitochondria from animals fasted post injury, studies using mitochondria in an excitotoxic damage model suggest that the beneficial actions of ketone bodies likely stem from their ability to enhance

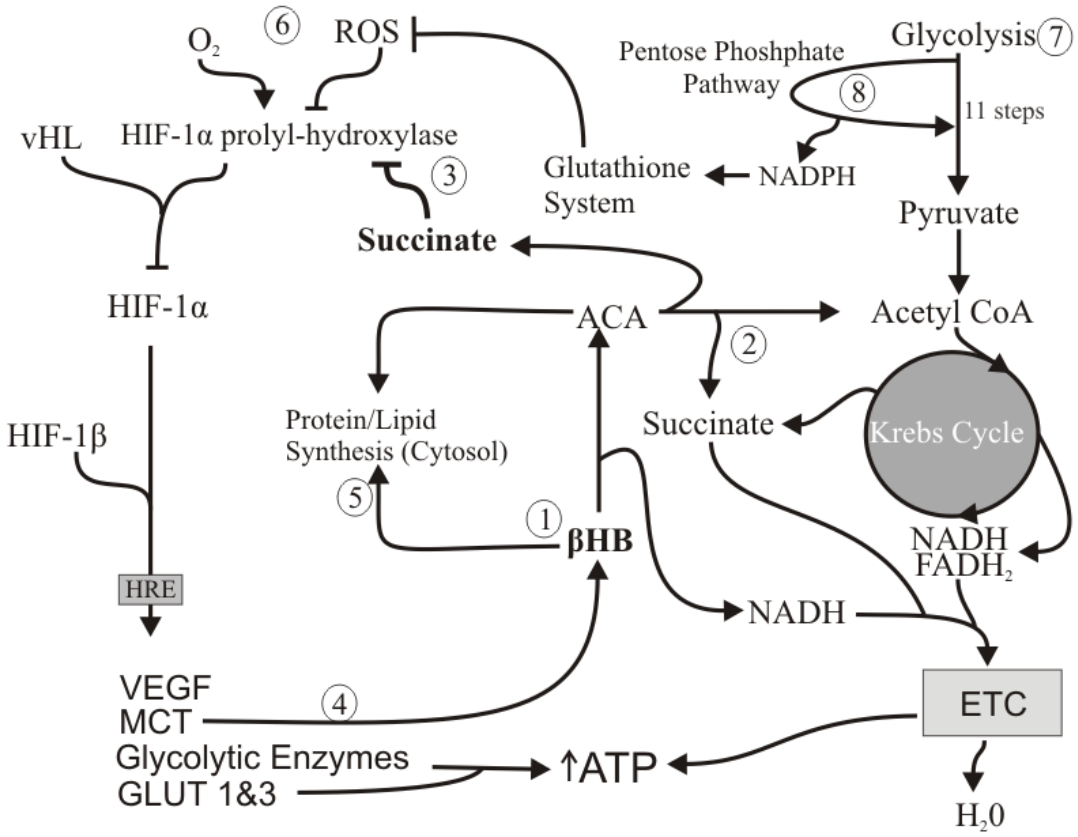


Figure 2.11 <u>Hypothesis of how ketones and HIF-1 work together to preserve mitochondrial function.</u>

1) Ketones (βHB and ACA) are utilized by mitochondria to produce Krebs cycle intermediates, which produce substrates for the ETC (NADH and $FADH_2$). This allows the mitochondria to produce sufficient energy, in the form of ATP, to maintain homeostasis. 2) The conversion of ketones into acetyl-CoA also produces succinate, which can be utilized by the Krebs cycle to produce energy. 3) However, succinate has also been shown to block HIF-1α prolyl-hydroxylases, which in turn stabilizes the levels of HIF-1α. This allows HIF-1α to dimerize with HIF-1β and travel to the nucleus, where it is responsible for the upregulation of proteins involved in angiogenesis, glucose uptake, glycolysis, and ketone uptake. 4) It is through the HIF-1 mediated upregulation of MCTs that we believed it is possible for increased ketone levels to increase their uptake into the cell for utilization as energetic substrates, as well as building blocks for (5) protein and lipid synthesis. 6) Oxygen is responsible for the activation of HIF-1α prolyl-hydroxylases, which alter HIF-1α, which allows the von Hipple-Lindau protein to target it for proteasome mediated degradation. ROS can also inhibit HIF-1α prolyl-hydroxylases, which may explain how HIF-1α expression is upregulated by injury. Ketone metabolism is more efficient than glycolysis in terms of enzymatic steps and ATP requirements. 7) Glycolysis requires 11 enzymatic steps, whereas ketone metabolism only requires 3 steps. However, because HIF-1 upregulates glycolytic enzymes the utilization of ketones by mitochondria could be shifting glycolytic intermediates into the (8) pentose pathways, which is responsible for the increase of NADPH levels and in turn support of the endogenous glutathione system.

mitochondrial function and reduce ROS production.

As the current management of adult patients with severe TBI generally includes fasting (i.e. use of i.v. normal saline without dextrose) for about 48 hours, our findings in this animal model support this approach, assuming appropriate monitoring for hypoglycemia. Our results also suggest the need for further exploration into the administration of alternative fuels at bench and bedside, and as such have begun to lay a preliminary mechanistic basis for the investigation of novel therapeutic interventions.

Chapter 3

UCP Mediated Free Fatty Acid Uncoupling of Isolated Cortical Mitochondria from Fasted Animals; Correlations to Dietary Metabolic Modulations

Introduction

Mitochondrial respiration is responsible for generating the majority of ATP used in normal cellular functions. This energy is produced by the electron transport chain (ETC), which translocates protons from the matrix to the intermembrane space, thereby creating a charge potential across the inner membrane ($\Delta\Psi$). Protons enter the F_1 subunit of ATPsynthase (Complex V), causing it to rotate, which then causes a conformational change in the F_o subunit thereby catalyzing the phosphorylation of ADP to ATP (Boyer 1997; Boyer 1999; Boyer 2000; Nicholls et al. 2000; Nicholls et al. 2002). These protons can only facilitate ATP production if the concentration of protons is higher within the intermembrane space than the matrix; and in fact the ATPsynthase can hydrolyze ATP if $\Delta\Psi$ reaches low levels in an attempt to recapitulate $\Delta\Psi$ (Sullivan et al. 2000). This process is the basis for oxidative phosphorylation or "cellular respiration", due to the resultant consumption of oxygen as the final electron acceptor (Nicholls et al. 2000; Nicholls et al. 2002). The maintenance of this system is critical to cellular survival, and its dysfunction ultimately leads to the initiation of cell death pathways (apoptosis/necrosis) (Sullivan et al. 2005).

The dysfunction of mitochondrial systems has been associated with many injury and disorder models, and has been described as the key cell death determinant in the recovery or loss of cellular integrity (Verweij et al. 1997; Xiong et al. 1997; Sullivan et al. 1998; Xiong et al. 1998; Cock et al. 2002; Sullivan et al. 2003; Tieu et al. 2003;

Brookes et al. 2004; Lifshitz et al. 2004; Patel 2004; Sullivan et al. 2005; Xiong et al. 2005). It has also been suggested that the preservation of mitochondrial function would thereby increase the ability of the cell to repair damage caused by these insults (Scheff et al. 1999; Sullivan et al. 1999; Lifshitz et al. 2004; Pandya et al. 2007). The perpetrators of a great deal of this damage are the highly volatile reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can oxidize proteins, lipids, and DNA (Nicholls et al. 2000; Brookes et al. 2004; Hall et al. 2004; Singh et al. 2006; Singh et al. 2007). These ROS/RNS are caused by increased electron back up within the ETC, which prolongs the reduction time of ETC intermediates thereby increasing the probability of electron escape and combination with oxygen or nitrogen.

Specific proteins within the mitochondrial inner membrane have recently been implicated in an endogenously regulated anti-ROS system, thought to function primarily to protect the brain from oxidative stress (Garlid et al. 1998; Klingenberg 1999; Richard et al. 2001; Echtay et al. 2002; Echtay et al. 2003; Mattiasson et al. 2003). These uncoupling proteins (UCPs) function to uncouple proton translocation from the phosphorylation of ADP; and in doing so alleviating the electron congestion within the electron transport chain (Negre-Salvayre et al. 1997; Echtay et al. 2002; Echtay et al. 2003; Sullivan et al. 2003). UCPs are upregulated through the peroxisome proliferator activating receptor (PPAR) pathway in response to shifts in metabolic substrate utilization. PPARs are members of the hormone receptor super family and are mainly involved in the regulation of lipid metabolism. (Staels et al. 1997; Pineda Torra et al. 1999; Debril et al. 2001; Kiec-Wilk et al. 2005). UCPs can be actively involved in lipid breakdown in both liver and adipocytes and their expression is upregulated by fasting,

possibly through the formation of free fatty acids via beta oxidation (Sullivan et al. 2003). UCPs are also believed to be activated by certain naturally occurring FFA derivatives; as well as endogenously synthesized free fatty acids (FFA), which are simultaneously released as a byproduct of triglycerides breakdown in adipocytes (Debril et al. 2001; Li et al. 2002; Grav et al. 2003; Sullivan et al. 2004; Shi et al. 2005).

The high fat/low carbohydrate ketogenic diet (KD) was originally developed as an attempt to mimic the physiological effects of fasting by inducing the production of ketone bodies, which have been suggested to be the neuroprotective mechanism of both fasting and KD (Bailey et al. 2005; Davis et al. 2008). However, during the production of ketones, free fatty acids are also produced as a byproduct of triglyceride breakdown. UCPs have been shown to be upregulated in response to ketogenic diet administration and could be involved in the efficacy of this dietary modulation, although it is not fully understood which FFAs are responsible for activation (Sullivan et al. 2004). Therefore, the purpose of our study was to investigate fasting induced modulation of FFA, as well as the ability of a variety of different FFA to uncouple oxidative phosphorylation and reduce ROS production in isolated cortical mitochondria with upregulated UCPs. Here we are using FFAs ranging from 4-22 carbons in length with varying levels of saturation. The results of our study should give us some insight into how diet modulates the metabolic breakdown of lipids and what influence this has on mitochondrial function.

The results from this study have shown that UCP2 expression can be upregulated by fasting in both naïve and injured animals. We have also investigated the ability of FFAs of various chain lengths to activate UCP mediated uncoupling and ROS production. As UCPs and their activation seem to be an integral part of fasting induced

neuroprotection we also have shown the upregulation of FFAs after fasting in serum and brain tissue. Collectively, our work has attempted to shed light on the role of specific FFAs within the UCP mediated uncoupling mechanism, as a potential target for therapeutic supplementation after injury.

Methods

Animals

All experiments were conducted using adult male Sprague-Dawley rats, which were housed 3 per cage in a facility which maintained a 12-hour light/12-hour dark cycle. All experimental animal procedures were approved by the University of Kentucky Animal Care and Use Committee. All animals used for mitochondrial isolation were fed a balanced diet *ad libitum* until 24hrs before mitochondrial isolation at which time they were fasted for 24hrs.

Mitochondrial Isolation

Cortical mitochondria were isolated from rats by differential centrifugation techniques as described previously in chapter 2. Prior to isolation naïve animals were fasted for 24 hrs in order to upregulate UCPs for FFA screen.

Western Blotting

For these studies a selective antibody for UCP2 (Everest Biotech, Oxfordshire, UK) was utilized to determine expression levels in isolated cortical mitochondria from fasted naïve, unfasted naïve, unfasted injured, and fasted injured animals. A protein assay

kit was used prior to loading (Pierce Rockford IL 61105), and samples were prepared as previously stated (Sullivan et al. 2004). The blots were incubated with primary goat anti-UCP2 and mouse anti-VDAC antibodies diluted 1:1000 and 1:10,000 respectively. Anti-goat (800nM) and conjugated goat anti- mouse IgG (700nM) (Rockland Immunochemicals Inc, Gilbertsville, PA) antibody were used at 1:25,000 and 1:10,000 respectively. Blots were subsequently imaged on a LiCore Odyssey imaging machine. Fluorescence values for UCP2 expression were divided by the corresponding VDAC values to control for protein concentration variability. Values are expressed as UCP2/VDAC in arbitrary units (AU).

Mitochondrial Respiration

After performing a BSA protein assay, a 30ug sample of mitochondria was placed in 250µL of KCL respiration buffer (125mM KCl, 2mM MgCl, 2.5mM KH_2PO_4, 20mM HEPES) inside a constantly stirred thermo-regulated sealed Oxytherm chamber. Oxygen within the chamber was measured using a Clark-type oxygen electrode. Oxygen consumption in response to the addition of substrates was measured in order to determine functionality of the mitochondria, along with the ability of the different FFA to uncouple this respiration from ATP production. Pyruvate (5mM) and Malate (2.5mM) were added in order to prime the Krebs cycle and build membrane potential. ADP (150µM) was added to assess oxidative phosphorylation capacity, which is an indication of the health of the electron transport chain. The mitochondria were then locked into state IV respiration by addition of oligomycin, an ATP synthase inhibitor; to create a baseline rate of oxygen consumption due to inner membrane leakage so that any increase in oxygen

consumption would be the result of uncoupling via FFA. Final concentrations of 60µM of FFAs of varying chain length were added after oligomycin during separate runs to activate UCPs. BSA (.6%) was added to sequester the exogenous FFAs to bring respiration back to baseline, and to assess any damage that the FFA may have caused to the mitochondrial ETC. "Good UCP activators" were characterized by FFAs that were able to both significantly increase respiration upon their addition and when sequestered the rate of respiration returned to baseline. For statistical purposes the increase in rate as a result of the addition of FFA as well as the rate resulting from the addition of BSA were expressed as % of the rate resulting from the addition of oligomycin (% of state IV). These rates were compared to each other using a student's t-test.

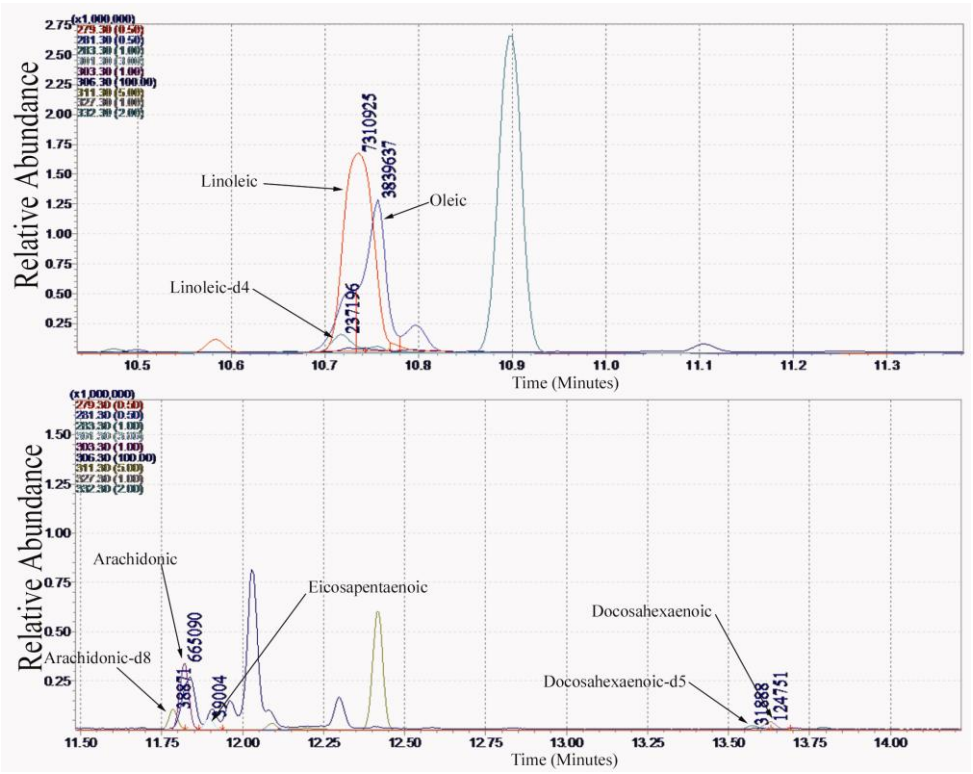
ROS Assay

Isolated cortical mitochondria from fasted animals were used to asses the ability of FFAs to attenuate ROS production in the presence of an electron transport chain complex inhibitor (Oligomycin). DCF (2, 7-dihydrodichlorofluorescein 10µM) and HRP (Horse Radish Peroxidase 10µM) were used as indicators of ROS production. A concentration of 60µM was used for each FFA in 50µL of KCl respiration buffer containing Pyruvate and Malate. Half of the samples were co-incubated with oligomycin so that the reduction of ROS would be correlated to the activation of UCPs instead of the activity of complex V. These samples were compared to their respective control samples, either with or without FFA, and expressed as % increase in ROS of control.

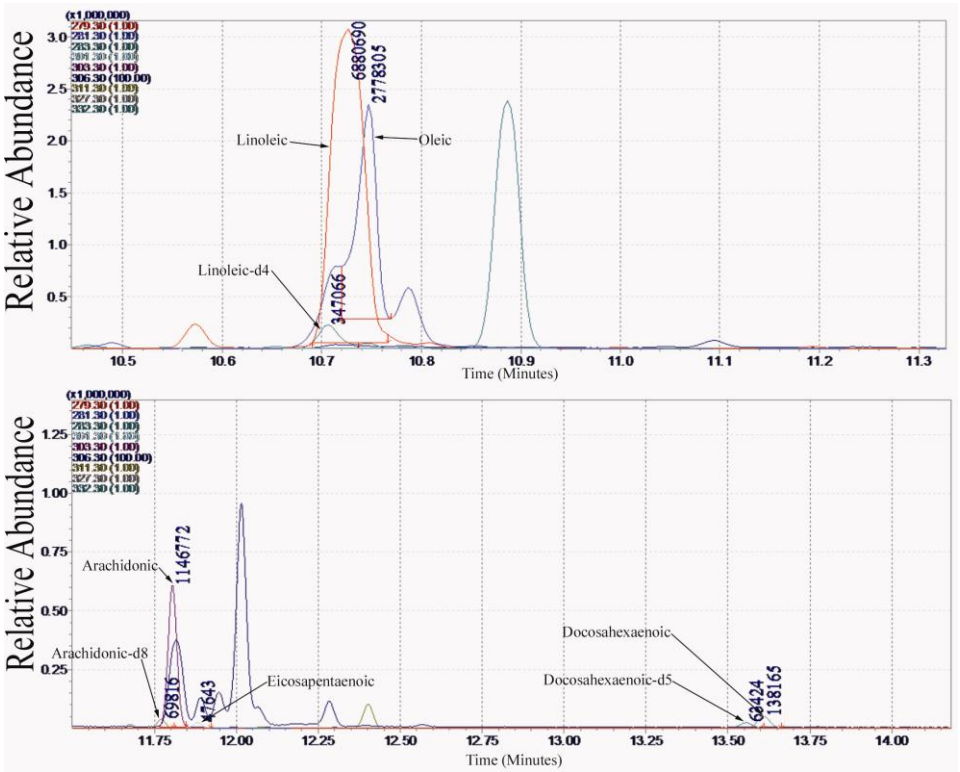
Gas Chromatography/Mass Spectrophotometry

Samples were taken from a separate cohort of animals that were either fasted or fed *ad libitum* for 24 hrs. Serum samples were taken at both 0hrs and 24 hrs, whereas CSF and Brain tissue were collected at 24hrs only. FFAs from serum samples and CSF were brought to an approximate pH of 2 with HCl. The samples were supplemented with Linoleic acid D_4, Arachidonic Acid (AA) D_8, and Docosahexaenoic acid (DHA)-D_5. Ethyl Acetate was then added as an extraction medium. The sample was vortexed for ~30 seconds and centrifuged at 2,000 rpm for 10 minutes. The organic layer was then removed and poured over Sodium Sulfate ($NaSO_4$), to remove residual water. This Ethyl Acetate extraction was then repeated and the organic layer was again poured over $NaSO_4$. The organic layers from each extraction step were then combined and the samples were evaporated under N_2 at 37°C. Samples were then reactive with 10% pentaflourobenzyl bromide (PFBB) and 10% di-isopropylethylamide (DIPE) for 20 minutes at 37°C. After the samples were brought to room temperature they were evaporated under N_2 at 37°C. Undecane was added to dissolve the extracts for injection on to the GC/MS. See figure 3.1 for an example GC/MS chromatogram.

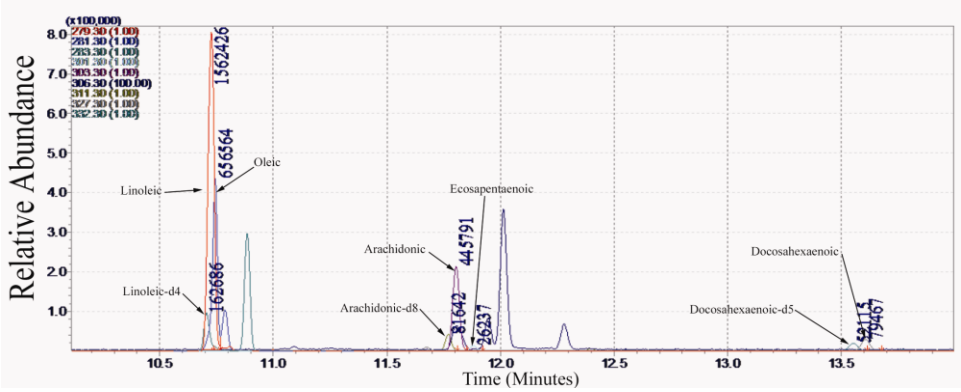
A .


Relative Abundance
Time (Minutes)
Linoleic
Oleic
Linoleic-d4
7310925
3839637
237196
Arachidonic
Eicosapentaenoic
Docosahexaenoic
Arachidonic-d8
Docosahexaenoic-d5
665090
38871
39004
31888
124751

B.


Relative Abundance
Time (Minutes)
Linoleic
Oleic
Linoleic-d4
6880690
2778305
347066
Arachidonic
Eicosapentaenoic
Docosahexaenoic
Arachidonic-d8
Docosahexaenoic-d5
1146772
69816
47643
60424
138165

C.



D.

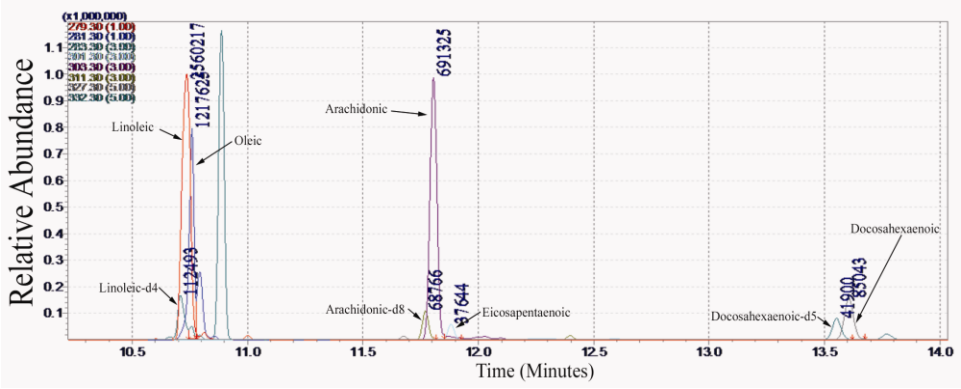


Figure 3.1 **Serum GC/MS chromatograms from a Fasted and Control animals**
This figure shows a representative trace for the serum GC/MS FFA content of Fasted and Control animals. A) Control baseline trace (0hr) B) Fasted baseline trace (0hr) C) Control 24hr trace D) Fasted 24hr trace. A and B were cut into 2 panels for ease of viewing.

Brain samples (~100mg) were homogenized in 10mL of cold Folch solution (CHCl3:CH3OH, 2:1 ratio), and brought to ~pH 2. Nitrogen was introduced and samples were uncubated for 30 minutes at room temperature. 4mLs of Normal Saline (0.9%) was added and samples were centrifuged at 2,000 rpm for 10 minutes. The organic layer was taken off and poured poured over Sodium Sulfate ($NaSO_4$), to remove residual water. This layer was then supplemented with Linoleic acid D_4, Arachidonic Acid (AA) D_8, and Docosahexaenoic acid (DHA)-D_5, and subsequently evaporated under N_2 at 37°C. The

ethylacetate extraction procedure was then followed as previously stated. Undecane was added to dissolve the extracts for injection on to the GC/MS.

Statistical Analysis

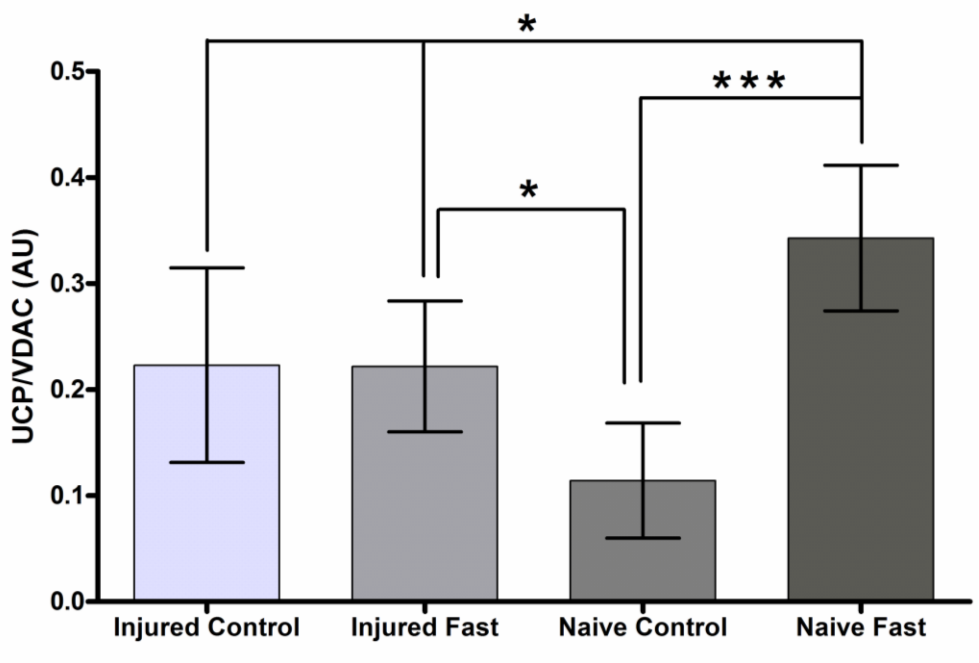
For respiration studies independent t-tests were used to determine the significance between FFA and BSA addition. ROS and Western blot studies used a One-way ANOVA with a student-newman-keuls post hoc when appropriate. GC/MS studies used paired (serum) and unpaired t-tests (brain and CSF) when appropriate. Significance was set at $p<0.05$.

Results

Uncoupling Protein 2 Expression

Our representative western blot shows that fasting increases the expression of UCP2, which is in accordance with stated literature that these proteins are upregulated in a fasted state (Li et al. 2002; Grav et al. 2003; Sullivan et al. 2004). Injury alone did not induce a significant increase UCP2 expression; however, injured fasted animals did show significantly increased mitochondrial UCP2 expression (Figure 3.2). Interestingly both injury groups were significantly decreased from fasted naïve levels, indicating that UCP2 expression is down regulated in response to injury. Injured control expression was not significantly different from either fasted injured or naïve control levels (Figure 3.2). This implies that fasting induces upregulation and activation of the UCP system, which would further implicate it in the neuroprotective mechanism of fasting. Additionally, this

A.



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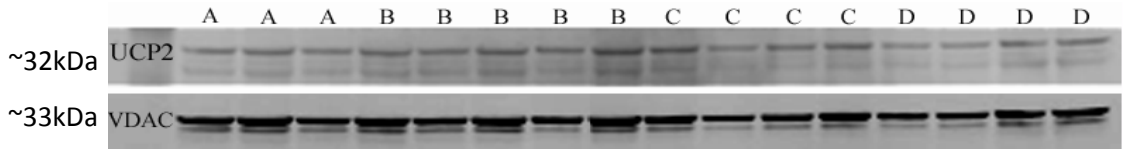

Figure 3.2 <u>Mitochondrial UCP2 expression is modulated after fasting and injury.</u>
A) Fasted animals showed significantly increased UCP2 protein expression levels compared to injured fasted, injured control, and naïve control animals. Naïve control animals showed significantly decreased UCP2 expression compared to both naïve and injured fasted animals. Injured control expression was not significantly different from injured fasted or naïve control levels. The UCP2 fluorescence level of each sample was divided by its corresponding VDAC fluorescence level as an internal control for protein concentration. Expression levels are expressed as arbitrary units (AU). (Injured Control n=3, Injured Fast n=5, Naïve Control n=4, Naïve Fast n=5) ANOVA $p<0.05$ $F_{3,\ 16}=8.5$, $R^2=0.66$, SNK $^{*}p<0.05$ $^{***}p<0.001$. B) Representative western blot, each band is at 32kDa. A= Injured Control, B= Injured Fasted, C= Naïve Control, D= Naïve Fasted.

indicates that we can use a fasting paradigm to up regulate UCP2 as a means to study the effects of FFA administration on mitochondrial respiration and ROS production.

Free Fatty Acid UCP Activation

It has been shown that both ROS and FFAs must be present in order to activate UCPs and that the duration of this activation is dependent upon the level of each of these

factors (Echtay et al. 2001; Echtay et al. 2002; Echtay et al. 2003). It is also known that the addition of oligomycin to isolated mitochondria causes increased ROS production due to maximization of membrane potential via the blockage of complex V (ATPsynthase) (Sullivan et al. 2003). Based upon this information, we used a 24hr fasting paradigm to upregulate UCPs in order to screen FFAs of different chain lengths and saturation states to determine their effect on uncoupling induction (Table 3.1 and Figure 3.3).

FFA	Carbons	Dbl Bonds
Butyric	4	0
Capronic	6	0
Octanoic	8	0
Valproic	8	0
Decanoic	10	0
9-Decanoic	10	1
Lauric	12	0
Cis-5-Doecenoic	12	1
Myristic	14	0
Myristoleic	14	1
Pentadecanoic	15	0
Palmitic	16	0
Palmitoleic	16	1
Heptadecanoic	17	0
Cis-10-Heptadecanoic	17	1
Linoleic	18	2
Oleic	18	1
Stearic	18	0
Arachidonic	20	4
Eicosapentaenoic	20	5
Docosahexaenoic	22	6

Table 3.1 Names and chain length of FFAs screened.
This table depicts the chain length and saturation state of each of the FFAs we screened for their ability to induce UCP mediated activation of respiration and ROS reduction.

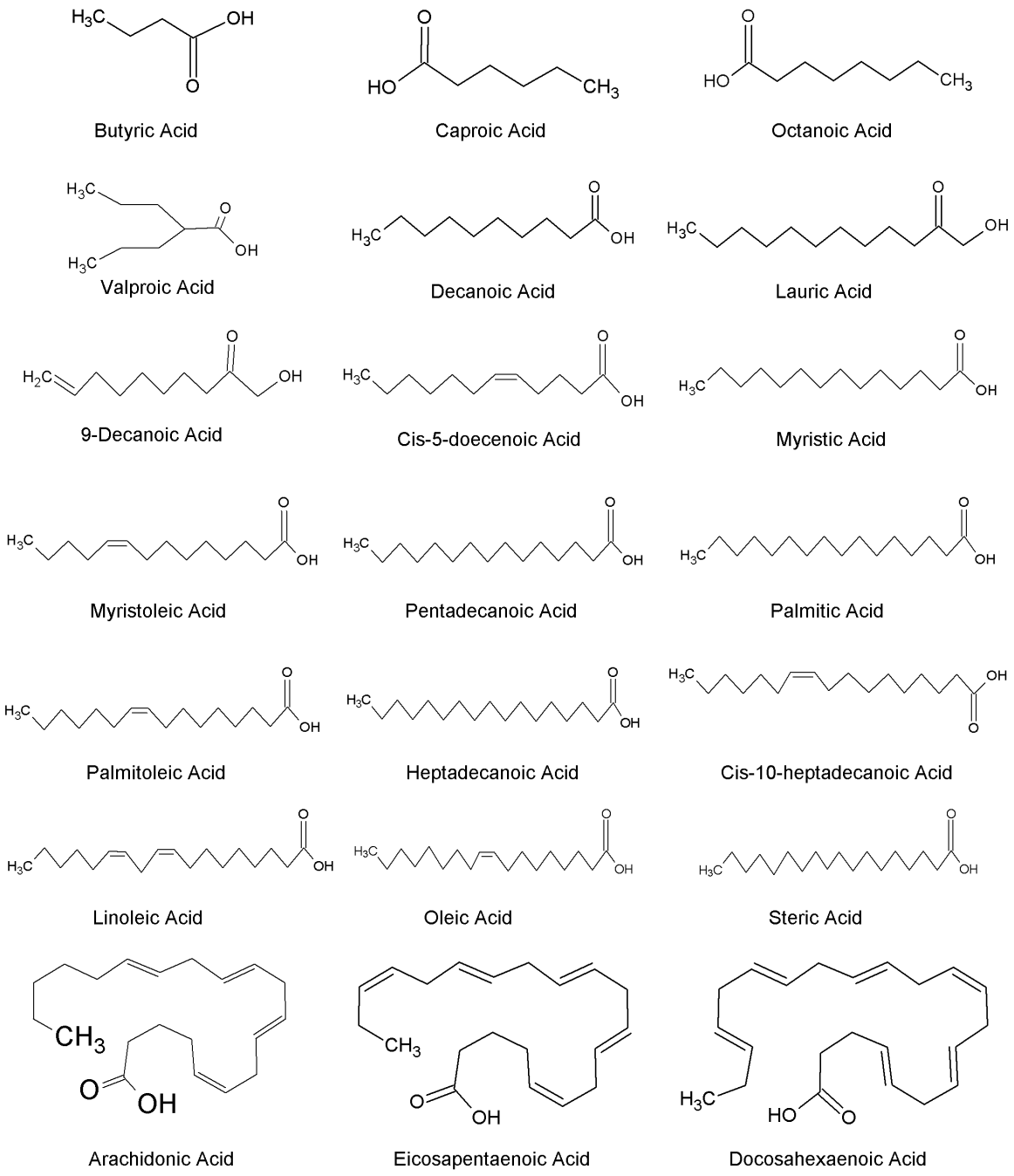

Figure 3.3 <u>Structures of individual FFAs screened.</u>
This figure depicts the individual structures of all FFAs tested in our studies. Pictures were created using ADC/ChemSketch Freeware, Advanced Chemistry Development, Inc.

Our results show that chain length is not an indicative factor in determining the ability of a FFA to activate UCPs, nor is saturation, within the scope of this study (Figure 3.4). In fact, we found FFAs that uncoupled mitochondria throughout the chain length spectrum. Palmitoleic (C16), Myristic (C14) and Butyric (C4) acid were all able to uncouple mitochondria effectively upon addition and with BSA addition returned to state IV levels; thereby indicating minimal damage to the inner membrane (Figure 3.4). DHA, EPA and AA also exhibited similar behavior upon addition to isolated mitochondria from fasted animals (Figure 3.4). Although many of the other FFAs we tested were able to increase

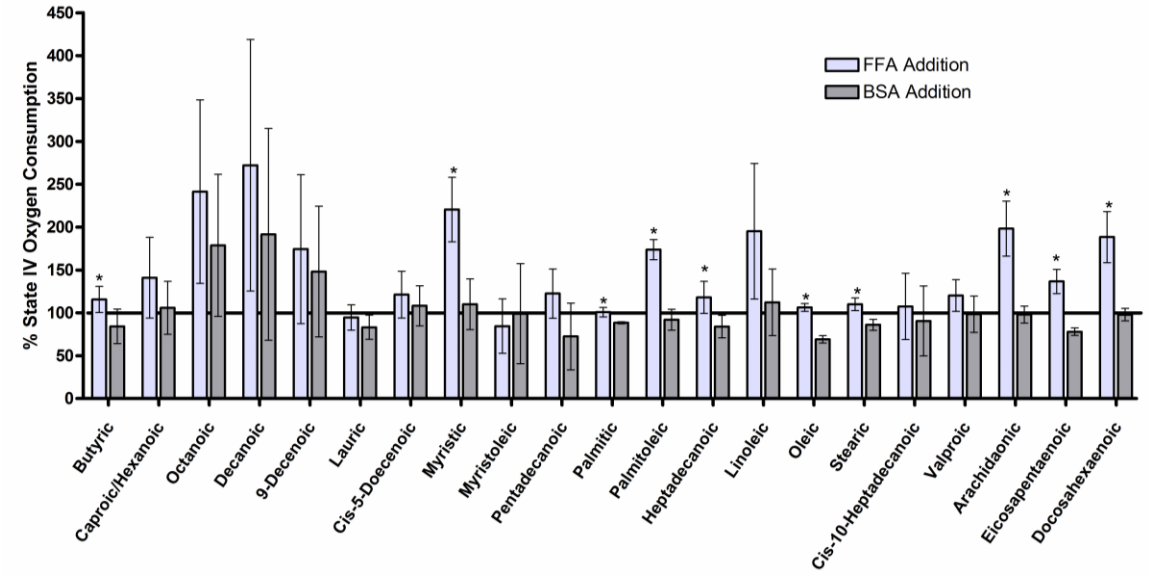

Figure 3.4: <u>Free fatty acids activate UCP mediated uncoupling of mitochondria.</u> Here we show the ability of various FFA to activate uncoupling in mitochondria isolated from fasted animals. Each FFA "change over state IV" was compared via t-test to its respective "recovery to state IV" rate. When we screened fatty acids for their ability to activate UCP mediated uncoupling we found no correlation between chain length/saturation and increased respiration. Butyric (t_6=2.5), Myristic (t_8=5.2), Palmitoleic (t_4=8.4), Heptadecanoic (t_6=3.0), Oleic (t_4=10.0), Stearic (t_6=1.5), Arachidonic (t_4=5.2), Eicosapentaenoic (t_4=6.8), and Docosahexaenoic (t_4=5.1) acids showed significant differences between the increase from state IV rate upon their addition and the recovery to state IV rate upon addition of BSA. This indicates that they are not damaging the membrane or membrane bound proteins of the ETC. *p<0.05 SD.

oxygen consumption after addition, BSA addition was not able to bring the rate of respiration back to basal state IV levels, which means that they were not "good UCP activators" and may have been damaging the inner or outer membrane and/or membrane bound proteins that comprise the ETC. Others showed a significant difference between FFA rate and BSA rate, however, the increase in rate was not increased above 100% of state IV, and thus we cannot call them "good UCP activators". Interestingly, Valproic acid, which has been implicated as a protective agent in seizure models, did not exhibit an ability to significantly uncouple mitochondria when compared to its BSA rate (Figure 3.4). This seems to indicate that Valproic acid is involved in an alternative mechanism of seizure attenuation. Although oleic (C18), Heptadecanoic (C17), and Stearic (C18) acid also showed marginal increases above 100% of state IV, but significant differences between FFA and BSA respiration rates, they could, in fact, be contributing to UCP mediated ROS reduction (Figure 3.4).

Free Fatty Acid Attenuation of Reactive Oxygen Species

In order to study the ability of FFAs to reduce ROS production we have utilized oligomycin due to its ability to maximize ROS production in isolated mitochondria, thereby allowing us to attribute the reduction of ROS production to the activation of UCPs. We co-incubated FFAs and isolated cortical mitochondria from fasted animals to determine their ability to attenuate ROS via UCP activation in the presence of a complex V inhibitor (Oligomycin). The FFAs selected for this either had been classically shown to be reducers of ROS or were shown to function as "good UCP activators".

In correlation with our respiration studies in which Valproic acid failed to increase the respiration rate above state IV, it also failed decrease to ROS production in the presence of oligomycin (Figure 3.5). Similarly, Cis-10-heptadecenoic, linoleic, heptadecanoic, palmitic, and stearic acid were also unable to attenuate ROS production (Figure 3.5). All of these FFA did show some modulation of mitochondrial respiration, however they were either not "good UCP activators" or did not increase respiration above state IV, all of which would indicate that they would not be able to attenuate ROS production through activation of UCPs thereby validating our assay. Although myristoleic acid was a robust activator of UCP mediated uncoupling and showed a 50% decrease in ROS production, due to high variability this level was not a significant decrease (Figure 3.5).

DHA, EPA, AA, Oleic, Myristic, Butyric, and Palmitoleic acid all showed significant decreases in ROS production, which is in agreement with their respective respiration studies(Figure 3.5). Interestingly, Oleic acid only increased respiration to a small degree, which suggests that its ability to attenuate ROS production could either be through a limited activation of UCPs or an alternative mechanism.

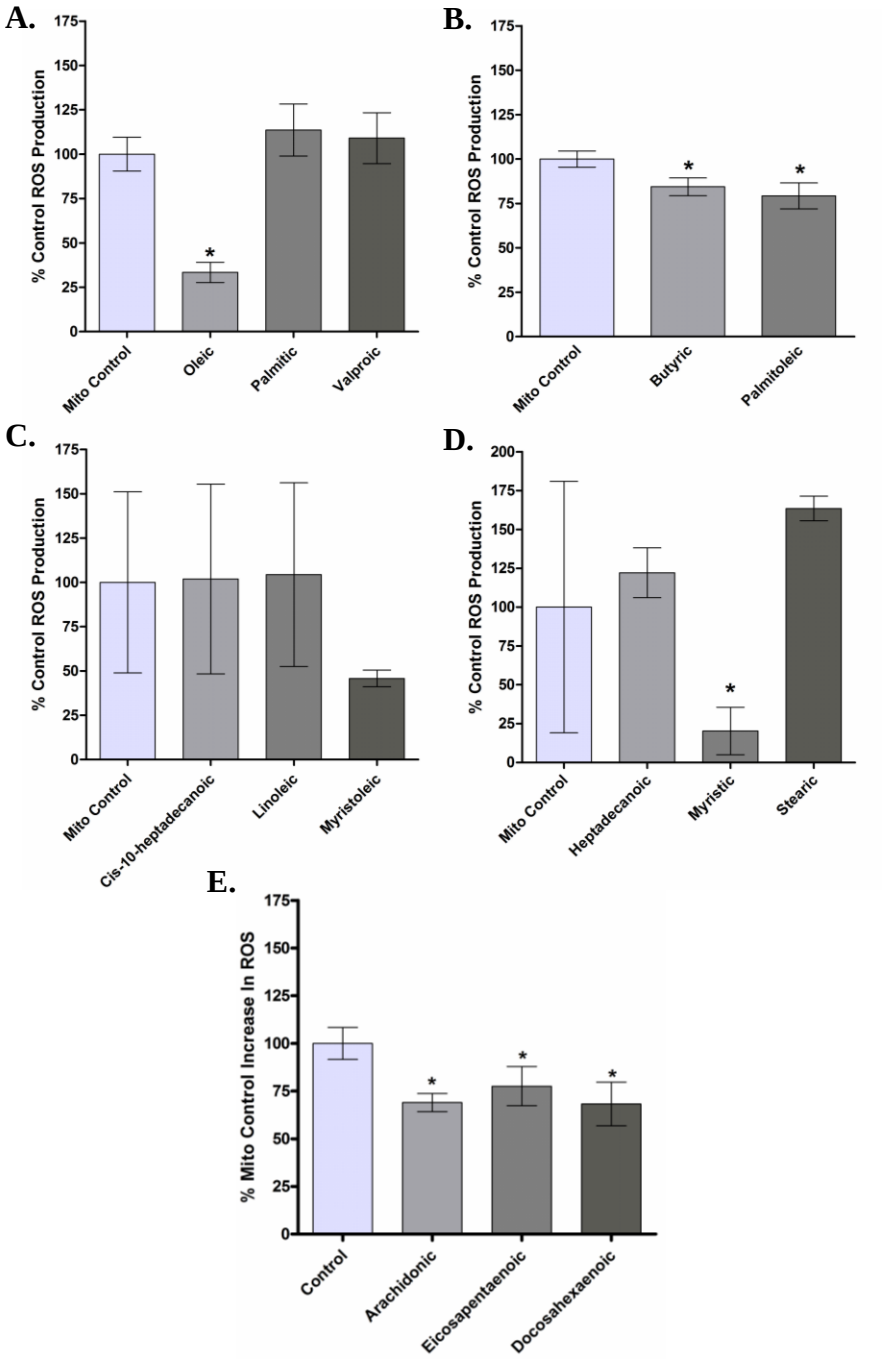

Figure 3.5 <u>Free fatty acids attenuate ROS production in isolated mitochondria</u>
Here we tested FFA from our respiration studies which were either "good activators of UCPs" or have been classically associated with antioxidant properties. Our results show that Myristic, Oleic, Butyric, and Palmitoleic significantly reduced ROS production. Of these, all significantly increased respiration, however Oleic did not seem to increase respiration very far above 100%. Myristoleic also reduced ROS production by 50%, however was not significant. ANOVA SNK A) $F_{3, 15}$=41 R^2=0.91; B) $F_{2, 8}$=11 R^2= 0.78; C) $F_{3, 11}$= 1.2 R^2= 0.30; D) $F_{3, 15}$= 6.3 R^2= 0.61; E) $F_{3, 11}$= 8.0 R^2= 0.75; *p<0.05 SD.

Fasting Induced Changes in Free Fatty Acid Levels *in Vivo*

It is known that fasting induces a metabolic shift to the upregulation of FFAs, which are used for various cellular functions. As our previous data has shown, they play a role in UCP activation and subsequent UCP mediated ROS reduction. In the aforementioned studies we used a broad range of FFAs, in terms of chain length and saturation. Unfortunately it is not known which FFAs are increased during fasting, so it is difficult to correlate our in vitro mitochondrial studies as a possible mechanism of fasting induced neuroprotection. Therefore, we measured the upregulation of five of our most probable FFAs (Linoleic, Oleic, Arachidonic, Eicosapentaenoic, and Docosahexaenoic) from our respiration and ROS studies to probe for in serum, brain tissue, and CSF of adult male Sprague Dawley rats.

Serum

In order to determine the levels of FFAs in serum we used both a control and fasted group, which included both a baseline serum sample and 24hr serum sample. Each of the groups 24hr samples, which are expressed as % of control level (baseline), were compared to their respective baselines using a paired t-test. This would allow for an accurate measure of increases within each animal as a result of dietary modulation. Control animals, those not fasted for 24hrs, did not show an increase in any of the measured FFA, indicating that any increase in these FFAs would be due to the implementation of fasting (Figures 3.6-3.10).

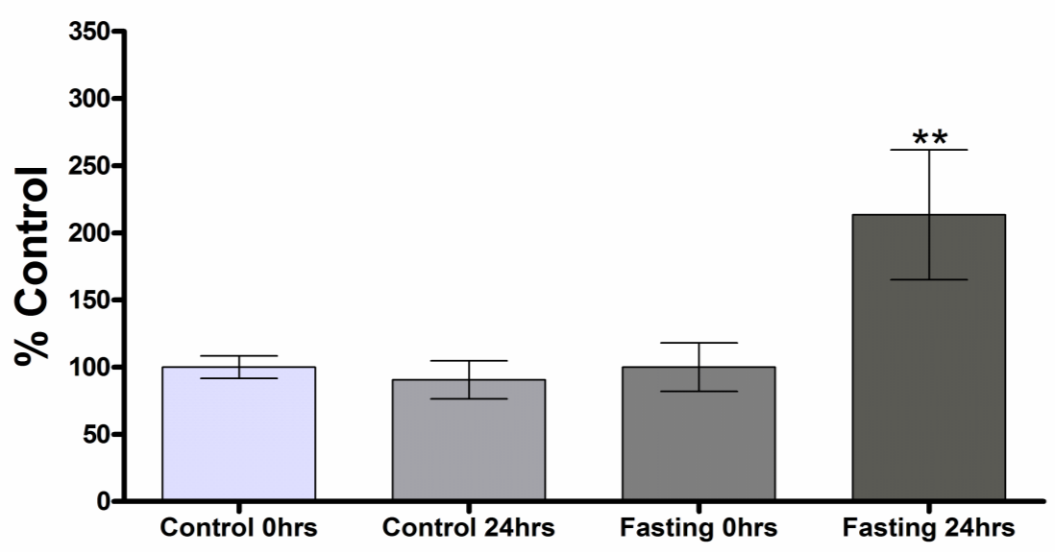

Figure 3.6 <u>The effect of fasting on serum levels of Linoleic acid.</u>
Animals fasted for 24 hrs showed a significantly increased level of serum Linoleic acid compared with 0hr baseline levels (t_4=4.5). There was no change in the levels of Linoleic acid in serum from unfasted control animals (t_3=1.1). Paired Student's t-test **p<0.01, n=4 (Control), 5 (Fasted) SD.

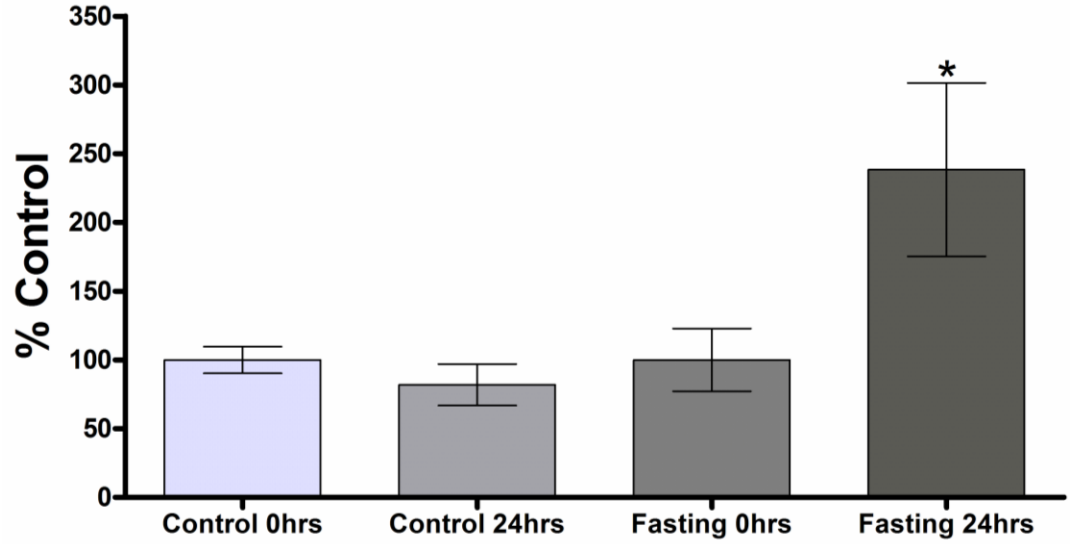

Figure 3.7 <u>The effect of fasting on serum levels of Oleic acid.</u>
Animals fasted for 24 hrs showed a significantly increased level of Oleic acid in serum compared with 0hr baseline levels (t_4=4.0). There was no change in the levels of Oleic acid in serum from unfasted control animals (t_4=1.9). Paired Student's t-test *p<0.05, n=5 SD.

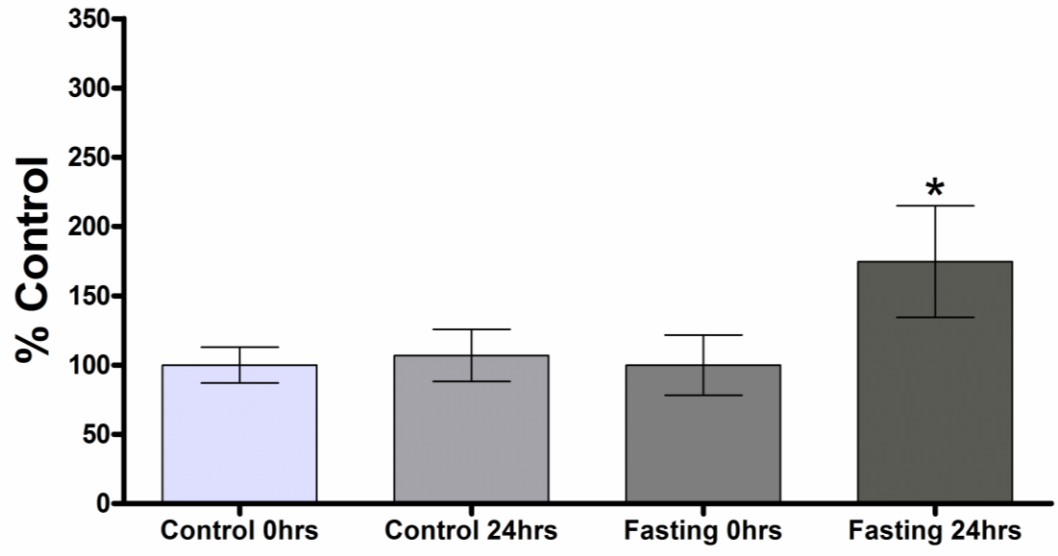

Figure 3.8 <u>The effect of fasting on serum levels of Arachidonic acid.</u>
Animals fasted for 24 hrs showed a significantly increased level of serum Arachidonic acid compared with 0hr baseline levels (t_4=2.9). There was no change in the levels of Arachidonic acid in serum from unfasted control animals (t_4=0.95). Paired Student's t-test *p<0.05, n=5 SD.

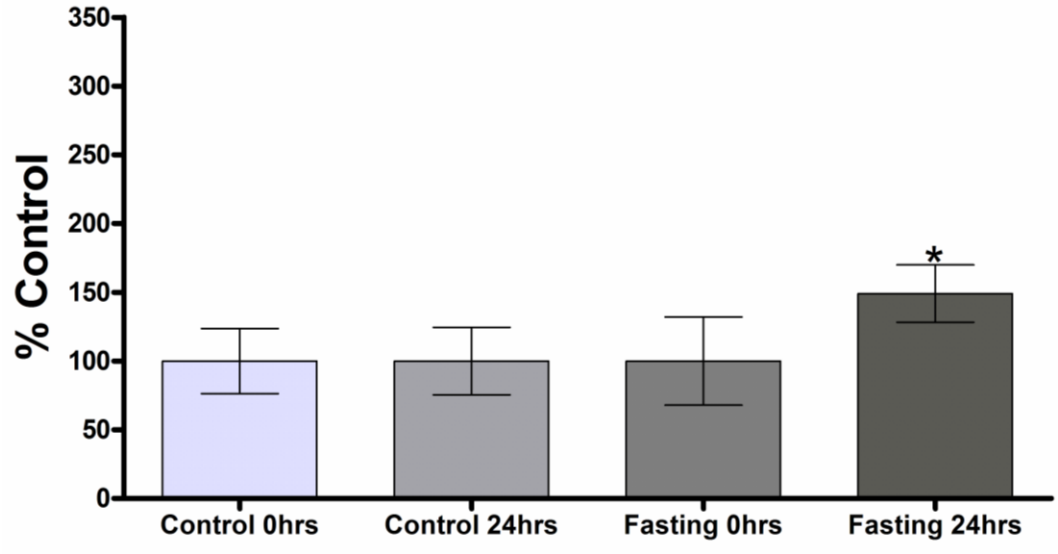

Figure 3.9: <u>The effect of fasting on serum levels of Eicosapentaenoic acid.</u>
Animals fasted for 24 hrs showed a trend toward an increased level of Eicosapentaenoic acid in serum compared with 0hr baseline levels (t_3=3.1). There was no change in the levels of Eicosapentaenoic acid in serum from unfasted control animals (t_4=0.305). Paired Student's t-test *p<0.05, n=4 (Fasted), 5 (Control) n=5 SD.

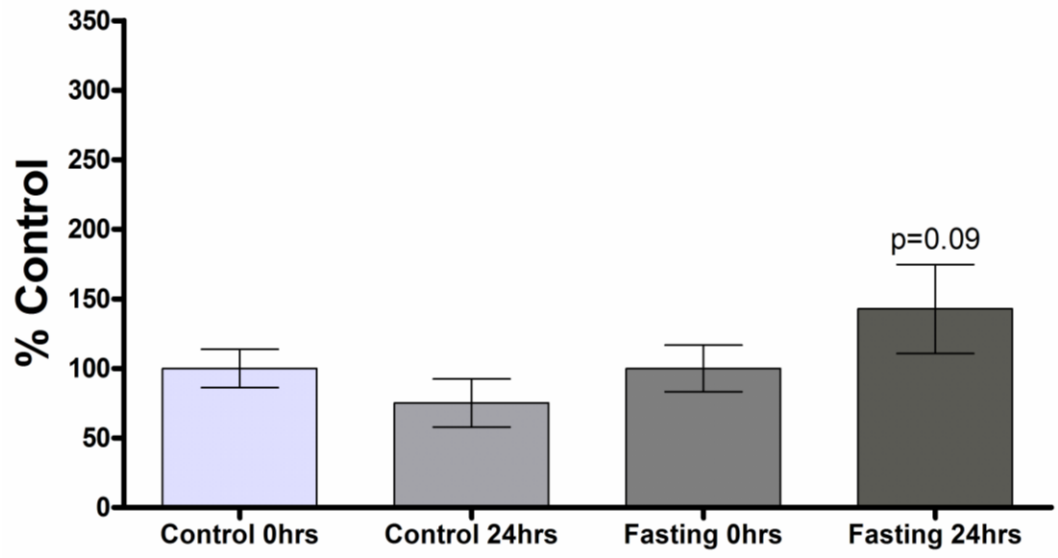

Figure 3.10 <u>The effect of fasting on serum levels of Docosahexaenoic acid.</u>
Animals fasted for 24 hrs showed a trend (p=.0974) toward an increased level of Docosahexaenoic acid in serum compared with 0hr baseline levels (t_4=2.2). There was no change in the levels of Eicosapentaenoic acid in serum from unfasted control animals (t_4=2.5). Paired Student's t-test n=5 SD.

Interestingly, although Linoleic acid did not show a significant increase in respiration or a decrease in ROS production, it was significantly increased in serum after 24hrs of fasting (Figures 3.6). Oleic, Eicosapentaenoic, and Arachidonic acids were also increased after a 24hr fast, which further suggests that they play role in fasting induced UCP mediated neuroprotection (Figures 3.7-3.8). Although Docosahexaenoic acid showed an increased level after 24hrs of fasting, this difference was not significant (Figures 3.10). However, the p value was 0.0974, which could indicate a trend in the upregulation of this FFA.

Brain and CSF

In order to determine the levels of FFAs in brain we used both a control and fasted group. However, because the harvesting of these samples inherently results in the

mortality of the animal we could only collect endpoint samples at 24 hours in each group. Therefore we have expressed our values as % Control, which was the unfasted group of animals, and have used an unpaired t-test to compare the changes of FFAs between groups.

As in our serum samples, Linoleic acid was increased in hippocampal and cortical tissue from animals fasted for 24hrs (Figure 3.11). In contrast to fasted serum samples, Oleic acid was not significantly increased in hippocampal tissue, but was increased in cortical tissue (Figure 3.12).Arachidonic acid also showed a significant increase in hippocampal tissue from fasted animals (Figure 3.13). EPA and DHA also remained unchanged in either brain region from fasted animals (Figures 3.14-3.15). CSF concentrations were unchanged for all of the FFAs measured in our study, which may indicate the uptake and utilization of these FFAs (Figures 3.11-3.15).

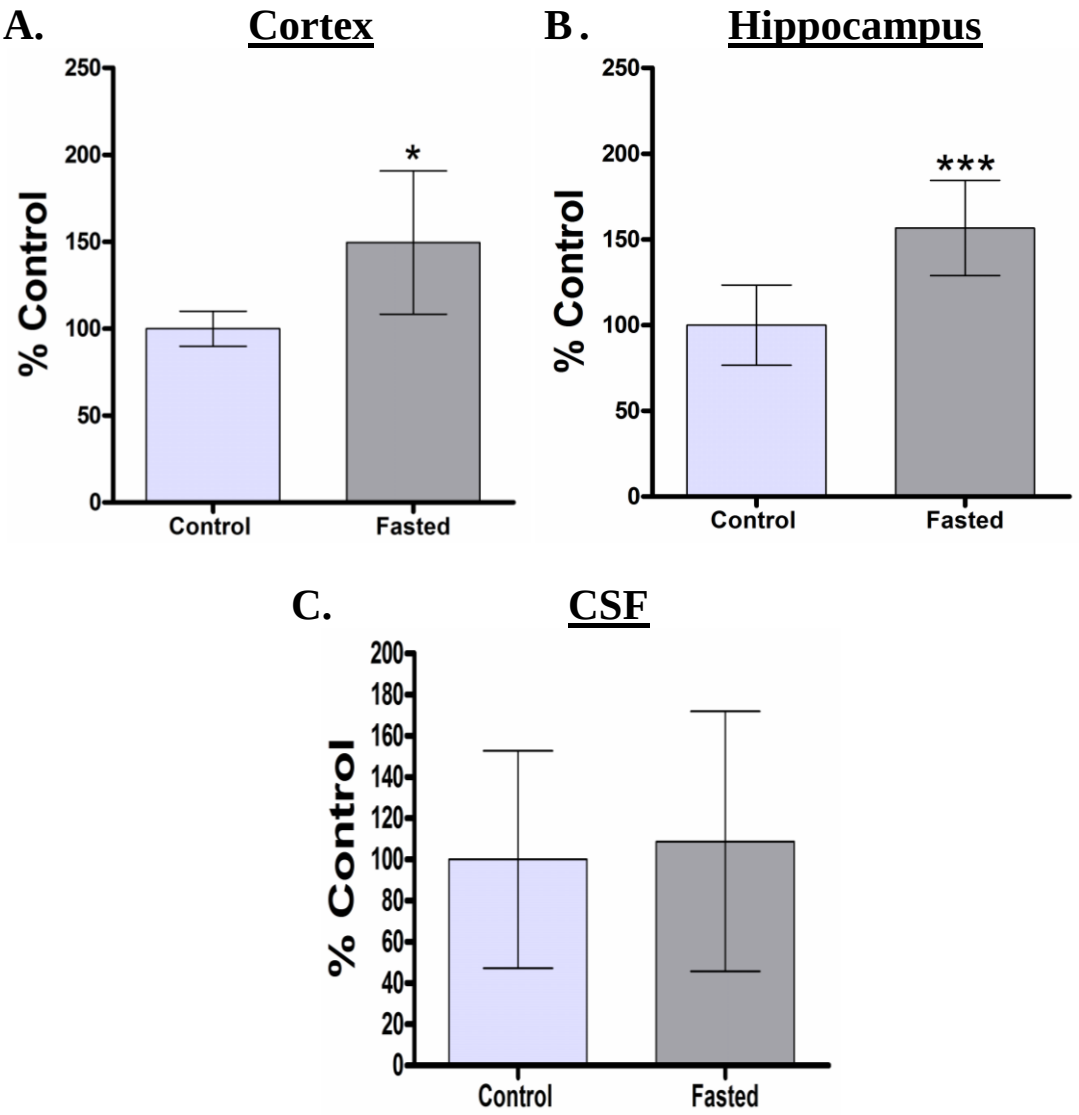

Figure 3.11 The effect of fasting on Linoleic acid levels in brain and CSF.
Animals fasted for 24 hours showed significantly increased levels of Linoleic acid in Cortical (t_8=2.6) and Hippocampal (t_7=3.3) tissue. However, these animals did not show significantly increased levels in CSF (t_6=0.21). Unpaired student's t-test, * $p< 0.05$, *** $p<0.001$ SD.

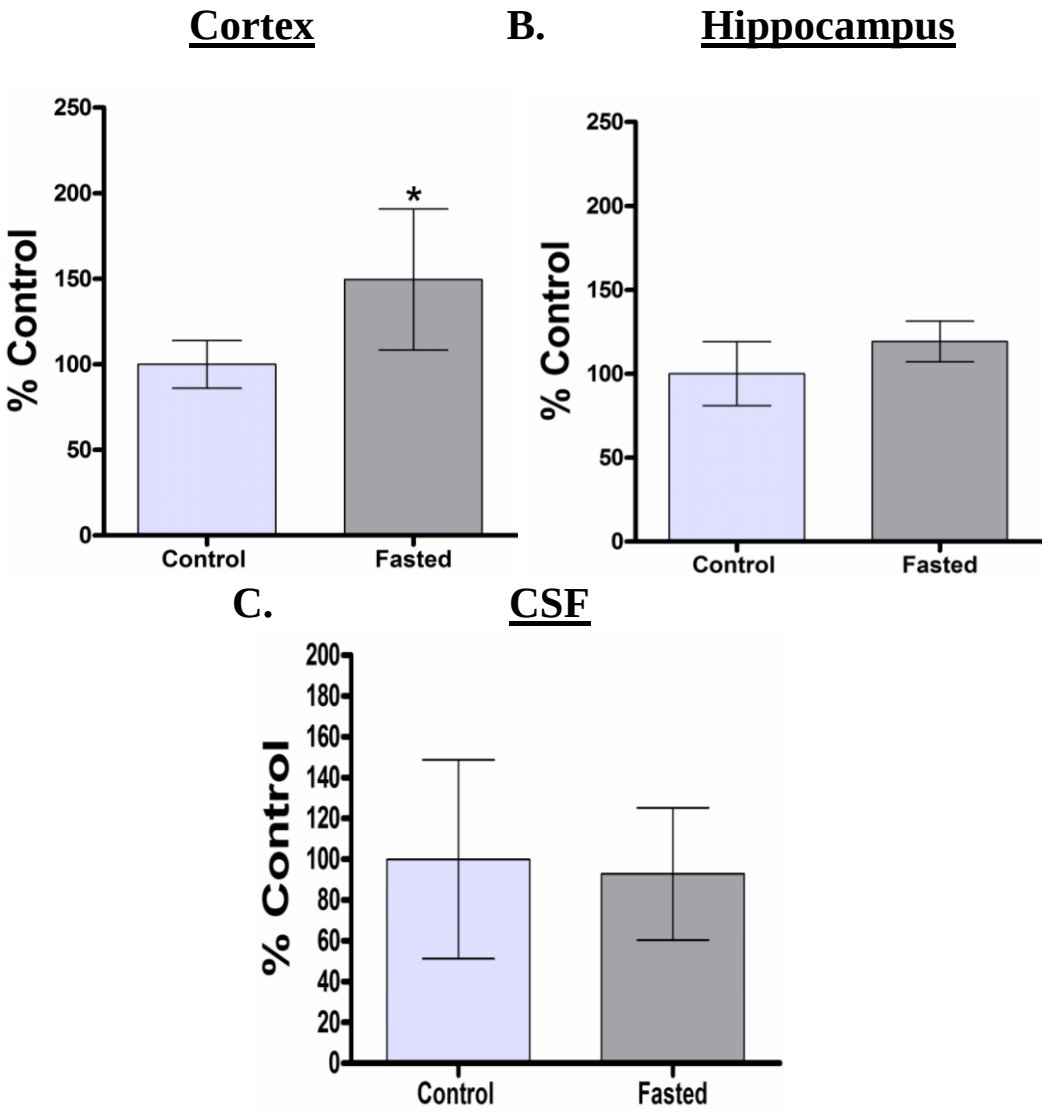

Figure 3.12 The effect of fasting on Oleic acid levels in brain and CSF.
Animals fasted for 24 hours showed significantly increased levels of Oleic acid in Cortical (A) tissue (t_8=2.5). However, these animals did not show significantly increased levels in Hippocampal (t_7=1.8) tissue (B) or CSF (C) (t_6=0.24). Unpaired student's t-test, * $p < 0.05$, SD.

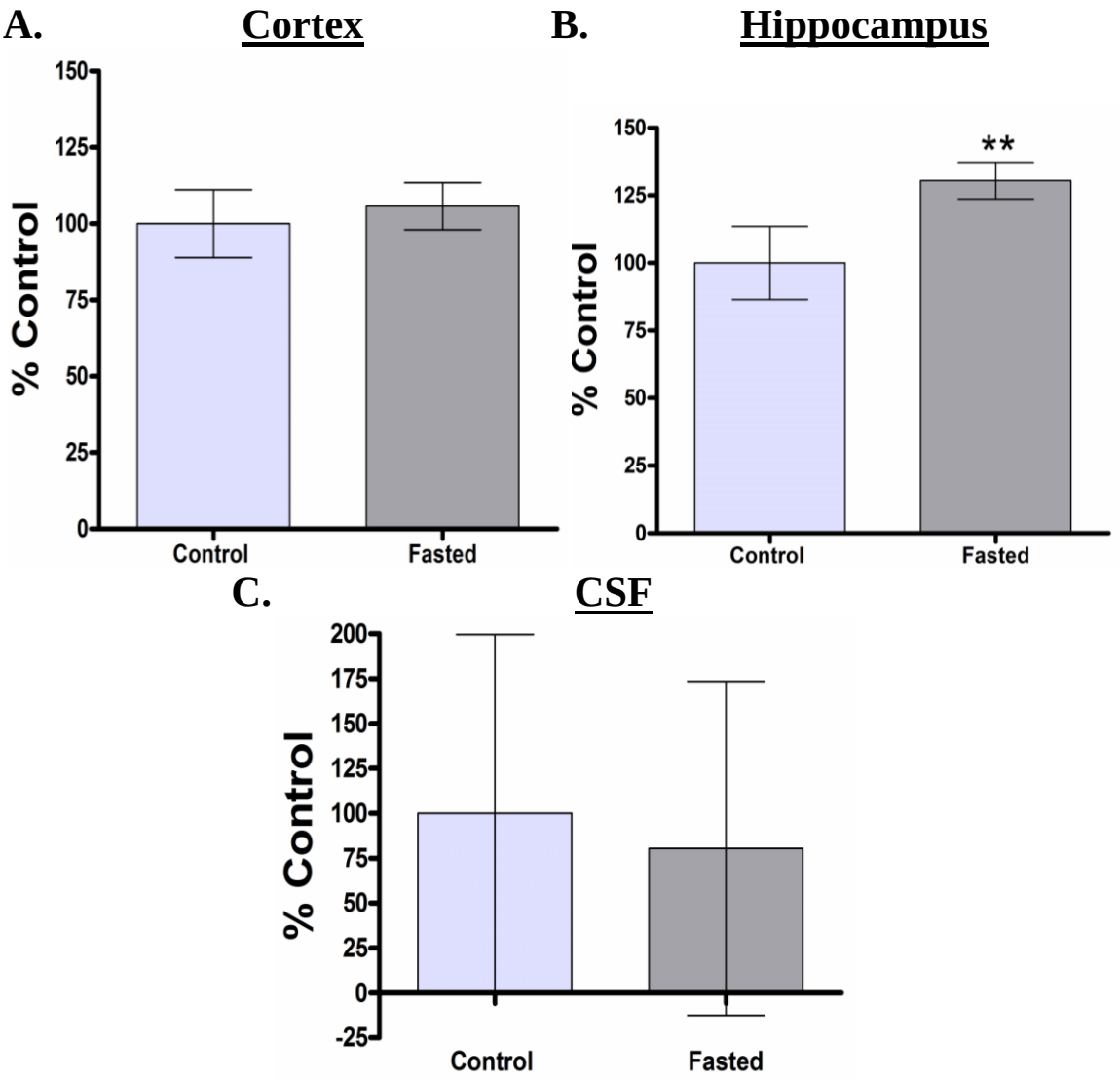

Figure 3.13 The effect of fasting on Arachidonic acid levels in brain and CSF.
Animals fasted for 24 hours showed significantly increased levels of Arachidonic acid in Hippocampal tissue (B) (t_6=4.0). However, these animals did not show significantly increased levels in Cortical tissue (A) (t_8=0.95) or CSF (C) (t_6=0.29). Unpaired student's t-test, ** $p < 0.01$, n=5 SD.

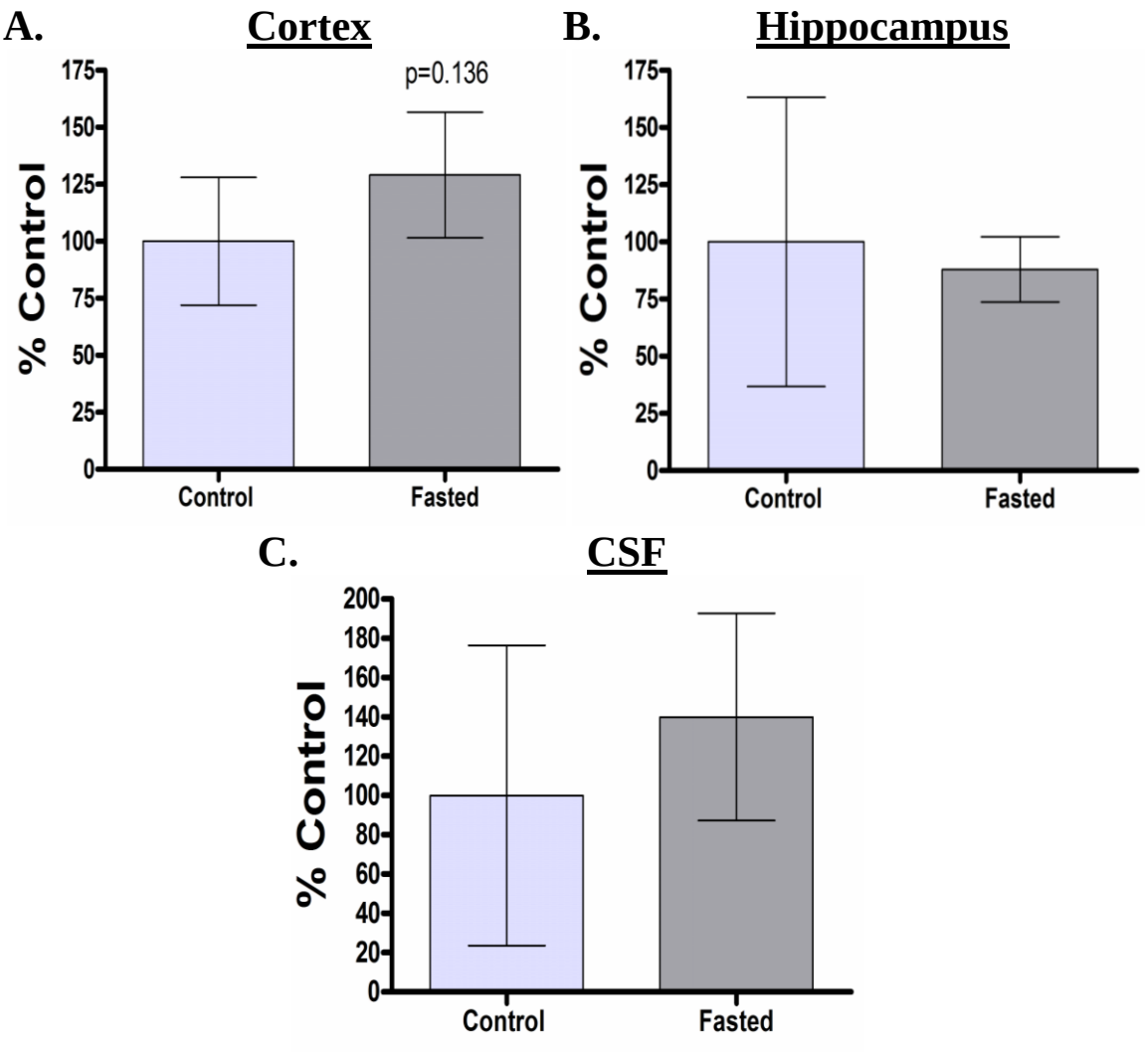

Figure 3.14 <u>The effect of fasting on Eicosapentaenoic acid levels in brain and CSF.</u> Animals fasted for 24 hours did not show significantly increased levels of Eicosapentaenoic acid in Cortical tissue (A) (t_8=1.7), Hippocampal tissue (B) (t_8=0.42), or CSF (C) (t_6=0.86). Unpaired student's t-test, n=5 SD.

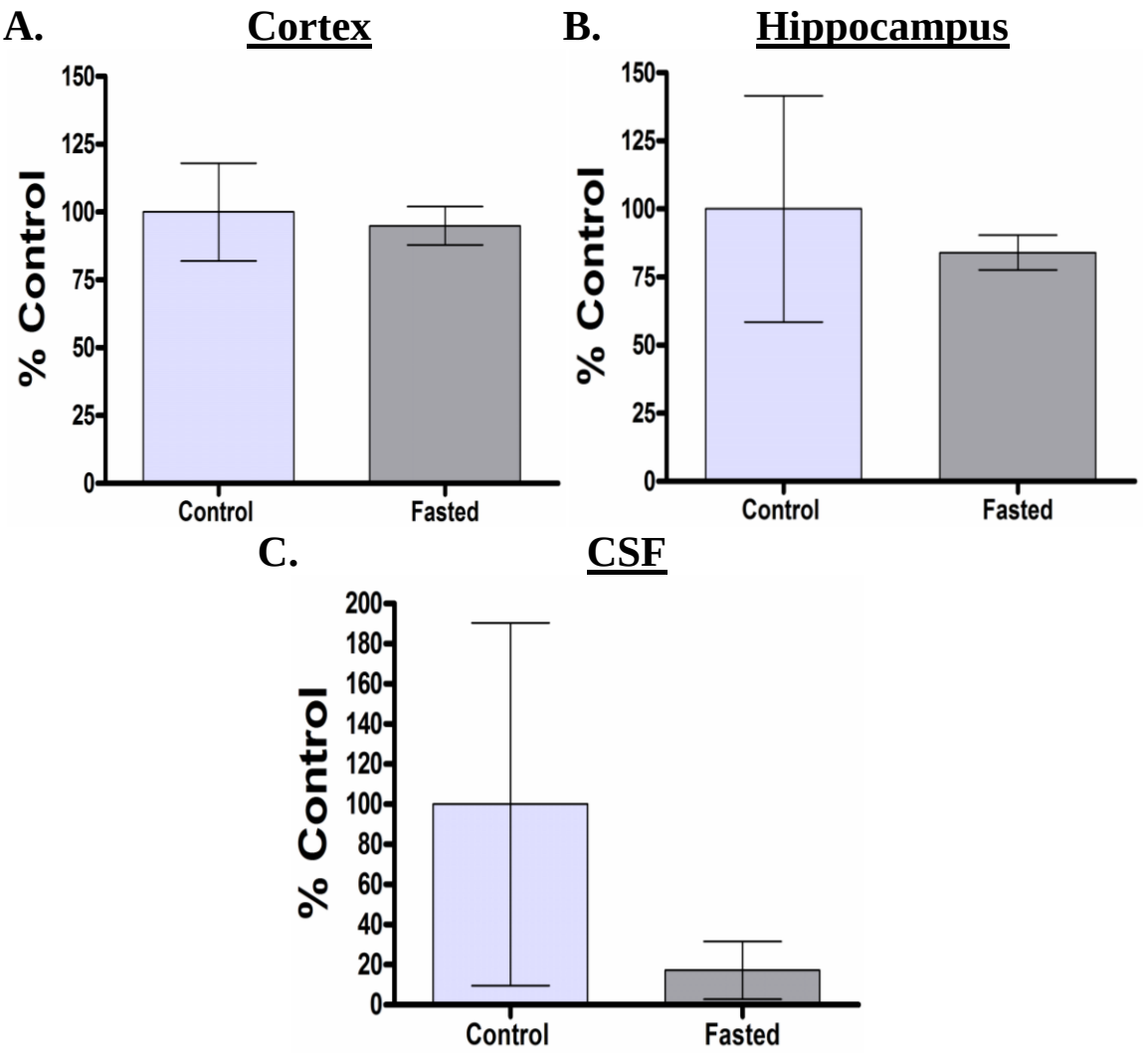

Figure 3.15 The effect of fasting on Docosahexaenoic acid levels in brain and CSF. Animals fasted for 24 hours did not show significantly increased levels of Docosahexaenoic acid in Cortical tissue (A) (t_8=0.59), Hippocampal tissue (B) (t_8=0.85), or CSF (C) (t_6=1.8). Unpaired student's t-test, n=5 SD.

Discussion

Classically, Uncoupling proteins have been characterized as an intregral part of a complex endogenously controlled system designed to protect mitochondrial function during times of increased oxidative stress (Garlid et al. 2001; Echtay et al. 2003; Mattiasson et al. 2003). This mechanism is an important component in every body system, and is particularly vital in the brain due to the lack of regeneration and the oxidative sensitivity of neural tissue. In times of neuronal insult and subsequent secondary injury cascades, the uncoupling mechanism operates to preserve mitochondrial function so that cellular repair mechanisms can enable the cell to recover and regain control of its functions (Nicholls 2002; Sullivan et al. 2003; Brookes et al. 2004; Lifshitz et al. 2004; Sullivan et al. 2004). The inhibition of these repair processes or the inundation of these mechanisms with overwhelming damage results in cognitive dysfunction due to lost or damaged tissue, which can decrease the ability of TBI patients to effectively re-enter and function in society.

In our studies, we have shown robust upregulation of UCP2, the most ubiquitously expressed UCP in the body, in neuronal mitochondria in response to fasting (Fig.3.2). These results indicate that we can upregulate UCP2, and possibly the brain specific uncoupling proteins UCP 4/5, using a fasting paradigm in order to study its function in response to FFA administration. Also, it should be noted that neuronal mitochondria are not equipped to use FFA oxidation for the production of energy (McKee et al. 2003), which could make this system uniquely suited for induction within the neuroprotective mechanism of fasting after injury.

Valproic acid has become an efficacious clinical treatment for both partial and generalized seizures; with its primary mechanism of action being the blockage of Ca^{2+} channel in order to prevent the release of aberrant amounts of neurotransmitter (Pellock et al. 2001). However, there have been other suggested mechanisms for valproic acid, such as increasing glutathione expression and activity via the upregulation of the glutamate-cysteine ligase (GCL), which is the rate limiting enzyme in Glutathione synthesis (Cui et al. 2007). As such, we investigated the possibility that valproic acid could act as an endogenous uncoupler of mitochondria as an additional alternative mechanism of action. Although valproic acid has been shown to be a neuroprotective agent, our results indicate that it is not involved in the UCP mediated anti-ROS mechanism. The failure of valproic acid to modulate ROS production may be due to the small time window in which it has been incubated in these studies; which may further implicate it in the modulation of the glutathione system rather than the UCP anti-oxidative mechanism.

Oleic acid, which constitutes ~55-80% of olive oil, has been previously shown to lower biomarkers of mitochondrial stress and improve antioxidant systems and DNA constitution when fed to rats over their life time (Quiles et al. 2006). It has been shown to increase Glutathione Peroxidase (GPx) activity and decrease ROS production in the presence of Antimycin A (Complex III Inhibitor), however these effects are blocked by GPx inhibitors thereby indicating that it is working through the support of the glutathione anti-oxidative system rather than UCP mediated ROS reduction (Duval et al. 2002). There are, however, also reports that indicate that oleic acid can promote the initiation of cell death pathways (Ishola et al. 2006). These contradictory findings may be the result of

concentration differences between studies, as high concentrations of FFA can lead to the inhibition of respiration and enhanced ROS production (Duval et al. 2002). As oleic acid is the metabolite of Palmitic and Stearic acid pathways, it could explain why these FFAs did not show robust induction of uncoupling or ROS reduction due to the lack of FFA oxidation taking place within the mitochondria (Clayton et al. 1991; McKee et al. 2003). However, in our studies Oleic acid did show a significant, although only ~10% increase in respiration. In our ROS studies oleic acid also significantly reduced ROS production, this could indicate that this small increase in uncoupling could have robust anti-ROS effects. In our fasted animals, oleic acid was significantly increased in serum and cortical tissue after 24 hrs of fasting; however we did not see an increase in hippocampal tissue, which suggests brain region specificity of upregulation.

In our studies, Butyric acid caused an increase in respiration and a decrease in ROS production, indicating an activation of UCPs. Interestingly butyric acid has also been linked with a paradoxical association to cell proliferation and apoptosis, which seems to, again, be dependent on concentration (Lupton 2004). Butyrate can also be shunted into ketogenesis pathways, which would create an alternative fuel source for mitochondria as well as reduce ROS production, contributing to the overall improved mitochondrial and cellular function (Lupton 2004; Kim et al. 2007; Maalouf et al. 2007). However, it is unlikely that it is being utilized in this pathway given the limited time window of our studies. Our results suggest that butyric acid could be an attractive agent to investigate in terms of a therapeutic regime designed to decrease ROS in numerous neurological disorders.

Myristic acid was found to be able to increase respiration and reduce ROS production via UCP mediated uncoupling. This FFA is a highly hydrophobic saturated fatty acid that easily becomes embedded within membranes, which makes it an ideal membrane localization agent for many enzymes (O'Neil 2001). This would be beneficial to the activation of UCPs due to their membrane bound functionality. Also, if the activation site of UCPs is within the membrane, this type of FFA would be well suited for targeting to that location. However, the exact mechanism of UCP function has been considered controversial; either being an activated pore structure or a platform for the protonation and "flip-flop" of FFA across the inner membrane (Garlid et al. 2000; Echtay et al. 2001; Garlid et al. 2001; Echtay et al. 2002). In either case, Myristic acid would be well suited for the activation of UCPs and translocation of protons due to its long chain length and localization characteristics.

Although the increase in respiration from state IV due to palmitic acid was significantly different from its BSA addition rate, this value was not increased above 100% of state IV nor was it able to significantly decrease ROS production. This fatty acid, which has been shown to be somewhat detrimental in terms of cholesterol levels, has also been shown to shut down the mitochondrial respiration and induce the formation of the mitochondrial permeability transition pore (MPTP) when used at high concentrations (Nestel et al. 1994; Penzo et al. 2002). However, at lower concentrations, similar to those we have used in our studies, it has not been shown to have a deleterious effect on mitochondria in terms of $\Delta\Psi$ and cytotoxicity (Penzo et al. 2002). Also, previous work from our lab has shown that palmitic acid is an activator of upregulated uncoupling proteins in isolated mitochondria from animals on the ketogenic diet

(Sullivan et al. 2004). However these studies used a higher concentration and animals were maintained on the ketogenic diet for 10-12 days, which could have an alternate effect on the upregulation of UCPs in terms of expression level and isoform upregulation. It was not shown to increase UCP expression in vitro through PPAR (Armstrong et al. 2001), which would limit its involvement to the activation of UCPs. Overall, this fatty acid could have beneficial or detrimental effects in regards to mitochondrial and cellular function depending on the amount and duration of exposure, however, in our studies it did not prove to increase respiration or decrease ROS production.

When we investigated the upregulation of certain FFAs during fasting, we found that the activation of UCP mediated uncoupling and ROS reduction by Oleic acid, EPA, and AA corresponded with increased serum levels after a 24hr fast. However, the upregulation of Linoleic acid in serum and brain tissue did not correspond with its ability to activate UCP mediated uncoupling and reduce ROS. This equivocal outcome of our studies could be explained by the fact that Linoleic acid can be converted into Arachidonic acid simply by adding a two carbon unit (McKee et al. 2003). Also, Linoleic has been shown to activate multiple isoforms of peroxisome proliferator activating receptors (PPAR), which is the primary mechanism of UCP upregulation and expression (Moya-Camarena et al. 1999; Moya-Camarena et al. 1999). Therefore, as Linoleic acid is increased in serum and brain tissue after fasting, but lacks direct effects on UCP activation, it may be utilized as a precursor of AA and a means by which the neural cells can upregulate the entire UCP system. Collectively, these data indicate that it is playing a role in the UCP mediated mechanism of fasting induced neuroprotection, and that certain FFAs could have play a therapeutic role in the treatment of TBI.

It has previously been shown that TBI increases Arachidonic acid levels in tissue taken from both the injury site and the surrounding penumbra (Scheff et al. 2004). The source of this FFA is believed to be the catalysis of membrane phospholipids by phospholipases. AA has been implicated as a detrimental factor in a number of tissues, where it has been shown to increase mitochondrial ROS in various mitochondrial bioenergetic manipulations (Cocco et al. 1999; Cao et al. 2004). This FFA, which is an essential fatty acid that is highly incorporated into membranes, also functions as the key substrate in the prostaglandin mediated second messaging (Armstrong et al. 2001; Halliwell et al. 2007). As we have shown in our studies, UCP2 expression is not significantly increased by injury alone, which would indicate that TBI induced FFAs are not involved in a uncoupling mediated protective mechanism. However, AA, as well as its downstream products EPA and DHA, has exhibited a role in the UCP mediated reduction of ROS suggesting a protective role within an uncoupling mechanism.

Manipulations in previous studies showing detrimental effects of AA did not induce the upregulation of UCPs, which would leave the mitochondria unable to utilize FFAs for ROS reduction, and as such could account for the conflicting results. Indeed, the fasting mediated upregulation of AA and EPA, as well as the AA precursor Linoleic acid, in serum and brain indicates that they may be involved within the previously described neuroprotective mechanism of fasting (Davis et al. 2008). Along with Oleic acid, which is also increased in serum and brain tissue after fasting, these FFAs have demonstrated the capability to upregulate hepatic UCP2 through a prostaglandin mediated PPARα induction pathway (Armstrong et al. 2001). This effect was not maximized until 36 hours after administration (Armstrong et al. 2001), which suggests

that prolonged upregulation of these FFAs, through the mechanism of fasting, is required to properly upregulate and activates UCPs.

ROS is generated as a result of the blockage of electron flow through the ETC, which causes the extension of the half-life of Q cycle intermediates, increasing the possibility of superoxide formation and oxidative stress. FFAs can be endogenously synthesized within the body via the breakdown of triglycerides, or they can be produced after injury due to activated lipases (Chan et al. 1982). The transient uncoupling of the ETC by UCPs has been suggested to be both ROS dependent and FFA activated; and that this uncoupling is a self regulated process due to the requirement of certain levels of both of these factors (Garlid et al. 1998; Garlid et al. 2001; Echtay et al. 2002; Echtay et al. 2003). Meaning, once the ROS levels decrease past a specific threshold, UCPs cease the translocation of protons into the matrix. This could explain why the expression of these proteins does not automatically lead to a total depolarization of the mitochondrial inner membrane, which would in turn initiate cell death pathways.

The production of 4-hydroxy-2-nonenal, a product of TBI induced lipid peroxidation, may be a mechanism designed to decrease oxidative damage through the activation of UCPs (Echtay et al. 2003; Echtay et al. 2007). However, this injury-mediated mechanism may not be sufficient to upregulate proper levels of UCPs or to adequately sustain their activation, and as such does not result in neuroprotection. Fasting could provide a sufficient mean of upregulation and activation of these proteins, which could be due to its ability to increase levels of FFAs involved in multiple pathways of regulation. Although we did not measure all of the FFAs that showed UCP activation, fasting could result in increased levels of these FFAs and as such could augment the

activation of UCPs. This system seems to be an innate defense mechanism, and due to the endogenous regulation, it presents an attractive site of intervention to alleviate mitochondrial damage and dysfunction. Although it would be ideal to identify all of the FFAs that are increased as a result of fasting, the identification of the FFAs within our studies has yielded interesting results, which has implicated PPAR and UCP in the underlying mechanism of fasting induced neuroprotection.

Conclusions

In light of our previous data showing that fasting is neuroprotective after TBI, the identification of the FFAs that are upregulated during fasting, increase UCP mediated mitochondrial respiration, and reduce ROS production is an important step in the further elucidation of this endogenous neuroprotective mechanism. This information could prove important in the development of potential therapies for various neurological insults and disorders, as well as add to the understanding of the mechanism underlying current therapeutic treatments such as the ketogenic diet. To this end, due to the endogenous regulatory nature of this system, therapies could potentially be designed to supplement specific FFAs or to induce their synthesis within the body after the onset of damage.

Chapter 4

The Effect of UCP2 Gene Dosing After Traumatic Brain Injury in a Mouse Model

Introduction

The basis of mitochondrial function is the utilization of the translocation of protons from the intermembrane space to the matrix coupled to the phosphorylation of ADP. This process relies on the function of the electron transport chain (ETC), which is a series of protein complexes responsible for creating a charge potential across the inner membrane. It is critical that this membrane potential ($\Delta\Psi$) be maintained within a specific range in order to ensure proper mitochondrial function (Nicholls et al. 2002). A large increase in $\Delta\Psi$ will cause a backup of electrons within the ETC, causing an increase in reactive oxygen species (ROS) production, which can damage mitochondrial proteins, lipids, and DNA (Cadenas et al. 2000). Conversely, a substantial decrease in $\Delta\Psi$ will cause the consumption of ATP through the reversal of the ATPsynthase in an attempt to recover $\Delta\Psi$, resulting in decreased ATP levels within the cell (Nicholls et al. 2000). The maintenance of adequate ATP levels is critical to the function of cellular ion translocators to maintain ionic balance, which is vital to neuronal function. Clearly, any major deviation in $\Delta\Psi$ can cause lasting and devastating effects on mitochondrial function and may lead to the initiation of cell death pathways, and ultimately cognitive dysfunction.

Fortunately, endogenously regulated mitochondrial uncoupling, mediated by members of the uncoupling protein (UCP) family, could alleviate increased $\Delta\Psi$ resulting from mitochondrial stress. The function of these proteins is to dissociate ATP production from the ETC in the mitochondria of muscle and fat tissues (Nicholls et al. 2000); subsequently leading to heat generation. UCPs translocate protons across the inner

mitochondrial membrane into the matrix, bypassing the ATPsynthase and dissipating mitochondrial $\Delta\Psi$ (Richard et al. 2001; Nicholls David et al. 2002). Their expression is believed to be upregulated by fasting mechanisms, and their function is activated by free fatty acids produced via beta oxidation (Dulloo et al. 2001; Sullivan et al. 2004).

Although UCP1 was the first isoform to be identified and is the most highly studied, it wasn't until the late 1990's that researchers found additional UCP1 homologs. Five mitochondrial UCPs have been identified in the human genome, and although all of the UCPs have a sequence homology, their physiological role(s) are unclear in regard to the CNS (Dulloo et al. 2001; Nicholls David et al. 2002). Among these characterized UCPs, UCP2, UCP4 and UCP5/BMCP1 have recently been shown to be significantly expressed in the CNS (Horvath et al. 1999; Arsenijevic et al. 2000; Diano et al. 2000; Kim-Han et al. 2001). UCP2 was also found to be localized ubiquitously throughout the body, and a substantial amount was found to be localized in various parts of the brain; including the hypothalamus (suprachiasmatic, paraventricular, dorsomedial, ventromedial nucleus and arcuate nuclei), brainstem, and limbic system; which suggests that UCP2 plays a role in neuroedocrine, behavioral, and autonomic functions (Horvath et al. 1999; Richard et al. 2001). This expression has been found to be mainly neuronal; although the identity of the type of neuron that is participating has been somewhat unclear. It has recently been accepted that neurons involved in the upregulation of UCP2 possess an atypical β_3 adrenergic receptor that releases noradrenalin in response to SNS signaling, which activates BAT thermogenesis and liberation of fatty acids (Dulloo et al. 2001; Nicholls 2001). Highlighting this pathway were studies showing animals treated with a β_3 agonist experiencing weight loss associated with UCP expression (Dulloo et al. 2001).

UCP2 knockout animals have an increased ability to secrete insulin, which may suggest a role for UCP2 in energy metabolism by functioning as a negative regulator of insulin secretion (Richard et al. 2001; Erlanson-Albertsson 2002).

Free radical production is a byproduct of ATP generation in mitochondria via the electron transport chain. Electrons escape from the chain and reduce O_2 to ($O_2^{-\cdot}$). Normally cells convert $O_2^{-\cdot}$ to H_2O_2 utilizing both manganese superoxide dismutase, which is localized to the mitochondria, and copper-zinc superoxide dismutase found in the cytosol. H_2O_2 is rapidly converted to H_2O via catalase and gluthione peroxidase, but has the potential to be converted to the highly reactive hydroxyl radical ($^{\cdot}OH$) via the Fenton reaction, underlying ROS neurotoxicity. $^{\cdot}OH$ rapidly attacks unsaturated fatty acids in membranes causing lipid peroxidation and the production of 4-hydroxynonenal (HNE) that conjugates to membrane proteins, impairing their function (Keller et al. 1997; Keller et al. 1997; Mark et al. 1997; Sullivan et al. 1998). Such oxidative injury results in significant alterations in cellular function. In particular, ROS induced lipid peroxidation and protein oxidation products may be particularly important in neurodegeneration (for review see (Mattson 1998)) and TBI (Braughler et al. 1985; Braughler et al. 1989; Braughler et al. 1992; Sullivan et al. 1998).

Mitochondrial ROS production is intimately linked to $\Delta\Psi$ such that hyperpolarization (high $\Delta\Psi$) increases and promotes ROS production (Skulachev 1996; Skulachev 1998; Votyakova et al. 2001). The underlying mechanism of this relationship is the alteration of the redox potential of ETC carriers (reduced) and prolonged semiquinone anion half-life time (high $\Delta\Psi$ prevents b_h oxidation of cytochrome b_1 in the

Q cycle). In other words, at a high $\Delta\Psi$, protons can no longer be pumped out of the matrix (against the electrochemical proton gradient) causing electron transport to slow/stall. This results in intermediates staying reduced longer and increasing the chance that the electrons will escape from these intermediates to increase ROS production. Since the magnitude of ROS production is largely dependent on--and correlates with--$\Delta\Psi$, even a modest reduction in $\Delta\Psi$ via increased proton conductance across the mitochondrial inner membrane (uncoupling) can reduce ROS formation (Skulachev 1996; Kim-Han et al. 2001; Votyakova et al. 2001). Endogenous mitochondrial uncoupling mediated by members of the UCP family could participate in the reduction of ROS production via this increased proton conductance. UCPs are activated by FFAs and superoxide, and inhibited by purine nucleotides, indicating that they are sensitive to both ROS and ATP levels (Echtay et al. 2002) (also see (Harper et al. 2001; Argiles et al. 2002; Zackova et al. 2002) for review).

Several hypotheses have been put forth concerning possible physiological roles of the UCPs including energy partitioning, energy balance and control of metabolism which may be pivotal in obesity and diabetes (for review see (Argiles et al. 2002; Jezek 2002)). Skulachev was the first to hypothesis that mild uncoupling could be beneficial as it causes a decrease in ROS production (Skulachev 1996). Several studies have now demonstrated roles for UCPs in modulating ROS production. UCP2 (Arsenijevic et al. 2000) and UCP3 (Vidal-Puig et al. 2000) knockout mice exhibit increased ROS in macrophages and muscle, respectively. Leptin-deficient mice have decreased levels of UCP2 and also show increased ROS production in macrophages (Lee et al. 1999). *In vitro* overexpression of UCP2 (Li et al. 2001) or UCP5/BMCP1 (Kim-Han et al. 2001)

decrease cell death following H_2O_2 exposure and ROS production respectively. Finally our lab has recently reported a neuroprotective role for UCP2 in excitotoxic cell death *in vivo* (Sullivan et al. 2003). These studies demonstrated that reducing UCP2 expression and activity, increases kainic acid induced mitochondrial ROS production and neuronal cell loss in p12 rats pups, which are classically resistant to excitotoxic insult (Sullivan et al. 2003). Together these studies have implicated UCPs, specifically UCP2, 4, and 5, to play a role in protecting the mitochondria from both increased calcium uptake and increased oxidative stress (Sullivan et al. 2003).

It is important to note that UCPs do not cause the complete uncoupling of mitochondrial function, which would result in total energy failure and the initiation of cell death pathways. Instead, they cause a mild transient reduction of $\Delta\Psi$ within a specific range, which allows for a dramatic decrease in ROS production. Also, because UCPs are activated by both ROS and FFAs, which includes lipid peroxidation product 4-hydroxy-2-nonenal (HNE) (Echtay et al. 2003; Echtay et al. 2007), the decrease in ROS levels would decrease activation of UCPs. This endogenous regulation of their activation makes them an attractive modulatory target for treatment after TBI. Therefore, in our current studies we have investigated the role of UCP2 by means of gene dosing in an attempt to elucidate its role in neuroprotection after TBI.

Materials and Methods

Animals

These experiments were conducted using 6-8 week old transgenic and knockout male mice, which have been extensively characterized (Horvath et al. 2003; Andrews et al. 2005) were housed 3 per cage in a facility which maintained a 12-hour light/12-hour dark cycle. All experimental animal procedures were approved by the Yale University Animal Care and Use Committee. All animals were fed a balanced diet *ad libitum* before and after injury. 6 animals were used per each of the 6 strains for tissue sparing assessments for a total of 36 animals for these studies. A subset of animals was used to assess oxidative damage markers within the injured tissue.

Surgical Procedures and Experimental Paradigms

These methods were performed as described previously in chapter 2. The main difference in these studies is that a pneumatically controlled impactor with a 3mm tip traveling at 3.5m/s compressed the exposed cortex 0.5mm in depth to deliver a moderate injury. Premade dental acrylic caps were secured with methyl-methacrylate over the exposed craniotomy and allowed to dry before closing the skin with surgical staples. Animals were allowed to recover in home cages and regained consciousness and mobility within 15-20 minutes.

Tissue Preparation and Tissue Sparing Assessment

These methods were performed as described previously in chapter 2.

Oxidative Damage Markers

A separate set of sections from the injured animals were used to determine the amount of oxidative damage within each group. Only homozygous (transgenic and knockout) and wild type (transgenic and knockout) groups were used for these studies. Tissue sections were rinsed with 0.2M Phosphate-buffered Saline (PBS), after which they were reduced by exposing them to a solution containing 0.1M $NaBH_4$ and 0.1M MOPS at pH 8.0. After rinsing sections in PBS, they were incubated in 0.3% H_2O_2 at room temperature (25°C). Tissue was then incubated in blocking buffer (0.2M PBS, 5.0% goat serum, 0.25% Triton X-100, and 1.0% dry milk) and subsequently incubated in blocking buffer containing polyclonal rabbit anti-HNE antibody (1:5000) and monoclonal mouse anti-3NT (1:1000) overnight (Calbiochem® Darmstadt, Germany). Sections were then incubated with fluorescent (800nM) secondary goat anti-rabbit antibody and (700nm) goat anti-mouse antibody (Rockland Immunochemicals Inc, Gilbertsville, PA). Sections were allowed to dry and imaged on a LiCore imager at 800nM.

Statistical Analysis

For all statistical comparisons, significance was set at $p < 0.05$. Data were evaluated using analysis of variance (ANOVA) or an unpaired t-test as appropriate. When warranted by the ANOVA, post hoc comparisons employed the Student-Neuman-Keuls analysis. Importantly, as the transgenic and knockout mice were bred on different background strains, we did not compare these groups within our statistical analysis.

Results

After a 0.5 mm CCI injury, heterozygous and homozygous UCP2 over expressing mice showed significantly increased neuroprotection in terms of tissue sparing compared to wild type littermates (Figure 4.1). However, homozygous and heterozygous UCP2 knockout animals did not show a significant difference in tissue sparing compared with their wildtype injured littermates (Figure 4.2). Although all of the groups were injured

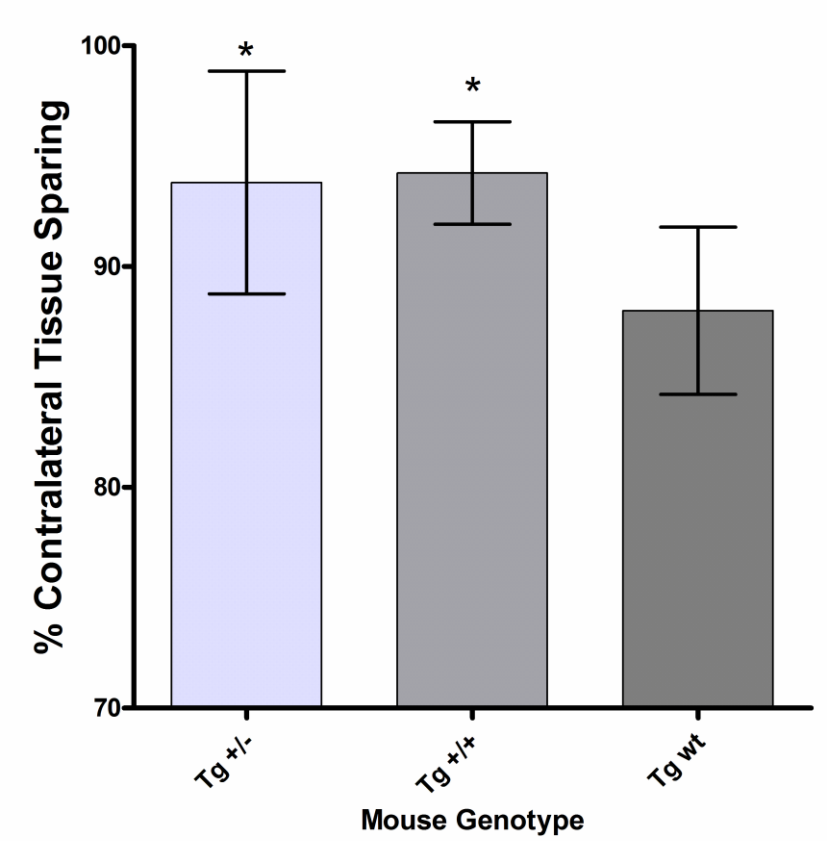

Figure 4.1: UCP2 overexpression increases neuroprotection after TBI.
Homozygous and heterozygous UCP2 transgenic overexpressing mice showed significantly increased tissue sparing after a 0.5mm CCI injury compared to their wild type littermates. ANOVA $F_{2,17}$=4.8, R^2=0.39, *p<0.05 SNK n=6/group SD.

and processed within the same experiment, the transgenic and knockout mice were bred on different background strains, and therefore were not statistically compared to each other.

In order to correlate increased tissue sparing with decreased cellular damage we assessed tissue for modulations in the levels of HNE and 3NT, which is a marker for lipid peroxidation and protein oxidation, respectively. This tissue assessment was performed with the same tissue we had used for the previously shown tissue sparing assessments. Unfortunately we failed to see UCP2 mediated modulation of HNE or 3NT levels in any

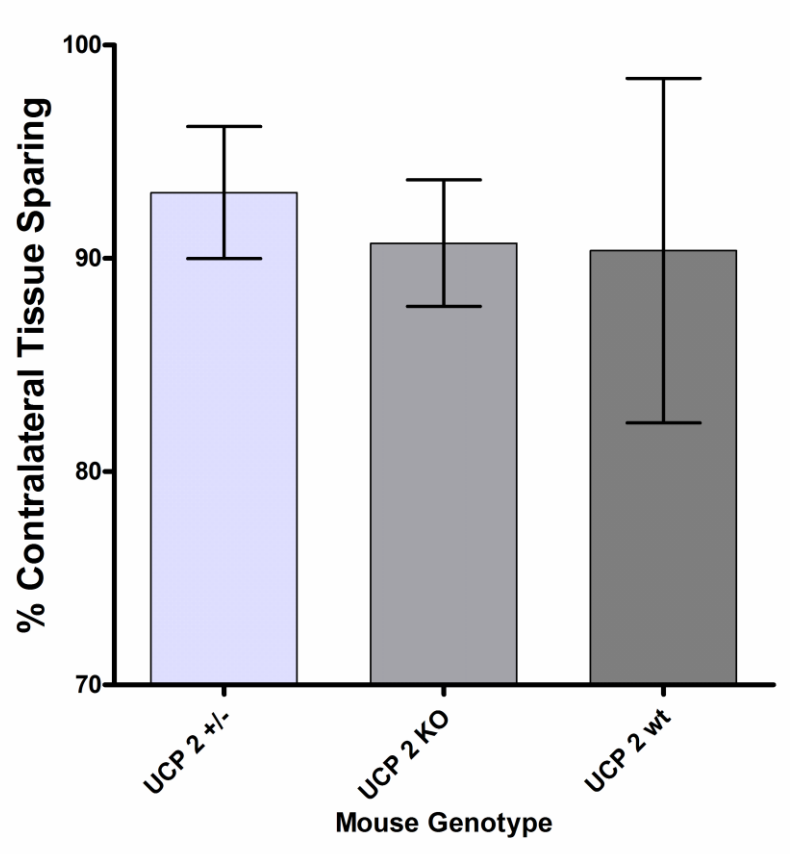

Figure 4.2: <u>UCP2 knockout does not decrease neuroprotection after TBI.</u>
Both Homozygous and heterozygous UCP2 knockout mice failed to show significantly different amounts of tissue sparing after a 0.5mm CCI injury compared to their wild type littermates. ANOVA $F_{2, 17}=1.1$, $R^2=0.12$, n=6/group SD.

of the groups (Figure 4.3). Homozygous overexpressing mice exhibited similar levels of HNE and 3NT at 7 days post injury compared to their wild type littermates. Although homozygous knockout animals would be expected to show increased levels of HNE and

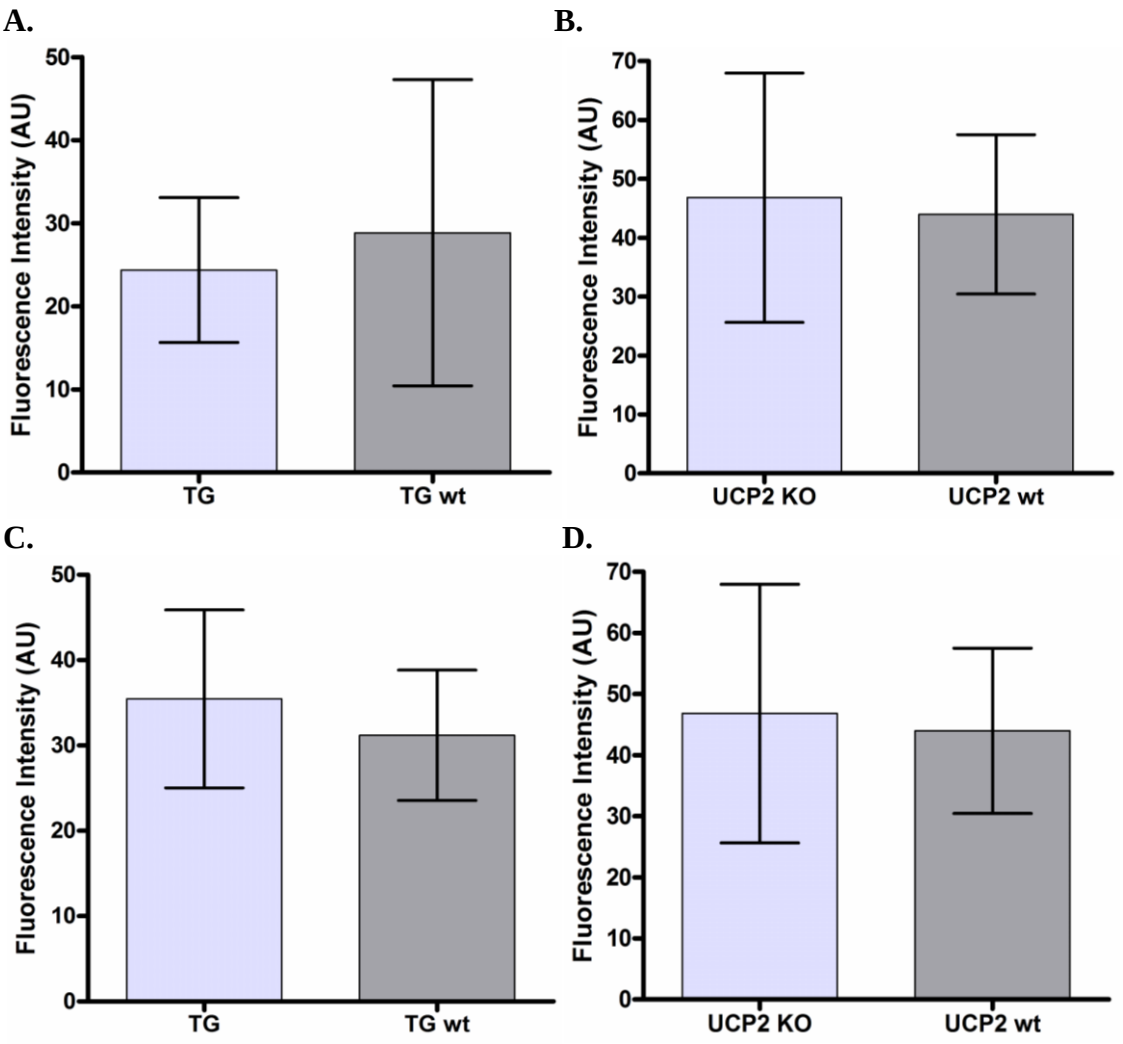

Figure 4.3: <u>Effect of UCP2 expression modulation on HNE production after traumatic brain injury.</u>
A) Mice overexpressing human UCP2 did not show decreased levels of HNE expression at 7 days post injury. $p>0.05$ $t_5=0.38$. B) Mice lacking UCP2 did not show a significant increase in HNE expression at 7 days post injury. $p>0.05$ $t_6=0.89$. C) Mice overexpressing human UCP2 did not show decreased levels of 3NT expression at 7 days post injury. $p>0.05$ $t_5=0.63$. D) Mice lacking UCP2 did not show a significant increase in 3NT expression at 7 days post injury. $p>0.05$ $t_6=0.23$. Unpaired students t-test, SD, n=4(KO+/+), 4(wt +/-), 3(TG+/+), and 4 (wt +/-).

3NT, they instead exhibited a similar level of each marker compared with their wild type littermates. This could indicate that UCP2 does not play a role in lipid peroxidation modulation; however, it could also indicate that by 7 days post injury HNE and 3NT levels have been reduced by intracellular systems.

Discussion

Our current results indicate that UCP2 can play a role in neuroprotection after TBI within a mouse model of CCI. Interestingly, we did see a significant increase in tissue sparing in our transgenic overexpressing mice after TBI, which indicates that increased UCP2 levels beneficially affect neuroprotection. However, we did not see a decrease in tissue sparing after TBI in mice that lacked UCP2. This may indicate a compensatory mechanism of other UCP isoforms (4/5) within these knockout animals. This compensation may also be present in our transgenic mice, and as such may prevent us from adequately assessing the effect of UCP2 modulation within neuroprotection after TBI. Interestingly, UCP2 overexpressing mice are less susceptible to stroke induced damage and cell death, which they propose is due to UCP2 mediated inhibition of MPTP and caspase dependent apoptosis (Mattiasson et al. 2003). Within this study they also found that transgenic UCP2 mice had decreased TBI induced lesion volume and improved neurological outcome compared to wild type (Mattiasson et al. 2003), however

our studies are a more rigorous assessment of the genetic influences of UCP2 expression on TBI induced tissue damage due to our gene dosing paradigm. Both studies are important to the understanding of the neuroprotective role of UCP2.

Due to the influence of UCP2 on the metabolic system, it may affect how animals consume food after injury. However, where it has been shown that mice overexpressing UCP2 actually consume more food (Horvath et al. 2003), the opposite has not been shown for UCP2 knockout mice. Therefore, we cannot attribute the induction of neuroprotection to a fasting related mechanism in either the knockout or transgenic animals. Also, because there is not a large drop in the availability of glycolytic substrate due to decreased food intake, both knockout and transgenic animals would lack fasting induced upregulation of FFA forcing their UCPs to rely on the endogenous production of lipid peroxidation byproducts (HNE) for activation (Echtay et al. 2007). Interestingly, there have been reports showing an increase in FFAs after TBI (Scheff et al. 2004), however this FFA profile may not be sufficient for UCP activation. Therefore, future studies with our UCP2 knockout and transgenic animal model utilizing fasting to upregulate these endogenous activators would give us a better understanding of the role of UCP2 within the mechanism of fasting induced neuroprotection. Also important to the elucidation of the neuroprotective role of UCP2 would be the development of knockout and overexpressing mice for the remaining UCP isoforms.

Although the two strains were not directly compared statistically, there seems to be some difference in their response to our injury model. Unfortunately, the responses of these mouse strains have not been extensively characterized in a CCI model of injury. Therefore, there could be strain effects on the level of cortical damage and what level of

injury constitutes a moderate injury. However, it is important to note that neither strain is impervious to kainic acid administration, which indicates these strains are indeed susceptible to secondary injury mechanisms (McKhann et al. 2003). Further studies comparing tissue sparing with multiple injury levels would need to be conducted for a full characterization these strains within our injury model. We feel that this strain difference would not affect our findings as these strains were not directly compared, however the terminology of "moderate injury" may not hold true for both strains.

Conclusion

These studies are the first to describe the effect of UCP2 expression modulation on tissue sparing after TBI. They indicate that the elevation of UCP2 expression after TBI could have profound effects on the sparing of tissue. Although more studies would be needed to correlate this tissue sparing with functional recovery, our results have implicated UCP2 as a potential therapeutic target for the attenuation of tissue loss after injury.

Chapter 5

Summary and Conclusions

The elucidation of the underlying mechanism of fasting-induced neuroprotection is an important step in the development of post-injury treatment therapies for acute TBI. This mechanism has the advantage of post-injury initiation, as well as the fact that it does not require the use of pharmaceutical agents. It utilizes endogenously upregulated factors to mediate neuroprotection, and as such may not carry the same potential side effects of many pharmaceutical interventions. To this end, the work done in these studies has attempted to discover how something as simple as fasting could have such substantial effects on neuroprotection, and may influence the future of clinical management of TBI.

Our studies have potential implications not only for the development of novel therapies for the treatment of TBI, but also for the revision of the current treatment protocols currently utilized to treat TBI patients. We have shown that insulin administration after injury can have highly detrimental effects on tissue sparing, and can increase mortality after moderate TBI. This is in agreement with studies showing detrimental outcome with high glucose administration (Robertson et al. 1991; Yager et al. 1992; Cherian et al. 1998). However, clinicians are in a difficult situation due to the need to provide metabolic substrates and at the same time minimize detrimental effects of hyperglycemia. Therefore, they are required to use insulin administration to regulate the levels of blood glucose in TBI patients. The problem with initiating insulin mediated hypoglycemia or normoglycemia is that it effectively shuts down ketogenesis, which we have shown in our current studies to be neuroprotective. Interestingly, although TBI results in a metabolic storm in which ATP levels decrease and glucose is highly consumed, the supplementation of metabolism with glycolytic substrates does not

translate to the preservation of tissue or beneficial outcome. In fact, it has been demonstrated that the utilization of glucose by the cell is shifted from ATP production into the pentose phosphate pathway (PPP), which is responsible for the production of NADPH and the preservation of the glutathione system (Bartnik et al. 2007; Dusick et al. 2007). This shift can be explained by the increased demand on the glutathione system as a result of the production of oxidative damage molecules after the initiation of the secondary injury cascade (Bartnik et al. 2007). Clearly, there is a great need for glycolysis to alter its enzymatic pathway in order to maintain mitochondrial anti-oxidative mechanisms. However, it also seems imperative that mitochondrial energy substrates must be provided in order to maintain mitochondrial function, as well as protect cellular energy production and prevent cell death and cognitive dysfunction. Therefore, a treatment protocol must be developed that can maintain glycolytic substrates, in order to maintain anti-oxidative mechanisms, as well as supplement metabolic energy production.

Oxidative damage has been shown to have significant effects on cellular damage and endogenous repair mechanisms at early time points after traumatic brain injury in both cortical and hippocampal regions (Ansari et al. 2008; Ansari et al. 2008). Reports of increased lactate levels after TBI could be linked to oxidative damage of the pyruvate dehydrogenase complex (PDHC), which is responsible for the conversion of Pyruvate into Acetyl CoA, the initial compound of the Krebs cycle. The PDHC is highly sensitive to oxidative damage resulting from neurological pathology, and as such can cause increased lactate formation and the depletion of energy within the cell (Martin et al. 2005). Therapies designed to overcome the PDHC block, using acyl-L-carnitine, indicate

that bypassing the enzymatic complex through Krebs cycle intermediate supplementation can stimulate aerobic metabolism by reducing lactate production and tissue acidosis arising from glycolitic metabolism (Martin et al. 2005). The shift to the PPP, coupled with the decreased ability of the cell to produce Krebs cycle intermediates, could lead to energy failure after injury. Fortunately, ketones are able to bypass the glycolytic block induced by oxidative damage mediated PDHC inactivation, supplying the Krebs cycle with intermediates for ATP production via oxidative phosphorylation (Martin et al. 2005). Our results indicate that ketones significantly increased tissue sparing after moderate TBI, as well as *in vitro* improvements in mitochondrial function in excitotoxicity models. This may indicate a central role in the neuroprotective mechanism of fasting for ketone metabolism.

Indeed, the utilization of ketones in the absence of fasting would allow the cell to complete its glycolytic shift to the PPP, while providing the necessary substrates for the production of energy (Figure 5.1). Interestingly, there is also evidence indicating that the utilization of ketones, and subsequent production of succinate, can stabilize HIF-1α and thereby upregulate HIF-1 genes (Puchowicz et al. 2008). This is also supported by studies showing mutations in succinate dehydrogenase, resulting in increased succinate levels, also cause HIF-1 mediated gene expression (Gottlieb et al. 2005; Selak et al. 2005). As these genes are highly involved in glycolytic enzyme and glucose transporter upregulation (Schofield et al. 2004; Sharp et al. 2004; Berra et al. 2006), this pathway could further support the shift to the PPP. Indeed there is a significant decrease in a key PPP enzyme glucose-6-phosphate dehydrogenase (G6PD), which coincides with an impairment of the endogenous glutathione system and increased oxidative markers after

TBI (Ansari et al. 2008). Interestingly, a putative HIF-1 binding site has been located on the promoter region of the rat G6PD gene (Gao et al. 2004), which indicates that succinate mediated stabilization of HIF-1 may lead to the maintenance of endogenous antioxidant systems and decreased oxidative damage, both of which has been seen in our current studies as well as cited literature. Indeed, ketone induced stabilization of HIF1α is also involved in the upregulation of anti-apoptotic Bcl2 genes, indicating a role in cell death regulation (Puchowicz et al. 2008). It is also important to note that HIF-1 induces the upregulation of monocarboxylate transporters (ketone transporters) (Knott et al. 1999; Zhang et al. 2005), which could further supplement this system with adequate ketogenic precursors. HIF-1 mediated upregulation of angiogenesis by VEGF (Schofield et al. 2004) could also alleviate TBI related hypoxia within the injury site, leading to decreased damage, and increased tissue recovery. In fact, this system seems to be both extensively interconnected, as well as highly regulated (Figure 5.1). Although we saw increased tissue sparing with our ketone administration, we only saw this effect at the lower dose of βHB, which could highlight the fact that HIF-1 can also upregulate pro-apoptotic genes (Koivunen et al. 2007), depending on the duration and intensity of signaling. This may be an important factor regarding treatment window and dosing regimen when translating this treatment into an effective therapy.

Although we have provided strong evidence for ketogenesis as the underlying mechanism of fasting after brain trauma, the amount of tissue sparing did not seem to be as robust as our fasting studies. This could be explained by the need for more extensive

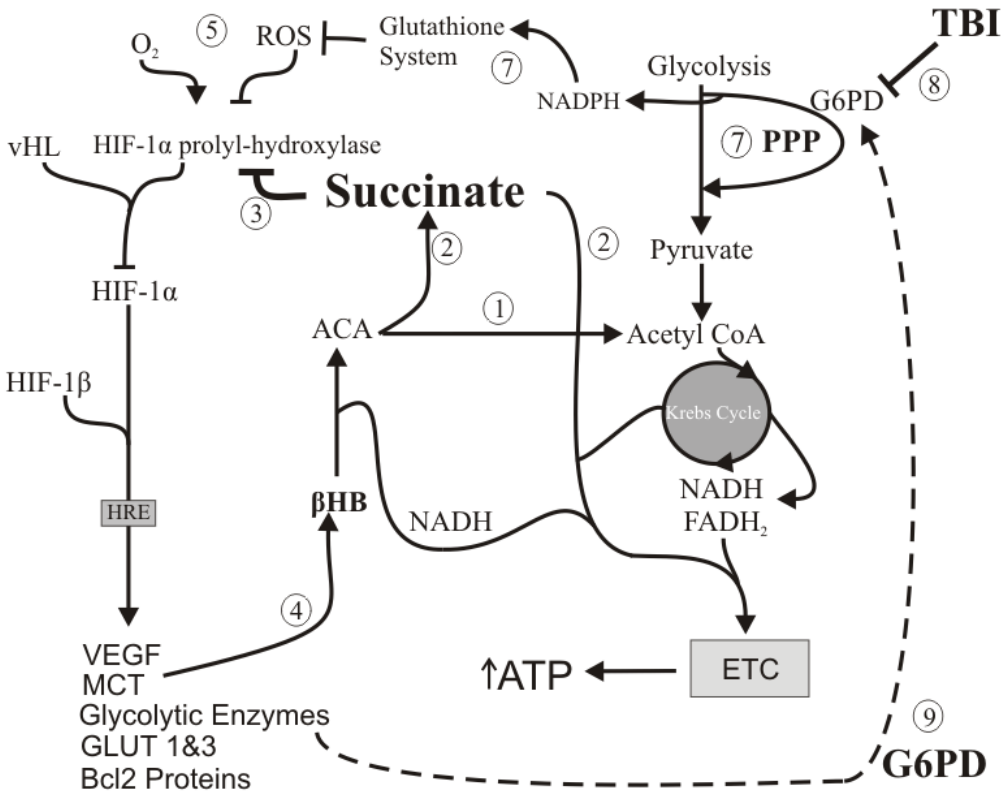

Figure 5.1 <u>Revised hypothesis of how ketones and HIF-1 work together to preserve mitochondrial function.</u>
1) Ketones (βHB and ACA) are utilized by mitochondria to produce Krebs cycle intermediates, which produce substrates for the ETC (NADH and $FADH_2$). This allows the mitochondria to produce sufficient energy, in the form of ATP, to maintain homeostasis. 2) The conversion of ketones into acetyl-CoA also produces succinate, which can be utilized by the Krebs cycle to produce energy. 3) Succinate has also been shown to block HIF-1α prolyl-hydroxylases, which in turn stabilizes the levels of HIF-1α. This allows HIF-1α to dimerize with HIF-1β and travel to the nucleus, where it is responsible for the upregulation of proteins involved in angiogenesis, glucose uptake, glycolysis, and ketone uptake. 4) It is through the HIF-1 mediated upregulation of MCTs that we believed it is possible for increased ketone levels to increase their uptake into the cell for utilization as energetic substrates. 5) Oxygen is responsible for the activation of HIF-1α prolyl-hydroxylases, which alter HIF-1α, which allows the von Hipple-Lindau protein to target it for proteasome mediated degradation. ROS can also inhibit HIF-1α prolyl-hydroxylases, which may explain how HIF-1α expression is upregulated by injury. HIF-1 upregulates glycolytic enzymes the utilization of ketones by mitochondria could be shifting glycolytic intermediates into the (7) pentose pathways, which is responsible for the increase of NADPH levels and in turn support of the endogenous glutathione system. 8) TBI induces oxidative damage of Glucose-6-phosphate dehydrogenase (G6PD), which causes a failure of the glutathione system due to a lack of NADPH production by the Pentose Phosphate Pathway (PPP). 9) HIF-1 upregulates G6PD, as well as other glycolytic proteins, thereby alleviating the glycolytic block induced by TBI, resulting in the restoration of the glutathione system.

ketone dosing studies or inherent differences between studies, however because we know that other factors are upregulated in response to fasting, we decided to investigate their possible role within this protective mechanism.

Our studies have indicated that fasting upregulates UCP expression in cortical mitochondria. These proteins are regulated through the peroxisome proliferator activating receptor (PPAR) pathway. The activation of the PPAR system is primarily through free fatty acid (FFA) ligand binding; which could explain the mechanism by which UCP expression may be increased (though not significantly) as FFA levels have been shown to be increased after TBI (Scheff et al. 2004), this. As UCPs are part of an endogenously regulated anti-ROS mechanism, we sought to determine their role in fasting induced neuroprotection. However, there has been little investigation into which of the FFAs activate uncoupling of mitochondria. Therefore, we screened a wide range of FFAs to determine their ability to induce uncoupling and reduce ROS production in cortical mitochondria from fasted animals. We also wanted to correlate their function with FFA upregulation in response to fasting in naïve animals. Interestingly, we found that FFAs that have been shown to increase after injury, such as Arachidonic (AA) and Oleic acids (Scheff et al. 2004), had an effect on UCP activation and ROS reduction. Oleic acid has been implicated in the preservation of the glutathione system, and our data showing its presence in cortical but not hippocampal tissue in response to fasting is in agreement with the literature in which oleic acid was measured after injury (Scheff et al. 2004). As oleic acid did not increase UCP mediated respirations in isolated mitochondria, it is most likely functioning to reduce ROS through the glutathione system. AA, which is a known activator of all PPAR isoforms (Jump 2002), and Linoleic acid (Moya-Camarena et al.

1999; Moya-Camarena et al. 1999), which can be converted into AA, could be highly involved in the PPAR mediated UCP upregulation. However, limited research has been done to investigate their role in direct UCP activation. Our results show that AA is able to activate UCP mediated ROS production and is significantly upregulated in the serum and hippocampal tissue of fasted animals, which may implicate it as a protective factor after injury. Interestingly, Eicosapenaenoic acid (EPA) and Docosahexaenoic acid (DHA), which are produced via AA metabolism, can also activate the UCP mediated anti-ROS mechanism in isolated cortical mitochondria. However, only EPA was significantly increased in fasted serum samples, where DHA showed a trend toward increased levels. Our studies represent the first measurements of specific FFAs in response to fasting, as well as a correlation to their activation of UCPs. These data implicate these specific FFAs in a UCP mediated protective mechanism within the neuroprotective mechanism of fasting.

Although we had indirectly implicated UCPs as a neuroprotective factor after TBI, we wanted to determine its role in the absence of fasting. To this end, we used UCP2 gene dosing to assess its effect on tissue sparing after moderate TBI in a mouse model of CCI. Our study indicates that UCP2 overexpression can be neuroprotective, whereas UCP2 knockout shows no detrimental effect on tissue sparing. This could indicate that other brain UCPs are compensating for the lack of UCP2 within our knockout animals, which have been shown to have consistent expression levels among knockout and wild type animals (Andrews et al. 2005). In addition to this compensation, without fasting mediated upregulation of FFAs the activation of UCPs must be through lipid peroxidation products, and as such may not be sufficient to cause a differentiation in

neuroprotection in our knockout mice. Indeed, in a future study in which injured transgenic and knockout mice are fasted, we may see a greater demarcation in tissue sparing among our genetic groups.

In our UCP study using transgenic and knockout mice we saw that increased UCP2 expression, as seen in fasted injured animals (Figure 3.2), resulted in neuroprotection. However, as we had seen in our ketone administration study, this effect is not as robust as that seen within our fasting study. Our modulations of UCP2 provide evidence that it is important in neuroprotection after injury, although it is difficult to isolate the activity of this specific protein as the underlying mechanism of fasting induced neuroprotection. It is more probable that these two mechanisms are working in a concerted effort to afford neuroprotection within our fasting mechanism (Figure 5.2). It has been shown that ketones have the ability to alter the redox potential of Co-enzyme Q_{10} (CoQ_{10}) from a reduced to oxidized form, which can decrease the probability of electron slippage and ROS formation (Maalouf et al. 2007). Interestingly, it has also been shown that UCP mediated proton translocation not only requires ROS and FFAs, but that oxidized CoQ_{10} is required for their activation as well (Echtay et al. 2000; Echtay et al. 2001; Echtay et al. 2002). In addition to this direct effect between ketones and UCP function, FFA activation of PPARα upregulates the production of mitochondrial HMG CoA sythetase, which is a key enzyme in ketone production (Jump 2002). As both ketones and UCPs are upregulated by fasting and that they seem to influence each other's metabolic and regulatory pathways, it is our assertion that it is the cooperation between ketones and UCPs that comprise the underlying mechanism of fasting induced neuroprotection. The development of treatment therapies that focus on the utilization of

these pathways, highlighting their interdependence, may lead to an efficacious attenuation of neurological deficits resulting from TBI pathology.

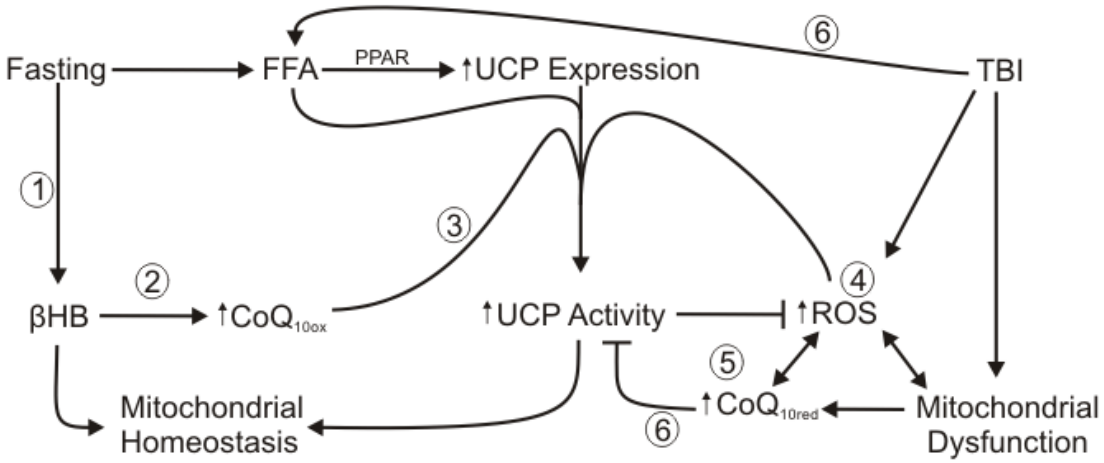

Figure 5.2 <u>Hypothesis of how ketones and UCPs work together as the underlying mechanism of fasting induced neuroprotection.</u>
1) Fasting initiates the upregulation of ketone bodies and free fatty acids (FFA). FFA can function to induce the upregulation of UCP expression via PPAR and to activate UCP mediated uncoupling and ROS reduction. 2) Ketones alter the redox state of Co-enzyme Q_{10} (Ubiquinone), which shifts it into an oxidized state. 3) This oxidized form of Co-enzyme Q_{10} (CoQ_{10ox}) is required for the activation of UCPs. 4) TBI causes an increase in ROS production, which is also required for activation of UCPs, and subsequently mitochondrial dysfunction. 5)The backup of electrons within the ETC due to increased membrane potential and mitochondrial function shifts Co-enzyme Q_{10} into a reduced state (CoQ_{10red}). This form of Co-enzyme Q_{10} has been shown to inhibit UCP function. 6) Any UCPs upregulated as a result of TBI would be unable to confer neuroprotection due to the lack of oxidized Co-enzyme Q_{10}, regardless of TBI induced FFA upregulation.

Appendix

1. 2'-7'-dichlorodihydro-fluorescein (DCF)
2. 4 hydroxy-2-nonenol (HNE)
3. 4-(2-hydrozyethyl)piperazine-1-ethanesulfonic acid potassium salt (HEPES)
4. 8-hydroxy-2-deoxyguanosine (8-ohdg)
5. Acetoacetate (ACA)
6. Adenine nucleotide translocase (ANT)
7. Adenosine diphosphate (ADP)
8. Adenosine triphosphate (ATP)
9. Analysis of variance (ANOVA)
10. Apoptosis activation factor-1 (Apaf-1)
11. Apoptosis inducing factor (AIF)
12. Atpsynthase (complex V)
13. Beta-hydroxybutyrate (βhb)
14. Bicinchoninic acid (BCA)
15. Bovine serum albumin (BSA)
16. Brain mitochondrial carrier protein-1 (BMCP-1)
17. Brown adipose tissue (BAT)
18. Calcium (Ca2+)
19. Calcium green 5N (cag5n)
20. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)
21. Carboxyl radical ($CO_3^{\cdot-}$)
22. Center for disease control (CDC)
23. Co-enzyme Q10 (coq_{10})
24. Controlled cortical impact (CCI)
25. Cytochrome-c Oxidase (complex IV)
26. Deoxyribonucleic acid (DNA)
27. Electron transport chain (ETC)
28. Endonuclease G (Endo G)
29. Ethylene glycol-bis (2-aminoethylether)-N,N,N'N'-tetraacetic acid (EGTA)
30. Excitatory amino acid (EAA)
31. Ferric iron (Fe^{3+})
32. Flavin adenine dinucleotides ($FADH_2$)
33. Free fatty acid (FFA)
34. Glutathione peroxidase (gpx)
35. HIF prolyl 4-hydrolase (P4H)
36. High density lipoprotein (HDL)
37. Horseradish peroxidase (HRP)
38. Hydrogen peroxide (H_2O_2)
39. Hydroxyl radical ($^{\cdot}OH$)
40. Hypoxia-inducible factor-1 (HIF-1)
41. Inner membrane (IM)
42. Intermembrane space (IMS)
43. Intracellular calcium ($[Ca^{2+}]_i$)
44. Ketogenic diet (KD)
45. Low density lipoprotein (LDL)
46. Magnesium (Mg+)
47. Magnesium chloride (mgcl)
48. Manganese superoxide dismutase (mnsod)
49. Membrane potential ($\Delta\Psi$)
50. Mitochondrial 3-hydroxy-3-methylglutaryl-coa synthase (mhs)
51. Mitochondrial permeability transition pore (mptp)
52. Monocarboxylate transporter (MCT)
53. Morris water maze (MWM)
54. NAD-Ubiquinone oxidoreductase (complex I)
55. Nicotinamide adenine dinucleotide (NADH)
56. Nitric oxide ($^{\cdot}NO$)
57. Nitrogen dioxide ($^{\cdot}NO_2$)
58. N-methyl-D-aspartate (NMDA)
59. Outer membrane (OM)
60. Oxygen (O_2)
61. Oxygen-dependent degradation domain (ODD)
62. Paraformaldehyde (PFA)
63. Pentose phosphate pathway (PPP)
64. Peroxisome proliferator activating receptor (PPAR)
65. Peroxisome proliferator response element (PPRE)

66. Peroxynitrite anion ($ONOO^-$)
67. Phosphate buffered saline (PBS)
68. Post traumatic stress disorder (PTSD)
69. Postconcussional syndrome (PS)
70. Potassium chloride (kcl)
71. Potassium phosphate monobasic anhydrous (KH_2PO_4)
72. Protons (H^+)
73. Pyruvate dehydrogenase complex (PDHC)
74. Reactive nitrogen species (RNS)
75. Reactive oxygen species (ROS)
76. Retinoid X receptor (RXR)
77. Shell shock (SS)
78. Sodium (Na+)
79. Sodium borohydride ($nabh_4$)
80. Standard deviation of the group mean (SD)
81. Standard error of the group mean (SEM)
82. Succinate dehydrogenase (complex II)
83. Superoxide ($O_2^{\cdot-}$)
84. Sympathetic nervous system (SNS)
85. Tetramethylrhodamine ethyl ester perchlorate (TMRE)
86. Thiazolidinediones (DZT)
87. Traumatic brain injury (TBI)
88. Triacylglycerol (Tg)
89. Ubiquinone-cytochrome-c-oxidoreductase (complex III)
90. Uncoupling protein (UCP)
91. United states (U.S.)
92. Units (U)
93. Vascular endothelial growth factor (VEGF)
94. Very low density lipoprotein (VLDL)
95. Voltage-dependent anion channel (VDAC)
96. Von Hipple Lindau tumor suppressor protein (vhl)
97. World war I (WWI)
98. A-amino-3-hydroxy-5-methyl-4-isozazole proprionic acid (AMPA)

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VITA

Laurie Michelle Helene Davis

Date of Birth: July 9, 1982

Place of Birth: Springfield, Missouri

Education: Bachelor of Science, 2003

Midway College

Publications:

Reed J. L., Dimayuga F. O., **Davies L. M.**, Keller J. N. and Bruce-Keller A. J. (2004) Estrogen increases proteasome activity in murine microglial cells. Neuroscience Letters **367,** 60-65.

Nukala VN, Singh IN, **Davis LM,** Sullivan PG. Cryopreservation of brain mitochondria: A novel methodology for functional studies. Journal of Neuroscience Methods. 2006 Apr 15;152(1-2):48-54.

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Awards:

2008 Best poster award from the Brain Injury Association of Kentucky (BIAK) at the Poster presentation at the University of Kentucky Neuroscience Day Symposium, Lexington Ky. March 2008: Honorarium Awarded.

2008 Travel funding awarded from "The International Symposium on Dietary Therapies for Epilepsy and Other Neurological Disorders" Phoenix AZ

2006-2008 Pre-doctoral fellowship through the NIH Blueprint Translational Neuroscience Training Grant "Therapeutic Strategies for Neurodegeneration".

2006 Competitive Travel Grant Recipient for the National Neurotrauma Society Symposium in St. Louis MO.

2005 6th Place poster award at the National Neurotrauma Society Symposium in Washington D.C.: Honorarium Awarded.

2005 Top 16 student posters at the National Neurotrauma Society Symposium in Washington, D.C.

2005 University of Kentucky Department of Physical Medicine & Rehabilitation 17th Annual Research Day Best Poster Award.

2004-2008 Competitive Travel Award to present research at national meeting from the University of Kentucky.

Professional Experience

2007-2008 Graduate student representative for the Department of Anatomy & Neurobiology; University of Kentucky

2007 Planning Committee for Graduate Student Interdisciplinary Conference. (Graduate Student Congress Organizer)

2007 ANA 511/814 Gross Anatomy for Medical Students. (**Lab TA).** University of Kentucky

2007 ANA 209- Guest Lecturer, Principals of Human Anatomy; University of Kentucky

2007 AHEC Health Careers Program: HCOP Camp for Rising High School Juniors. Co-lecturer for the Anatomy and Neurobiology block. University of Kentucky

2007 ANA 611/811 Gross Anatomy for Physical Therapy and Physician Assistant students. (**Lab TA).** University of Kentucky

2007-2008 Mentor for TRY-IT outreach program; University of Kentucky

2007-2008 Brain Awareness Week-Public Neuroscience Outreach Program; University of Kentucky

2007-2008 Science Fair Judge- Kentucky State Science Fair; Richmond, Ky

2005 Co-organizer of the University of Kentucky department of Anatomy and Neurobiology "Molecular and Cellular Basis of Brain Aging" Training Grant; Mitochondrial Symposium. (Student organized)

2005 ANA 802: Neurobiology for Physical Therapy students. (TA) University of Kentucky

2005-2008 Member of National Neurotrauma Society

2005-2008 Member of Society for Neuroscience

2005-2008 Member of Society for Neuroscience Bluegrass Chapter

2004-2006 Mentor for Girls in Science Program; University of Kentucky

2004-2008 Graduate Student, Department of Anatomy & Neurobiology; Spinal Cord & Brain Injury Research Center (SCoBIRC) University of Kentucky

Poster Presentations

Davis, LM, Bosken, JM, Rho, JM, Sullivan, PG. "Polyunsaturated Fatty Acid Activation of Uncoupling Protein Mediated Reduction of Reactive Oxygen Species" Poster presentation at the 2008 Poster presentation at Kentucky Spinal Cord and Brain Injury Trust Symposium, Lexington Ky. June 2008.

Davis, LM; Pauly, JR; Sebastian, AH; Readnower, RD; Rho, JM, Sullivan, PG. "Acute Post Injury Fasting Is Neuroprotective Following Traumatic Brain Injury: Potential Mechanisms and Implications for Treatment." Poster presentation at the University of Kentucky Neuroscience Day Symposium, Lexington Ky. March 2008.

Davis, LM; Pauly, JR; Sebastian, AH; Readnower, RD; Rho, JM, Sullivan, PG. "Acute Post Injury Fasting Is Neuroprotective Following Traumatic Brain Injury: Potential Mechanisms and Implications for Treatment." Poster presentation at the 2007 Society for Neuroscience annual meeting November 2007 San Diego CA.

Readnower, RD; Sebastian, AH; **Davis, LM**; Goldstein,GA; Sullivan, PG. "Administration of a Novel, Cell-Permeant Glutathione Precursor, TOVA, following Traumatic Brain Injury Increases Tissue Sparing and Reduces Oxidative Stress." Poster presentation at the National Neurotrauma Society meeting in Kansas City, MO, July 2007

Davis, LM; Sebastian, AH; Readnower, RD; Rho, JM Sullivan, PG. "Acute Post Injury Fasting Is Neuroprotective Following Traumatic Brain Injury; Potential Mechanisms and Implications for Treatment." Poster presentation at the National Neurotrauma Society meeting in Kansas City, MO, July 2007

Davis, LM; Pauly, JR; Sebastian, AH; Readnower, RD; Rho, JM, Sullivan, PG. "Fasting Induced Neuroprotection; Mechanism and Implications for the Treatment of Traumatic Brain Injury." Poster presentation at Kentucky Spinal Cord and Brain Injury Trust Symposium, Louisville Ky. June 2007

Davis, LM; Sebastian, AH; Rho, JM; Sullivan, PG. "Fasting Induced Neuroprotection; Mechanism and Implications for the Treatment of Traumatic Brain Injury." Poster

presentation at the University of Kentucky Department of Physical Medicine & Rehabilitation 17th Annual Research Day, Cardinal Hill Rehabilitation Hospital, Lexington Ky. May 2007.

Davis, LM; Sebastian, AH; Rho, JM; Sullivan, PG. "Fasting Induced Neuroprotection; Mechanism and Implications For The Treatment Of Traumatic Brain Injury." Poster presentation at the University of Kentucky Neuroscience Day Symposium, Lexington Ky. March 2007.

Davis, LM; Sebastian, AH; Rho, JM; Sullivan, PG. "Role of Ketones (ß-hydroxybutyrate and Acetoacetate) and Mitochondria in Fasting-Induced Neuroprotection after Traumatic Brain Injury." Poster presentation at the National Neurotrauma Society meeting in St. Louis, MO. July 2006.

Davis, LM; Sebastian, AH; Rho, JM; Sullivan, PG. "Role of Ketones (ß-hydroxybutyrate and Acetoacetate) and Mitochondria in Fasting-Induced Neuroprotection after Traumatic Brain Injury." Poster presentation at Kentucky Spinal Cord and Brain Injury Trust Symposium, Lexington Ky. June 2006.

Davis, LM; Sebastian, AH; Rho, JM; Sullivan, PG "Post-Injury Fasting is Neuroprotective after Traumatic Brain Injury in Rodents; Possible Mechanisms and Implications**."** Poster presentation at the University of Kentucky Neuroscience Day Symposium, Lexington Ky. March 2006.

Davis, LM; Sebastian, AH; Rho, JM; Sullivan, PG "Post-Injury Fasting is Neuroprotective after Traumatic Brain Injury in Rodents; Possible Mechanisms and Implications**."** Poster presentation at the 2007 Society for Neuroscience annual meeting November 2007 Washington DC.

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Laurie M. Davis