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ABSTRACT OF DISSERTATION

Lesley Knight Gilmer

The Graduate School
University of Kentucky
2009

AGE MAY BE HAZARDOUS TO OUTCOME FOLLOWING TRAUMATIC BRAIN INJURY: THE MITOCHONDRIAL CONNECTION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
Anatomy and Neurobiology Department at the
University of Kentucky

By
Lesley Knight Gilmer

Lexington, Kentucky

Director: Dr. Stephen W. Scheff, Professor of Anatomy and Neurobiology

Lexington, Kentucky

2009

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ABSTRACT OF DISSERTATION

AGE MAY BE HAZARDOUS TO OUTCOME FOLLOWING TRAUMATIC BRAIN INJURY: THE MITOCHONDRIAL CONNECTION

Older individuals sustaining traumatic brain injury (TBI) experience a much higher incidence of morbidity and mortality. This age-related exacerbated response to neurological insult has been demonstrated experimentally in aged animals, which can serve as a model to combat this devastating clinical problem. The reasons for this worse initial response are unknown but may be related to age-related changes in mitochondrial respiration.

Evidence is shown that mitochondrial dysfunction occurs early following traumatic brain injury (TBI), persists long after the initial insult, and is severity-dependent. Synaptic and extrasynaptic mitochondrial fractions display distinct respiration capacities, stressing the importance to analyze these fractions separately. Sprague-Dawley and Fischer 344 rats, two commonly used strains used in TBI and aging research, were found to show very similar respiration profiles, indicating respiration data are not strain dependent. Neither synaptic nor extrasynaptic mitochondrial respiration significantly declined with age in naïve animals. Only the synaptic fraction displayed significant age-related increases in oxidative damage, measured by 3-nitrotyrosine (3-NT), 4-hydroxynonenal (4-HNE), and protein carbonyls (PC). Alterations in respiration with age appear to be more subtle than previously thought. Subtle declines in respiration and elevated levels of oxidative damage may not to be sufficient to produce detectable deficits until the system is challenged.

Following TBI, synaptic mitochondria exhibit dysfunction that increased significantly with age at injury, evident in lower respiratory control ratio (RCR) values and declines in ATP production rates. Furthermore, synaptic mitochondria displayed increased levels of oxidative damage with age and injury, while extrasynaptic mitochondria only displayed significant elevations following the insult. Age-related synaptic mitochondrial dysfunction following TBI may contribute to an exacerbated response in the elderly population.

KEYWORDS: Aging, Cortical Contusion Injury, Mitochondria, Respiration, Traumatic Brain Injury

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AGE MAY BE HAZARDOUS TO OUTCOME FOLLOWING TRAUMATIC BRAIN INJURY: THE MITOCHONDRIAL CONNECTION

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DISSERTATION

Lesley Knight Gilmer

University of Kentucky

2009

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A dissertation submitted in partial fulfillment of the
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Lexington, Kentucky

2009

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I would like to dedicate this dissertation to my dad, Richard Thomas Gilmer. (1937-2005)

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Chapter 1: Introduction

Is age a good thing or a bad thing?

Experience is simply the name we give our mistakes. — Oscar Wilde

If you live to be one hundred, you've got it made. Very few people die past that age. — George Burns

Age -- that period of life in which we compound for the vices that we still cherish by reviling those we no longer have the enterprise to commit. — Ambrose Bierce

You know you're getting old when everything hurts, And what doesn't hurt doesn't work. — Hy Gardner

Old age isn't so bad when you consider the alternative. — Maurice Chevalier

How pleasant is the day when we give up striving to be young -- or slender. — William James

The older I grow the more I distrust the familiar doctrine that age brings wisdom. — H.L. Mencken

The young do not know enough to be prudent, and therefore they attempt the impossible -- and achieve it generation after generation. — Pearl Buck

I refuse to admit I'm more than fifty-two, even if that does make my sons illegitimate. — Nancy Astor

The first half of our lives is ruined by our parents, and the second half by our children. — Clarence Darrow

Being an old maid is like death by drowning -- a really delightful sensation after you have ceased struggling. — Edna Ferber

The contents of Chapter Two have previously been published as an original article in the *Journal of Neurotrauma* titled, "Early mitochondrial dysfunction following cortical contusion injury," by Lesley. K. Gilmer, Kelly. N. Roberts, Kelly M. Joy, Patrick. G. Sullivan, and Stephen W. Scheff, 26:1271–1280, Copyright © 2009. This work is reprinted with the kind permission of Mary Ann Leibert, Inc.

The contents of Chapter Four have previously been resubmitted (December 2009) as an original article in the *Mechanisms of Aging of Development* titled, "Age-related Changes in Mitochondrial Respiration and Oxidative Damage in the Cerebral Cortex of the Fischer 344 Rat," by Lesley K. Gilmer, Mubeen A. Ansari, Kelly N. Roberts, and StephenW. Scheff.

The contents of Chapter Five have previously been submitted (November 2009) as an original article in the *Journal of Neurotrauma* titled, "Aging May be Hazardous to Outcome following Traumatic Brain Injury: the Mitochondrial Connection," by Lesley K. Gilmer, Mubeen A. Ansari, Kelly N. Roberts, and StephenW. Scheff.

Rationale for Dissertation

Mitochondrial respiration has been implicated to decline in the brain with age and also becomes dysfunctional following TBI. Increased mortality rates, longer hospitalizations periods, and a higher incidence of lasting motor and cognitive deficits occur in older individuals suffering a TBI. Experimentally, aged animals also exhibit this exacerbated response to TBI, mimicking what is seen in the clinic. The reasons underlying this age-related deficit are unknown, but may be related to changes in mitochondrial respiration. The following experiments focus on determining possible age-related declines in mitochondrial respiration. Age-related declines in mitochondrial respiration may contribute to greater dysfunction seen following injury. Understanding possible mechanisms that explain age-specific responses to TBI will lead to development of effective therapeutic interventions.

Central Hypothesis

During the early phase of the secondary injury cascade following TBI, there is an increase mitochondrial dysfunction following TBI in aged animals (22-24 mos), relative to younger animals. Increased mitochondrial dysfunction may be one mechanism explaining the exacerbated response (e.g. increased cortical tissue loss and mortality) following TBI seen with age. In order to test this hypothesis, the following experiments were performed:

Specific Aim 1. Establish an experimental model for early bioenergetic mitochondrial dysfunction following TBI.

Specific Aim 2. Demonstrate that mitochondrial dysfunction following TBI occurs in both synaptic and extrasynaptic fractions in rats and is not strain dependent.

Specific Aim 3. Determine possible age-related alterations in mitochondria bioenergetics in naïve animals.

Specific Aim 4. Determine if there is an early age-related increase in synaptic or extrasynaptic mitochondrial dysfunction following TBI.

Mitochondria

Components

All mitochondria have five main specialized structures that enable energy production, depicted in Figure 1.1. The main structural components are:

1. **The outer membrane** is needed for compartmentalization of the specific activity of mitochondria from the cytosol.
2. **The inner membrane** contains all the necessary components of the electron transport chain (ETC).
3. **The inner membrane space** is where the protons (H^+) are pumped to create the proton motive force which drives adenosine triphosphate (ATP) production.
4. **Cristae** are the functional internal compartments formed by the numerous invaginations and foldings of the inner membrane. This is the site where cellular respiration occurs.
5. **The matrix** is where soluble enzymes catalyze the oxidation of pyruvate, the breakdown products of glucose which occurs in the cytosol, into energy rich products that drive the ETC. The matrix is also the location of DNA, known as mitochondrial DNA (mtDNA), needed for complete production of several complexes of the ETC.

Primary functions

Two primary functions of mitochondria are cellular energy production via the ETC and regulation of cytosolic calcium [1]. All eukaryotic organisms rely heavily on mitochondria for two main functions: their metabolic needs and regulation of cellular levels of calcium. Mitochondria also serve several other functions in addition to ATP production and calcium regulation including cell signaling that can determine cell survival or death.

Figure 1.1. Key structural components of mitochondria.

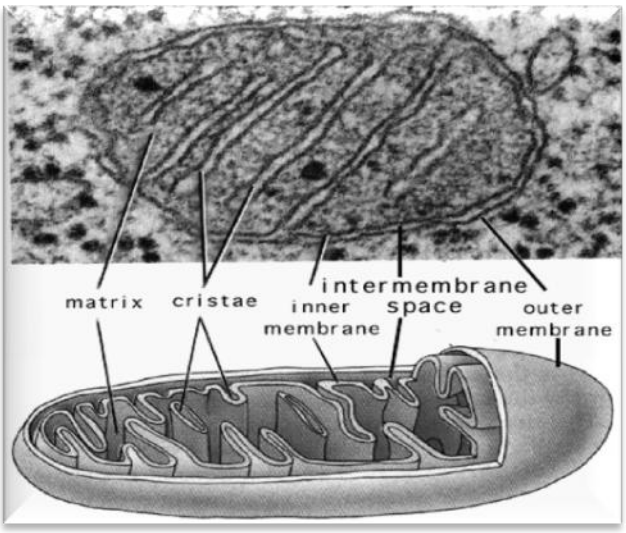

Figure 1.1. An electron micrograph of a single mitochondrion (top) with a simplistic cartoon demonstrating major components of mitochondrion (bottom). http://academic.brooklyn.cuny.edu/biology/bio4fv/page/mito.gif

Cellular respiration

Cellular respiration is a step-wise harvesting of energy through a series of biochemical reactions. Mitochondria are known as the powerhouse of the cell because of their unique ability to generate a large amount of ATP. Production of ATP occurs by breaking glucose down through a series of reactions known as the tricarboxylic acid cycle (also known as the Kreb's cycle). Cellular respiration is the process of breaking down food ultimately into carbon dioxide and water. Cellular respiration can be divided into four major reaction pathways:

1.) Glycolysis
2.) Tricarboxylic Acid Cycle (TCA cycle) or Kreb's cycle
3.) ETC
4.) Oxidative phosphorylation

Glycolysis

The energy released from these sequential break-down reactions is harvested in the form of ATP which can then be used in any cellular activity that requires energy. Glycolysis occurs in the cytoplasm by initially breaking down glucose molecules into two pyruvate molecules. Glycolysis produces 4 molecules of ATP and 2 of nicotinamide adenine dinucleotide (NADH), a coenzyme that is conserved in all living cells and required to drive complex 1 of the ETC. Glycolysis uses 2 molecules of ATP to complete reaction; the net is 2 ATP and 2 NADH molecules.

TCA cycle

To continue the subsequent aerobic reactions, pyruvate and NADH are imported inside the mitochondria, specifically the matrix. Here catabolism continues to convert the pyruvate into acetyl CoA before it enters the TCA cycle. Converting pyruvate into acetyl CoA also produces 2 molecules of NADH and 2 carbon dioxide (CO_2) for every pyruvate converted; so a total of 4 NADH and 4 CO_2 molecules are produced. For both acetyl CoA that enter the TCA cycle, 6 NADH and 2 flavin adenine dinucleotide ($FADH_2$) molecules, another energy-carrying reduced coenzyme, 2 ATP, and 4 CO_2 molecules are generated. These molecules drive the ETC. During the oxidation of pyruvate and the TCA cycle, there are 8 NADH, 2 $FADH_2$, 2 ATP, and 6 CO_2. It is interesting to note that NADH can be made in the cytosol and the mitochondrial matrix, whereas $FADH_2$ is only made in the matrix. Up to this point, production of these necessary energy-rich molecules does not require oxygen.

ETC

The ETC operates by a step-wise transferring of energy rich electrons from NADH and $FADH_2$ donors to lower energy acceptor molecules (e.g. complex 1-4). Before the process can be explained, an introduction of some of the main key players would be helpful. The two protein complexes that utilize these electron donors are NADH dehydrogenase (complex 1) which uses NADH and succinate dehydrogenase (complex 2) which utilizes only $FADH_2$. Both of these protein complexes serve as separate starting

points of the ETC; the rest of the chain operates regardless of which complex (1 or 2) initiated the chain. The remaining carrier molecules (ubiquinone and cytochrome *c*) and protein complexes [cytochrome bc_1 (complex 3) and cytochrome c oxidase (complex 4)] simply finish harvesting the remaining energy from the electrons (received from either complex 1 or 2) until they can pass the "spent" electrons to molecular oxygen (O_2).

The pathways for the electrons to pass through the ETC are as follows:

complex 1 ↘
ubiquinone → complex 3 → cytochrome *c* → complex 4 → O_2
complex 2 ↗

To clarify, complex 1 takes an electron from NADH and complex 2 accepts from $FADH_2$. Before the energy rich electron is passed on, complexes 1, 3, and 4 use a small amount of the energy to pump protons (H^+) into the inner mitochondrial membrane to establish a proton electrochemical gradient (Δp). Since complex 2 utilizes $FADH_2$, which has less energy than NADH, it is unable to pump H^+ and simply passes the lower energy electron straight to ubiquinone. Ubiquinone (complex 1 or 2→3) and cytochrome c (complex 3→4) simply carry the electron to the next complex that continues to harvest a small amount of energy with a concomitant transfer of protons into the inner membrane space. A total of 10 H^+ ions are transferred to the intermembrane space (4 H^+ from complex 1, 4 H^+ from complex 3, and 2 H^+ from complex 4). Finally, oxygen is ready to accept the "spent" electrons inside the matrix. Oxygen is an ideal candidate to accept these energy depleted electron acceptors due to its electronegative properties.

Oxidative phosphorylation

Oxidative phosphorylation is the term for the process of phosphorylating ADP into ATP, driven by the Δp [2]. Three to four protons are passed down their electrochemical gradient back into the mitochondrial matrix through the ATP synthase, complex 5 to synthesize 1 molecule of ATP [3]. Figure 1.2 is a simplistic drawing depicted in coupling

of the ETC to oxidative phosphorylation. Oxidative phosphorylation production of 32 ATP molecules, which puts the grand total to 36 ATP molecules produced from the complete breakdown of one glucose molecule.

Figure 1.2. Overview of electron transport chain coupled to oxidative phosphorylation

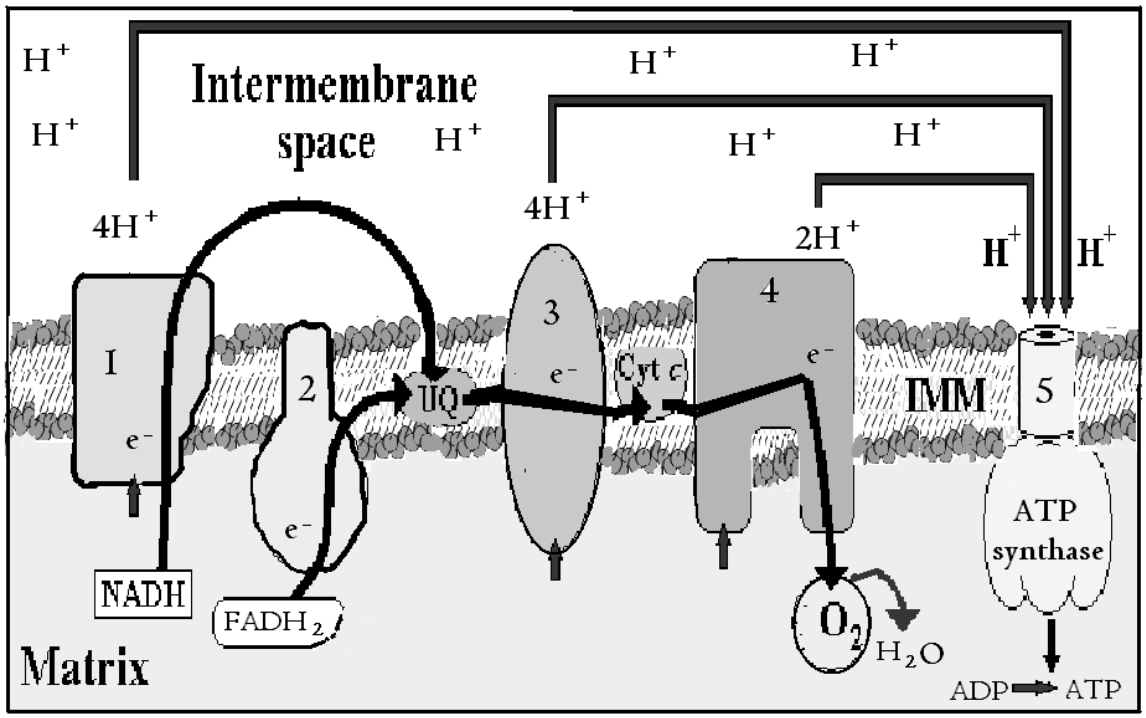

Figure 1.2. A simplified schematic of key events involved in the electron transport chain coupled to oxidative phosphorylation. UQ ubiquinone

Calcium sequestration

Calcium is very tightly regulated since it is used widely as a signaling molecule. Calcium is a secondary messenger that activates many signaling pathways and is a known second messenger that can regulate metabolism in the cell [4]. Under physiological conditions, the extracellular concentration of free ionic calcium is 1-2 mM and cellular levels are usually between 50 and 100 nM [4], while levels inside mitochondria are in the µM range [5]. Three dehydrogenases linked to the Kreb's cycle are activated by intramitochondrial calcium concentration [6] as well as F_0F_1ATPase [7].

Although calcium is necessary for proper functioning of mitochondria, a little goes a long way. Mitochondria can modulate calcium signaling by either releasing

additional calcium into the intracellular space (propagating) or by sequestering even slight increases of calcium (inhibiting). As levels rise above the physiological range they become even more involved [1]. The degree of calcium loading into mitochondria is dependent on the frequency and duration of stimulation [8]. For lower enduring calcium concentrations (200-300 nM) the calcium uniporter is activated, while larger bursts of calcium (>350 nM) activate the rapid mode of calcium uptake (RaM) [6]. Calcium uptake by RaM operating at least 300 times faster than that of the calcium uniporter is inhibited very quickly after it starts, whereas the uniporter continues to operate for prolonged amounts of time. The ability of mitochondria and other organelles (e.g. endoplasmic reticulum) to regulate calcium with such a precise spatiotemporal control underscores the important roles calcium plays in the cell.

Although calcium can positively regulate metabolism, under certain pathological conditions (e.g. trauma), large amounts of calcium influx can overwhelm mitochondria's buffering capacities and actually inhibits oxidative phosphorylation [9]and directly affect the rate of ROS production [10]. Excessive calcium loads can also trigger assembly of the mitochondrial permeability transition pore (mPTP). Even though the composition of the mPTP is still debated, there is consensus that the key components of the pore are the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D [11]. Assimilation of these proteins located in the inner and outer mitochondrial membrane establishes a large conductance channel for matrix solutes < 1.5kDa. Formation of the mPTP has catastrophic cellular consequences as a result of the dissipation of mitochondrial proton gradients, release of cytochrome c, and initiation of apoptosis [10-14].

Cell Signaling

Mitochondria play a large role in cellular signaling, including cell survival and death pathways. Although mitochondrial signaling is very important to cellular function, the breadth of this topic is out of the scope of this dissertation project, but has been reviewed [15]. Instead, a few key mitochondrial signaling molecules are mentioned due to their significant roles in proper maintenance and regulation of mitochondrial

respiration as well as cell survival. Cell death is a basic cellular event that occurs by either apoptosis or necrosis, two distinct mechanisms. Apoptosis, or programmed cell death, is necessary for survival and plasticity, while necrosis is an accidental death due to a lack of available ATP indicative of energy failure, details of the apoptosis pathway have been reviewed [16, 17]. Cytochrome *c* (cyt *c*) and mitochondrial nitric oxide synthase (mtNOS) are two vitally important molecules that play dual roles in cellular respiration and these two cell survival/death signaling pathways.

Cyt *c* and mtNOS are molecules that are found to be associated with the inner mitochondrial membrane and are directly or indirectly modulated by levels of calcium. Cyt *c* is intimately linked to mitochondrial respiration by transferring electrons between complex 3 and 4 of the ETC. A decline in respiration could occur due to the fact a portion of cyt *c* is loosely bound to the inner mitochondrial membrane [18] and could be released during mitochondrial turnover, pathological increases of intercellular calcium, or due to oxidative damage leading to "leaky" membranes. Regardless of the cause or mechanism for the release of cyt c, it is a potent stimulus for initiation of apoptosis [19, 20]. Oxygen uptake is regulated by mtNOS that can reversibly inhibit cytochrome oxidase (complex 4) [21] and the production of superoxide ($O_2^{\bullet -}$) from the ETC [22]. The extent of activation of mtNOS is dependent on physiologic or pathological conditions, but can produce significant suppression of mitochondrial respirations and elevations of oxidative stress [23]. It is believed that following certain pathological events (e.g. trauma) these molecules can ultimately determine cell fate. For example, following trauma, a sustained elevation of intracellular calcium levels triggers mitochondrial sequestration leading to activation of mtNOS. Activated mtNOS suppresses respiration and increases oxidative damage that compromises mitochondria membrane integrity, enabling escape/release of cyt *c* [24]. Once released from the inner mitochondrial membrane, cyt *c* can activate caspase 9 (also known as the initiator caspase), which belongs to a family of cysteine-aspartic proteases that are essential for apoptosis since they are able to cleave many different proteins within the cell. If caspase 9 activates caspase 3 and 7 (executioner proteins) apoptosis is inevitable. There are many other possible mechanisms to initiate apoptosis secondary to cyt *c* release [11, 25]. There is a tight regulation of cyt *c* but their

activities change with age [26-28] and are effected greatly under pathological conditions (e.g. trauma) [16, 17]. Altered activity or dysregulation of either of these molecules can have profound effects on cell survival following perturbation.

Production and turnover

The size and shape of mitochondria vary across tissue types, but the greatest variation is their distribution inside the cell. Regional energy demands often dictate the number, size, and shape of mitochondria. The average half-life of neuronal mitochondria is approximately 24 days. Damaged mitochondria are either degraded or rebuilt to replace defective components [29]. New mitochondria are synthesized through a complex process involving the nuclear and mitochondrial genome [30]coupled with binary fission of pre-existing mitochondria[31]. Along with fission, mitochondria can be revitalized by fusing with other mitochondria to maintain appropriate morphology and functional integrity [32]. On the other hand, mitochondria can be degraded when tagged with ubiquitin via autophagic vacuoles (termed mitophagy) to rid the cell of dysfunctional mitochondria or mitochondria that are burdened with heavy loads of oxidative damage [31].

Mitochondrial Aging

Key physiological alterations that occur with age

Aging is a complex process of maturation involving every organelle and every organ system. Aging involves a variety of genetic, biological, and environmental factors. Several physiological alterations that occur with age that underlie the aging process in central nervous system (CNS) have been reviewed [33]. There are several neurological and physical deficits associated with normal aging, even though there is no overt tissue loss [34, 35]. The distinctions between normal aging and neurodegenerative disease are difficult to define. For example, changes in memory and cognition seen with normal aging are much less severe than in neurodegenerative diseases, are not pathophysiological, and rarely progress into dementia [36]. Similarly, physical or motor

deficits are associated with normal aging, but not an inevitable aspect [37]. Typically, discrimination between physiological and pathological brain aging emerges when groups of individuals are compared [38]. One central theme in much of the aging literature is that there is a loss of homeostatic reserve, the capacity of neurons to respond to cellular stressors, with age. Furthermore, the decline in the homeostatic reserve does not affect the functional resting state of the neurons or the base-line level of neuronal activity, but the decline is only realized under excessive activity or some type of stressor (e.g. injury or neurodegenerative disease) [33].

There are no shortages of theories that explain the aging process from molecular up to the systemic level, reflecting the complexity of the process. Though brain aging is very complex, free radical damage is considered key to the progression of aging. Free radical damage to cellular components, especially to those necessary for energy production, can result in an increased reliance on compensatory mechanisms to maintain energy production that can set up an energy crisis situation [39]. A few theories that explain age-related cellular alterations are briefly mentioned, with an emphasis on how these changes can in turn disrupt normal mitochondrial respiration. Deterioration of mitochondrial function has been proposed as a primary contributor to the aging process [40]. Age-related declines in enzymatic activities of the TCA cycle and complexes of the ETC have been demonstrated [33, 41-54]. Determination of functional alterations in mitochondrial respiration are addressed and discussed further in Chapter 4.

Aging theories implicating mitochondria

Free Radical Theory of Aging

The free-radical theory of aging was first postulated by Denham Harman and since then has been the basis of a wealth of research for the past 50 years [55]. The theory maintains that critical cellular components are under constant attack by free radicals (FR), which are defined as any chemical species that contains one or more unpaired electrons [56]. Having unpaired electrons alters the molecule's reactivity, usually causing them to take electrons from other molecules. Alterations to electron numbers can result in

structural damage and altered function in many components inside the cell. Superoxide radical ($O_2^{\cdot-}$), hydroxl radical ($OH^{\cdot}$), and nitric oxide ($NO^{\cdot}$) are the most common cellular free radicals [57, 58]. These endogenously produced FR are thought to contribute to age-related functional declines seen in normal aging, as well as in degenerative diseases [59].

Mitochondrial Theory of Aging

Determining the primary source of FR production was integral in understanding cellular mechanisms in aging and to determine if slowing their production increases lifespan. The absolute dependency of mammalian systems on the healthy mitochondria and accumulating evidence of age-related changes in mitochondria compelled Harman to modify his free radical theory into the mitochondrial theory of aging [60]. In essence, the mitochondrial theory of aging postulates that FR damage to mtDNA accumulates with age, resulting in altered genetic blueprints. These altered blueprints lead to the production of abnormal components of the ETC that are dysfunctional, gradually culminating in cellular energy deficits [61]. Mitochondria are known to be a major source of FR since they are inevitably produced during normal cellular respiration [62]. Certain species can be less toxic, meaning they can diffuse some distance before they attack cellular components relatively far from where they were produced. These less toxic species and free radicals are collectively called reactive oxygen species (ROS) and reactive nitrogen species (RNS) [63]. A few examples of ROS are: superoxide ($O_2^{\cdot-}$**)**, hydroxyl radical ($OH^{\cdot}$**)**, and hydrogen peroxide (H_2O_2). Nitric oxide (NO), nitrogen dioxide ($\cdot NO_2$), and peroxynitrite ($ONOO^-$) are examples of RNS. ROS and RNS are capable of inflicting oxidative changes to lipids, proteins, and nucleic acids [58].

Mitochondria are particularly susceptible to oxidative damage since ROS are generated by components of their ETC [64], specifically complex 1 and 3. There is a 5 to 10 times higher steady state of $O_2^{\cdot-}$concentration present in mitochondria making them susceptible to accumulating oxidative damage [65]. ROS generation has been shown to decrease antioxidant capacities in the brain [66, 67]. If the antioxidants are lower than oxidants produced a subsequent accumulation of oxidative damage can occur in mitochondria. Mitochondria that have accumulated oxidative damage with age actually

produce ROS at an accelerated rate [68, 69]. Since mtDNA are close in proximity to the source of FRs and lack certain repair mechanisms, they are particularly sensitive to oxidative damage. In isolated from liver of Sprague Dawley rats, mtDNA has been shown to accumulate 16 times more damage than nuclear DNA with age [70, 71], but may not accumulate in the same way in brain tissue [72]. In addition to the high level of damage, there is no evidence that mitochondria are capable of repairing DNA damage, compared to the nucleotide-base-excision seen in nuclear DNA, which causes mtDNA damage to be more prevalent and persist longer [73]. Oxidative damage to mtDNA can result in production of aberrantly made components necessary for respiration.

There are several caveats to interpreting MTA and the research revolving around this theory. Different tissues can produce various amounts of free radicals, respond to, and accumulate various amounts of damage differently. The degree of damage that is allowed to collect varies across tissue types [74]. Since most tissue types have very different metabolic demands, it is likely that mitochondrial respiration rates are responsible for these differences. The higher the metabolic activity the more free radicals are produced since they are a normal byproduct of aerobic respiration. Over many years, production of free radicals increase, there is a decrease in antioxidant protection, or the damage they inflict begins to exceed the cell's ability to repair the resulting damage [75]. This condition, known as oxidative stress, plays a substantial role in aging and a variety of neurodegenerative diseases.

There is a large amount of experimental evidence that established an age-related decline in mitochondrial function. The evidence for mitochondrial dysfunction that occurs with aging is based on both enzymatic assays of several components of the ETC and functional assays from intact isolated mitochondria. Support for the mitochondrial theory of aging has been obtained from enzymatic assays of the components of the ETC using a variety of animal species and tissue types including heart [76-78], skeletal muscle [79-84], liver [59, 62, 85-89], and brain [47, 50, 52, 90, 91]. Although many of the early enzymatic studies clearly demonstrated age-related changes, results from one tissue type are often inappropriately generalized to other tissue types [59, 86, 92-97]. The idea that activities of the ETC decline at a uniform rate across different tissue types remains

controversial [48, 98]. The central nervous system (CNS) is unique from other organs and likely to be more susceptible to aging since it is extremely active metabolically, utilizes a large amount of oxygen, has a high content of free fatty acids that can be easily damaged by free radicals, and has limited regenerative properties. Some reports insinuate that significant enzymatic declines reflect functional declines without actually monitoring respiration in intact mitochondria [33, 40, 99-102]. With numerous reports showing a decline in components of the ETC, this fueled functional studies to determine if these deficits culminate in an overall functional decline in intact mitochondria. Several groups have indeed reported evidence supporting a functional decline in mitochondrial respiration with age [103, 104].

Many of these early studies confirmed the validity of the mitochondrial theory of aging and defined the time course and the extent of mitochondrial dysfunction in various species and tissue types. With that said, these early studies produced a broad-based assumption about mitochondrial aging that lacked species, strain, tissue and mitochondrial fraction specificity. Like many other tissues and organs in the body, the CNS is composed of many heterogeneous cell types that vary cytoarchitecturally and biochemically. Distinct regions of the CNS display differences in mitochondrial bioenergetics based on both enzymatic [42, 105, 106] and respiration parameters [68, 107-112]. Due to cellular diversity, the CNS may age very differently than other systems. A portion of this dissertation attempts to address these specificity issues in order to better understand how mitochondria age in the CNS.

Mitochondrial-Lysosomal Axis Theory of Aging

Until mitochondria are replaced in the cell, worn-out or dysfunctional mitochondria are utilized to maintain metabolic needs. These mitochondria are likely to have a significantly higher proportion of damaged lipids, proteins, and components of their ETC before they are degraded. The structural damage may not be sufficient to produce overt changes in respiration under normal circumstances, but may produce greater levels of ROS. Brunk and Terman [113] suggests that insufficient renewal of mitochondria probably has the most serious consequences, in terms of energy production and additional

ROS formation. Brunk and Terman maintain that an age-related decline in mitophagy and autophagy of other organelles through lysosomal machinery may impede normal cellular repair that would be exacerbated under pathological conditions. Therefore, this theory was termed the mitochondrial-lysosomal axis theory of aging.

Several groups have shown an age-related accumulation of incompletely degraded mitochondria, known as lipofuscin in neuronal tissue [113-115]. Worn-out cellular components would not be in optimal working condition until they are replaced by newly synthesized organelles and would be more susceptible to cellular stressors. Neuronal mitochondria have slower turnover rates than many other cell types [29], making proper turnover imperative due to the extended time mitochondria are utilized before being turned over. Logically, mitochondria in the synaptic terminal would have even longer turnover rates due to their being a greater distance from the nucleus. This spatial separation from the nuclear synthetic machinery may make synaptic mitochondria more susceptible to dysfunction, especially with age-related declines in turnover rate.

Mitochondria on the brink of dysfunction and age-related declines in turnover could be an ideal scenario for an exacerbated cellular response to perturbation. A decline in mitochondrial fission/fusion activity in postmitotic cells is associated with a concomitant decline of membrane potential and intact mtDNA [116]. These mitochondria would be less equipped to handle cellular perturbation than properly repaired mitochondria. The accumulation of damage to mtDNA can lead to damage to the mitochondrial genome resulting in aberrant production or even loss of certain mitochondrial gene products [85]. This in turn may lead to an increased production of ROS that contributes to an age-dependent decline of respiratory function, especially in postmitotic cells [117]. Molecular defects can lead to altered expression of the mtDNA-encoded genes or impairment in biogenesis of mitochondria also causing deficiencies in energy metabolism [67]. One possible theory that describes a mechanism for how oxidative damage could accumulate in mtDNA is known as the “survival of slowest”. This theory proposes that a large deletion in mtDNA could prevent assembly of the superoxide-generating enzyme complexes resulting in diminished $O_2^{\cdot-}$ production. These mitochondria would be able to avoid degradation due to minimal mitochondrial

membrane damage compared to that in mitochondria with normal $O_2^{\cdot-}$ production, which is how lysosomes recognize dysfunction or worn out organelles [118]. Even though these mitochondria are malfunctioning and able to produce little ATP, lysosomes would not target them for degradation because of the minimal damage to the mitochondrial membrane, giving these mitochondria an advantage for survival.

Traumatic Brain Injury

Traumatic brain injury (TBI) is defined by the Center for Disease Control as a blow, jolt, or penetrating injury to the head that disrupts the function of the brain. Experimental focal TBI results in a rapid and significant loss of neurons in the tissue immediately below the site of the impact. TBI consists of a primary insult resulting from the biomechanical forces directly damaging neuronal tissue. The depth of the insult is directly proportional to the extent of neuronal tissue loss that ensues.

Epidemiology

In the United States, the Center for Disease Control estimates that 1.5 million people sustain head injuries each year [119] and this is the leading cause of death and life-long disability in young adults [63] with an annual cost over 60 billion dollars in medical expenses and loss of productivity [120]. These numbers are expected to continually rise, underscoring the necessity for an effective therapy.

PRIMARY INSULT VERSUS SECONDARY INJURY CASCADES

There has been a wealth of knowledge obtained through studying the pathological events that occur following experimental TBI. The initial insult or blow to the head is defined as the primary injury. There are many different models of experimental TBI; most include either a focal or diffuse head injury. This thesis utilizes the cortical contusion injury model (CCI) that subjects animals to a focal insult, which results in a rapid and significant loss of neurons in the tissue immediately below the site of the impact, core and penumbral regions. Extensive tissue loss is partially due to a disruption

of mitochondrial respiration occurring rapidly after the insult [121, 122]. Similar mitochondrial dysfunctions occurs in experimental models of diffuse head injury (e.g. fluid percussion injury model), but to a lesser degree [123]. Detrimental secondary injury cascade evolve over a period of hours to weeks after the trauma [124], this may offer opportunities for intervention due to this prolonged time course [125, 126]. Secondary injury cascades are believed by many to be more detrimental than the initial insult itself [127], necessitating research focused on defining mechanisms responsible for tissue loss and functional impairments and how to modulate these processes.

Key pathological events

Several key secondary damage cascades following TBI sets the stage for continued cellular dysfunction. Following the insult, there is an over activation of *N*-methyl-D-aspartate (NMDA) receptors, glutamate is released in large quantities, and excitotoxicity ensues. Associated with this excitotoxic event there is a large influx of cytosolic calcium that overloads mitochondria, which results in metabolic dysfunction and increases in oxidative damage [126-129]. Neuronal survival is intimately linked to mitochondrial homeostasis, due to the fact that mitochondria supply the CNS with a majority (80-85%) of ATP and regulate calcium within the cell [130]. As a result of these two functions, mitochondria normally operate close to their physiological peaks and are very sensitive to cellular perturbations. Mitochondria have been shown to be unable to sequester high levels of calcium influx [131] or maintain appropriate ATP levels [126] following TBI, since both functions utilize the proton gradient [132-134]. It is unknown if there are differences in respiratory capacities of mitochondria in neuronal and astroglial cells. In this dissertation, several bioenergetic parameters were collected from two distinct populations of mitochondria, synaptic and extrasynaptic. Synaptic mitochondria are located in synapses coming from both dendrites (neuronal input) and pre- and post synaptic neuronal terminals (neuronal output). During the homogenization process these synapses are pinched off and the lipid bilayer closes up around the synapse to create what is known as a synaptosomes. Synaptosomes contain mitochondria that is located in the synapse and can be isolated using various sucrose gradients. Synaptosomes can be

ruptured to release mitochondria trapped in the lipid bilayer and therefore analyzed separately from mitochondria from other regions. Extrasynaptic mitochondria are mitochondria from all regions, excluding the synapses. Extrasynaptic mitochondria are isolated from different cell types and regions of those cell types. Extrasynaptic mitochondria are predominately isolated from astrocytes and microglia due to the relative abundance (estimates range from 3 to 10 times more) compared to neurons [135]. Nevertheless, mitochondria from the soma and axons of neurons do contribute to this fraction of mitochondria, but we are assuming that the respiration from this fraction is primarily from astroglia cells isolated from the cortex.

There is a growing body of research that documents the structural and functional damage that occurs to mitochondria following TBI [72, 121, 122, 125, 136-145]. Significant respiration changes are seen as early as 15 to 30 min post-injury [142, 145], with peak mitochondrial dysfunction at 12 to 24 h [122, 146]. Changes in mitochondrial bioenergetics can persist for up to 14 days [122]. There is limited information concerning whether or not early mitochondrial dysfunction is an all or none event [141, 147] or if dysfunction occurs on a gradient, which is addressed in this dissertation. Furthermore, acute (1 and 3 h) bioenergetic changes in mitochondrial respiration following TBI were characterized. Alterations in respiration following TBI were also determined in synaptic and extrasynaptic mitochondria separately. Regardless of extent, the damage that mitochondria sustain causes them to become contributors to secondary damage [121, 138]. This vicious cycle of secondary damage can continue for weeks after the initial injury. Several therapeutic interventions that target stabilizing mitochondria have shown promising results by reducing overall neuronal tissue damage as well as enhancing neurological outcome following TBI [121, 137, 141, 148, 149]. Finsterer [150] reviewed several drugs that might be utilized to improve aspects of mitochondrial bioenergetics in many respiratory chain diseases (e.g., epilepsy, dementia, etc), some of which may be effective in TBI as well.

Traumatic Brain Injury in the aged population

Epidemiology

Currently, aged individuals sustaining a TBI are treated solely according to guidelines derived from research primarily in young individuals [151]. The census for 2000 showed that close to 35 million people are age 65 years or older in the United States (U.S), over 12% of the total population [152]. Even though serious injuries are thought to be primarily a problem in younger individuals, 25% of all hospital traumatic admissions involve older adults [153]. Individuals 65 and older account for more than 80,000 TBI-related emergency department visits each year, with those 75 and older having the highest rates of morbidity and mortality [151]. Unfortunately, clinical studies have shown that age negative effects outcome of brain injury at every level of injury severity [154]. Although many possible factors could theoretically contribute to the disparity of outcomes following TBI between young and aged individuals, none has fully explained the adverse effects of age [155]. There are reports that even with a less severe insult elderly patients have a worse functional outcome and higher mortality rate [156]. With 32 million baby boomers over the age of 50, one-fifth of the U.S. population will be between the ages of 66 and 84 by the year 2030 [151]. These numbers underscore the growing need for research that reveal underlying mechanisms that lead to these more negative outcomes in the geriatric population following TBI. One recent clinical study showed that the overall crude mortality rate of penetrating trauma was 2.6 times that of blunt trauma [157]. This clinical data indicates the relevance of the head injury model utilized in these experiments, a focal penetrating blow to the brain (cortical contusion injury model), as a great research tool to understand age-specific responses to TBI. A portion of this dissertation was dedicated to characterizing mitochondrial dysfunction following TBI in young (3 – 5 mos) and aged (22 – 24 mos) rats. A lack of understanding the cellular mechanisms that result in age-specific outcomes to TBI is currently compromising our efforts in developing effective treatments.

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Chapter 2: Early Changes in Mitochondrial Respiration Following Traumatic Brain Injury

Specific Aim 1 will test the hypothesis that mitochondrial dysfunction occurs early and in an injury-dependent manner following traumatic brain injury.

Introduction

Experimental Traumatic Brain Injury (TBI) results in a rapid and significant loss of neurons in the tissue immediately below the site of the impact. TBI consists of a primary insult resulting from the biomechanical forces directly damaging neuronal tissue. The depth of the insult is directly proportional to the extent of neuronal tissue loss that ensues in young animals. The primary insult is then followed by secondary cascades that evolve over a period of hours to weeks after the trauma [124] and may offer opportunities for intervention [125]. Secondary damage is believed to be much more detrimental than the initial insult itself, which drives research to focus on what causes these impairments and how they can be minimized. In particular, mitochondria sustain considerable structural and functional damage following TBI [122, 142-145, 158]. Neuronal survival is intimately linked to mitochondrial homeostasis. Mitochondria supply the central nervous system with energy (ATP) as well as regulating calcium within the cell [130]. Several therapeutic interventions that target stabilizing mitochondria have shown promising results by reducing overall neuronal tissue damage as well as enhancing neurological outcome following TBI [121, 137, 141, 148, 149, 159]. Finsterer [150] reviewed several drugs that could be utilized to improve aspects of mitochondrial bioenergetics in many respiratory chain diseases (e.g., epilepsy, dementia, etc), some of which may be effective in TBI as well.

Experimental models of TBI have shown that mitochondrial damage occurs rapidly. Significant respiration changes are seen as early as 15 to 30 min post-injury [142, 145], with peak mitochondrial dysfunction at 12 to 24 h [122, 146]. Changes in mitochondrial bioenergetics can persist for up to 14 days [122]. Some of the very early changes in mitochondrial bioenergetics may represent part of the primary injury cascade

and not be amenable to pharmacologic intervention. There is limited information concerning the interaction between injury severity and early mitochondrial dysfunction. Two relatively recent studies, using transgenic mice, reported no significant alterations in mitochondrial respiration at 24 h following a moderate insult, while a severe injury produced a significant functional deficit [141, 147]. The present study explored possible early mitochondrial bioenergetic changes (1 h and 3 h) at three different injury severities (mild, moderate, and severe).

Materials and Methods

Surgical procedures

This study used young adult male Sprague-Dawley (SD) rats (Harlan Labs, Indianapolis, IN; *n* = 63; 275-300 g), which were housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water.

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee.

Animals were subjected to a moderate unilateral cortical contusion as previously described [75, 160]. Anesthesia was induced with isoflurane (5%) and reduced to 2.0% after subjects were placed in a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA). All injuries were produced using a pneumatic controlled cortical impact device (TBI 0310, PSI, Fairfax Station, VA) with a soft stop Bimba cylinder (Bimba Manufacturing, Monee, IL). The head was positioned in the horizontal plane with the nose bar set at - 5. Following a midline incision exposing the skull, a 6.0 mm craniotomy was performed with a Michelle trephine (Miltex, Bethpage, NY) placed lateral to the sagittal suture and centered between bregma and lambda. The skull cap at the craniotomy was carefully removed without damaging the underlying dura, and the exposed cortex was injured according to the parameters of nine randomly assigned groups: (I) *Naïve*. No anesthesia or any type of surgical intervention (*n* = 7); (II - III) *Sham*. Rats subjected to a craniotomy alone without injury and assessed at 1 (*n* =7) and 3 h (*n* = 6); (IV - V) *Mild injury* (0.5 mm cortical displacement) assessed at 1 (*n* = 7) and 3 h (*n* = 6) post-injury; (VI – VII) *Moderate injury* (1.0 mm cortical displacement) assessed at 1 (*n* = 6) and 3 h

(n = 7) post-injury; (VIII – IX) *Severe injury* (1.5 mm cortical displacement) assessed at 1 (n = 8) and 3 h (n = 6) survival. After injury, Surgicel (Johnson & Johnson, Arlington, TX) was laid on the dura and the skull disk replaced. A small amount of dental acrylic was applied over the skull disk and allowed to harden. The skin was sutured with wound clips and the animals returned to their cages for post-operative recovery. Animals were maintained at 37°C.

Total mitochondria isolation

Mitochondrial isolation protocol and all procedures were performed on ice. Animals were briefly gassed (less than 1 minute) with CO_2 until flaccid, decapitated and the brains rapidly removed. The cortices were dissected and a 10-mm (diameter) circular punch encompassing the site of impact and penumbral tissue and a corresponding contralateral cortex at the site of the impact were extracted. The cortices were placed in separate glass dounce homogenizers with 4 mL of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Tissue punches were homogenized and mitochondria isolated by differential centrifugation. Homogenate was centrifuged twice at 1300 × g for 3 min in a microcentrifuge at 4°C. The resulting supernatant was diluted with isolation buffer containing EGTA and centrifuged at 13,000 × g for 10 min. The resulting pellet was resuspended in 500 μL of isolation buffer with EGTA and subjected to a pressure of 1,200 psi for 10 min inside the nitrogen cell disruption bomb (Parr Instrument Company, Moline, IL) at 4◦C. After rupturing the synaptosomes, the samples were brought up to a final volume of 2 mL with isolation buffer containing EGTA, and centrifuged at 13,000 × g for 10 min. The pellet was resuspended in isolation buffer without EGTA and centrifuged at 10,000 ×g for 10 min. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of 10 mg/mL or higher. The protein concentration was determined using the bicinchoninic acid protein (BCA) assay kit (Pierce, Rockford, IL).

Mitochondria respiration measurements

Mitochondrial functionality was assessed using an Oxytherm Clark-type oxygen electrode (OXYT1/ED, Hansatech Instruments, Norfolk, UK). Mitochondrial protein (~180 µg taken from uninjured tissue and ~200 µg from injured tissue) were placed in the sealed Oxytherm chamber containing respiration buffer (125 mM KCl, 0.1% BSA, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, pH 7.2) and was continuously stirred at a constant temperature of 37° C. To ensure all mitochondrial respiration assays contained equal amounts of respiring mitochondria, measured by state II respiration rates (10 – 15 nmoles of oxygen/ min), on average 20 µg more mitochondrial protein was needed when isolated from the injured cortex. The total volume of respiration buffer and mitochondrial protein placed in the chamber was 250 µL.

Rates of oxygen utilization were defined as amount of oxygen (nmoles) utilized over time (min) on consecutive administrations of oxidative substrates, similar to those described previously [146]. The oxidative substrates were injected into the Oxytherm chamber sequentially as follows: **state I** – mitochondria with respiration buffer and no substrates added so respiration was minimal; **state II** (pyruvate/malate) - addition of 2.5 µL of 2.5 mM pyruvate and 5 mM malate (P/M) and allowed to reach a steady, constant rate; **state III** (ADP) – addition 1.25 µL of 150 µM ADP allowed to reach maximum rate; **state IV** (oligomycin) – addition of 1.0 µL of 1 µM oligomycin allowed to reach minimum rate; **state V-FCCP**– addition of 1.0 µL of 1 µM FCCP allowed to reach maximum rate; 0.2 µL of rotenone was added to shut down complex 1 activity resulting in cessation of oxygen utilization; **state V-succinate** - addition of 2.5 µL of 10 mM succinate and allowed to reach maximum rate. State V (succinate) represents maximum complex 2 activity since FCCP is still present in the assay system. Figure 2.1 depicts a typical mitochondria respiration trace depicting the sequence of substrate additions and subsequent oxygen utilization rates.

Overall oxygen utilization rate was determined by measuring the amount of oxygen consumed throughout all states of respiration divided by the time elapsed and amount of mitochondrial protein present in the assay. This measurement serves as an index of their overall respiration capacities and can detect overt alterations in respiration.

The respiratory control ratio (RCR) was determined by dividing the rate of oxygen utilization for state III (ADP) by state IV (oligomycin). RCRs tell how coupled the electron transport chain (ETC) is to ATP production. The states of respiration (III-V), overall oxygen utilization rates, and RCRs were all collected to better define respiration capabilities. To ensure the quality of the isolation procedure, mitochondria isolated from contralateral cortex had to produce RCRs of ≥ 5. If the RCRs of < 5 both the ipsi- and contralateral hemispheres would be excluded from this study. All mitochondrial samples were able to produce an RCR ≥ 5 when taken from the contralateral hemisphere. Respiration capabilities of mitochondria isolated from the contralateral cortex served as an internal control for extent of dysfunction as a result of the injury in the ipsilateral cortex. Previous studies, as well as our own, have shown no early changes in mitochondria respiration in the contralateral hemisphere following TBI [161-163] or focal cerebral ischemia [164], supporting the use of the contralateral cortex as an appropriate "internal" control.

Statistical analyses

All data are reported as group means ± SD. Overall oxygen utilization and rates (states II through VI) are plotted as percent of contralateral rate to show extent of damage dependent on injury severity and time post-injury. Actual rates of all states of respiration are also reported to show relative respiration capabilities across groups and to show extent of biological variance. Respiration states (I – V) and state V (succinate) via Complex 2 were calculated as nmol of oxygen/min/mg of mitochondria in 250 µL of respiration buffer, described previously [142]. RCRs, are an index of overall health of the mitochondria, and reported for both ipsi- and contralateral sides separately.

Figure 2.1. Typical respiration trace from a sham and a severely injured rat.

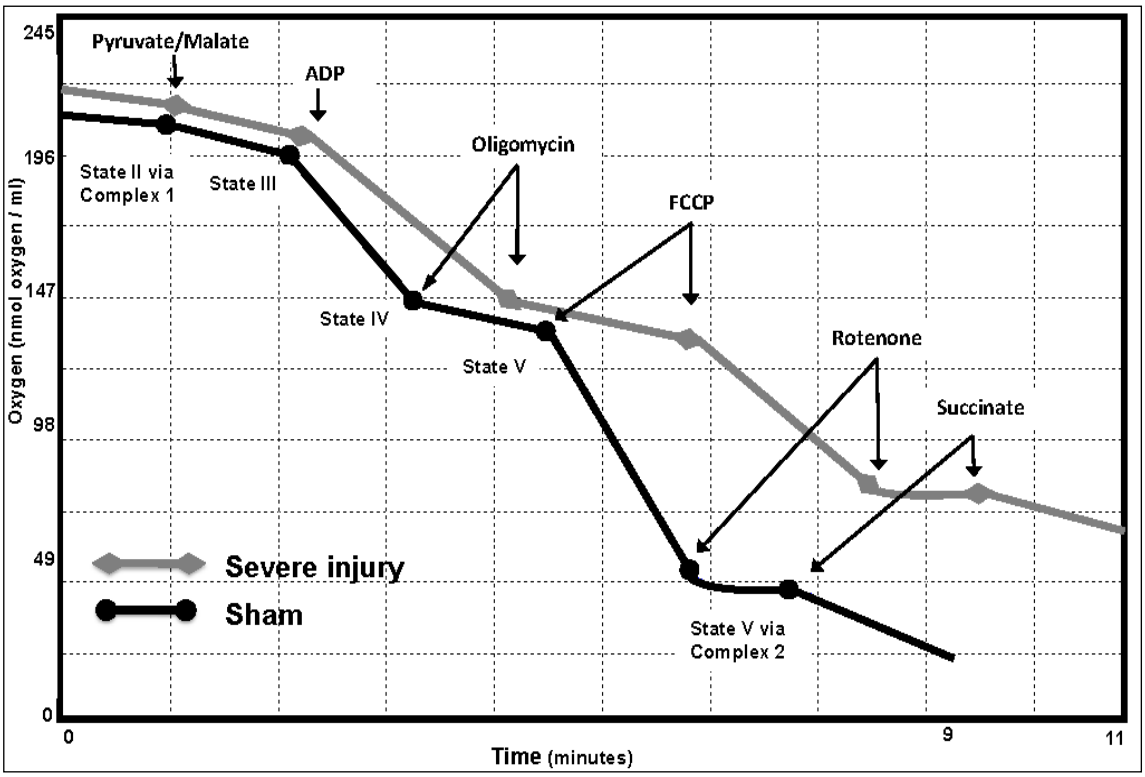

Figure 2.1. Typical respiration trace of ipsilateral mitochondria isolated at 1 or 3 h following a sham surgery or a rat receiving a severe injury. **state I**: no substrates for respiration have been added; no respiration apparent. **state II**: addition of P/M; basal rate of respiration. **state III**: addition of ADP completes necessary substrates needed for operation of the ETC; high level of oxygen utilization indicated ADP is getting converted into ATP. **state IV**: addition of oligomycin; return of basal rate of respiration since the ATP synthase is shut down and no electrons are allowed to return to matrix. The ETC continues only to maintain mitochondrial membrane potential due to loss of protons back into the matrix. **state V-FCCP**: addition of FCCP; represents maximum rate of respiration, causes uncoupling of the ETC to ATP synthesis by allowing protons to rush back into the matrix. Rotenone is added to shut down complex 1-driven respiration. **state V-succinate**: addition of succinate: maximum rate of respiration via complex 2 due to FCCP still being present in the respiration chamber.

A paired *t* test was utilized to detect possible hemispheric differences in naïve animals. An unpaired *t* test was used to compare the ipsilateral sides of 1 and 3 h post-surgery sham animals to investigate the possible suppression of mitochondrial function due to the surgery (anesthesia and craniotomy). To determine if sham animals were different than naïve rats, respiration rates from 3 h post-surgery sham animals were compared to naïve rats using an unpaired *t* test. To show that the contralateral hemisphere could be used as an internal control for typical respiration and reduce biological variance, an unpaired *t* test was used to compare sham animals to contralateral tissue. Two separate *t* tests were performed to compare mitochondrial respiration of sham animals to contralateral tissue from severely injured animals of the same time post-injury (1 h contra to 1 h shams and then 3 h contra to 3 h shams). A two-way ANOVA (injury severity X time) was used to determine the extent of mitochondrial dysfunction following injury. All ANOVAs were followed by a Student-Newman-Keuls post-hoc analysis. The alpha level was set at 0.05 for significance.

Results

Naïve animals. Statistical analysis using a paired comparison *t* test failed to reveal any significant hemispheric differences among naïve animals for any of the dependent measures of mitochondrial bioenergetics: RCR ($t_6 = 0.13$, $p > 0.1$), overall oxygen utilization ($t_6 = 0.96$, $p > 0.1$), P/M ($t_6 = 0.23$, $p > 0.1$), ADP ($t_6 = 0.93$, $p > 0.1$), oligomycin ($t_6 = 0.86$, $p > 0.1$), FCCP ($t_6 = 1.6$, $p > 0.1$), and succinate ($t_6 = 1.6$, $p > 0.1$). These results support the idea that both hemispheres have the same mitochondrial respiration capacities.

Sham surgery animals. Animals were subjected to both anesthesia and craniotomy then killed at 1 or 3 h post-surgery. For each bioenergetic parameter (e.g. total oxygen utilization), *t* tests were carried out to probe for alterations in mitochondrial function due to the general surgical procedure and no significant differences were found. A paired comparison statistical analysis failed to identify any significant differences in mitochondrial bioenergetics between the two hemispheres due to the surgical process ($p >$

0.1). These results indicate that the craniotomy and surgical procedures do not alter mitochondrial bioenergetics.

Comparison of naïve and sham animals. The cortex immediately beneath the sham craniotomy site was also compared to values obtained from naïve animals. An unpaired *t* test failed to identify any significant differences in respiration capabilities between naive and sham animals at either 1 or 3 h post-surgery ($p > 0.1$). These results support the use of sham animals as an appropriate control.

Comparison of sham versus contralateral tissue taken from injured animals. Two separate ANOVAs were used to compare the contralateral side of injured animals to sham operated animals at 1 and 3 h post-injury. No significant differences ($p > 0.1$) in respiration capabilities were observed, indicating that the contralateral hemisphere of injured animals reflects that observed in the sham animals and can be used as an internal control for each subject. Since mitochondria from the contralateral hemisphere are unaffected by the insult, we compared ipsilateral respiration rates as a percent contralateral side to reduce biological variance.

Injury-induced mitochondria bioenergetics:

Mitochondrial amounts used for analysis. Equal amounts of mitochondrial protein isolated from naïve, sham, or contralateral tissue of injured animals could produce similar, consistent respiration traces. The average amount of mitochondrial protein taken from uninjured cortical tissue used to test mitochondrial functionality was 180.1 µg ± 25 ($n = 39$) compared to 205.5 ± 27 µg from the site of injury. An paired t test showed these amounts of mitochondrial protein used for respiration analysis were significantly greater for the injured hemisphere [$t_{39} = 5.2$, $p < 0.0001$].

Table 2.1. Respiratory Control Ratio (RCR) in naïve cortices and contralateral to injury.

Injury Group	1 h Post-injury			3 h Post-injury		
	Mean	**± SD**	**n**	**Mean**	**± SD**	**n**
Naive	6.4 ± 0.6 (n = 7)					
Sham	6.7	0.7	7	6.7	0.4	6
0.5 mm	6.4	0.6	7	6.9	0.6	6
1.0 mm	6.4	0.5	6	6.7	0.7	7
1.5 mm	6.4	0.5	8	6.8	0.6	6

Table 2.1. RCR values (state III / state IV) are an overall index of the overall health of isolated mitochondria. RCRs above 5 are indicative of healthy mitochondria that have a tight coupling of the electron transport chain with ATP production. No significant differences existed between RCR values of mitochondria isolated from contralateral tissue of sham animals or injury groups and naïve rats. Data verifies that mitochondria taken from the contralateral hemisphere could be used as a suitable internal control that would reduce biological variance.

Overall oxygen utilization. The overall rate of oxygen utilization demonstrated a significant effect for injury severity [$F(3, 45) = 102.54$, $p < 0.0001$], but not for time post-injury [$F(3, 45) = 0.196$, $p > 0.1$] (Figure 2.2). Post-hoc analysis showed all injured groups were significantly lower than sham animals ($p < 0.05$) and, that as the injury severity increased, oxygen utilization significantly declined ($p < 0.05$).

State II respiration (pyruvate/malate). A two-way ANOVA (time X injury severity) revealed no significant effect for time post-injury [$F(1, 45) = 1.065$, $p > 0.1$] or for the severity of injury [$F(3, 45) = 0.114$, $p > 0.1$]. All preparations contained equal amounts of respiring mitochondria determine by state II respiration (10-15 nmoles oxygen/min) as baseline. This gave all groups equal ability to produce similar respiration traces, regardless of the amount of mitochondrial protein added, unless damage to components of the ETC is compromising activity as a result of the injury. By simply adding the same amount of mitochondrial protein to each respiration assay, we would be

Figure 2.2 Injury-severity dependent alterations to overall oxygen utilization rates.

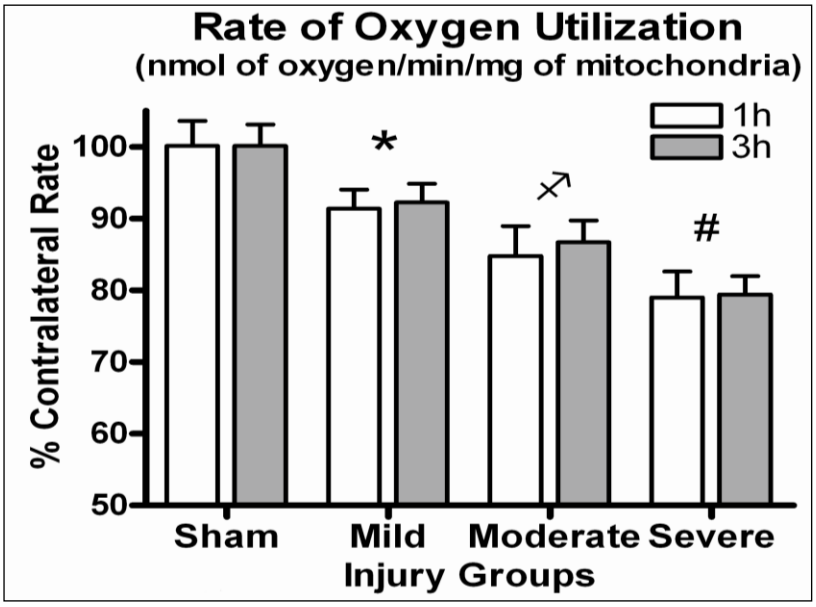

Figure 2.2. Overall oxygen utilization rate. As injury severity increases the overall oxygen utilization significantly drops, indicating a decline in mitochondrial respiration following injury. Bars represent group means ± SD. * $p < 0.01$ compared to sham, ↗ $p < 0.05$ compared to mild, and # $p < 0.01$ compared to moderate injury.

assuming that all preparations contain equal amounts of debris and functional mitochondria.

State III respiration (ADP). Figure 2.3 depicts how efficiently ADP is utilized to make ATP. A two-way ANOVA was significant for effect for injury severity [$F(3, 45) = 38.982$, $p < 0.0001$], but not for time post-injury [$F(1, 45) = 1.145$, $p > 0.1$]. The magnitude of state III decline was directly proportional to mitochondria's ability to utilize ADP. No significant interaction between injury severity and time post-injury existed [$F(3, 45) = 0.495$, $p > 0.1$]. Post-hoc test revealed that all injured groups were significantly lower than sham operated controls ($p < 0.05$) and that as the injury severity increased the ability to utilize the ADP significantly decreased ($p < 0.05$).

Figure 2.3. Injury-severity dependent decline in ATP production rates.

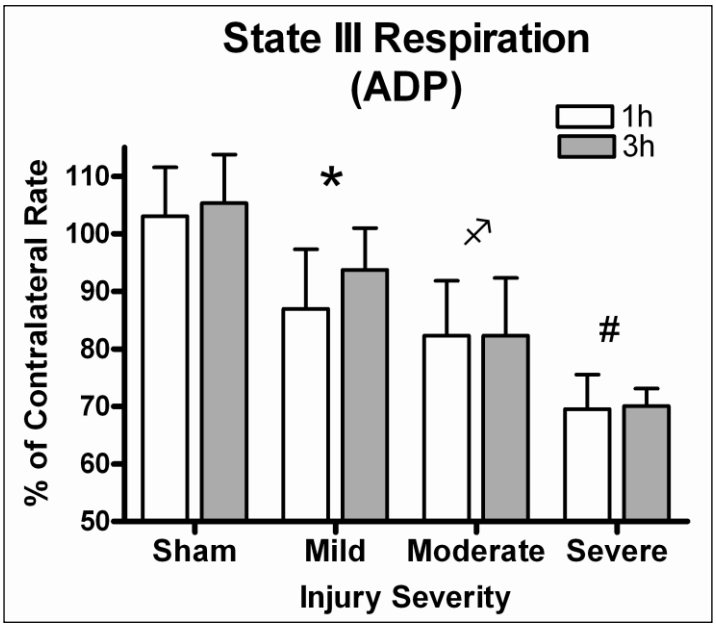

Figure 2.3. State III (ADP) rates. The ability of mitochondria to produce ATP significantly declines as a function of injury severity. Bars represent group means ± SD. * $p < 0.05$ compared to sham, ↗ $p < 0.05$ compared to mild, # $p < 0.05$ compared to moderate injury.

Oligomycin (state IV respiration). State IV respiration indicates inner mitochondrial membrane integrity. A two-way ANOVA revealed a significant increase in state IV effect for injury severity [$F(3, 45) = 10.46$, $p < 0.0001$], but not for time post-injury [$F(1, 45) = 0.627$, $p > 0.1$] (Figure 2.4). Post-hoc analysis revealed that rats receiving a moderate injury were significantly different from sham animals. State IV rates were significantly greater in animals receiving a severe insult compared to rats sustaining a moderate injury ($p < 0.05$).

RCR (state III / state IV respiration). A decline in coupled respiration, assessed by RCR values, were evident following TBI. A two-way ANOVA revealed a significant effect for injury severity [$F(3, 45) = 77.006$, $p < 0.0001$], but not for time post-injury [$F(3, 45) = 2.022$, $p > 0.1$]. There was no significant interaction ($p > 0.1$) (Figure 2.5). Post-hoc analysis revealed that all injured groups had significantly lower RCR values than

Figure 2.4. Injury-severity alterations to inner mitochondrial membrane integrity.

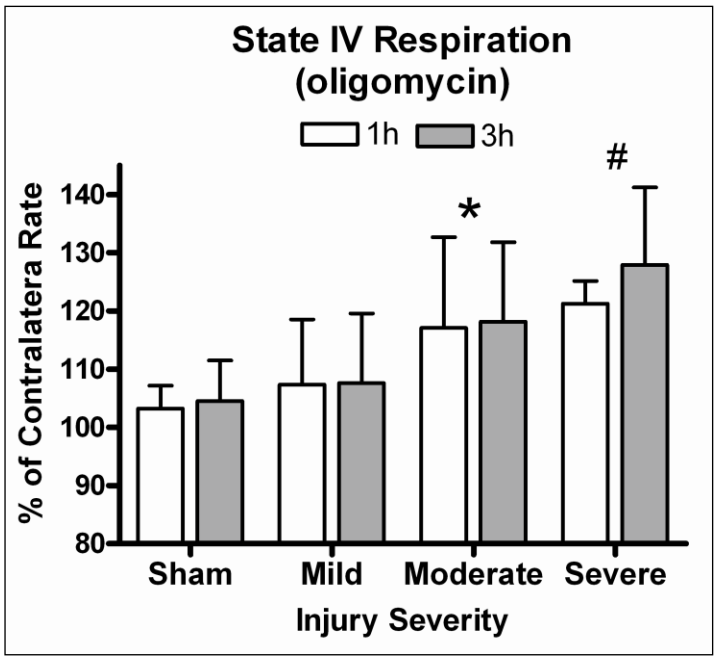

Figure 2.4. Oligomycin rates. The inner membrane becomes increasingly damaged, causing more of the proton to be lost back into the matrix, resulting in less of the proton gradient being coupled with ATP production. Bars represent group means ± SD.
* $p < 0.05$ compared sham and # compared to moderate injury.

sham operated controls ($p < 0.05$) and as injury severity increased the RCR values significantly decreased ($p < 0.05$).

FCCP (state V respiration). The metabolic poison FCCP was introduced into the Oxytherm chamber to monitor state V respiration. A two-way ANOVA revealed a significant effect for injury severity [$F(3, 45) = 34.492$, $p < 0.0001$], but not for time post-injury effect [$F(3,45) = 0.349$, $p > 0.1$] (Figure 2.6). Post-hoc testing revealed that all injury severity groups significantly differed from sham controls ($p < 0.05$) and as injury severity increased complex-driven maximum rate of respiration significantly declined ($p < 0.05$).

Figure 2.5. Injury-severity dependent reduction in coupled respiration.

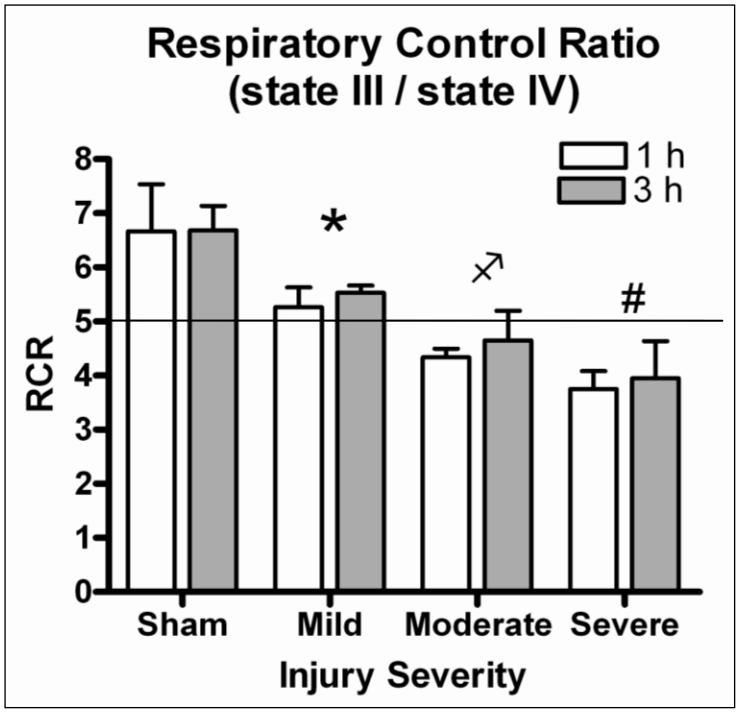

Figure 2.5. Respiratory Control Ratio (RCR). RCR is an index of how coupled respiration is to ATP production. Regions above line on graph (RCR of 5) represents indicates well-coupled mitochondria, below line represents dysfunctional mitochondria. RCRs significantly drop with increasing injury severity. Data suggests mitochondria are displaying an uncoupling of respiration from ATP production, meaning a decreased ability of mitochondria to produce ATP. Bars represent group means ± SD. * $p < 0.05$ compared to sham ↗ $p < 0.05$ compared to mild # $p < 0.05$ compared to moderate.

Succinate (state V respiration via complex 2). Succinate was added to the chamber to study complex 2 (state V) respiration. A two-way ANOVA was not significant for time post-injury effect [$F(1, 45) = 0.195$, $p > 0.1$], but was significant for injury severity effect [$F(3, 45) = 51.416$, $p < 0.001$] (Figure 2.7). Post-hoc analysis revealed that all injured groups were significantly lower than sham operated controls ($p < 0.05$) and as the injury severity increased complex 2-driven maximum rate of respiration significantly decreased ($p < 0.05$).

Discussion

The present study performed a detailed analysis of mitochondrial bioenergetics following a mild, moderate, and severe unilateral cortical contusion injury (CCI). The results from this study are the first to demonstrate alterations in mitochondrial respiration with a mild injury. Animals subjected to a moderate and severe insult show an even greater mitochondrial dysfunction. Changes were observed as early as 1 h post-trauma and were equivalent at 3 h. We chose 1 and 3 h because these are times in the literature that conform to times when pharmacologic interventions are often carried out. These results support the idea that mitochondria are affected very early and precede many of the TBI injury cascades, including oxidative damage [165]. The fact that we can demonstrate functional mitochondrial respiration at early times after trauma suggests that therapeutic interventions aimed at assisting mitochondria need to be initiated early for possible neuroprotection.

Figure 2.6. Injury-severity dependent decline in maximum respiration (complex 1).

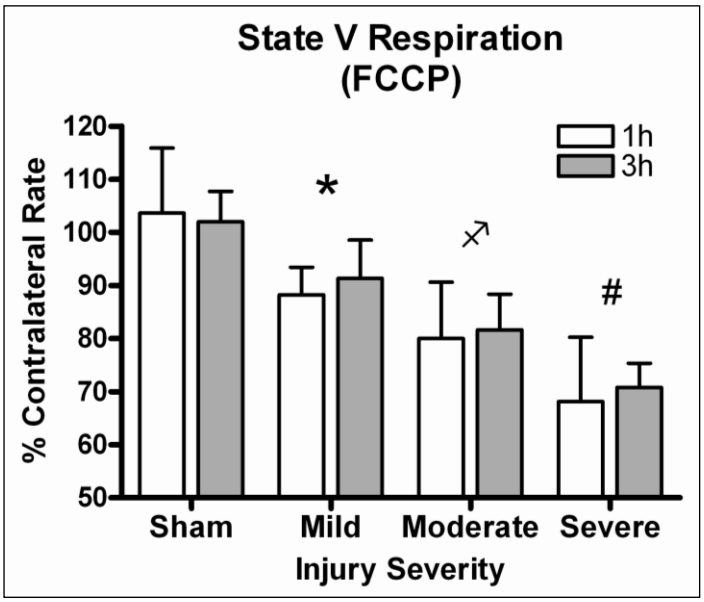

Figure 2.6. FCCP rates. Maximal respiration capability drops with increasing injury severity. Bars represent group means ± SD. * $p < 0.05$ compared to sham ↗ $p < 0.05$ compared to mild # $p < 0.05$ compared to moderate.

Our results differ from previous studies that have reported a lack of severity-dependent changes in mitochondrial respiration. Xiong et al. reported no significant alterations in mitochondrial bioenergetics 24 h following a moderate CCI in mice [141, 147], when deficits have been reported to be the greatest following a severe CCI in rats [122]. Discrepancies may be a result of species differences, mitochondrial purification procedures, or whether or not pooling tissue from multiple animals was used. Previous studies have shown that significant TBI-related behavioral dysfunction can occur without obvious tissue destruction and cavity formation [166, 167], suggesting that a mild injury disrupts normal brain biochemical and physiological function. A mild injury has also been shown to activate cortical astrocytes despite the lack of any obvious tissue destruction[168].

Figure 2.7. Injury-severity dependent decline in maximum respiration (complex 2).

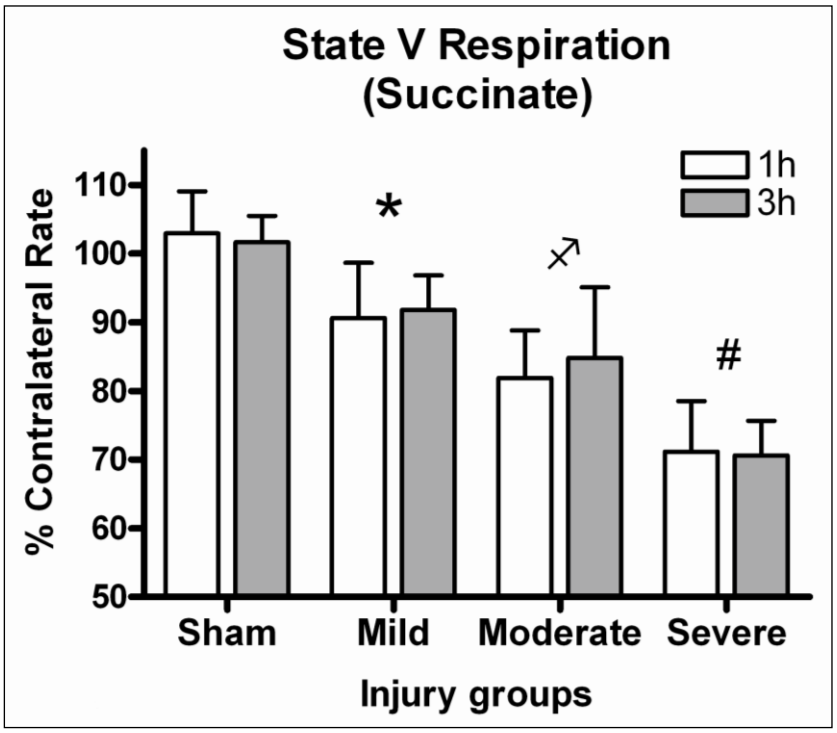

Figure 2.7. Succinate rates. Not only is complex 1 driven respiration effected by injury, but complex 2 as well. Bars are group means ± SD. * $p < 0.05$ compared to sham $p < 0.05$ compared to mild # $p < 0.05$ compared to moderate.

In the present study, bioenergetic measurements are reported as a percentage of that subject's uninjured contralateral hemisphere. Multiple experiments were undertaken to demonstrate that the contralateral uninjured cortex represents conditions normally observed in naïve animals. This provides a common baseline to compare results from multiple different experiments. As a further indicator of mitochondrial quality, only preparations in which the RCR value for the contralateral hemisphere was above 6.0 were used, a value equivalent to that observed in the sham operated groups (see Table 1). Previous reports have noted that an RCR above 5 indicates viable mitochondria [122, 142, 161].

In every mitochondrial preparation, including those from naïve animals, a portion of these organelles can be damaged or non-functional and still contribute to the overall protein concentration. Using the same amount of protein for each respiration assay assumes that the total protein content is primarily mitochondrial and any alterations in bioenergetics are attributable to a failure of total functioning organelles. Following more severe injuries, a larger portion of the sample contains debris or non-functional mitochondria making it difficult to accurately measure respiration rates. In efforts to try to maintain a consistent amount of functional mitochondrial protein, all groups were afforded the same starting potential to produce equivalent respiration traces. This was accomplished by adding an amount of mitochondrial protein that resulted in standardized level of oxygen utilization during state II respiration (~10-15 nmol O/min). Statistical analysis revealed no differences in state II respiration between any experimental groups ($p > 0.1$). In the present study, up to 14% more mitochondrial protein had to be used for injured cortex to maintain a standard level of state II respiration. This minor addition of sample for even the most severely injured animals suggests a high level of purity with our preparations considering the magnitude of injury for some animals. Other studies using a similar mitochondrial isolation protocol report a relatively high variance (240%) in the amount of mitochondrial protein used for respiration analysis following a severe CCI in SD rats [122] and mice [141, 147]. The necessity of using increased amounts of mitochondrial protein to obtain equivalent state II respiration rates indicates that some

component(s) of the mitochondrial respiration chain sustained some type of structural and/or functional damage as a result of the insult.

Total oxygen utilization rate was monitored to determine if there were overt respiration changes occurring in tissue immediately below the area of impact. This parameter takes into account all states of the mitochondrial respiration combined. Figure 2.2 clearly shows an injury-dependent decline in the overall rate of oxygen utilization indicative of a significant bioenergetic malfunction. Other studies report an early decline in mitochondrial oxygen utilization following TBI [122, 137, 169] but did not compare differences due to injury severity. In this study, a mild injury produces approximately a 10% decline the oxygen utilization compared to sham operated controls, while a severe injury results in a 20% or greater deficit. Total oxygen utilization rate declined as a function of injury severity, but changes between 1 and 3 h were not found. While measurement of overall oxygen utilization rate signifies a general overview of mitochondrial function it cannot determine which particular complex(s) are significantly affected following injury. The robust nature of the injury-dependent decline in total oxygen utilization suggests that multiple respiration states are involved. It is necessary to investigate which complexes and states of respiration are most involved.

State III respiration is demonstrated by adding ADP following a stabilization of P/M oxygen utilization. As protons cross the inner membrane, the ETC increases and consequently more oxygen is consumed as ATP is synthesized. In this study, state III respiration significantly declined as a function of injury-severity (Figure 2.3). More severely injured animals showed a 30% reduction in state III respiration, while animals with a mild injury showed a 10% reduction. Since the amount of ADP available was constant, a decline would signify a decline in ATP production, a situation that would be deleterious for cells attempting to regain homeostasis following the injury. There were no significant differences between 1 and 3 h post-trauma for state III respiration.

A previous study reported as much as a 43% drop in cortical ATP levels 15 min following a mild controlled cortical impact (CCI) and a 51% drop in severely injured SD rats [126]. ATP levels were restored by 40 min post-trauma in animals with a mild injury, but remained significantly reduced in animals receiving a severe insult. Sullivan et al

[145] reported an approximately 30% drop in ATP levels 30 min following a moderate CCI in cortical synaptosomes that was not restored until 6 h post-trauma. More severe injuries place higher energy demands on neuronal tissue, which are compounded by greater mitochondrial respiration deficits. A lack of ATP production could be partially responsible for the progressive tissue loss that quickly begins in the cortex following TBI.

One direct cellular consequence of a significant drop in ATP would be an inability to acquire calcium homeostasis following TBI. Excitatory amino acids (EAA) play an important role in the mechanisms of secondary injury following TBI [145, 170-172]. Elevated EAAs increase intracellular calcium concentrations by mechanisms involving activation of NMDA-receptor/ion channels, the AMPA receptor, and voltage operated calcium channels. In order to maintain calcium homeostasis and neuronal survival, calcium must be removed from the cytosolic compartment via the calcium ATPase at the plasma membrane. This extrusion of calcium is primary mediated through the calcium ATPase, which requires large amounts of ATP. If the deficits in ATP production are large enough, cell death is inevitable.

Calcium influx can also cause significant alterations in membrane potential that reflect an intermediate unstable state of mitochondria, which may lead to or reflect mitochondrial dysfunction [173]. The adenine nucleotide translocator (ANT) typically exchanges ATP in the matrix for cytosolic ADP across the inner mitochondrial membrane, but can its ability can be altered by oxidative stress or a large influx of calcium. Damage occurs to ANT following TBI [161] may be responsible for the decline in ATP production as well as enhanced formation of the devastating mitochondrial permeability transition pore [174, 175].

Intact healthy mitochondria maintain a proton gradient primarily for synthesis of ATP, which is the dominant pathway for reentry of protons into the mitochondrial matrix [176]. Oligomycin is a metabolic poison that binds ATP synthase and inhibits ADP-stimulated respiration; following its addition mitochondria are locked into state IV respiration, where extent of proton leakage into the matrix can be determined. State IV indicates the extent that the proton motive force is coupled with ATP production versus maintaining the basal metabolic rate. With an isolated mitochondrial preparation, an

increase in oxygen utilization during state IV indicates an uncoupling of the ETC to ATP production as well as the magnitude of damaged to the inner mitochondrial membrane.

Figure 2.4 demonstrates an injury-related increase in state IV respiration that was most pronounced at the most severe levels (~20%). A mild injury produced oligomycin respiration rates that were equivalent to sham operated controls, while a moderate injury produces evidence that changes are occurring within the mitochondria. Others have reported state IV respiration being twice as high following a severe TBI in mice [142]. A short (12 -20 min) ischemic/reperfusion event has also been shown to result in an increase in state IV respiration, by ~25% [177]. The lack of any time-dependent changes is indicative of a very rapid injury to the mitochondria within the first hour, which may have important implications in formulating therapeutic interventions.

The overall functionality of the mitochondria is often stated in terms of the RCR. This ratio is a measure of how coupled mitochondrial respiration is to ATP production. A high RCR (5 to 10) indicates fully functional organelles while low ratios (< 5) indicate more of the proton motive force is being lost back into the matrix or used for processes other than ATP production. As shown in Figure 2.5, even a mild injury significantly reduced the RCR with further reductions observed with increased injury severity. Low RCRs indicate that the ability to produce ATP efficiently is significantly compromised, which would have consequences for injured neurons trying to regain cellular homeostasis. Several other studies support a reduction in RCRs at 1 h [122, 178] as well as 3 h following a TBI [142, 161]. The extended mitochondrial dysfunction that persists for at least 3 h after the initial insult may be amenable to pharmacological intervention.

FCCP is a pure uncoupler that acts as a protonophore, which allows the protons to freely pass back into the matrix, bypassing the ATP synthase at an extremely rapid rate. FCCP is added to determine maximum respiration capabilities of the ETC in its attempts to restore the dissipated proton gradient. We found a 10% reduction in maximum respiration abilities in animals receiving mild injuries, while animals subjected to a severe insult dropped 30% (Figure 2.6). State V (FCCP) is important to assess, especially following an injury, because neurons and supporting cells depend heavily on mitochondria to produce large amounts of ATP and sequester the large influx of calcium.

Due to the decline in cellular homeostasis following injury and higher energy demands, mitochondria are probably operating near capacity. Other studies report reductions of state V (FCCP) by more than 50% by 3 h post-trauma in rats [161]; similar reductions were seen in mice [142].

Following the addition of rotenone, which acts as a competitive inhibitor to block complex 1-driven respiration, succinate was added to determine if complex 2-driven respiration is affected by TBI. State V (succinate) was affected proportionally as a function of injury severity (Figure 2.7). A mild injury produced approximately a reduction of 7% in state V (succinate) respiration, 15-20% in a moderate and 30% following a severe injury. Data indicates both complex 1- and 2-driven respiration are significantly affected by TBI that could quickly evolve into a metabolic crisis situation where neurons require ATP for survival.

Several studies support complex 2 dysfunction following TBI. Xiong et al [122] showed impairments to state III respiration by complex 1-driven respiration were more severe (~ 45% drop from sham rates), but complex 2-driven respiration was still affected by the injury (~ 25% drop from sham rates). Similarly, maximal increases in state IV respiration via complex 1 were 155% of sham values and 125% of shams for complex 2, demonstrating that complex 2 is not impervious to damage. Verweij et al. [137] reported a significant decrease in state III respiratory rates, RCRs, and P/O ratios with either succinate or NAD-linked substrates in SD rats following a severe unilateral CCI. Both complex 1 and 2 maximum respiration have been shown to drop by approximately 50% following a moderate unilateral CCI in SD rats [161]. Significant changes in both NAD-linked and succinate-driven respiration have also been reported following ischemic/reperfusion injury [177, 179] as well as spinal cord contusion injury [180].

When analyzing changes in mitochondrial bioenergetics following trauma, we realized that damage to mitochondria could occur at any number of sites in the molecular machinery, which could be responsible for declines in respiration. A few of the possible sites of malfunction are transporter proteins responsible for importing substrates inside mitochondria [181]; enzymes needed to initiate the tricarboxylic acid cycle (TCA), such as pyruvate dehydrogenase (PDH) [182]; various complexes of the ETC used for

production of proton motive force [142]; or the ANT that exchanges ADP for ATP across the inner mitochondrial membrane [161]. Future studies will have to probe the molecular and biochemical mechanisms for both the functional and structural changes that occur within the mitochondria following TBI.

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Chapter 3: Two Rat Strains One Problem

Specific Aim 2 will test the hypothesis that there are no significant differences in mitochondrial respiration of Sprague-Dawley and Fischer 344 rats. To carry out this experiment, several controls must be taken into consideration. First, we need to determine if a further purification step will eliminate the injury effect seen early following TBI in total mitochondria. Furthermore, we need to establish whether there are respiration differences between synaptic and extrasynaptic mitochondrial fractions. Finally, it is necessary to determine if synaptic and extrasynaptic mitochondria respond differently to traumatic brain injury.

Introduction

The central hypothesis of this thesis project is that an early age-related increase in mitochondrial dysfunction following Traumatic Brain Injury (TBI) contributes to the exacerbated response (e.g. increased tissue loss, morbidity, and mortality) in aged animals. Before we could test this hypothesis, several experiments had to be performed. The objectives of this study were: **I.** Confirm mitochondrial dysfunction following TBI using a highly purified isolation procedure **II.** Determine if there are inherent respiration differences between the two mitochondrial fractions that can be isolated from cortical tissue (synaptic and extrasynaptic) **III.** Determine if these two mitochondrial fractions exhibit distinct responses following TBI **IV.** Determine if Sprague-Dawley (SD) and Fischer 344 (F344) rats exhibit strain differences in terms of mitochondrial bioenergetics in synaptic and extrasynaptic mitochondrial fractions. A better understanding of these issues will strengthen our understanding of mitochondrial dysfunction following TBI and determine whether experimental findings of mitochondrial respiration could be applicable to both of these commonly used strains of rats. These findings are imperative in producing effective therapeutic interventions that could modulate the sequelae that occurs in multiple organisms, not just one strain.

Establishing a time frame and extent of mitochondrial dysfunction after TBI is vitally important to our understanding of mechanisms underlying cortical tissue loss. In Chapter 2, we demonstrated that mitochondrial dysfunction occurs in an injury-severity dependent manner following TBI [183]. Several laboratories have also demonstrated very early respiratory alterations following trauma [17, 121, 140, 142, 183]. For these reasons,

our laboratory is interested in the early events following trauma that are likely to have profound effects on cell survival much later.

Currently, most studies that look at mitochondrial dysfunction following TBI use total isolated mitochondria, which include a mixture of mitochondria from neurons and glia from a variety of locations (e.g. soma, axons, and synapse). Our laboratory is interested in determining if mitochondrial bioenergetics are inherently different in certain regions of the cell (e.g. surrounding nucleus, axon, or synapse) or cell type (neurons and glia). Unfortunately at this time, we are only able to isolate neuronal synaptic mitochondria, which are trapped in lipid bound vesicles called synaptosomes, and an extrasynaptic fraction, which originates from neurons, soma and axons, and glial cells [184, 185]. Glial cell density is approximately twice the neuronal population in rat cortex [186] suggesting that the extrasynaptic fraction is predominantly neuronal [134, 135]. Regardless of the exact composition of the extrasynaptic mitochondria (neuronal versus glial), this fraction represents a distinct spatial location than the mitochondria located in the synapse. Besides being derived from different spatial locations, these two populations of mitochondria may also display different respiration capacities.

Potential differences between synaptic and extrasynaptic mitochondria would mean that they may also exhibit distinct responses to injury. Synaptic mitochondria are expected to be more sensitive to damage than extrasynaptic mitochondria, because of their decreased ability to buffer calcium [134]. There is also experimental evidence indicating that the electron transport chain (ETC) of synaptic and extrasynaptic mitochondria require very different degrees of inhibition before a significant decline in energy production (ATP) is realized [109, 111, 187]. Furthermore, synaptic mitochondria are under higher calcium loads due to synaptic transmission [8], which could stress their respiratory capabilities. Once these basic respiration questions are answered, we can then determine whether SD and F344 rats have similar respiration profiles that would enable us make comparisons among these two strains of rat. SD rats are largely used in TBI studies, while F344 rats are mainly used in aging research. Again, the central hypothesis of this thesis project was that there is an age-related decline in mitochondrial respiration following TBI, which has an age and an injury component. There is a much experimental

data that each of these strains offer and it would be useful to know if there is some degree of overlap in terms of the respiratory function among these commonly utilized rats.

Previously, our laboratory produced evidence for mitochondrial dysfunction following TBI in total mitochondria. In this study, we were able to demonstrate this dysfunction in total mitochondria following TBI using additional purification techniques following the isolation procedures. Isolated synaptic (from neuronal and peripheral astrocytic processes) and extrasynaptic (from neuronal and astroglial somal and axonal processes) mitochondrial fractions in naïve animals were compared to show respiratory differences that exist. The response of these two mitochondrial fractions after TBI was monitored at two early time points post-injury (1 and 3 h). Lastly, mitochondrial bioenergetics of synaptic and extrasynaptic mitochondrial fractions from SD and F344 rats were compared to confirm a lack of strain differences.

Materials and Methods

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee.

The following experiment was conducted to test the hypothesis that the injury effect will be apparent following an additional purification step.

Early dysfunction observed following TBI in purified total mitochondria

Animals

This study used young adult male Sprague-Dawley (SD) rats (Harlan Labs, Indianapolis, IN; n = 16; 275-300 g), which were housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water.

Surgical procedure

Cortical contusions were carried out under isoflurane anesthesia (2%) as previously described [183]. All injuries were produced using a pneumatic controlled cortical impact device (TBI 0310, Precision Systems and Instrumentation, Fairfax

Station, VA) with a soft stop Bimba cylinder (Bimba Manufacturing, Monee, IL). A severe injury was administered to each animal at a depth of the impact of 2.3 mm cortical displacement. The impactor tip size (5 mm beveled), dwell time (400 ms), and velocity (3.5 m/s) were held constant. Animals were randomly assigned to either: 1 (n = 8) or 3 h (n = 8) survival time.

Purified total mitochondria isolation

Mitochondrial isolation protocol and all procedures were performed on ice. Animals were briefly gassed with CO_2 until flaccid, decapitated and the brains rapidly removed. The cortices were dissected and a 10 mm punch of the ipsilateral cortex, containing injury and penumbra, and corresponding contralateral tissue was collected. Mitochondria were isolated separately from the injured (ipsilateral) and uninjured (contralateral) cortex. Mitochondria was isolated from either 1 animal (n = 1) or 2 animals (n = 2) in a given day, which always included one contralateral mitochondrial sample to ensure consistency and quality of isolation procedures. The contralateral sample was used as a baseline to compare respiration changes in the ipsilateral cortex, for either n = 1 or n = 2 for that day. We obtained contralateral respiration data from n = 5 (1 h) and n = 5 (3 h) instead of using sham animals, to reduce biological variance. Previous work has demonstrated that respiration is unaltered in the contralateral hemisphere at 3 h post-injury, and is equivalent to respiration seen in sham or naïve animals [183]. The 10 mm punch of the cortex (either ipsi- or contralateral) were placed in separate glass dounce homogenizers with 4 mL of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Tissue punches were homogenized and mitochondria isolated by differential centrifugation. The homogenate was centrifuged twice at 1300 × g for 3 min in a microcentrifuge at 4°C. The resulting supernatant was diluted with isolation buffer containing EGTA and centrifuged at 13,000 × g for 10 min. The resulting pellet was resuspended in 500 μL of isolation buffer with EGTA and subjected to a pressure of 1,200 psi for 10 min inside the nitrogen cell disruption bomb (Parr Instrument Company, Moline, IL) at 4◦C. After rupturing the synaptosomes, the samples were brought up to a final volume of 2 mL with isolation

buffer containing EGTA, and centrifuged at 13,000 × *g* for 10 min. The supernatant was discarded; the pellet was resuspended with isolation buffer with EGTA to a total volume of 500 μL; and samples were place on a discontinuous Ficoll gradient (F5415 Ficoll solution Type 400, 20% in H_2O, Sigma, St. Louis, MO), composed of 2 layers (2 mL of a 7.5% on top of 2 mL of 10% Ficoll cut with isolation buffer with EGTA). The final volume of the gradient was ~ 4.5 mL in the 7 mL Beckman tubes (344057, Fullerton, CA). Samples were placed in a Beckman SW 55 Ti swinging bucket rotor and centrifuged at 32,000 rpm for 30 min at 4°C. The pellet containing total mitochondria (synaptic and extrasynaptic) was collected at the bottom of the Ficoll gradient, resuspended in isolation buffer with EGTA, and centrifuged at 13,000 x *g* for 10 min to wash out the Ficoll. The mitochondrial pellet was resuspended with isolation buffer without EGTA and centrifuged at 10,000 ×*g* for 10 min to wash out the calcium chelator EGTA. The pellet was resuspended in isolation buffer without EGTA and centrifuged at 10,000 ×*g* for 10 min. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of 10 mg/mL or higher. The protein concentration was determined using the bicinchoninic acid protein (BCA) assay kit (Pierce, Rockford, IL).

The following experiment was conducted to test the hypothesis that there are differences in respiration between synaptic and extrasynaptic mitochondrial fractions.

Comparison of synaptic and extrasynaptic mitochondrial respiration

Animals

This study used naïve, young adult male Sprague-Dawley (SD) rats (Harlan Labs, Indianapolis, IN; *n* = 7; 275-300 g) which were housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water.

Synaptic and extrasynaptic mitochondria isolation

The cortex used for respiration analysis was placed in an all-glass dounce homogenizer with 4 mL of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Tissue was homogenized and mitochondria were extracted by differential centrifugation. The homogenate was spun twice at 1300 × *g* for 3 min in an Eppendorf microcentrifuge at 4°C and the supernatant was transferred to new tubes. The resulting supernatant was topped off with isolation buffer with EGTA and spun at 13,000 × *g* for 10 min. The supernatant was discarded; the pellet was resuspended with isolation buffer with EGTA to a total volume of 500 μL; and samples were place on a discontinuous Ficoll gradient (F5415 Ficoll solution Type 400, 20% in H_2O, Sigma, St. Louis, MO), composed of 2 layers (2 mL of a 7.5% on top of 2 mL of 10% Ficoll cut with isolation buffer with EGTA). The final volume of the gradient was ~ 4.5 mL in the 7 mL Beckman tubes (344057, Fullerton, CA). Samples were placed in a Beckman SW 55 Ti swinging bucket rotor and centrifuged at 32,000 rpm for 30 min at 4°C. The gradient produced two separate mitochondrial fractions: the extrasynaptic mitochondrial pellet (bottom) and synaptic mitochondria trapped in synaptosomes located at the interphase of the two Ficoll layers. Synaptosomes were collected in 2.5 mL tubes, resuspended, and washed in isolation buffer with EGTA by centrifugation at 13,000 × *g* for 10 min to remove Ficoll from sample. The extrasynaptic mitochondrial pellet was collected in 500 µL tubes and also resuspended and washed using isolation buffer with EGTA. Both fractions were washed with fresh buffer a second time to ensure that the Ficoll was eliminated. Synaptosomes were resuspended in isolation buffer with EGTA, and mitochondria released using a nitrogen cell disruption bomb made by Parr Instrument Company (Moline, IL) at 1,200 psi for 10 min. Disrupted synaptosomes were placed on a new Ficoll gradient and returned to the ultracentrifuge and spun at 32,000 rpm for 30 min at 4°C. After removal of the Ficoll gradient, the synaptic mitochondria pellet was resuspended in isolation buffer with EGTA and centrifuged at 13,000 x *g* for 10 min. Both synaptic and extrasynaptic mitochondrial fractions were then resuspended with isolation buffer without EGTA and centrifuged at 10,000 ×*g* for 10 min to wash out the calcium chelator (EGTA). The final mitochondrial pellets were resuspended in isolation buffer without

EGTA to yield a concentration of 10 mg/mL or higher. Protein concentration was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL) by measuring absorbance at 560 nm with a Molecular Devices microplate reader (Sunnyvale, CA).

The following experiment was conducted to test the hypothesis that synaptic and extrasynaptic mitochondria elicit a distinct response following TBI.

Early synaptic and extrasynaptic mitochondrial dysfunction following TBI

Animals

This study used young adult male Sprague-Dawley (SD) rats (Harlan Labs, Indianapolis, IN; n = 16; 275-300 g), which were housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water.

Surgeries

Cortical contusions were carried out under isoflurane anesthesia (2%) as previously described [183]. All injuries were produced using a pneumatic controlled cortical impact device (TBI 0310, Precision Systems and Instrumentation, Fairfax Station, VA) with a soft stop Bimba cylinder (Bimba Manufacturing, Monee, IL). A moderate injury was administered to each animal at a depth of the impact of 2.0 mm cortical displacement. The impactor tip size (5 mm beveled), dwell time (400 ms), and velocity (3.5 m/s) were held constant. Animals were randomly assigned to either: 1 (n = 8) or 3 h (n = 8) survival time.

Synaptic and extrasynaptic mitochondria isolation

Details are described above.

The following experiment was conducted to test the hypothesis that there are no differences between mitochondrial respiration of Sprague-Dawley and Fischer 344 rats.

Respiration comparison of Sprague-Dawley and Fischer 344 rats

This study utilized male Sprague-Dawley (SD) rats (Harlan Labs, Indianapolis, IN; *n* = 17; 275-300 g) and Fischer 344 (F344) rats (National Institute of Aging - Harlan Labs, Indianapolis, IN; *n* = 20) at two distinct ages of development: young (3 - 5 mos) and middle-aged (12 - 14 mos). Rats were housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water. All rats were naïve (not subjected to any type of operation, testing, or prior medication) and placed into one of 4 groups based on their strain and age: **I**. Young SD (n = 7); **II**. Young F344 (n = 10); **III**. Middle-aged SD (n = 10); **IV**. Middle-aged F344 (n = 10).

Synaptic and extrasynaptic mitochondria isolation

Details for these procedures are identical to that described above.

Mitochondria respiration measurements for all studies

Mitochondrial functionality was assessed using an Oxytherm Clark-type oxygen electrode (OXYT1/ED, Hansatech Instruments, Norfolk, UK). Samples were placed in the sealed Oxytherm chamber containing respiration buffer (125 mM KCl, 0.1% BSA, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, pH 7.2) and continuously stirred at 37° C. The rate of oxygen consumption was defined as the slope of the response of isolated mitochondria to the consecutive administrations of oxidative substrates as previously described [183], with minor adjustments. One adjustment had to be made to the previous protocol due to the highly level of purity the Ficoll gradient offers compared to differential centrifugation alone. In this study ~60 µg of mitochondrial protein was used for each respiration analysis compared to using ~180 µg previously. The amount of oligomycin administered was reduced from 1 µL to 0.4 µL, because the original amount was too high and was poisoning the mitochondria. Two additions of adenosine diphosphate (ADP), instead of one addition, were administered to ensure accurate state III respiration measurements. The modified protocol of the substrates injected into the Oxytherm chamber sequentially as follows: 2.5 µL of pyruvate/ malate (P/M; 2.5 mM);

1.25 µL of ADP (150 µM) added twice in 1-min intervals; 0.5 µL oligomycin (1 µM); 1µL carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone *(*FCCP; 1 µM); 0.2 µL rotenone (1 µM) added to shut down complex 1 activity resulting in cessation of oxygen utilization; and finally 2.5 µL succinate (10 mM).

To ensure that respiration analysis for each mitochondrial sample contained equal amounts of respiring mitochondria, a standardized state II respiration (10-15 nmoles oxygen/min) was maintained as a baseline for all groups. This gave all groups equal ability to produce similar respiration traces, regardless of the amount of mitochondrial protein added. Respiration differences following the standardized state II respiration rates would indicate alterations to components of the ETC as a result of the insult.

Overall oxygen utilization rate was determined by measuring the amount of oxygen consumed throughout all states of respiration divided by the time elapsed and amount of mitochondrial protein used for the respiration assay. The overall oxygen utilization rate can detect alterations in the time mitochondria respond to substrate additions, which serve as a general index of respiration capacities. States II – V-FCCP are mitochondrial respiration parameters driven by complex 1, while complex 2-driven respiration was measured separately by shutting down complex 1 with rotenone, to produce state V-succinate. Respiration states were calculated as nmol of oxygen/min/mg of mitochondria in 250 µL of respiration buffer, described previously [142]. The respiratory control ratio (RCR) was determined by dividing the rate of oxygen utilization for state III (ADP) by state IV (oligomycin). RCRs indicate how coupled the ETC is to ATP production. All isolated mitochondria, regardless of whether or not the fractions of mitochondrial were separated out (total or synaptic and extrasynaptic), were able to produce RCRs ≥ 5 from contralateral (uninjured tissue). Maintaining high RCR values in all samples derived from uninjured tissue indicates that the mitochondria were not damaged during the isolation procedure. The states of respiration (III, IV, V-FCCP, and V-succinate), overall oxygen utilization rates, and RCRs were all collected to fully characterize respiration capabilities.

Statistical analyses

Early dysfunction observed following TBI in purified total mitochondria

All data are reported as group means ± SD. Two-way repeated measures ANOVA (hemisphere X time post-injury) were performed on the mitochondrial protein amounts utilized and each respiration parameter (overall oxygen utilization rates, RCR values, state II thru state V-succinate). All states of respiration were graphed for both ipsi- and contralateral cortices to show relative respiration capabilities across groups, consistency, and extent of biological variance (Figure 2).

Comparison of synaptic and extrasynaptic mitochondrial respiration

All data are reported as group means ± SD. Paired *t* tests were used to compare all respiration parameters of synaptic and extrasynaptic mitochondrial fractions. A Bonferroni correction factor was implemented to adjust for multiple comparisons.

Early synaptic and extrasynaptic mitochondrial dysfunction following TBI

All data are reported as group means ± SD. All states of respiration are reported for both synaptic and extrasynaptic mitochondria were graphed to show relative respiration capabilities across groups, consistency, and extent of biological variance. Separate two-way repeated measures ANOVA (hemisphere X time post-injury) were performed on the amounts of synaptic and extrasynaptic mitochondrial protein used for respiration assessment. Each respiration parameter (overall oxygen utilization rates, RCR values, state II thru state V-succinate) was analyzed in the same manner. All ANOVAs were followed by a Student-Newman-Keuls post-hoc analysis. The alpha level was set at 0.05 for significance.

Respiration comparison of Sprague-Dawley and Fischer 344 rats

All data are reported as group means ± SD. Separate two-way ANOVA (strain X age) were performed to compare the amounts of mitochondrial protein used for respiration assessment, oxygen utilization rates, RCR values, and all states of respiration for the synaptic and extrasynaptic fractions.

Results

Early dysfunction observed following TBI in purified total mitochondria

Amounts of Mitochondrial protein used for respiration analysis. The average amount of total mitochondrial protein used to assess respiration capabilities in animals with a 1 h post-injury survival was 58.5 µg ± 7.4 (contralateral) and 61.6 ± 8.5 (ipsilateral), while rats with a 3 h survival was 66.1 µg ± 5.9 (contralateral) and 67.1 ± 11.4 (ipsilateral). A two-way repeated measures ANOVA (hemisphere X time post-injury) showed there was no differences in the amounts of mitochondrial used for respiration analysis regardless of hemisphere analyzed [$F(1, 14) = 1.005$, $p > 0.1$] or time post-injury [$F(1, 14) = 3.100$, $p > 0.1$].

Overall oxygen utilization rate. A two-way repeated measures ANOVA revealed there was a significant injury effect on the overall oxygen utilization rate in [$F(1, 14) = 44.420$, $p < 0.0001$], while the time post-injury had no effect [$F(1, 14) = 0.033$, $p > 0.1$]. Post-hoc analysis revealed that the overall oxygen utilization rates in the ipsilateral hemispheres were significantly lower than the rates seen in the contralateral cortex ($p < 0.05$). Data reported in Figure 3.1.

RCR (state III / state IV respiration). The RCR is an index of how coupled the electron transport chain (ETC) is to ATP production. A two-way repeated measures ANOVA revealed a significant injury effect on RCR values [$F(1, 14) = 103.342$, $p < 0.0001$], while the time post-injury had no effect [$F(1, 14) = 0.479$, $p > 0.1$]. Post-hoc

Figure 3.1. Alteration in overall oxygen utilization following TBI in purified total mitochondria.

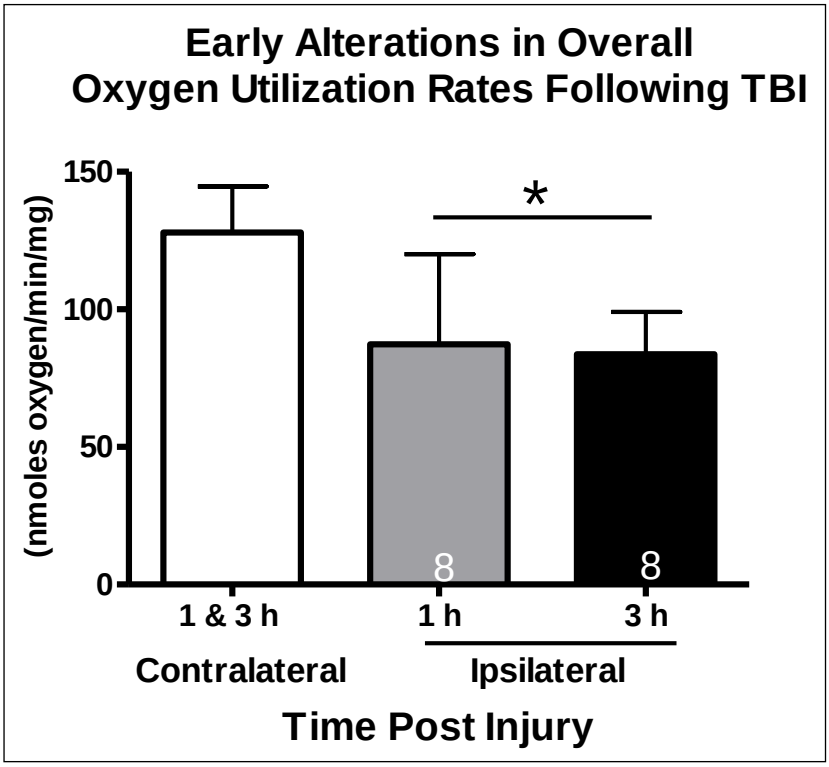

Figure 3.1. A significant decline in respiration, measured by overall oxygen utilization, was observed at both 1 and 3 hr post-injury in total mitochondria isolated from Sprague-Dawley rats sustaining a TBI compared to contralateral rates. Contralateral rates from 1 & 3 h post-injury were combined for graph only. Bars represent group means ± SD, while numbers in bars represent animals used per group. * $p < 0.05$ compared to the contralateral rates of each animal.

analysis revealed that the RCR values in the ipsilateral hemispheres were significantly lower than the contralateral cortex values ($p < 0.05$). Data reported in Figure 3.2.

State II respiration (pyruvate/malate). State II respiration rates are used as an indicator of basal complex 1-driven respiration. Rates of state II respiration were quantified in Figure 3.3. A two-way repeated measures ANOVA revealed there was a significant injury effect in the ipsilateral hemisphere on state II respiration rates in total

mitochondria [F (1, 14) = 2.534, $p > 0.1$], while the time post-injury had no effect [F (1, 14) = 2.901, $p > 0.1$].

State III respiration (ADP). State III respiration is an indicator of the efficiency of ATP production via complex 1. Rates of state III respiration were quantified in Figure 3.3. A two-way repeated measures ANOVA revealed a significant injury effect on state III respiration rates [F (1, 14) = 103.342, $p < 0.0001$], while the time post-injury had no effect [F (1, 14) = 0.479, $p > 0.1$]. Post-hoc analysis revealed that the state III rates in the ipsilateral hemispheres were significantly lower than those from the contralateral cortex ($p < 0.05$).

State IV respiration (oligomycin). State IV is able to give insight in the integrity of the inner mitochondrial membrane as well as how much of the basal respiration rate is being lost to processes other than ATP production. Rates of state IV respiration were quantified in Figure 3.3. A two-way repeated measures ANOVA revealed there were no significant alterations to state IV respiration rates following TBI [F (1, 14) = 1.645, $p > 0.1$] or as a function time post-injury [F (1, 14) = 0.504, $p > 0.1$].

State V-FCCP respiration. In the presence of an uncoupler, FCCP, protons can freely pass into the mitochondrial matrix from the inner membrane space which drastically drops the proton motive force used to drive ATP synthesis. Therefore, state V-FCCP is an indicator of maximum respiration capabilities of the ETC via complex 1 in attempts to maintain a proton gradient. Rates of state V-FCCP respiration were quantified in Figure 3.3. A two-way repeated measures ANOVA revealed a significant injury effect on state V-FCCP respiration rates [F (1, 14) = 50.496, $p < 0.0001$], while the time post-injury had no effect [F (1, 14) = 0.164, $p > 0.1$]. Post-hoc analysis revealed that the state V-FCCP rates in the ipsilateral hemispheres were significantly lower than those observed in the contralateral cortex ($p < 0.05$).

Figure 3.2. Alterations in RCR values following TBI in purified total mitochondria.

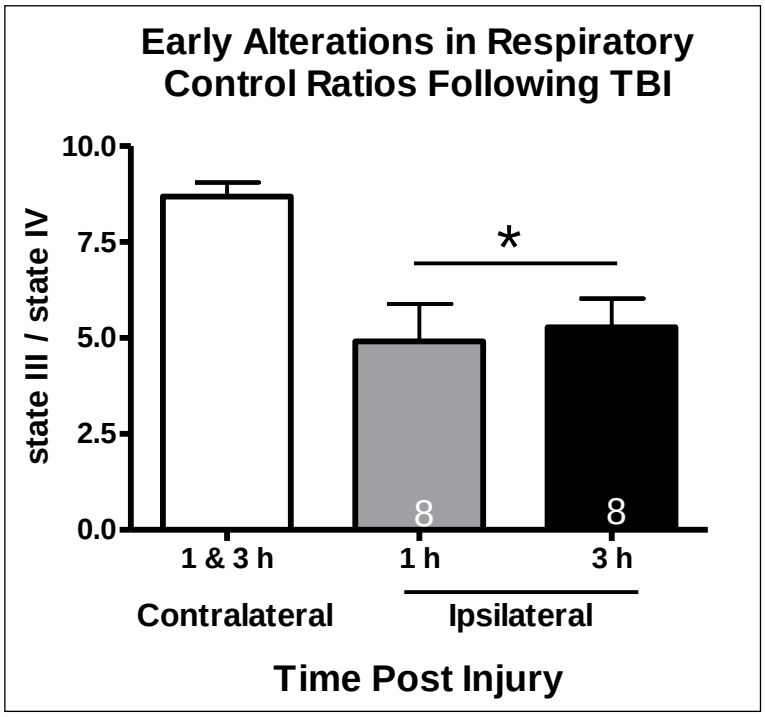

Figure 3.2. A significant decline in coupled respiration, measured by RCR values, were observed at both 1 and 3 hr post-injury in total mitochondria isolated from Sprague-Dawley rats sustaining a TBI compared to contralateral rates. Contralateral rates from 1 & 3 h post-injury were combined for graph only. Bars represent group means ± SD, while numbers in bars represent animals used per group. * $p < 0.05$ compared to the contralateral rates of each animal.

State V-succinate respiration. Once FCCP is added in the respiration chamber, it is maintained in the mitochondrial inner membrane allowing protons to continually pass into the mitochondrial matrix. Therefore, state V-succinate is an indicator of maximum respiration capabilities of the ETC via complex 1 in attempts to maintain a proton gradient. Rates of state V-succinate respiration were quantified in Figure 3.3. A two-way repeated measures ANOVA revealed a significant decline in state V-succinate respiration rates following TBI [$F(1, 14) = 35.715$, $p < 0.0001$], while the time post-injury had no effect [$F(1, 14) = 0.178$, $p > 0.1$]. Post-hoc analysis revealed that the state V-succinate rates in the ipsilateral hemispheres were significantly lower than those observed in the contralateral cortex ($p < 0.05$).

Figure 3.3. Early dysfunction observed following TBI in purified total mitochondria.

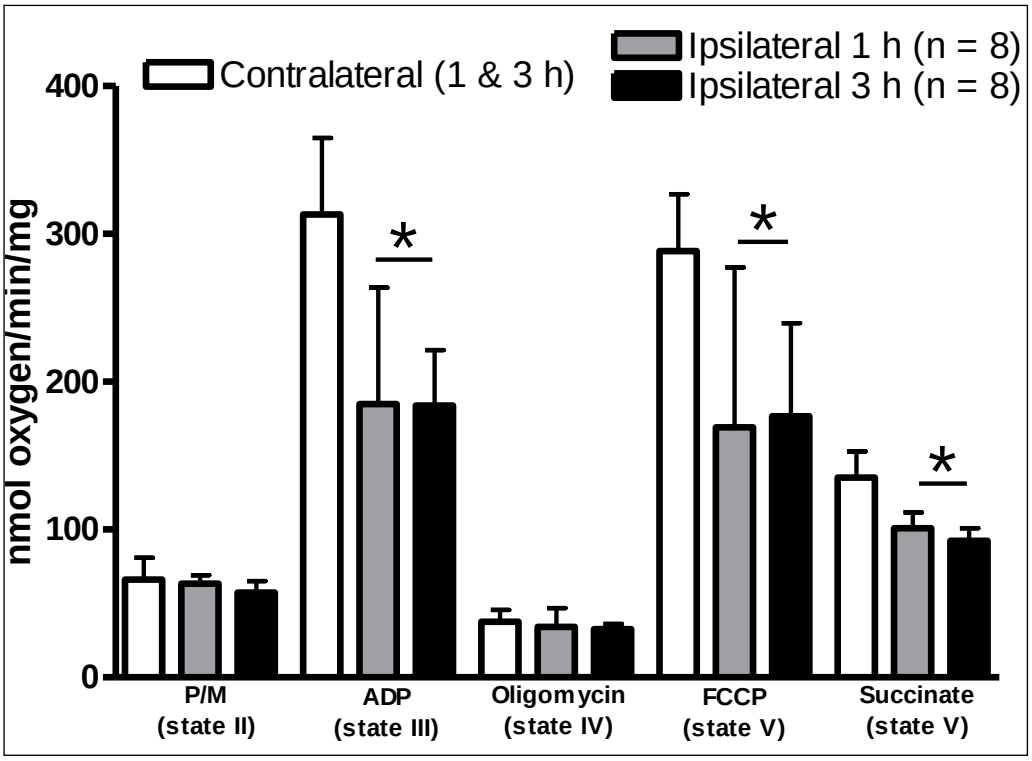

Figure 3.3. Dysfunction observed in total mitochondrial respiration isolated from Sprague-Dawley rats that have sustained a TBI. States III, V – FCCP, and V– succinate rates of respiration displayed significant alterations at both 1 and 3 hr post-injury, while no significant alterations were evident between 1 and 3 h post-injury. Contralateral rates from 1 & 3 h post-injury were combined for graph only. Bars represent group means ± SD, while numbers in bars represent animals used per group * $p < 0.05$ compared to the contralateral rates of each animal.

Comparison of synaptic and extrasynaptic mitochondrial respiration

Amounts of mitochondrial protein used for respiration analysis. The average amount of total mitochondrial protein used to assess respiration capabilities was 62.3µg ± 12.5 (synaptic) and 63.3 µg ± 17.5 (extrasynaptic). A paired *t*-test revealed no difference in the amount of mitochondrial protein used for respiration analysis for either fraction ($t_6 = 0.1001$, $p > 0.1$).

Overall oxygen utilization rate. The mean overall oxygen utilization rates are displayed in Figure 3.4. A paired *t*-test revealed that extrasynaptic mitochondria displayed significantly higher overall oxygen utilization rates compared to the synaptic fraction ($t_6 = 2.463$, $p < 0.05$).

Figure 3.4. Overall oxygen utilization rate comparison between synaptic and extrasynaptic mitochondria isolated from naïve Sprague-Dawley rat cortices.

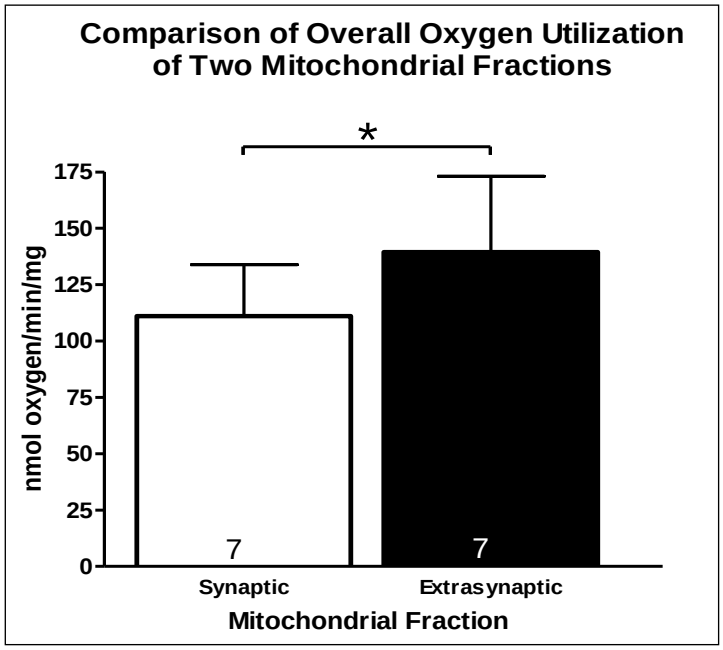

Figure 3.4. A significant difference exist between respiration capacities, measured by the overall oxygen utilization rate, of synaptic and extrasynaptic isolated mitochondria from naive Sprague-Dawley rats. Bars represent group means ± SD, while numbers in bars represent animals used per group * $p < 0.05$ a paired *t* test comparison.

RCR (state III / state IV respiration). A paired *t*-test revealed no significant difference in RCR values between synaptic and extrasynaptic mitochondria ($t_6 = 2.148$, $p > 0.05$). The average RCR value for synaptic mitochondria was 7.4 ± 0.9, while the extrasynaptic fraction produced an average RCR value of 8.2 ± 0.9.

States of respiration. Quantification of all states of respiration is displayed in Figure 3.5. Paired *t* tests were used to determine if there are significant differences in any state of respiration between synaptic and extrasynaptic mitochondria, listed in Table 3.1.

Table 3.1. Paired t-test results with p values comparing synaptic and extrasynaptic mitochondrial respiration capacities.

Paired t-test values with *p* values for each state of respiration					
	State II – P/M	State III - ADP	State IV - oligomycin	State V- FCCP	State V - succinate
Synaptic Versus Extrasynaptic	$t_6 = 0.6011$ $p > 0.1$	$t_6 = 3.345$ $p < 0.05$	$t_6 = 1.1002$ $p > 0.1$	$t_6 = 2.905$ $p < 0.05$	$t_6 = 2.694$ $p < 0.05$

Figure 3.5. Comparison of synaptic and extrasynaptic mitochondrial respiration isolated from cortical tissue of naïve Sprague-Dawley rats.

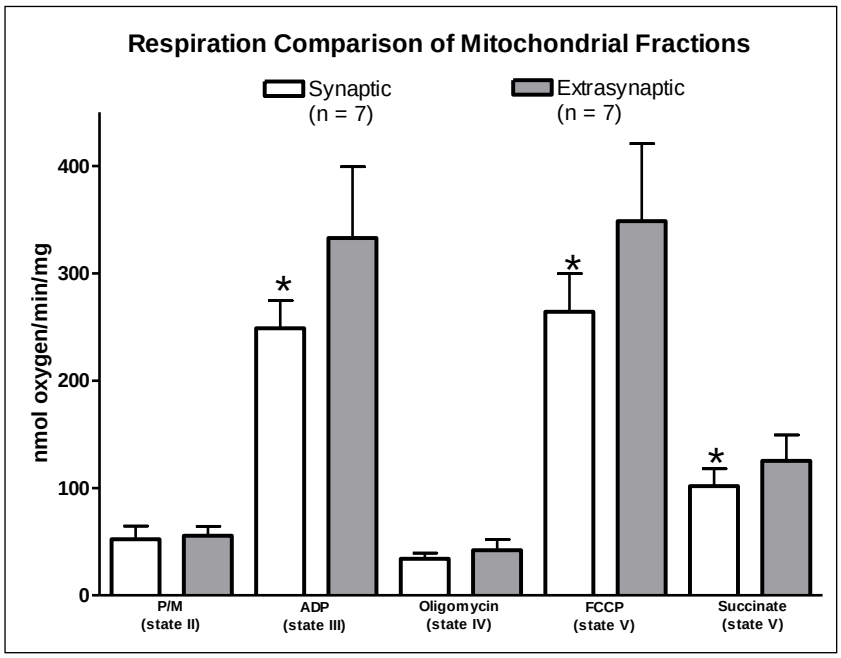

Figure 3.5. A bioenergetic analysis of synaptic and extrasynaptic mitochondrial respiration isolated from Sprague-Dawley rat cortices. Bars represent group means ± SD. * $p < 0.05$ using paired *t*-tests comparisons and Bonferroni correction factor.

Early synaptic and extrasynaptic mitochondrial dysfunction following TBI

Amounts of Mitochondrial protein used for respiration analysis. The average amounts of mitochondrial protein (mean ± SD) used for respiration analysis are displayed in Table 3.2. For synaptic mitochondria, a two-way repeated measures ANOVA showed there were no differences in the amounts of mitochondrial used for respiration analysis regardless of hemisphere analyzed [$F(1, 9) = 1.480$, $p > 0.1$] or time post-injury [$F(1, 9) = 2.316$, $p > 0.1$]. For extrasynaptic mitochondria, a two-way repeated measures ANOVA revealed no differences in the amounts of mitochondrial used for respiration analysis regardless of hemisphere analyzed [$F(1, 9) = 2.392$, $p > 0.1$] or time post-injury [$F(1, 9) = 0.450$, $p > 0.1$]. The additional purification step likely eliminates a majority of the cellular debris.

Table 3.2. Amounts of mitochondrial protein used for respiration analysis of synaptic and extrasynaptic fractions.

Mitochondrial Protein (µg) Used for Respiration Analysis (mean ± SD)				
	Contralateral		**Ipsilateral**	
	1 h post-injury	3 h post-injury	1 h post-injury	3 h post-injury
Synaptic	58.0 ± 13.2	63.9 ± 8.5	59.3 ± 6.5	70.2 ± 12.3
Extrasynaptic	57.2 ± 15.4	63.7 ± 8.1	62.9 ± 15.9	65.4 ± 6.7

Overall oxygen utilization rate. Alterations in the overall oxygen utilization rate were apparent following TBI in both mitochondrial fractions (Figure 3.6). A two-way repeated measures ANOVA revealed there was a significant injury effect on the overall oxygen utilization rate in synaptic mitochondria [F (1, 9) = 17.016, $p < 0.005$], while the time post-injury had no effect [F (1, 9) = 1.6709, $p > 0.1$]. Post-hoc analysis revealed that the overall oxygen utilization rates in the ipsilateral hemispheres were significantly lower than the rates seen in the contralateral cortex ($p < 0.05$). A two-way repeated measures ANOVA revealed there was a significant injury effect on the overall oxygen utilization rate in extrasynaptic mitochondria [F (1, 9) = 32.682, $p < 0.0005$], while the time post-injury had no effect [F (1, 9) = 0.427, $p > 0.1$]. Post-hoc analysis revealed that the overall oxygen utilization rates in the ipsilateral hemispheres were significantly lower than the rates seen in the contralateral cortex ($p < 0.05$).

Figure 3.6. Alterations in overall oxygen utilization rates following TBI in Sprague-Dawley rats.

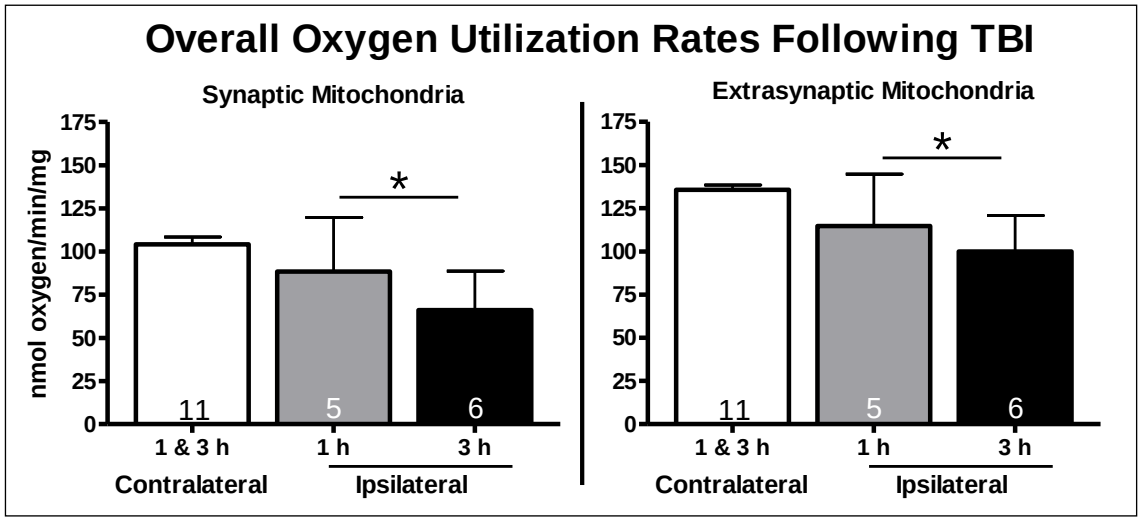

Figure 3.6. Synaptic and extrasynaptic mitochondrial respiration dysfunction observed in isolated following TBI. The overall oxygen utilization rated declined significant at both 1 and 3 hr post-injury, no differences were evident between 1 and 3 h post-injury. Contralateral rates from 1 & 3 h post-injury were combined for graph only. Bars represent group means ± SD, while numbers in bars represent animals used per group. * $p < 0.0005$ compared to the contralateral rates of each animal.

RCR (state III / state IV respiration). RCR values declined significantly following TBI in both mitochondrial fractions (Figure 3.7). A two-way repeated measures ANOVA revealed a significant injury effect on RCR values in synaptic mitochondria [F (1, 9) = 37.529, $p < 0.0005$], while the time post-injury had no effect [F (1, 9) = 0.238, $p > 0.1$]. Post-hoc analysis revealed that the RCR values in the ipsilateral hemispheres were significantly lower than the contralateral cortex values ($p < 0.05$). A two-way repeated measures ANOVA revealed a significant injury effect on RCR values in extrasynaptic mitochondria [F (1, 9) = 13.013, $p < 0.005$], while the time post-injury had no effect [F (1, 9) = 0.228, $p > 0.1$]. Post-hoc analysis revealed that the RCR values in the ipsilateral hemispheres were significantly lower than the contralateral cortex values ($p < 0.05$).

Figure 3.7. Alterations in RCR values following TBI in Sprague-Dawley rats.

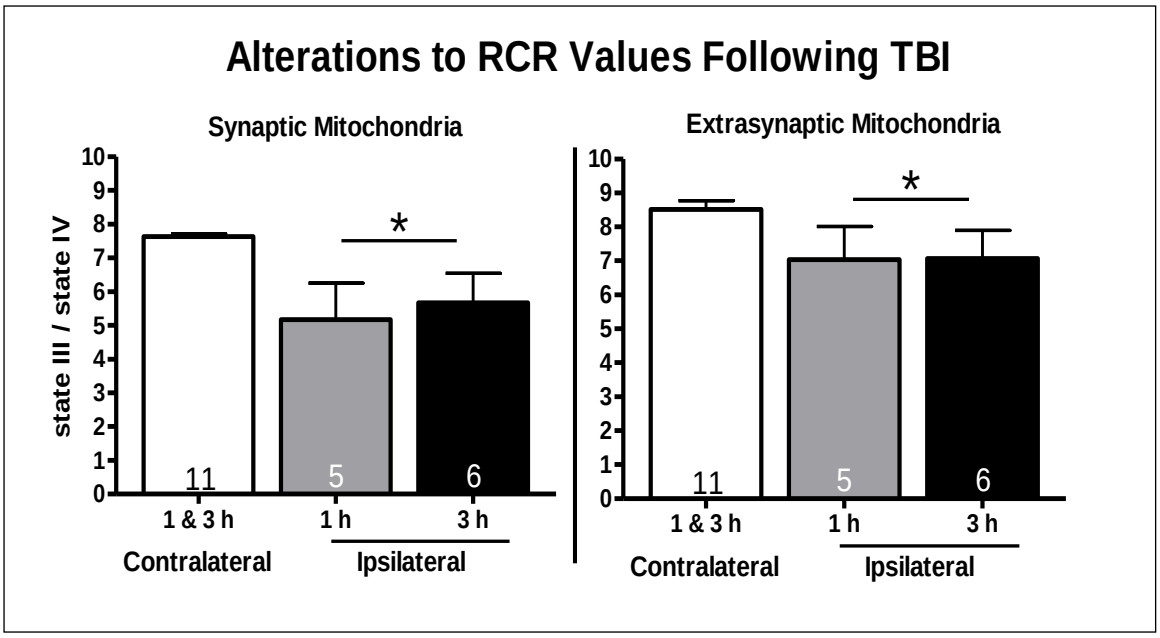

Figure 3.7. Synaptic and extrasynaptic mitochondrial respiration dysfunction observed in isolated following TBI. The overall oxygen utilization rated declined significant at both 1 and 3 hr post-injury, no differences were evident between 1 and 3 h post-injury. Contralateral rates from 1 & 3 h post-injury were combined for graph only. Bars represent group means ± SD, while numbers in bars represent animals used per group. * $p < 0.0005$ compared to the contralateral rates of each animal.

States of respiration. Quantification of all states of respiration (states II – V-succinate) following TBI for synaptic and extrasynaptic mitochondrial fractions are displayed in Figure 3.8.

State II (pyruvate/malate respiration. A two-way repeated measures ANOVA revealed there was no significant alterations to state II respiration rates in synaptic mitochondria regardless of hemisphere [$F(1, 9) = 0.164$, $p > 0.1$] or time post-injury [$F(1, 9) = 0.019$, $p > 0.1$]. The same was true for the extrasynaptic mitochondrial fraction; there were no significant alterations to state II respiration rates regardless of hemisphere [$F(1, 9) = 0.723$, $p > 0.1$] or time post-injury [$F(1, 9) = 0.056$, $p > 0.1$].

State III (ADP) respiration. A two-way repeated measures ANOVA revealed a significant injury effect on state III respiration rates in synaptic mitochondria [$F(1, 9) = 45.701$, $p < 0.0001$], while the time post-injury had no effect [$F(1, 9) = 1.384$, $p > 0.1$]. Post-hoc analysis revealed that the state III rates in the ipsilateral hemispheres were significantly lower than those from the contralateral cortex ($p < 0.05$). A two-way repeated revealed a significant injury effect on state III respiration in extrasynaptic mitochondria [$F(1, 9) = 28.645$, $p < 0.0005$], while the time post-injury had no effect [$F(1, 9) = 1.352$, $p > 0.1$]. Post-hoc analysis revealed that the state III rates in the ipsilateral hemispheres were significantly lower than those from the contralateral cortex ($p < 0.05$).

State IV (oligomycin) respiration. A two-way repeated measures ANOVA revealed there were no significant alterations to state IV respiration rates following TBI in synaptic mitochondria [$F(1, 9) = 0.667$, $p > 0.1$] or time post-injury [$F(1, 9) = 2.345$, $p > 0.1$]. A two-way repeated measures ANOVA revealed there were no significant alterations to state IV respiration rates following TBI in extrasynaptic mitochondria [$F(1, 9) = 0.048$, $p > 0.1$] or time post-injury [$F(1, 9) = 0.690$, $p > 0.1$].

State V-FCCP respiration. A two-way repeated measures ANOVA revealed a significant injury effect on state V-FCCP respiration rates in synaptic mitochondria [$F(1, 9) = 16.801$, $p < 0.005$], while the time post-injury had no effect [$F(1, 9) = 1.430$, $p > 0.1$]. Post-hoc analysis revealed that the state V-FCCP rates in the ipsilateral hemispheres were significantly lower than those observed in the contralateral cortex ($p < 0.05$). A two-way repeated measures ANOVA revealed a significant injury effect on state V-FCCP respiration rates in extrasynaptic mitochondria [$F(1, 9) = 7.343$, $p < 0.05$], while the time post-injury had no effect [$F(1, 9) = 2.148$, $p > 0.1$]. Post-hoc analysis revealed that the state V-FCCP rates in the ipsilateral hemispheres were significantly lower than those observed in the contralateral cortex ($p < 0.05$).

State V-succinate respiration. A two-way repeated measures ANOVA revealed a significant decline in state V-succinate respiration following TBI in synaptic mitochondria [$F(1, 9) = 38.532$, $p < 0.0005$], while the time post-injury had no effect [$F(1, 9) = 0.661$, $p > 0.1$]. Post-hoc analysis revealed that the state V-succinate rates in the ipsilateral hemispheres were significantly lower than those observed in the contralateral cortex ($p < 0.05$). A two-way repeated measures ANOVA revealed a significant decline in state V-succinate respiration following TBI in extrasynaptic mitochondria [$F(1, 9) = 5.293$, $p < 0.05$], while time post-injury had no effect [$F(1, 9) = 0.648$, $p > 0.1$]. Post-hoc analysis revealed that the state V-succinate rates in the ipsilateral hemispheres were significantly lower than those observed in the contralateral cortex ($p < 0.05$).

Figure 3.8. Early alterations in synaptic and extrasynaptic mitochondrial respiration following TBI in Sprague-Dawley rats.

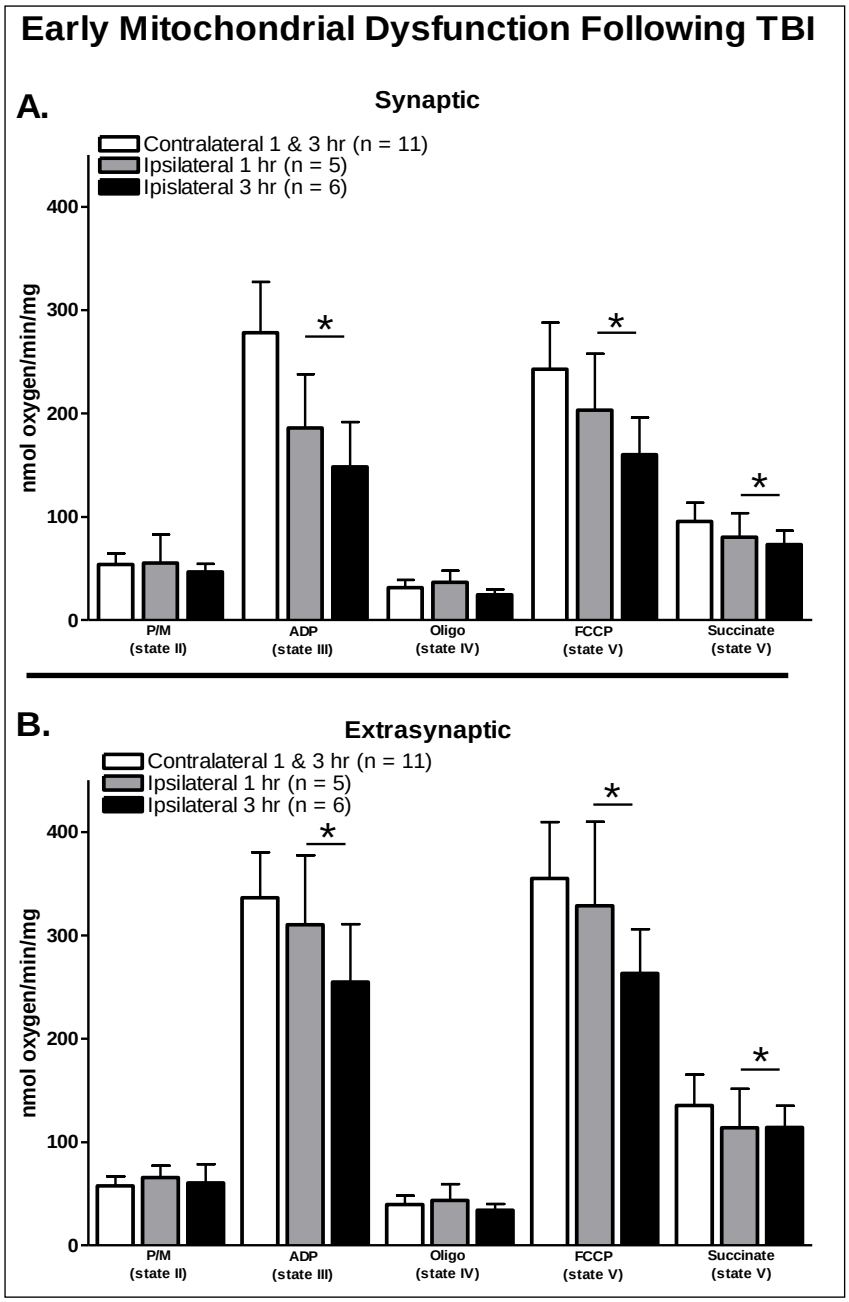

Figure 3.8. Alterations in synaptic and extrasynaptic mitochondrial respiration observed at 1 and 3 h post-injury in Sprague-Dawley rats. A significant decline in several respiration parameters was apparent in at both time points in each mitochondrial fraction. Contralateral rates from 1 & 3 h post-injury were combined for graph only. Bars represent group means ± SD. * $p < 0.05$ compared to the contralateral rates of each animal.

Respiration comparison of Sprague-Dawley and Fischer 344 rats

Amounts of Mitochondrial protein used for respiration analysis. The average amounts of mitochondrial protein (mean ± SD) that were used for respiration analysis are displayed in Table 3.3. For synaptic mitochondria, a two-way ANOVA (strain X age) showed there were no differences in the amounts of mitochondrial used for respiration analysis regardless of rat strain [F (1, 33) = 2.999, $p > 0.1$] or age [F (1, 33) = 2.118, $p > 0.1$]. For extrasynaptic mitochondria, a two-way ANOVA revealed no differences in the amounts of mitochondrial used for respiration analysis regardless of rat strain [F (1, 33) = 0.057, $p > 0.1$] or age [F (1, 9) = 0.021, $p > 0.1$].

Table 3.3 Mitochondrial protein amounts used in respiration analysis of Sprague-Dawley and Fischer 344 rats.

Mitochondrial Protein (µg) Used for Respiration Analysis (mean ± SD)				
	Young (3 - 5 mos)		Middle Aged (12-14 mos)	
	SD	**F344**	**SD**	**F344**
Synaptic	62.3 ± 12.5	56.8 ± 7.3	69.9 ± 14.1	61.1 ± 14.2
Extrasynaptic	59.1 ± 10.0	58.9 ± 8.5	60.0 ± 9.4	58.9 ± 8.5

Overall oxygen utilization rate. There were no significant difference existed in the overall oxygen utilization rates across strain or age (Figure 3.9). A two-way ANOVA revealed no significant differences in overall oxygen utilization rates of synaptic mitochondria regardless of strain [F (1, 33) = 2.019, $p > 0.1$] or age group [F (1, 33) = 1.168, $p > 0.1$]. Likewise, no significant differences existed in overall oxygen utilization rates of extrasynaptic mitochondria regardless of strain [F (1, 33) = 1.033, $p > 0.1$] or age group [F (1, 33) = 1.174, $p > 0.1$].

Figure 3.9. Comparison of overall oxygen utilization rates in synaptic and extrasynaptic mitochondria isolated from cortical tissue of naïve Fischer 344 and Sprague-Dawley rats.

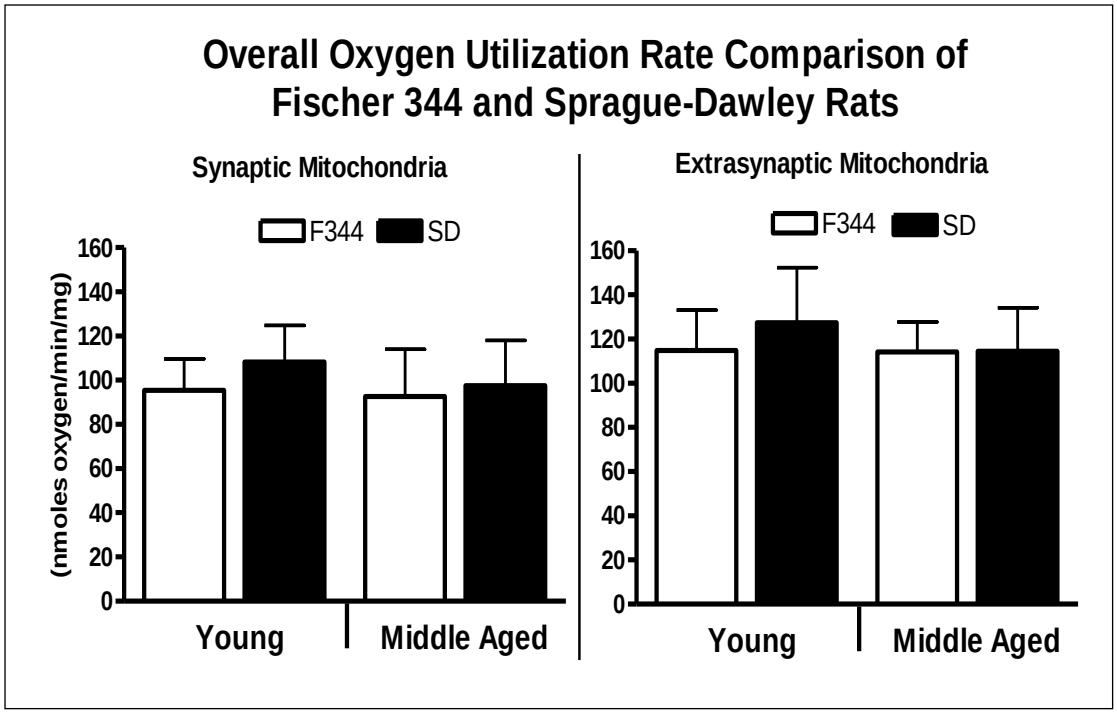

Figure 3.9. Comparison of overall oxygen utilization rates from mitochondria isolated from cortical tissue of F344 and SD rat at distinct stages of development, young and (3 - 5 mos) and middle aged (12-14 mos). Bars represent group means ± SD.

RCR (state III / state IV respiration). There were no significant difference existed in RCR values of a given mitochondrial fraction (synaptic or extrasynaptic) across strain or age (Figure 3.10). A two-way ANOVA revealed no significant differences in state overall oxygen utilization rates in synaptic mitochondria across strain [$F(1, 33) = 0.623$, $p > 0.1$] or age groups [$F(1, 33) = 0.001$, $p > 0.1$]. Likewise, a two-way ANOVA revealed no significant differences in overall oxygen utilization rates in extrasynaptic mitochondria across strain [$F(1, 33) = 1.145$, $p > 0.1$] or age groups [$F(1, 33) = 3.058$, $p > 0.1$].

Figure 3.10. Comparison of RCR values of synaptic and extrasynaptic mitochondria isolated from cortical tissue of naïve Fischer 344 and Sprague-Dawley rats.

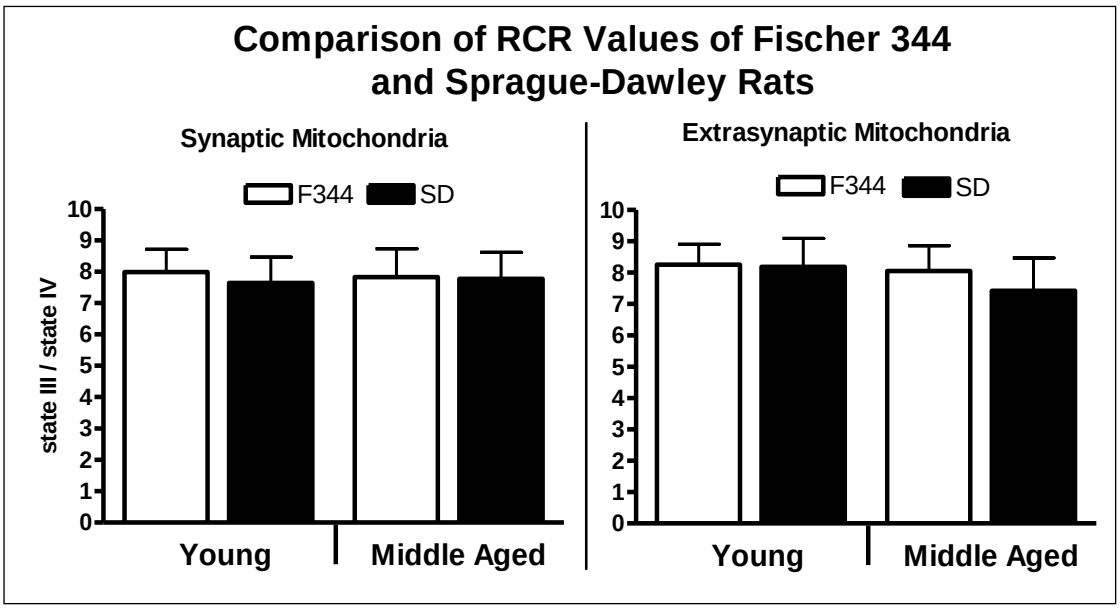

Figure 3.10. Comparison of RCR values from mitochondria isolated from F344 and SD rat at distinct stages of development, young and (3 - 5 mos) and middle aged (12-14 mos). Bars represent group means ± SD.

States of respiration. Quantification of all states of respiration for both mitochondrial fractions of young and middle aged SD and F344 rats are displayed in Figure 3.11.

P/M (state II respiration via complex 1). A standard state II respiration rate (10 – 15 nmol/min was used as an indicator of basal respiration regardless of mitochondrial protein utilized per assay. A two-way ANOVA failed to reveal any significant differences in state II respiration of synaptic mitochondrial across strain [F 1, 33) = 2.376, $p > 0.1$] or age group [F (1, 33) = 0.702, $p > 0.1$] Likewise, no differences were observed in state II respiration in extrasynaptic mitochondrial across strain [F 1, 33) = 0.354, $p > 0.1$] or age group [F (1, 33) = 1.728, $p > 0.1$].

ADP (state III respiration). The efficiency of each mitochondrial group to produce ATP was measured. A two-way ANOVA failed to reveal any significant differences in state III respiration of synaptic mitochondrial across strain [F 1, 33) = 2.152, $p > 0.1$] or age group [F (1, 33) = 0.003, $p > 0.1$]. There were also no significant differences in state III respiration were observed in extrasynaptic mitochondrial across strain [F 1, 33) = 1.441, $p > 0.1$] or age group [F (1, 33) = 3.047, $p > 0.1$].

Oligomycin (state IV respiration). Potential strain and age-related differences in state IV respiration was assessed. Mitochondria were able to maintain the proton gradient indicating preservation of inner membrane integrity in both rat strains and age groups. A two way ANOVA revealed no significant changes in state IV respiration of synaptic mitochondrial across strain [F 1, 33) = 3.325, $p > 0.05$] or age group [F (1, 33) = 0.399, $p > 0.1$]. The ANOVA also failed to reveal any differences in state IV respiration of extrasynaptic mitochondria across strain [F (1, 33) 1.660, $p > 0.1$] or age group [F (1, 33) = 0.519, $p > 0.1$].

FCCP (state V respiration via complex 1). Maximum respiration capacities for both fractions of mitochondria were quantified. A two-way ANOVA revealed no significant changes in state V-FCCP respiration of synaptic mitochondrial across strain [F 1, 33) = 2.033, $p > 0.1$] or age group [F (1, 33) = 0.062, $p > 0.1$]. A two-way ANOVA also failed to reveal any differences in state V-FCCP respiration of extrasynaptic mitochondria across strain [F 1, 33) = 0.594, $p > 0.1$] or age group [F (1, 33) = 0.134, $p > 0.1$].

Succinate (state V respiration via complex 2). Maximum respiration capabilities via complex 2 of both fractions of mitochondria were quantified. A two ANOVA revealed no significant differences in state V-succinate respiration of extrasynaptic mitochondria across strain [F (1, 33) = 0.944, $p > 0.1$] or age groups [F (1, 33) = 0.364, $p > 0.1$]. No significant differences existed in state V-succinate of synaptic mitochondria regardless of strain [F (1, 33) = 1.609, $p > 0.1$] or age group [F (1, 33) = 2.175, $p > 0.1$].

Figure 3.11. Respiration comparison of young and middle aged Sprague-Dawley and Fischer 344 rats.

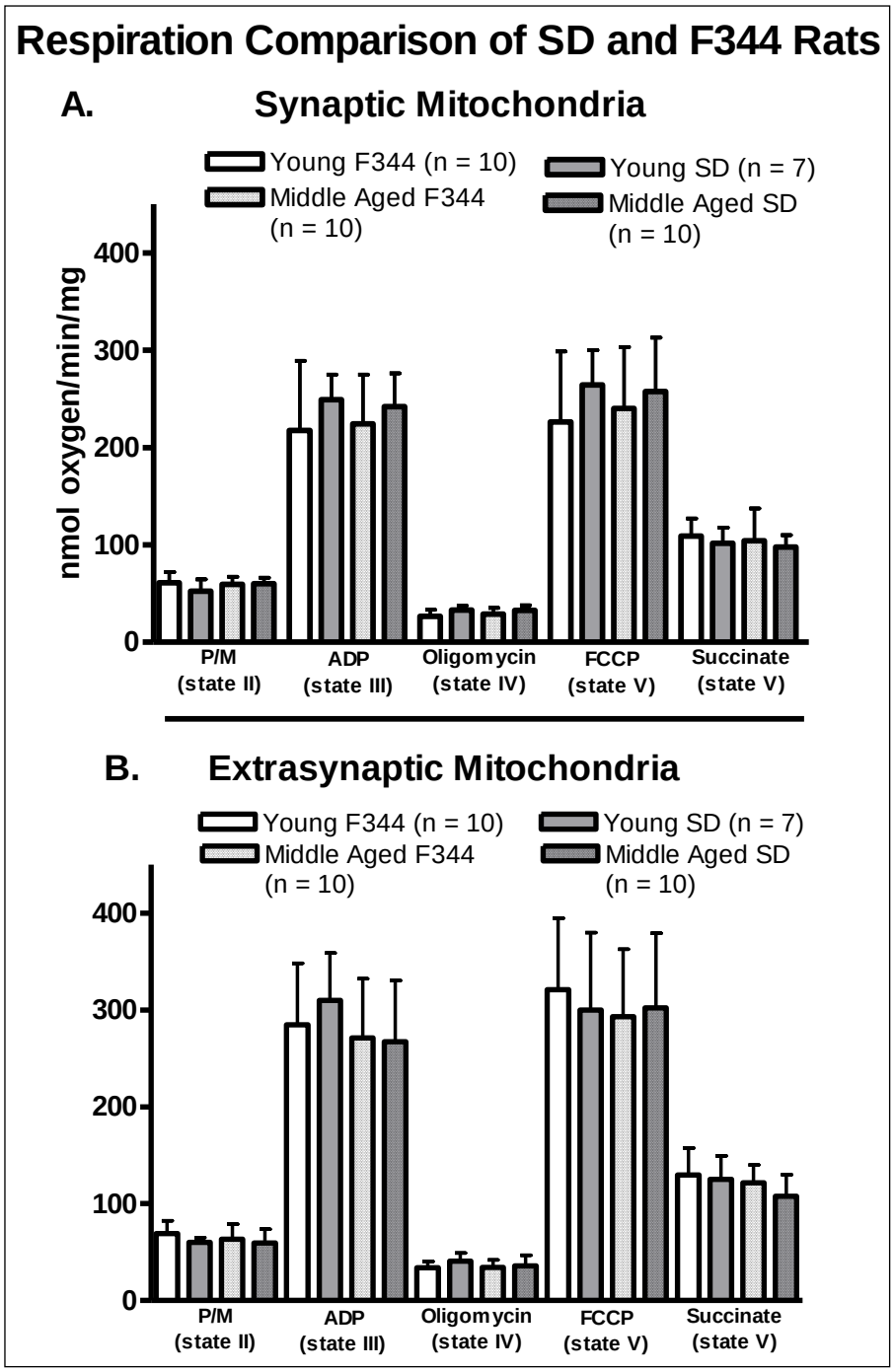

Figure 3.11. A bioenergetic comparison of isolated, cortical mitochondrial respiration from **A**. young and **B**. middle aged Fischer 344 and Sprague-Dawley rats. No significant differences were found between these two strains for any respiration parameter. Bars represent group means ± SD.

Discussion

There were several questions addressed in the present set of experiments. We were able to determine that the mitochondrial dysfunction that is known to occur early following TBI is not lost with the additional purification procedure using a Ficoll gradient. The data confirms the previous finding of mitochondrial dysfunction, with no significant alterations between 1 and 3 h post-injury (Figure 3.3). The addition purification step was implemented to enable the separation of synaptic and extrasynaptic mitochondrial fractions. Significant respiration differences between these two distinct populations of mitochondria isolated from naïve animals was demonstrated (Figure 3.5). Both synaptic and extrasynaptic mitochondria displayed respiration changes following TBI. We were able to observe significant respiration alterations in both mitochondrial fractions at 1 and 3 h post-injury, while time post-injury had no significant effect. Both mitochondrial fractions seem to be transitioning into a greater dysfunctional state at 1 h post-injury, state III and V-FCCP respiration rates were noticeably lower (Figure 3.8), even though differences were not significant. Evidence was provided that mitochondrial respiration in SD and F344 rats is very similar at two ages of development (Figure 3.11). At least in terms of respiration analysis, there is data indicating a lack of strain differences.

Alterations to mitochondrial bioenergetics occur following TBI [122, 125, 137, 139-142, 147, 159, 188]. These studies utilized total mitochondria that gave insight to the time frame and extent of dysfunction, but did not take into consideration potential differences between mitochondrial fractions. It is well established that mitochondrial respiration varies in different regions of the brain as well as in different mitochondrial fractions [105, 109, 134, 185, 187, 189-194]. Besides displaying lower respiration capacity, synaptic mitochondria have also been shown to be able to buffer less calcium [134] and have a higher vulnerability permeability transition due to higher cyclophilin D content [194] compared to extrasynaptic mitochondria. Deficits in one fraction could be much different than the other, which could skew our understanding of the temporal dysfunction following TBI.

Data from this study indicates that respiration analysis from total mitochondria following injury would be a less accurate measurement of dysfunction than parceling out the two fractions. Extrasynaptic mitochondria retain higher respiration capacities in states III and V-FCCP following TBI compared to the synaptic fraction, evident by a much smaller decline in respiration at 1 h post-injury (Figure 3.8), which is likely due to inherently higher respiration capacities (Figure 3.5).

One of the goals of this study was to transition from using SD to F344 rats. Unfortunately, there has been a disconnect between TBI and aging research due to the use of two different strains of rats. TBI studies predominantly utilize SD rats, while aging studies often use F344 rats. It was a concern that these strains may have inherently different respiration rates, which could limit findings to one particular strain. Data from this study revealed that SD and F344 rats display a high degree of similarity in their respiration profiles at two distinct ages of development (3 - 5 and 12-14 mos). Perhaps both of these strains would also display similar respiration profiles at later stages of life (22 - 24 mos and older). Since these two strains show no differences in normal respiration capabilities, the response to a pathological condition (e.g. TBI) may also be similar among both rat strains. This study provides evidence that there high degree of overlap among these strains of rats and either strain could be used to determine the typical mitochondrial alterations that occur with aging or following injury. If there is a common mitochondrial response between SD or F344 rats to age and injury, there is a high probability that similar responses would be conserved in other species.

The next portion of the thesis determines whether or not there are age-related declines in synaptic and extrasynaptic mitochondrial bioenergetics in naïve F344 rats. Determining if there is a significant age-related decline in mitochondrial respiration would give us valuable insight and one possible explanation to why older animals display an exacerbated response (e.g. increased tissue loss, morbidity, and mortality) to TBI [75]. Since it is well established that older individuals have significantly higher incidences of morbidity and mortality and take much longer to recovery following many types of neurological insults [119, 120, 195-197], we have a great model to study experimentally what is seen in the clinic. Discovering potential mechanisms that underlie this serious

problem is necessary in developing effective treatments specifically for the geriatric population.

Acknowledgements

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Chapter 4: Age-Related Changes in Mitochondrial Respiration

Specific Aim 3 will test the hypothesis that there are age-related decline in synaptic and extrasynaptic mitochondrial respiration in naïve Fischer 344 rats.

Introduction

The free-radical theory of aging, first postulated by Harman [55], gave a molecular mechanistic explanation for why physiological systems decline with aging. This theory maintains that critical cellular components are under constant attack by various types of free radicals (FR), and that endogenously produced oxidants overcome endogenous antioxidants. Mitochondria are a major source of FR generated during normal cellular respiration [62, 70, 71, 198].

The absolute dependency of mammalian systems on mitochondria and their inherent production of FR compelled Harman to modify his theory into the mitochondrial theory of aging [60]. The mitochondrial theory of aging postulates that cellular aging is the product of mutations in the mitochondrial DNA (mtDNA) genome as a result of oxidative damage. As a result of accumulating damage to mtDNA, the mitochondrial blueprints are markedly altered, perpetuating production of the aberrant ETC components. Altered cellular components can lead to a decline in normal metabolic activity, specifically energy production. Depending on the severity of mitochondrial decline dysfunction (mitochondrial or cellular) may occur. Mitochondrial dysfunction has been implicated in more than just normal aging as being a primary contributor to many clinical problems (e.g. renal dysfunction, liver disease) and neurodegenerative diseases such as Parkinsonism and Alzheimer's disease [199-201].The gradual and perpetual cycle of accumulation of damaged cellular components necessary for energy production creates an energy crisis situation within the cell leading to its eventual dysfunction and demise [39].

A brief review of brain aging in context of mitochondrial aging research is given to reveal how our study is unique and contributes to this field. Many studies have shown significant age-related declines in mitochondrial enzymatic activity, especially in

complex 1 and 4, isolated from whole brains of mice [50, 53, 202, 203], rats [47, 51, 52, 90, 91, 204, 205], and primates [45]. Conversely, there are a few groups reporting no age-related declines in the activity of these complexes in rodents [43, 48]. These studies do not address possible regional differences in the brain.

Others have analyzed a specific brain region and/or a particular mitochondrial fraction for declines in enzymatic activity. Desmukh et al [189] reported no significant age-related changes in complex 1 activity in either synaptic or extrasynaptic mitochondria isolated from whole rat brain. A later study [41] also reported no age-related declines in complex 4 activity of synaptic and extrasynaptic mitochondria in the frontal cortex, hippocampus, or striatum of rats. Synaptic mitochondria isolated from the whole brain of mice showed significant decreases in complex 4 and 5 activities, while extrasynaptic mitochondria manifested significant declines in complexes 1, 2 + 3, and 4 [46]. Subsequent studies by Genova et al [206] and Cocco et al [207] reported age-related declines in complex 1 activity in extrasynaptic mitochondria isolated from the cortex of rat but failed to analyze the synaptic fraction. Sharman and Bondy [49], using mice, failed to find any age-related declines in enzymatic activities of cortical synaptic mitochondria. However, Navarro et al [208] showed significant declines in mitochondrial enzymatic activity of complex 1 and 4 in tissue isolated from the cortex and hippocampus of rats. Although several groups report declines in enzymatic activities of certain ETC complexes with age, it is unclear if these enzymatic changes translate into a functional decline in respiration. It has been reported that components of the ETC are present in excess and substantial inhibition of certain components is probably necessary before a decline in ATP production is realized [39, 95, 209].

The present study evaluated possible age-related changes in bioenergetics of synaptic and extrasynaptic mitochondria separately, since several groups have reported heterogeneity in these two fractions [109, 134, 185, 187, 189-194]. Several complex 1- and 2-driven respiration parameters were quantified from cortical synaptic and extrasynaptic mitochondria. Levels of oxidative damage were also determined from the same groups of rats in both mitochondrial fractions and in the post mitochondrial

supernatant (PMS), structures outside mitochondria, to determine if levels of oxidative damage impact possible functional changes that occur with age.

Materials and Methods

Animals

Adult male Fischer 344 rats (National Institute of Aging - Harlan Labs, Indianapolis, IN; n = 34) were used in this study and housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water. All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. All rats were naïve (not subjected to any type of operation, testing, or prior medication) and placed into one of three groups based on their age: (I). Young – 3-5 mos (n = 11); (II). Middle Aged – 12-14 mo (n = 13); and (III). Aged – 22-24 mo (n = 10).

Synaptic and extrasynaptic mitochondria isolation

Animals from multiple age groups were randomly selected each day to ensure homogeneity of procedures. Animals were briefly gassed with CO_2 until flaccid, decapitated, and the brains rapidly removed. All subsequent procedures were performed on ice including the entire mitochondrial isolation protocol. Cortical tissue from one hemisphere was randomly chosen for measuring levels of oxidative damage in the PMS and the other was used for respiration analysis. Unused synaptic and extrasynaptic mitochondria samples leftover from respiration analysis were then probed for markers of oxidative damage.

The cortex used for respiration analysis was placed in an all-glass dounce homogenizer with 4 mL of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Tissue was homogenized and mitochondria were extracted by differential centrifugation. The homogenate was spun twice at 1300 × g for 3 min in an Eppendorf microcentrifuge at 4°C and the supernatant was transferred to new tubes. The resulting supernatant was topped off with isolation buffer with EGTA and spun at 13,000 × g for 10 min. The supernatant was

discarded; the pellet was resuspended with isolation buffer with EGTA to a total volume of 500 μL. Both fractions were washed with fresh buffer a second time to ensure that the Ficoll was eliminated. Synaptosomes were resuspended in isolation buffer with EGTA, and mitochondria released using a nitrogen cell disruption bomb made by Parr Instrument Company (Moline, IL) at 1,200 psi for 10 min. Disrupted synaptosomes were placed on a new Ficoll gradient and returned to the ultracentrifuge and spun at 32,000 rpm for 30 min at 4°C. and samples were place on a discontinuous Ficoll gradient (F5415 Ficoll solution Type 400, 20% in H_2O, Sigma, St. Louis, MO), composed of 2 layers (2 mL of a 7.5% on top of 2 mL of 10% Ficoll cut with isolation buffer with EGTA). The final volume of the gradient was ~ 4.5 mL in the 7 mL Beckman tubes (344057, Fullerton, CA). Samples were placed in a Beckman SW 55 Ti swinging bucket rotor and centrifuged at 32,000 rpm for 30 min at 4°C. The gradient produced two separate mitochondrial fractions: the extrasynaptic mitochondrial pellet (bottom) and synaptic mitochondria trapped in synaptosomes located at the interphase of the two Ficoll layers. Synaptosomes were collected in 2.5 mL tubes, resuspended, and washed in isolation buffer with EGTA by centrifugation at 13,000 × *g* for 10 min to remove Ficoll from sample. The extrasynaptic mitochondrial pellet was collected in 500 µL tubes and also resuspended and washed using isolation buffer with EGTA. After removal of the Ficoll gradient, the synaptic mitochondria pellet was resuspended in isolation buffer with EGTA and centrifuged at 13,000 x *g* for 10 min. Both synaptic and extrasynaptic mitochondrial fractions were then resuspended with isolation buffer without EGTA and centrifuged at 10,000 ×*g* for 10 min to wash out the calcium chelator (EGTA). The final mitochondrial pellets were resuspended in isolation buffer without EGTA to yield a concentration of 10 mg/mL or higher. Protein concentration was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL) by measuring absorbance at 560 nm with a Molecular Devices microplate reader (Sunnyvale, CA).

Mitochondrial respiration measurements

Mitochondrial functionality was assessed using an Oxytherm Clark-type oxygen electrode (OXYT1/ED, Hansatech Instruments, Norfolk, UK). Mitochondria (~60 µg) were placed in the sealed Oxytherm chamber containing respiration buffer (125 mM KCl,

0.1% BSA, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, pH 7.2) and continuously stirred at 37° C. The rate of oxygen consumption was defined as the slope of the response of isolated mitochondria to the consecutive administrations of oxidative substrates as previously described [183], with minor adjustments. One adjustment had to be made to the previous protocol due to the high level of purity the Ficoll gradient offers compared to differential centrifugation alone. In this study ~60 µg of mitochondrial protein was used for each respiration analysis compared to using ~180 µg previously. The amount of oligomycin administered was cut in half, from 1 µL to 0.4 µL, because the original amount was too high and was poisoning the mitochondria. Two additions of adenosine diphosphate (ADP), instead of one addition, were administered to ensure accurate state III respiration measurements. The modified protocol of the substrates injected into the Oxytherm chamber sequentially as follows: 2.5 µL of pyruvate/ malate (P/M; 2.5 mM); 1.25 µL of ADP (150 µM) added twice in 1-min intervals; 0.5 µL oligomycin (1 µM); 1µL carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP; 1 µM); 0.2 µL rotenone (1 µM) added to shut down complex 1 activity resulting in cessation of oxygen utilization; and finally 2.5 µL succinate (10 mM) depicted in Figure 4.1.

States II - V are mitochondrial respiration parameters driven by complex 1 (P/M, ADP, oligomycin, and FCCP), while complex 2-driven respiration was measured separately by shutting down complex 1 with rotenone, to produce state V respiration (succinate). Figure 4.1 is a schematic drawing of a typical mitochondria respiration trace depicting the sequence of substrate additions and subsequent oxygen utilization rates. The overall oxygen utilization rate was determined by measuring the amount of oxygen consumed throughout all states of respiration divided by the time elapsed and amount of mitochondrial protein used for the respiration assay. The oxygen utilization rate can detect alterations in the time mitochondria respond to substrate additions, which serve as an index of their overall respiration capacities. The respiratory control ratio (RCR) was determined by dividing the rate of oxygen utilization for state III (ADP) by state IV (oligomycin). RCRs indicate how coupled the ETC is to ATP production. The states of respiration (III-V), overall oxygen utilization rates, and RCRs were all collected to further define respiration capabilities. All isolated mitochondrial fractions, regardless of

age, were able to produce RCRs of ≥ 5, indicating that the mitochondria were not damaged during the isolation procedure. Actual respiration rates for all states of respiration, RCRs, and overall oxygen utilization rates were plotted as a function of mitochondrial fraction and age.

Markers of oxidative damage quantification

Estimation of protein carbonylation (PC). Levels of PC were assessed as described previously [165] from the PMS, synaptic, and extrasynaptic mitochondria. All samples were normalized to 4mg/ml, then combined with sodium dodecyl sulfate (SDS) and dilute 2,4-dinitrophenylhydrazine (DNP). Samples were incubated at room temperature for 20 min then neutralized with 2 M Tris in 30% glycerol. The resulting samples were loaded on a nitrocellulose membrane and run on a slot-blot apparatus. Membranes were blocked in 3% bovine serum albumin and PBS/Tween for 1 h and incubated with a 1:100 dilution of anti-DNP polyclonal rabbit antibody in PBS/Tween for 1 h. Membranes were washed in PBS/Tween and incubated for 1 h with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. Each membrane was washed in PBS/Tween and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image. Nonspecific binding of antibody to the membrane was not observed. The intensity of PC staining was quantified by arbitrary optical density and data reported as percent of the young age group values.

Figure 4.1. Typical respiration trace of synaptic and extrasynaptic mitochondrial fractions.

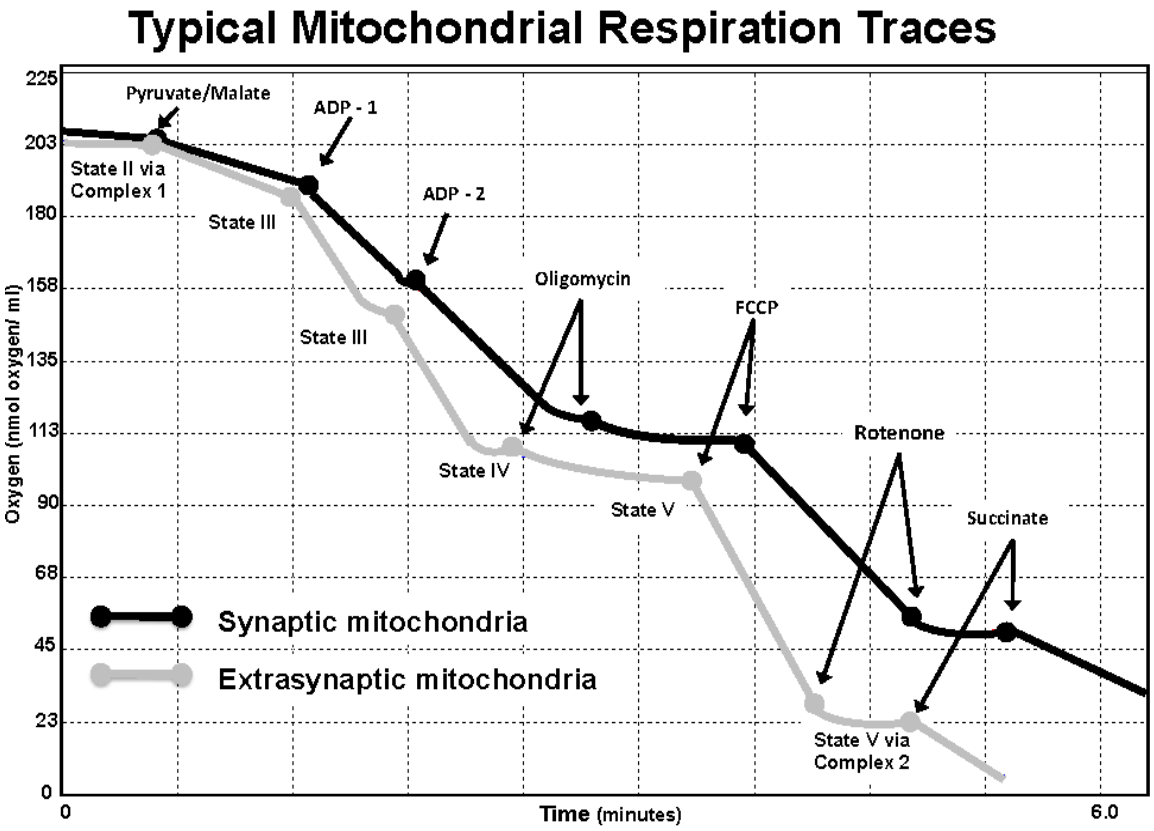

Figure 4.1. Typical oxygen utilization traces of synaptic and extrasynaptic mitochondria isolated from a naïve cortex of an aged animal. **state I**: no substrates for respiration added; no oxygen utilization apparent. **state II**: addition of P/M; basal rate of respiration. **state III**: two separate additions of ADP; each addition connects the ETC with oxidative phosphorylation; high level of oxygen utilization indicated ADP being converted to ATP. **state IV**: addition of oligomycin; electrons are blocked from returning into the matrix through the ATP synthase, the ETC slows only to maintain mitochondrial membrane potential that is lost through inner membrane into the matrix, and oxygen utilization is greatly reduced. **state V**: addition of FCCP; results in an uncoupling of the ETC to ATP synthesis, represents maximum rate of respiration since no bottle-necking occurs at ATP synthase - protons are allowed to rush back into the matrix. Rotenone is then added to shut down complex 1-driven respiration. **state V (succinate)**: addition of succinate; maximum rate of respiration via complex 2 due to presence of FCCP still in the system.

Estimation of 3-nitrotyrosine (3-NT; an index of protein nitration) and 4-hydroxynonenal (4-HNE; an index of lipid peroxidation). The PMS, synaptic, and extrasynaptic mitochondria samples were normalized with 12% SDS and modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature. Samples (250ng) were loaded into a well on a nitrocellulose membrane in a slot-blot apparatus under vacuum. Membranes were blocked with 3% bovine serum albumin in PBS/Tween for 1 h, and then incubated with either a 1:2000 dilution of anti-3-NT polyclonal rabbit antibody or a 1:5000 dilution of anti-HNE polyclonal goat antibody in PBS/Tween for 1.5 h. Each membrane was washed in PBS/Tween for 5 min three times after incubation. Membranes were incubated for 1 h, after washing, with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in 1:8000 ratio. Membranes were washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image as above. Nonspecific binding of antibody to the membrane was not observed. The intensities of 4-HNE and 3-NT staining were quantified by arbitrary optical density and data reported as percent of the young age group values.

Statistical analyses

All data are reported as group means ± SD. The states of respiration are reported as nmol O_2 utilized / min / mg of mitochondrial protein. A two-way repeated measure ANOVA (age X mitochondrial fraction) was used to probe for possible differences in respiration data. Based on evidence that synaptic and extrasynaptic mitochondria have very different respiration properties [109, 111, 187, 210], we expected to find differences between the two fractions of mitochondria.

A two-way repeated measure ANOVA (age X mitochondrial fraction) was used to compare the levels of oxidative damage. Separate analysis were performed for each of the measured of oxidative damage.

A Pearson product-moment correlation coefficient was use to probe possible associations between the levels of each marker of oxidative damage and the overall oxygen utilization rate and RCR value in each subject.

When appropriate, all ANOVAs were followed by a Student-Newman-Keuls post-hoc analysis to determine individual group differences. The alpha level was set at 0.05 for significance.

Results

Age-related changes in mitochondria bioenergetics

Mitochondrial protein levels. The average amount of mitochondrial protein used to assess respiration capabilities was extrasynaptic: 59.5 µg ± 8.3 (young), 59.0 ± 12.6 (middle aged), and 65.5 ± 9.9 (aged), synaptic: 53.8 µg ± 13.5 (young), 62.2 ± 13.3 (middle aged), and 67.4 ± 10.2 (aged). The ANOVA failed to reveal a significant age effect [$F(2, 29) = 2.413$, $p > 0.1$] or a significant effect for mitochondrial fraction [$F(1,29) = 0.619$, $p > 0.1$]. In all experiments, the amounts of mitochondrial protein used produced respiration traces similar to the one depicted in Figure 4.1.

Overall oxygen utilization rate. There were no significant age-related changes in overall rate of oxygen utilization [$F(2, 29) = 0.126$, $p > 0.1$]. However, the analysis did reveal a significant main effect for mitochondrial fraction [$F(1, 29) = 6.537$, $p < 0.01$] (Figure 4.2). The mean overall oxygen utilization rates of extrasynaptic mitochondria were significantly higher than those from the synaptic fraction. There was no significant interaction between age and mitochondrial fraction ($p > 0.1$).

P/M (state II respiration via complex 1). An ANOVA revealed no significant age-related differences in state II respiration [$F(2, 29) = 0.288$, $p > 0.1$] or a significant main effect for mitochondrial fraction [$F(1, 29) = 1.724$, $p > 0.1$] (Figure 4.3a).

Figure 4.2 Effects of age on overall oxygen utilization rates in mitochondrial respiration.

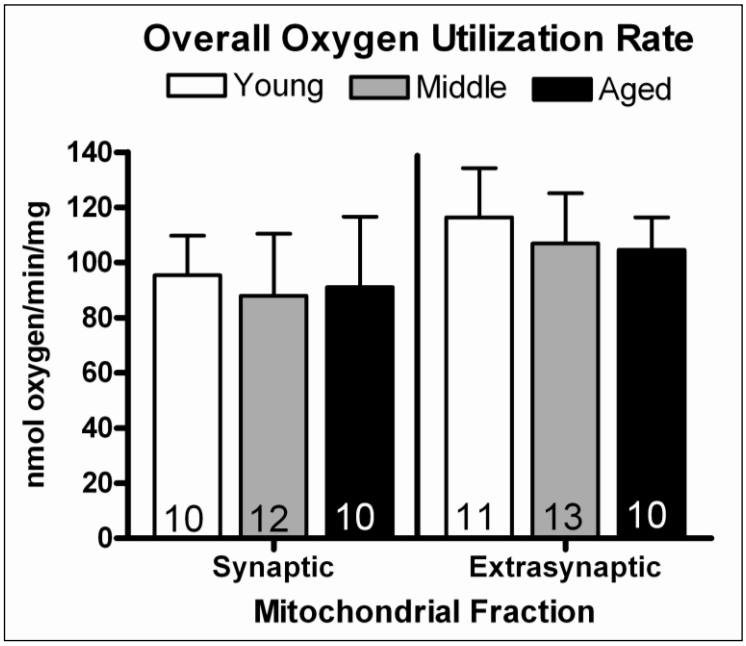

Figure 4.2. No overt age-related alteration in overall oxygen utilization rate was apparent in either mitochondrial fraction. Bars represent group means ± SD; numbers inside the bars represent sample size.

Since the amount of mitochondrial protein utilized for each assay and state II respiration rates were not significantly different, the data indicates that all mitochondrial groups contain the same ability to produce similar respiration traces.

ADP (state III respiration). Figure 4.3b depicts how efficiently ADP was utilized for ATP synthesis in both fractions of mitochondria across age groups. An ANOVA failed to reveal any significant age-related changes in state III respiration [$F(2, 29) = 0.158$, $p > 0.1$]. The analysis did reveal a significant main effect for mitochondrial fraction [$F(1, 29) = 17.220$, $p < 0.0005$]. Extrasynaptic mitochondria were significantly higher than those seen in the synaptic fraction.

Oligomycin (state IV respiration). Possible age-related changes in state IV respiration were assessed by the addition of oligomycin. An ANOVA revealed no significant age-related changes [$F(2, 29) = 0.8714$, $p > 0.1$] but a significant main effect for mitochondrial fraction [$F(1,29) = 13.007$, $p < 0.005$] (Figure 4.3c). These data indicate aging does not deteriorate the inner membrane to the point where there are functional impairments in the membrane potential in either synaptic or extrasynaptic mitochondria. Regardless of age, mitochondria were able to maintain the proton gradient indicating preservation of inner membrane integrity. Extrasynaptic mitochondria had significantly higher values indicating an inherently higher control over respiration in the synaptic fraction. There was no significant age by mitochondrial fraction interaction.

RCR (state III / state IV respiration). The RCR is an index of how coupled the ETC is to ATP production. No age-related changes in the overall condition of the mitochondria were apparent [$F(2, 29) = 0.450$, $p > 0.1$]. There was a significant main effect for mitochondrial fraction [$F(1, 29) = 4.188$, $p > 0.05$] (Figure 4.3d). Synaptic mitochondria displayed significantly lower RCRs than the extrasynaptic fraction.

FCCP (state V respiration). Maximum respiration capabilities of both fractions of mitochondria were quantified by charging the ETC with the addition of all the necessary substrates (P/M and ADP) and subsequently bypassing the ATP synthase with the addition of FCCP. FCCP, a pure uncoupler that acts as a protonophore, allows the protons built up in the inner membrane space to freely pass back into the matrix at a rapid rate. The ETC quickly attempts to replenish this sudden decline in the electrochemical gradient, which results in a high level of oxygen consumption via complex 4 (Figure 4.3e). An ANOVA revealed no significant age-related change in maximum rates of respiration [$F(2, 29) = 0.008$, $p > 0.1$]. The repeated measure ANOVA detected a significant main effect for mitochondrial fraction [$F(1,29) = 14.598$, $p < 0.001$].

Extrasynaptic mitochondria had significantly higher levels of oxygen utilization than synaptic following addition of FCCP.

Succinate (state V respiration via complex 2). Rotenone was added specifically to inhibit complex 1, allowing succinate driven respiration to be measured independently. Rotenone works by competitively inhibiting the transfer of electrons from iron-sulfur centers of complex 1 to ubiquinone. An ANOVA revealed no significant age-related changes in maximum complex 2-driven respiration [$F(2, 29) = 0.359$, $p > 0.1$] (Figure 4.3f). There was a significant main effect for mitochondrial fraction [$F(1, 29 = 12.771$, $p < 0.005$] with extrasynaptic displaying significantly higher values than synaptic.

Age-related changes in levels of oxidative damage

Estimation of PC. An ANOVA detected a significant age-related difference in the levels of PC in the cortical PMS fraction [$F(3, 21) = 13.462$, $p < 0.0005$] (Figure 4.4a). Post-hoc analysis revealed that the PMS of middle and aged rats contained higher levels of PC compared to young ($p < 0.05$). Synaptic and extrasynaptic fractions of mitochondria were also probed for levels of PC, quantified as a percent of the mean value for the young group, and compared across age groups using two-way repeated measures ANOVA. Analysis revealed no significant difference in PC between mitochondrial fractions [$F(2, 21) = 4.846$, $p > 0.1$], but did significantly differ across age groups [$F(2, 21) = 4.846$, $p < 0.05$] (Figure 4.4b). The level of PC in the synaptic fraction did not significantly correlate with the overall oxygen utilization rate ($r = 0.014$, $p > 0.5$) or the RCR values ($r = 0.086$, $p > 0.5$). In the extrasynaptic fraction there was positive significant correlation between the levels of PC with the overall oxygen utilization rate ($r = 0.475$, $p < 0.01$), but no significant correlation with RCR values ($r = 0.004$, $p > 0.9$).

Estimation of 4-HNE. An ANOVA detected a significant age-related change in 4-HNE in the cortical PMS [$F(2, 21) = 23.2$, $p < 0.0001$]. Post-hoc analysis revealed a

Figure 4.3. Effects of age on several bioenergetic parameters.

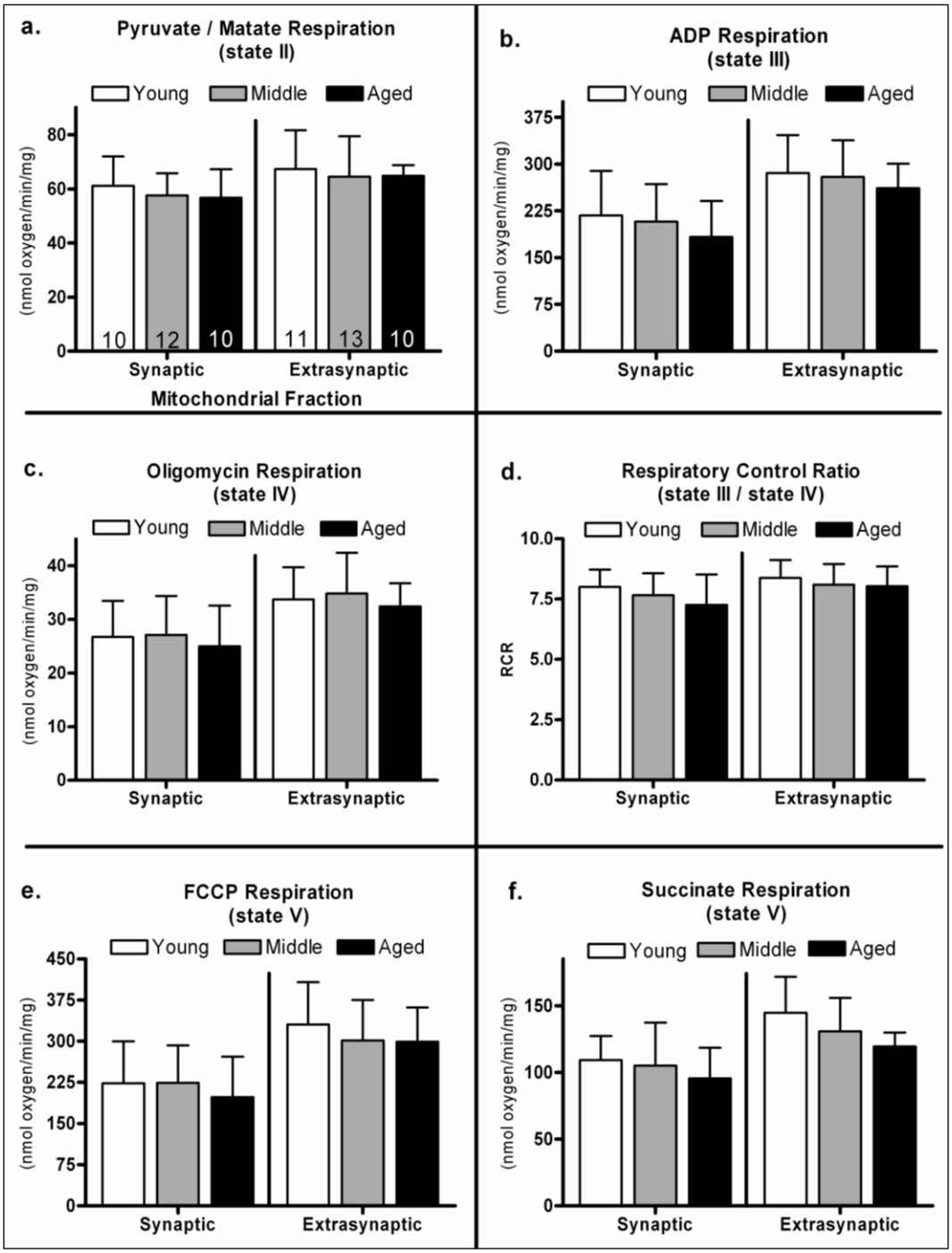

Figure 4.3. Actual states of respiration for synaptic and extrasynaptic fractions. **a.** state II – P/M **b.** state III – ADP **c.** state IV – Oligomycin **d.** state V – FCCP **e.** state V – Succinate. **f.** respiratory control ratio (RCR) – which is an index of how coupled respiration is to ATP production and is calculated by dividing state III by state IV. Bars represent group means ± SD; numbers inside the bars represent sample size.

significant increase in 4-HNE in middle aged and aged animals compared to young (Figure 4.4c). The synaptic and extrasynaptic fractions of mitochondria were probed for levels of 4-HNE and compared across age groups using two-way repeated measures ANOVA. Analysis revealed a significant difference in 4-HNE, measured as a percent of the mean value for the young group, between mitochondrial fractions [$F(2, 21) = 7.662$, $p < 0.05$], but were not significantly different when compared across age groups [$F(2, 21) = 2.009$, $p > 0.1$] (Figure 4.4d). Post-hoc revealed synaptic mitochondria displayed significantly higher levels of 4-HNE compared to the extrasynaptic mitochondria. The level of 4-HNE in the synaptic fraction had no correlation with the overall oxygen utilization rate ($r = 0.024$, $p = 0.91$), but not with the RCR values ($r = 0.014$, $p = 0.95$). In the extrasynaptic fraction there was positive significant correlation between the levels of PC with the overall oxygen utilization rate ($r = 0.598$, $p = 0.001$) or the RCR values ($r = 0.250$, $p = 0.21$). Post-hoc revealed synaptic mitochondria displayed significantly higher levels of 4-HNE compared to the extrasynaptic mitochondria. The level of 4-HNE in the synaptic fraction demonstrated no significant correlation with the overall oxygen utilization rate ($r = 0.024$, $p > 0.9$) or the RCR values ($r = 0.014$, $p > 0.9$). In the extrasynaptic fraction there was a positive significant correlation between the levels of PC with the overall oxygen utilization rate ($r = 0.598$, $p < 0.001$), but no correlation was apparent with RCR values ($r = 0.250$, $p > 0.2$). Synaptic mitochondria displayed levels of 4-HNE equivalent to that in the extrasynaptic fraction of young animals ($p > 0.1$).

Estimation of 3-NT. 3-NT is used as a biomarker of reactive nitrogen species formation [211] specifically peroxynitrite-induced protein modification [212]. An ANOVA detected an age-related change in 3-NT levels in the cortical PMS [$F(2, 21) = 6.439$, $p < 0.01$]. Post-hoc analysis revealed a significant increase in aged animals compared to young but not to middle aged subjects (Figure 4.4e). A two-way repeated measures ANOVA revealed a significant difference in 3-NT levels between mitochondrial fractions [$F(2, 21) = 5.59$, $p < 0.01$] and when compared across age groups [$F(2, 21) = 4.016$, $p < 0.05$] (Figure 4.4f). The level of 3-NT in the synaptic fraction had no correlation with the overall oxygen utilization rate ($r = 0.254$, $p = 0.23$)

or the RCR values (r = 0.033, p = 0.88). In the extrasynaptic fraction there was positive significant correlation between the levels of 3-NT with the overall oxygen utilization rate (r = 0.420, p = 0.03), but no correlation was found with the RCR values (r = 0.096, p = 0.64). Post-hoc analysis revealed a significant increase in middle aged animals in the extrasynaptic fraction ($p < 0.05$) and aged subjects in the synaptic fraction ($p < 0.05$) compared to young animals. The level of 3-NT in the synaptic fraction had no correlation with the overall oxygen utilization rate (r = 0.254, $p > 0.2$) or the RCR values (r = 0.033, $p > 0.8$). In the extrasynaptic fraction there was a significant positive correlation between the levels of 3-NT with the overall oxygen utilization rate (r = 0.420, $p < 0.05$), but no significant correlation was found with the RCR values (r = 0.096, $p > 0.6$).

Discussion

This study is the first to perform a comprehensive analysis of possible age-related changes in mitochondrial respiration in the cortex of Fischer 344 (F344) rats. Two different fractions of mitochondria were analyzed, synaptic and extrasynaptic, in rats representing three different stages of development. The analysis demonstrated robust mitochondrial respiration in the oldest subjects (22-24 mos) that were not significantly different from younger (3-5 mos) cohorts, in either mitochondrial fraction. Across all age groups, synaptic mitochondria displayed significantly lower respiration capacities compared to the extrasynaptic fraction. Age-related increases in oxidative damage were observed in the synaptic and extrasynaptic mitochondria and brain parenchyma.

The evidence for age-related functional decline in neuronal mitochondria is controversial and lacks conformity to a specific brain region, use several different species and strains. There are also important issues concerning pooling of tissue and number of subjects used for evaluation. A relatively early study [213] assessed total mitochondria in the whole brain from F344 rats and reported a significant decline in only state III respiration. RCR and state III respiration declines have been observed in Swiss CD-1/UCadiz mice [202] and Wistar rats [91, 204, 205]. However, Weindruch [214] found no significant changes in these parameters in C57BL/6J mice and Meng [215] failed to observe these changes in F344 rats.

Several laboratories using young adult animals have reported differences in the bioenergetics of synaptic and extrasynaptic mitochondrial fractions [105, 109, 111, 187]. Synaptic mitochondria may display higher respiration control [176], even though oxidative metabolism is the major source of ATP production in the synapse [210], due to their intricate role in maintaining cytoplasmic calcium levels to allow repetitive stimulation needed for neurotransmission [216]. Examinations of age-related functional differences in synaptic and extrasynaptic mitochondrial fractions isolated from whole brain preparations have reported significant declines in both fractions [189, 190, 217, 218]. These studies are controversial since the results were obtained by pooling tissue from multiple animals and subsequently replicating findings from this common pool of tissue and treating the results as a large sample size. A study by Leslie et al [219] using Long Evans rats failed to find a functional decline in extrasynaptic mitochondria. Relatively few studies limited their evaluations to a specific region of the brain. Navarro et al [208] reported a significant age-related decline in total cortical and hippocampal mitochondria, showing complex 1- and 2-driven respiration deficits (RCRs, state III, and state IV). Cocco et al (2005) reported a significant age-related decline in cortical state III respiration by both complex 1 (43%) and complex 2 (25%) in extrasynaptic mitochondria using Wistar rats. The present set of observations are unique in that the analyses were limited to the cortex and carefully evaluated both synaptic and extrasynaptic mitochondrial bioenergetics. While we failed to find any age-related differences in mitochondrial bioenergetics, we did observe significant differences between synaptic and extrasynaptic mitochondria in support of the previously mentioned studies.

Several possibilities may account for the lack of age-related bioenergetic changes in cortical mitochondria. Our study compared aged F344 rats 22-24 mos old to young (3-5 mos) and middle aged (12-14 mos) subjects. A previous study showed that naïve F344 rats begin to die spontaneously at around 20 months of age [220] as a result of aging. F344 rats 22-24 mos old have been frequently used in aging research, have been extensively characterized, and display many age-related motor and cognitive deficits [220-222]. One possible reason no differences were observed with age could be that the worn out or damaged mitochondria from older animals may have been eliminated during

Figure 4.4 Effects of age on levels of oxidative damage.

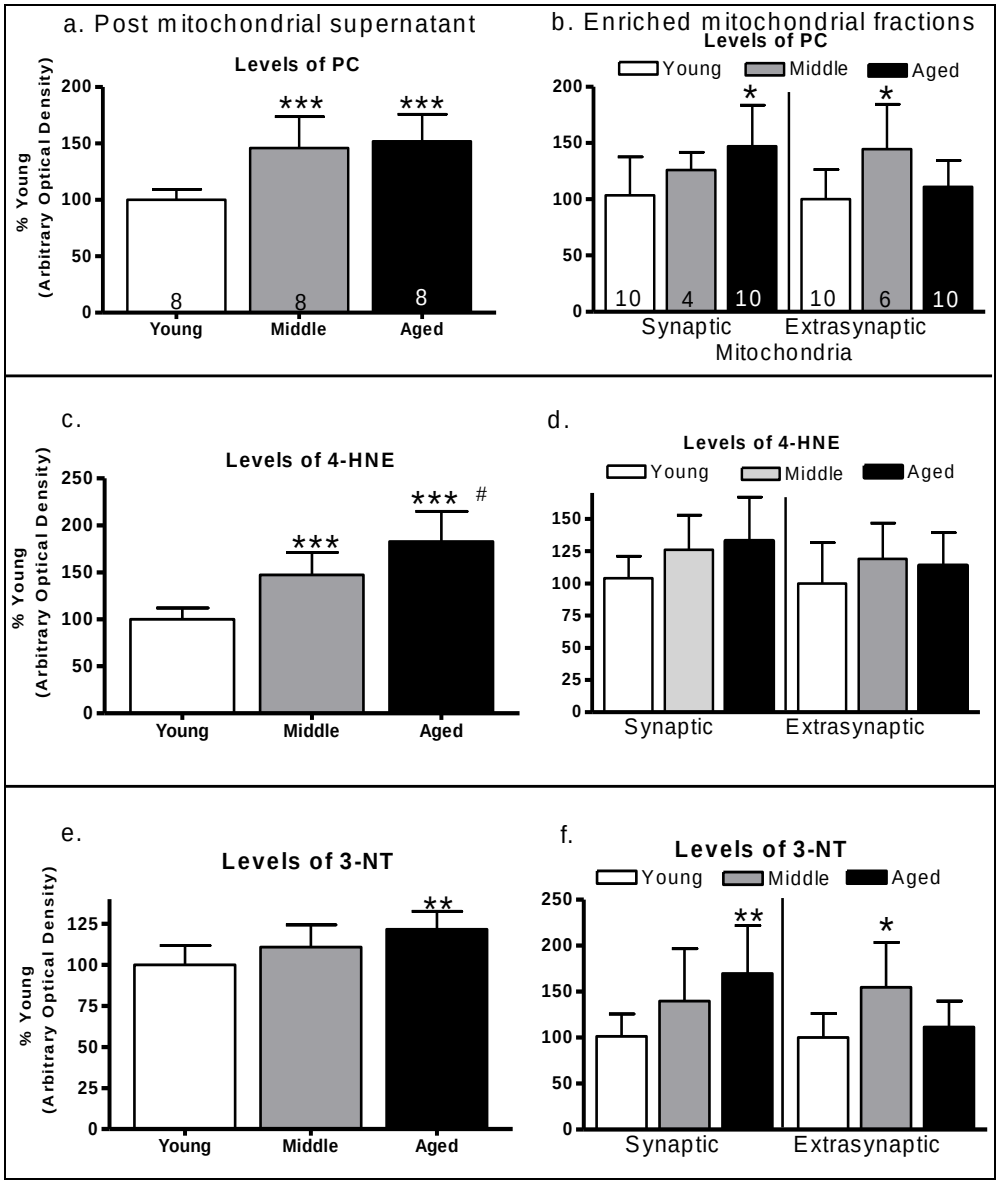

Figure 4.4. Markers of oxidative damage; expressed as a percent of values observed in young animals. Relative optical densities of protein carbonyls (PC) were determined in the **a.** post mitochondrial supernatant (PMS) and in **b.** both mitochondrial fractions. Relative levels of 4-hydroxynonenal (4-HNE)-protein adducts, the most prevalent toxic peroxidation product formed during oxidant stress, were assessed in the **c.** PMS and **d.** both mitochondrial fractions. Relative levels of nitrotyrosine (3-NT), a biomarker of reactive nitrogen species formation, were measured in the **e.** PMS and **f.** both mitochondrial fractions. Bars represent group means ± SD; numbers inside bars represent the sample size. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to young. # $p < 0.01$ compared to middle aged rats.

the isolation process. Long et al [90] reported that these rats display age-related mitochondrial enzymatic impairments and increased levels of oxidative damage at 24 mos of age. Meng et al [215] reported no significant declines in mitochondrial membrane potential or membrane integrity in F344 rats 26 mos of age, while several groups report deficits in considerably older (28-32 mos) animals [207, 213, 219]. LaFrance et al [110] reported no age-related declines in membrane potential or sensitivity to calcium of cortical mitochondria at 24 mos in F344 rats, but did find significant alterations in animals 33 mos of age. A lack of significant age-related respiration changes in aged F344 rats may indicate mitochondria are more resilient than previously thought and alterations in respiration may not occur until more advanced ages. Since 33 mos are well beyond the normal lifespan of F344 male rats, the findings may represent observations from a unique population and not representative of normal aging.

Aging studies have used several different species of rats and report age-related declines in not only mitochondrial enzymatic activity but also respiration parameters. Numerous early studies assessed aged Sprague-Dawley rats [41, 189, 190, 217], although a few used Long Evans [219] and F344 rats [213, 218]. Thus the F344 rat used in the present set of experiments is not a unique strain and cannot account for the lack of age-related functional decline in mitochondria.

In the present set of experiments, 10-13 individual animals were analyzed for each age group, making our sample size larger than most aging studies assessing intact functional parameters. A larger sample size often reduces variability and protects against a type II statistical error. Typically the sample size per age group in mitochondrial aging studies were 5-6 and could result from the pooling of a larger number of animals, especially when synaptic mitochondria were evaluated, (e.g. [189, 218]). In some experiments the reported sample size was extremely small [213, 218]. Meng et al [215] used 8-9 F344 rats/age group and failed to find any significant age-related changes in membrane potential and membrane integrity, supporting our findings.

The purification process of the mitochondria may explain the differences in our findings from those reported previously. While it is difficult to compare the quality of the mitochondrial preparation between studies, the RCR is one reasonable method. The

Figure 4.5. Correlations between the levels of oxidative damage and respiration.

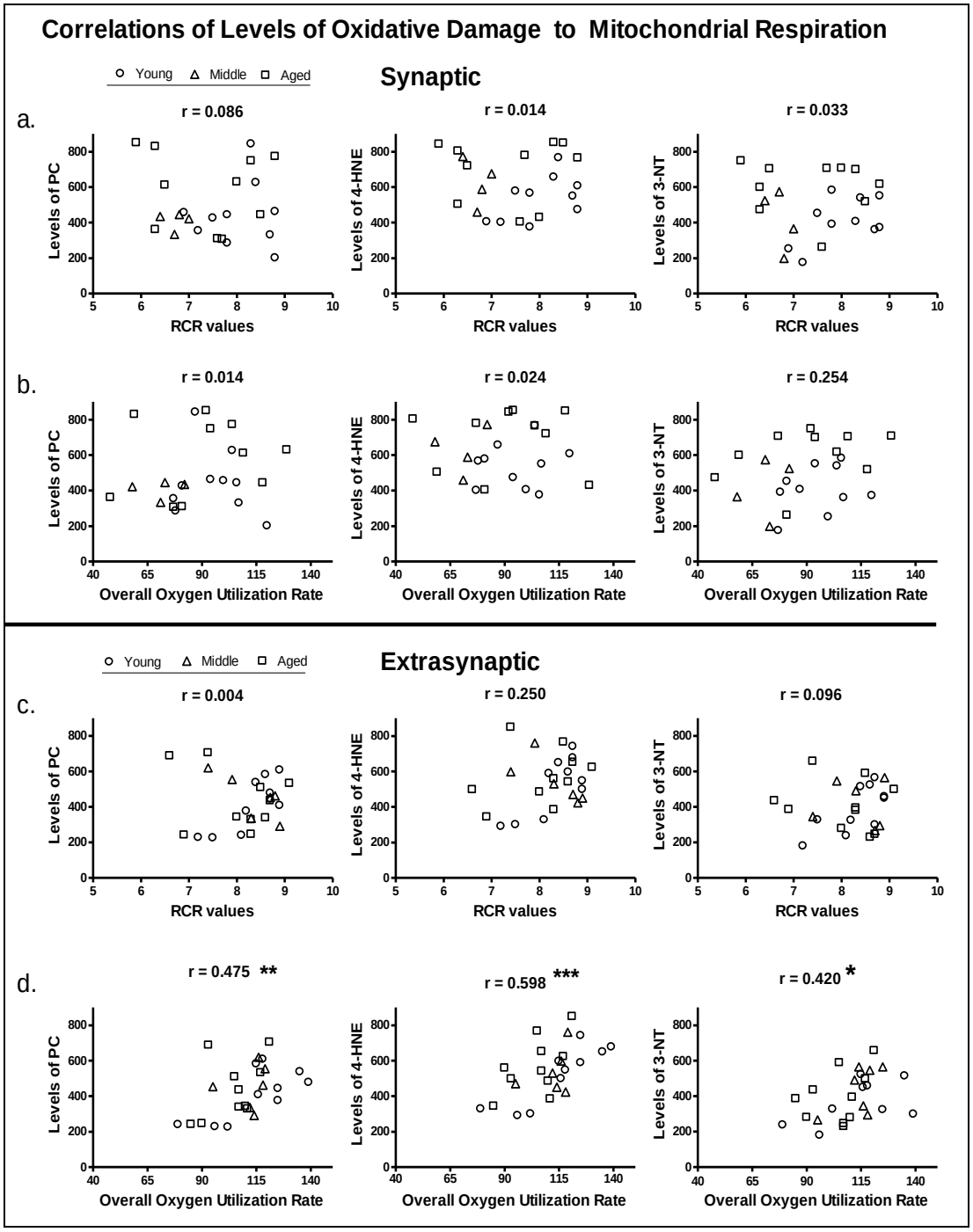

Figure 4.5. The relationship between the levels of oxidative damage (PC, 4-HNE, and 3-NT) and the RCR values in **a.** synaptic mitochondria and **c.** extrasynaptic mitochondria. The relationship between the levels of oxidative damage and the overall oxygen utilization rates in **b.** synaptic mitochondria and **d.** extrasynaptic mitochondria. Symbols represent individual values. * $p < 0.05$, ** 0.01, *** 0.005.

RCR is a good indicator of overall mitochondrial function because it measures how quickly mitochondria can produce ATP (state III) as well as the ability to maintain the proton gradient when not making ATP (state IV). In intact cells of naïve tissues, the ETC is tightly coupled to ATP production (high RCR). Declines in RCR values indicate an uncoupling of the ETC from ATP production (low RCR). The RCR has a range of 0 (totally nonfunctional) to 10 (well-coupled and functional). Several studies have reported mitochondrial RCR values as being much higher than 5 in naïve and uninjured central nervous tissue, demonstrating that the mitochondria are tightly coupled with little damage from isolation procedures [134, 142, 161, 183, 188, 223]. The mean RCR in the present study ranged from 7.2 to 8.4 for young and middle aged, and aged cohorts for both synaptic and extrasynaptic fraction, with no significant age-related declines. These values support the idea that the mitochondria used in our study were of high quality. Several other groups have reported no age-related decline in RCR values of rodents [189, 190, 214, 215, 219], while others report declines ranging from 7-33% [106, 189, 190, 202, 217]. Almost all prior aging studies evaluating possible changes in mitochondrial bioenergetics had RCR values of 5 or lower in young animals [106, 190, 202, 213, 215, 224]. The range for RCR values in much of the aging literature among young, naive animals was 1.3 [215] to 6.1 [189]. Low RCR values (below 5) have been reported when mitochondria in young animals have sustained significant damage [122, 142, 183]. Low RCR values not only signify uncoupling of the ETC but also possible disruption of the mitochondrial membranes. Considering that the animals are naïve and not subjected to any type of injury, low RCR values may indicate mitochondrial damage could have occurred during the isolation process. We were unable to detect any age-related changes in the RCR in either the synaptic or extrasynaptic fractions.

In addition to assessing possible age-related changes in respiration, levels of oxidative damage were quantified in the same animals. Three well-characterized markers of oxidative damage (4-HNE, 3-NT, PC) were quantified in the PMS and in purified synaptic and extrasynaptic mitochondria. In these experiments, the levels seem to vary between the PMS and mitochondrial fractions, but in general increased with age. All three markers of oxidative damage were elevated in the PMS with age indicates that

reactive oxygen species, primarily formed in mitochondria, are capable of diffusing out from mitochondria and attacking surrounding cell structures [225]. Though mitochondria may be a main source for oxidative damage, it does exclude other possible sources of this oxidative damage (e.g. lysosomes, phagocytes, peroxisomes, etc). Synaptic mitochondria displayed significant increases in levels of PC and 3-NT in the aged group, while only middle aged animals showed increases in these markers in the extrasynaptic fraction. There were no significant increases in 4-HNE found in either mitochondrial fraction, but higher levels were observed with age.

Previous research links an age-related increase in oxidative damage to a number of lipids, proteins, and enzymes in F344 rats [66]. Oxidative damage is likely to be responsible for alterations in mitochondrial respiration observed with aging [59, 62, 226] due to chemical-physical alteration in membranes [227] and/or electron carriers [44]. Structural alterations to components of the ETC explain increased free radical generation with age, but the extent of oxidative damage needed to produce respiration declines remains unexplained.

PC are proteins that have been oxidized by reactive oxygen species and are chemically stable, making them fitting markers of oxidative stress. In the PMS, PC levels were increased 46% (middle) and 52% (aged) compared to young rats. An age-related increase in protein oxidation to cellular components was observed in the fraction outside of mitochondria. Several other groups report significant elevations of PC in neuronal tissue, supporting our findings. Similar increases of PC in F344 rats 24 (~30%) compared to rats 1 mo old [228] was obtained from whole brain homogenates. Aged mice (19 mos) display approximately a 57% increase [53, 202, 203], while Wistar rats (23 mos) were found to show a 69% increase compared to young animals [52]. Age-related increases in PC levels were observed in the synaptic fraction, 42% (aged) compared to young values. Damaged ETC protein complexes may explain the subtle functional declines in the respiration observed in this mitochondrial fraction (e.g. state III and V-FCCP). Extrasynaptic mitochondria displayed a significant increase in PC levels in only middle aged animals (45%) which likely represents the heterogeneity of the middle aged population, meaning some rats would survive to older ages and others would not. A

potential skewing of the data may have occurred in the middle aged group due to limited available mitochondrial samples (n = 4 to 6, instead of n = 10). This may be responsible for higher oxidative damage levels in extrasynaptic mitochondria of middle aged rats compared to aged rats. (Figure 4.4). Extrasynaptic mitochondria from aged animals, on the other hand, contained only 10% higher PC isolated than young rats.

4-HNE is one of the most prevalent and toxic products generated during lipid peroxidation (LP) formed during oxidative stress [229]. 4-HNE exerts cytotoxic effects primarily by modifying intracellular proteins [230]. LP can modify molecular membrane structure resulting in altered membrane asymmetry and fluidity [34]. The levels of 4-HNE significantly increased in an age-dependent manner in the PMS (46% in middle aged and an 82% in aged rats). Conversely, increases of LP in either fraction of mitochondria were not significant, although a 28% (synaptic) and 14% (extrasynaptic) increase was observed in aged animals. Reports regarding the extent of LP that occurs with aging are conflicting, which probably stems from the use of several different assay methodologies, animal type, and tissue examined. Some studies have reported no age-related alterations of LP in Wistar rats [231], while others observed significant elevations in several distinct fractions of the cerebral cortex [52, 226, 232] of these aged rats. Tian et al [228] observed no age-related increases of LP in aged F344 rats, while noting a significant elevation in PC, suggesting that LP may not be the most sensitive measure of oxidative stress.

Several reactive nitrogen species are derived from nitric oxide (NO), one of which is peroxynitrite ($ONOO^-$). A number of oxidation and nitration products are produced from the reaction of $ONOO^-$ with cellular macromolecules. One such product, 3-NT, is routinely used as a marker of $ONOO^-$ formation *in vivo* [233]. $ONOO^-$ is formed by the biradical reaction of NO and O_2^-. A number of oxidation and nitration products are produced from the reaction of $ONOO^-$ with cellular macromolecules. One such product, 3-NT, can be used as a marker of $ONOO^-$ formation *in vivo* [234]. Our data show a significant increase in 3-NT levels in the PMS of aged animals (17%) compared to young levels. Only synaptic mitochondria from aged animals displayed significant increases of 3-NT levels (71%), but middle aged animals did have elevated levels (38%) compared to

young rats. In the extrasynaptic fraction, only middle aged animals displayed significantly higher 3-NT levels (55%) alluding to the possibility that the middle aged animals represent an extremely heterogeneous population. The middle aged group may represent a subgroup of rats that were experiencing high levels of oxidative stress that would not survive to older ages, due to the limited amount of samples available for respiration analysis (Figure 4.4). Shin et al [235] reported that several areas of the brain displayed increased levels of 3-NT in 24-29 mos old Sprague-Dawley rats, with the cerebral cortex having almost twice the levels of young animals (4-6 mos).

An interesting finding from this study was that the levels of oxidative damage (PC, 4-HNE, and 3-NT) did not significantly correlate with the overall functionality, measured by RCR values, in either fraction of mitochondria (Figure 5). On the other hand, extrasynaptic mitochondria showed a positive correlation between the levels of oxidative damage and the overall oxygen utilization rate, whereas this correlation was not apparent in the synaptic fraction (Figure 5). Data indicates that mitochondria with the highest respiration capacity also accumulating the most oxidative damage. Extrasynaptic mitochondria display significantly higher respiration than synaptic mitochondria, indicative of inherent differences in these two fractions of mitochondria.

Extrasynaptic mitochondria may be utilized heavily for ATP production and when they accumulate oxidative damage they are replaced or repaired quickly. One possible explanation for not observing significant increases in oxidative damage in extrasynaptic mitochondria from aged animals are these animals represent a group of rats that have maintained proper mitochondrial maintenance and turnover. The significant increase in damage observed in the middle aged animals may represent a crucial turning point in whether the animal will survive to older ages if they have the capacity to make necessary repairs or not. Synaptic mitochondria display a lower respiration, which may indicate that they are utilized more for calcium buffering than ATP production in the synapse. Perhaps synaptic mitochondria accumulate more oxidative damage as long as the damage is not significantly affecting the calcium buffering ability. The relatively large distance of the synapse from the cell body may also determine how often maintenance or replacement of mitochondria occurs.

Data in this report suggest that the mitochondria have not sustained sufficient amounts of damage that manifests into significant functional changes at 22-24 mos of age. Stuart et al [236] supports this idea in that OGG1 mice, deficient in 8-oxodG removal, which allows high levels of mtDNA lesions to accumulate (20-fold higher than wild-type), did not show respiration deficits. Therefore it is possible mitochondria from 22-24 mos old F344 rats may be able to withstand a greater amount of oxidative damage than previously thought before major functional respiration declines occur in a naïve state. Having several compensatory mechanisms in place for increasing ATP production may offset minor deficiencies in oxidative phosphorylation [39]. Having an excess of active respiratory complexes that can be used as reserves, direct chemical regulation of active complexes, or varying concentrations of intermediate metabolites can increase ATP production within mitochondria. Systemic approaches to increase ATP synthesis would be to promote glycolysis, enhance oxygen delivery by the circulatory system, or use phosphocreatine if mitochondria are exhibiting minor deficits.

In conclusion, this study found no significant age-related declines in mitochondrial bioenergetics in the cortex. Nevertheless, the aging rodent cortex accumulated higher levels of oxidative damage especially in the synaptic fraction. Data from this study suggest that the accumulation of oxidative damage with aging is not sufficient to result in significant bioenergetic changes in naïve rats 22-24 mos of age. Discrepancies that exist on the role mitochondria play in aging and the extent of dysfunction underscore the necessity of gathering structural, individual subunit enzymatic activities, as well as intact functional data across many brain regions and mitochondria fractions. Our data show that synaptic mitochondria display a high degree of respiratory control in agreement with other studies reporting a relatively large range (< 25% up to 80%) of the maximal respiratory capacity depending on synaptic activity [109, 237-239]. Respiratory control is extremely important in maintaining the specialized functions of synaptic mitochondria. Synaptic mitochondria may maintain more of a basal respiration state in readiness for neurotransmission (e.g. calcium influx, ATP production for vesicle transportation and maintenance of synaptic membrane potential, etc), increasing the probability of free radical formation [240] and explaining the elevation of oxidative

damage apparent in the synaptic fraction of aged rats. Future studies could examine respiration changes of F344 rats of older ages, but significant bioenergetic deficits may only be apparent in pathological condition or neurodegenerative diseases. It would also be useful to determine if aged F344 rats (22-24 mos of age) show greater mitochondrial dysfunction after challenging the CNS (e.g. insult) to see if mitochondria of aged animals are in fact more sensitive to cellular perturbation.

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Chapter 5: Age-Related Changes in Mitochondrial Respiration Following Traumatic Brain Injury

Specific Aim 4 will test the hypothesis that there is an age-related increase in synaptic and extrasynaptic mitochondrial dysfunction following TBI.

Introduction

Traumatic brain injury (TBI) is a serious public health care concern affecting over 55 million people worldwide [120] and 1.4 million each year in the United States alone [119]. Elderly individuals, 65 or older, account for a disproportionate amount of TBI cases and a staggering incidence for morbidity and mortality [197]. Hospitalization and deaths from TBI are higher among older individuals than any other age group [195]. TBI among older adults is an under-recognized health problem even though mortality rates are comparable to Alzheimer's Disease, diabetes, and influenza [196], necessitating geriatric research.

Many reports confirm that age negatively effects outcome following trauma both clinically [153, 155, 241] and experimentally in ischemia [242], intracerebral hemorrhage injury[243], closed head injury[244], and neurotrauma [243-249]. Hukkelhoven et al [250] reported older age is continuously associated with a more negative outcome following TBI, with the probability for poor outcome increasing by 40-50% for every decade following 39 years of age. Data suggests that, as individuals age, they become more susceptible to cellular perturbation or have lowered thresholds for dysregulation, which would increase the likelihood for an exacerbated responses given the same insult. Determining cellular mechanisms for the age-related deficits seen following TBI is imperative before effective therapies can be produced.

There have been several studies that have revealed possible explanations to why older individuals fair worse following TBI. Older patients usually have a higher occurrences of subdural hematomas [155, 241], preexisting co-morbid medical conditions [153], and are often taking multiple medications that can exacerbate the injury and compromise recovery. Several other age-related systemic changes that could affect outcome following TBI have been reviewed [251].

One reasonable cellular explanation for some of these age-related differences may be related to alterations in mitochondrial respiration in the cortex and from elevated levels of oxidative damage. Several theories of aging implicating mitochondria allude to reactive oxygen species (ROS), which are produced during normal cellular respiration, as a possible driving force underlying the aging process. ROS can cause oxidative damage to several components of mitochondria including the electron transport chain (ETC) [95, 209, 252], lipid membranes [104], and DNA [70]. Oxidative damage to mitochondrial components has been shown to accumulate in an age-dependent manner in certain mitochondrial fractions [253] and can alter several mitochondrial and cellular functions [254]. This accumulation of oxidative damage in mitochondria associates with an increase in ROS production in several regions of the brain [255]. Age-related increased oxidative stress alters endogenous antioxidant defenses and leaves the brain more susceptible to oxidative damage [256].

Research has implicated mitochondrial respiration decline as a mechanistic explanation for the observed functional decline and cellular senescence exhibited in normal aging in many tissue types [209]. The extent of mitochondrial decline that occurs with normal aging is much less pronounced than that seen in pathological conditions [150]. There is an ongoing debate concerning the magnitude of decline in mitochondrial respiration that occurs during normal aging [253]. Some groups report extensive age-related declines in mitochondrial respiration [91, 202, 204, 205, 207, 213]. Our laboratory and others [189, 215 , 219, 253, 257] previously reported no significant age-related changes in cortical mitochondrial bioenergetics in naïve rats, while significant elevations in oxidative damage were apparent [253]. It is possible that under naive conditions, the subtle age-related declines in mitochondrial respiration are not sufficient to impair resting energy demands, but perhaps when the system is challenged deficits become apparent.

Mitochondria display a significant amount of dysfunction early following TBI [17, 122, 145, 183, 258]. This dysfunction contributes to the progression of several other secondary cascades that ensue for many days after the insult [128], particularly since they are the major source of damaging ROS [259]. The theory suggested by Signorini [260], that the repair mechanisms of the brain decline with age, may implicate mitochondria as

an important variable. Higher levels of oxidative damage to machinery inside mitochondria of aged subjects, particularly in the synaptic fraction (GILMER, 2009), may impede their ability to produce ATP resulting in greater cellular dysfunction. Increased mitochondrial dysfunction may be a mechanistic explanation to why aged individuals require longer recovery times and experience increased morbidity and mortality following many different neurological insults. This study probed possible age-related declines in mitochondrial respiration and determined levels of oxidative damage that accumulate following TBI.

Materials and Methods

Animals

Male Fischer 344 (F344) rats (National Institute of Aging - Harlan Labs, Indianapolis, IN; $n = 40$) maintained on a standard diet (Taklad 2918) were used in this study. Animals were housed in group cages (2 per cage) on a 12-h light/dark cycle with free access to food and water. All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. Rats were assigned to groups based on their age: Young: 3-5 mo (n = 20); Aged: 22-24 mo (n = 20). Different age groups were randomly selected each day to ensure consistency of surgeries and mitochondrial isolation procedures.

Surgical procedures

Cortical contusion injury (CCI) was carried out under isoflurane anesthesia (2%) as previously described [183]. All injuries were produced using a pneumatic controlled cortical impact (CCI) device (TBI 0310, Precision Systems and Instrumentation, Fairfax Station, VA) with a hard stop Bimba cylinder (Bimba Manufacturing, Monee, IL). A moderate injury was given to both young and aged rats (1.7 mm cortical displacement depth, 5 mm impactor tip diameter (beveled), 400 ms dwell time, and a 3.5 m/s velocity). Animals were maintained at 37°C and allowed to survive for 3 h.

Synaptic and extrasynaptic mitochondria isolation

Animals were briefly gassed with CO_2 until flaccid, decapitated and the brains rapidly removed. All subsequent procedures were performed on ice including the entire mitochondrial isolation protocol. A 10 mm punch of the ipsilateral cortex, containing injury and penumbra, and corresponding contralateral tissue was collected. To isolate enough mitochondria for respiration analysis and measurements of oxidative damage, tissue was pooled from two animals subjected to identical injury parameters. Mitochondria were isolated separately from the injured (ipsilateral) and control (contralateral) cortex. Respiration data was collected from either 2 animals (n = 1) or 4 animals (n = 2) in a given day, which always included contralateral mitochondrial sample as a control (synaptic and extrasynaptic) to ensure consistency and quality of isolation procedures. The contralateral sample was used as for baseline to compare respiration changes in the ipsilateral cortex. Previous work has demonstrated that respiration is unaltered in the contralateral hemisphere at 3 h post-injury, and is equivalent to respiration seen in sham or naïve animals [183].

The cortex used for respiration analysis was placed in an all-glass dounce homogenizer with 4 mL of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Tissue was homogenized and mitochondria were extracted by differential centrifugation. The homogenate was spun twice at 1300 × *g* for 3 min in an Eppendorf microcentrifuge at 4°C and the supernatant was transferred to new tubes. The resulting supernatant was topped off with isolation buffer with EGTA and spun at 13,000 × *g* for 10 min. The supernatant was discarded; the pellet was resuspended with isolation buffer with EGTA to a total volume of 500 μL; and samples were place on a discontinuous Ficoll gradient (F5415 Ficoll solution Type 400, 20% in H_2O, Sigma, St. Louis, MO), composed of 2 layers (2 mL of a 7.5% on top of 2 mL of 10% Ficoll cut with isolation buffer with EGTA). The final volume of the gradient was ~ 4.5 mL in the 7 mL Beckman tubes (344057, Fullerton, CA). Samples were placed in a Beckman SW 55 Ti swinging bucket rotor and centrifuged at 32,000 rpm for 30 min at 4°C. The gradient produced two separate mitochondrial fractions: the extrasynaptic mitochondrial pellet (bottom) and synaptic

mitochondria trapped in synaptosomes located at the interphase of the two Ficoll layers. Synaptosomes were collected in 2.5 mL tubes, resuspended, and washed in isolation buffer with EGTA by centrifugation at 13,000 × *g* for 10 min to remove Ficoll from sample. The extrasynaptic mitochondrial pellet was collected in 500 µL tubes and also resuspended and washed using isolation buffer with EGTA. Both fractions were washed with fresh buffer a second time to ensure that the Ficoll was eliminated. Synaptosomes were resuspended in isolation buffer with EGTA, and mitochondria released using a nitrogen cell disruption bomb made by Parr Instrument Company (Moline, IL) at 1,200 psi for 10 min. Disrupted synaptosomes were placed on a new Ficoll gradient and returned to the ultracentrifuge and spun at 32,000 rpm for 30 min at 4°C. After removal of the Ficoll gradient, the synaptic mitochondria pellet was resuspended in isolation buffer with EGTA and centrifuged at 13,000 x *g* for 10 min. Both mitochondrial fractions (synaptic and extrasynaptic) were resuspended with isolation buffer without EGTA and centrifuged at 10,000 ×*g* for 10 min to wash out the calcium chelator (EGTA). The final mitochondrial pellets were resuspended in isolation buffer without EGTA to yield a concentration of 10 mg/mL or higher. Protein concentration was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL) by measuring absorbance at 560 nm with a Molecular Devices microplate reader (Sunnyvale, CA).

Mitochondrial respiration measurements

Mitochondrial functionality was assessed using an Oxytherm Clark-type oxygen electrode (OXYT1/ED, Hansatech Instruments, Norfolk, UK). Mitochondria (~60 µg isolated from contralateral cortex and ~68 µg from ipsilateral tissue) were placed in the sealed Oxytherm chamber containing respiration buffer (125 mM KCl, 0.1% BSA, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, pH 7.2) and continuously stirred at 37° C. To ensure that all preparations contained equal amounts of respiring mitochondria, a standardized state II respiration rate was used as baseline (~10-15 nmol oxygen/min). This gave all groups equal ability to produce similar respiration traces, regardless of the amount of mitochondrial protein added. With this method, alterations to different aspects

of the bioenergetics following state II respiration would indicate actual damage to components of the ETC.

For each state of respiration, the rate of oxygen consumption was defined as the slope of the response of the mitochondria to consecutive administrations of respiration substrates, as previously described [183] with minor modifications. The amount of oligomycin administered was 0.4 µL, and two additions of adenosine diphosphate (ADP) were administered to ensure accurate state III respiration measurements. The modified protocol of the substrates injected into the Oxytherm chamber sequentially were as follows: 2.5 µL of pyruvate/ malate (P/M; 2.5 mM); 1.25 µL of ADP (150 µM) added twice in 1-min intervals; 0.4 µL oligomycin (1 µM); 1µL carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP; 1 µM); 0.2 µL rotenone (1 µM) added to shut down complex 1 activity resulting in cessation of oxygen utilization; and 2.5 µL succinate (10 mM).

The overall oxygen utilization rate was determined by measuring the amount of oxygen consumed throughout all states of respiration divided by the time elapsed and amount of mitochondrial protein present in the assay. This measurement serves as an index of their overall respiration capacities. States II - V are mitochondrial respiration parameters driven by complex 1 (P/M, ADP, oligomycin, and FCCP), while state V-succinate represents maximum complex 2-driven respiration. Maximum respiration capabilities were quantified by supplying the ETC with all the necessary substrates then bypassing the restricting ATP synthase via FCCP. Addition of FCCP, a pure uncoupler that acts as a protonophore, allowed protons in the inner membrane space to freely pass into the matrix. The ETC tries to maintain the proton gradient, which results in a high level of oxygen utilization during state V respiration. Following addition of FCCP, rotenone was added to specifically inhibit complex 1 to allow for measurement of maximum complex 2-driven respiration independently. Inhibiting complex 1 respiration increases the signal-to-noise ratio during complex 2 state V respiration.The respiratory control ratio (RCR) was determined by dividing the rate of oxygen utilization for state III (ADP) by state IV (oligomycin). RCR values indicate how coupled the ETC is to ATP production. Both isolated mitochondrial fractions (synaptic and extrasynaptic) from

uninjured tissue, regardless of age, were able to produce RCRs ≥ 5 indicating that the isolation procedure does not damage mitochondria. Overall oxygen utilization rates, RCR values, and percent of contralateral rates for states III-V were plotted as a function of injury and age.

Markers of oxidative damage quantification

Estimation of protein carbonylation. Following respiration analysis, unused synaptic and extrasynaptic mitochondria samples were probed for levels of oxidative damage. Levels of protein carbonyls (PC) were assessed as described previously[165]. All samples were normalized to 4 mg/ml, then combined with sodium dodecyl sulfate (SDS) and dilute 2,4-dinitrophenyl hydrazine. Samples were incubated at room temperature for 20 min then neutralized with 2 M Tris in 30% glycerol. The resulting samples were loaded on a nitrocellulose membrane and run on a slot-blot apparatus. Membranes were blocked in 3% bovine serum albumin and PBS/Tween for 1 h and incubated with a 1:100 dilution of anti-DNP polyclonal rabbit antibody in PBS/Tween for 1 h. Membranes were washed in PBS/Tween and incubated for 1 h with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. Each membrane was washed in PBS/Tween and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image. There was no nonspecific binding of antibody to the membrane observed. The intensity of protein carbonyl staining was quantified by optical density and data reported as percent of the young age group values.

Estimation of 3-nitrotyrosine (an index of protein nitration) and 4-HNE (an index of lipid peroxidation). Ficoll purified synaptic and extrasynaptic mitochondria samples were normalized with 12% SDS and modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature. Samples (250 ng) were loaded into a well on a nitrocellulose membrane in a slot-blot apparatus under vacuum. Membranes were blocked with 3% bovine serum

albumin in PBS/Tween for 1 h, and then incubated with either a 1:2000 dilution of anti-3-nitrotyrosine (3-NT) polyclonal goat antibody or a 1:5000 dilution of anti-HNE polyclonal rabbit antibody in PBS/Tween for 1.5 h. Each membrane was washed in PBS/Tween for 5 min three times after incubation. Membranes were incubated for 1 h, after washing, with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in 1:8000 ratio. Membranes were washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image as above. Nonspecific binding of antibody to the membrane was not observed. The intensities of 4-HNE and 3-NT staining were quantified by optical density and data reported as percent of the young age group values.

Statistical Analyses

Data from mitochondrial fractions (synaptic and extrasynaptic) were expected to be different based upon previous literature demonstrating structural and functional differences in these fractions [109, 111, 134, 187, 194, 210]. All data are reported as group means ± SD.

A two-way repeated measures analysis of variance (ANOVA) (age X hemisphere) was used to compare the amounts of mitochondrial protein used for respiration analysis of each fraction separately.

All respiration parameters for each sample were assessed 2 to 3 times, and the average values were used in the final analysis. There was not enough sample to verify respiration findings on three occasions, so samples were excluded from analysis (1 young synaptic, 1 aged synaptic, and 1 aged extrasynaptic). Actual values were reported for both overall oxygen utilization rate and RCR values since they are calculated measurements that are obtained from two or more states of respiration. These two measurements were analyzed with a two-way repeated measures ANOVA (age X mitochondrial fraction) for both the contra- and ipsilateral hemispheres. A Student-Newman-Keuls post-hoc analysis was used with alpha set at 0.05. Previous work by our laboratory has shown that mitochondrial respiration does not decline with normal aging in Fischer 344 rats [253],

therefore respiration data collected from the contralateral hemisphere of young and aged rats were used as an internal control respectively.

All states of respiration (II, III, IV, V-FCCP, and V-succinate) were measured as nmol oxygen utilized/min/mg of mitochondrial protein and then normalized as a percent of the contralateral rate for each of the respiration state. Reporting data as the percent of contralateral respiration rate minimizes biological variance and serves as an appropriate internal control for normal respiration capabilities [183]. Using percent contralateral also ensures that possible age-related changes are not overshadowed by the injury effect. States of respiration was compared using a two-way repeated measures ANOVA (age X mitochondrial fraction). A Student-Newman-Keuls post-hoc analysis was used with alpha set at 0.05.

Markers of oxidative damage (4-HNE, 3-NT, PC) were measured in both synaptic and extrasynaptic mitochondrial fractions and analyzed separately. The levels of oxidative damage were expressed as the percent observed in the uninjured hemisphere of young animals. A two-way repeated measure ANOVA (age X hemisphere) was used to determine the extent of oxidative damage following injury. A Student Newman-Keuls post-hoc test was used with alpha set at 0.05.

Results

Mitochondrial respiration measurements

Mitochondrial amounts used for respiration analysis. The average amount of extrasynaptic mitochondrial protein used to assess respiration capabilities from young animals: 64.9 µg ± 11.2 (contralateral); 68.5 µg ± 15.9 (ipsilateral) and in aged rats: 54.9 µg ± 7.6 (contralateral); 67.0 µg ± 9.0 (ipsilateral). A two-way repeated measures ANOVA (age X hemisphere) revealed a significant main effect for hemisphere [$F(1, 17) = 5.987$, $p < 0.05$], but not for age [$F(1, 17) = 2.243$, $p > 0.1$]. Post-hoc testing revealed that significantly more mitochondria were used for respiration in the ipsilateral cortex. The average amount of synaptic mitochondrial protein used in young animals: 84.0 µg ±

15.1 (contralateral); 92.1µg ± 22.9 (ipsilateral) and in aged rats: 62.1 µg ± 13.0 (contralateral); 94.0 µg ± 12.0 (ipsilateral). A two-way repeated measure ANOVA (age X hemisphere) revealed a significant main effect for hemisphere [F (1, 16) = 20.518, $p < 0.001$], but not for age [F (1, 27) = 2.523, $p > 0.1$]. Post-hoc testing revealed significantly greater amounts of synaptic mitochondria was also used in respiration analysis of the isplateral (injured) hemisphere.

Figure 5.1 shows distinct respiration profiles in both mitochondrial fractions (synaptic and extrasynaptic) for both young and aged animals expressed as nmol/oxygen utilized per min per milligram protein. Both age groups displayed similar profiles following injury. It is important to note that the baseline levels (contralateral hemisphere) for the synaptic fractions are significantly lower compared to the extrasynaptic fraction, indicating a significant difference in respiration between these two mitochondrial fractions as previously described [253].

Overall oxygen utilization rate. Significantly lower overall oxygen utilization rates were apparent in the ipsilateral hemisphere compared to the contralateral hemisphere ($p < 0.05$), regardless of age. Figure 5.2a shows that following TBI there are significant decline in overall oxygen utilization rates in both mitochondrial fractions, while no significant age-related decline existed. A repeated measure ANOVA was utilized for the ipsilateral hemisphere (age X mitochondrial fraction) revealed a significant main effect for mitochondrial fraction [F (1, 16) = 156.817, $p < 0.0001$], while age had no effect [F (1, 16) = 0.870, $p > 0.1$]. Post hoc analysis revealed that extrasynaptic mitochondria displayed significantly higher overall oxygen utilization rates than the synaptic fraction, regardless of age. A repeated measure ANOVA was also used for the contralateral hemisphere and revealed a significant main effect for mitochondrial fraction [F (1, 16) = 199.027, $p < 0.0001$], while age had no effect [F (1, 16) = 0.052, $p > 0.1$]. Post hoc analysis revealed that extrasynaptic mitochondria displayed significantly higher overall oxygen utilization rates than the synaptic fraction, regardless of age.

State II respiration (pyruvate/malate). State II respiration (10 - 15 nmoles oxygen/min) was used as baseline that gave all groups an equal ability to produce similar respiration profiles, regardless of the amount of mitochondrial protein added. The percent contralateral state II respiration rates were compared with a repeated measure ANOVA (age X mitochondrial fraction) revealed a significant main effect for mitochondrial fraction [$F(1, 16) = 5.929$, $p < 0.05$], while age had no effect on state II respiration rates [$F(1, 16) = 3.853$, $p > 0.1$]. Post-hoc analysis revealed that extrasynaptic mitochondria displayed significantly higher state II respiration, regardless of age.

State III respiration (ADP). Figure 5.2c shows changes in state III respiration (percent of the contralateral hemisphere) following injury in both fractions of mitochondria. A repeated measure ANOVA (age X mitochondrial fraction) revealed a significant main effect for mitochondrial fraction [$F(1, 16) = 13.229$, $p < 0.005$], while age had no effect on state III respiration rates [$F(1, 16) = 2.532$, $p > 0.1$]. However, there was a significant age X mitochondrial fraction [$F(1, 16) = 9.899$, $p < 0.01$] (Figure 5.1). Post-hoc analysis revealed that extrasynaptic mitochondria displayed significantly higher state III respiration than the synaptic fraction and aged synaptic state III respiration levels were significantly lower than young synaptic values.

State IV respiration (oligomycin). Changes in of state IV respiration rates (percent of the contralateral hemisphere) following TBI are depicted in Figure 5.2d. A repeated measure ANOVA (age X mitochondrial fraction) revealed no main effect for mitochondrial fraction [$F(1, 16) = 0.778$, $p > 0.1$] or age [$F(1, 16) = 0.001$, $p > 0.1$] (Figure 5.1).

RCR (state III/state IV respiration). The RCR is an index of how coupled the ETC is to ATP production. Figure 5.2b shows alterations in RCR values following injury in both fractions of mitochondria. A repeated measures ANOVA was utilized to compare the RCR values from the ipsilateral hemisphere (age X mitochondrial fraction) revealed a significant main effect for mitochondrial fraction [$F(1, 16) = 9.144$, $p < 0.01$] and age

Figure 5.1. Effects of age and TBI on mitochondrial respiration in F344 rats following TBI.

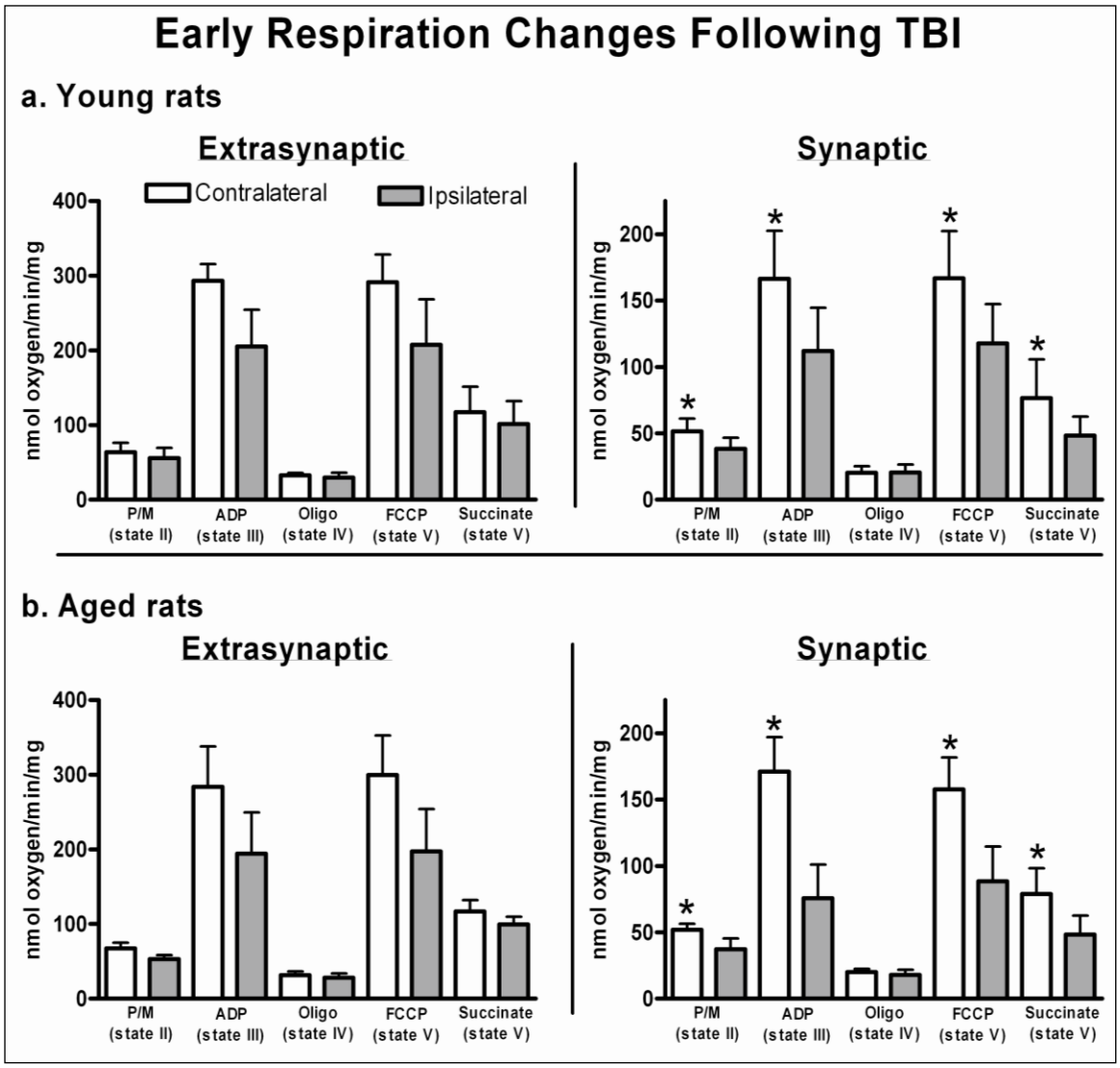

Figure 5.1. Quantification of all states of respiration early (3 h) after TBI for both **a**. extrasynaptic and **b**. synaptic mitochondrial fractions isolated from rat cortices. No significant age-related changes were apparent in any respiration parameter in extrasynaptic mitochondria, but respiration was significantly higher than the synaptic fraction, note differences range of y-axis between mitochondrial fractions. A significant decline in state III respiration was observed in the synaptic fraction with age (quantified in Figure 3.2c). Respiration in extrasynaptic mitochondria was significantly higher than those observed in the synaptic fraction of the same hemisphere indicating a baseline shift in respiration between these two fractions. Bars represent group means ± SD. * $p < 0.05$ compared to respiration of extrasynaptic mitochondria from the same hemisphere.

[F (1, 16) = 9.599, p < 0.01]. Post-hoc testing revealed extrasynaptic mitochondria display significantly higher RCR values from those seen in the synaptic fraction, regardless of age. Post hoc analysis revealed that RCR values of young animals were significantly higher than those of aged animals, regardless of mitochondrial fraction. A repeated measures ANOVA was utilized to compare the RCR values from the contralateral hemisphere (age X mitochondrial fraction) revealed a significant main effect for mitochondrial fraction [F (1, 16) = 8.367, $p < 0.01$], but no main effect for age [F (1, 16) = 1.975, p > 0.1] data not shown. Post-hoc testing revealed extrasynaptic mitochondria display significantly higher RCR values from those seen in the synaptic fraction, regardless of age.

FCCP respiration (state V). Figure 5.2e depicts the alterations (percent of the contralateral hemisphere) to state V respiration following injury. A repeated measure ANOVA (age X mitochondrial fraction) revealed no main effect for mitochondrial fraction [F (1, 16) = 2.075, $p > 0.1$] or age [F (1, 16) = 1.750, p > 0.1] (Figure 5.1).

Succinate respiration (state V via complex 2). Changes state V-succinate respiration rates (percent of the contralateral hemisphere) following TBI are depicted in Figure 5.2f. A repeated measure ANOVA (age X mitochondrial fraction) revealed a significant main effect for mitochondrial fraction [F (1, 16) = 15.240, $p < 0.001$] but not for age [F (1, 16) = 0.303, p > 0.1] (Figure 5.1). Post hoc analysis revealed that extrasynaptic mitochondria displayed significantly higher state V-succinate respiration than the synaptic fraction, regardless of age.

Age-related changes in levels of oxidative damage following TBI

PC. An analysis of the extrasynaptic fraction using a two-way repeated measure ANOVA (age X hemisphere) revealed a significant elevation in the levels of oxidized

Figure 5.2. Effects of age and TBI on several bioenergetic parameters.

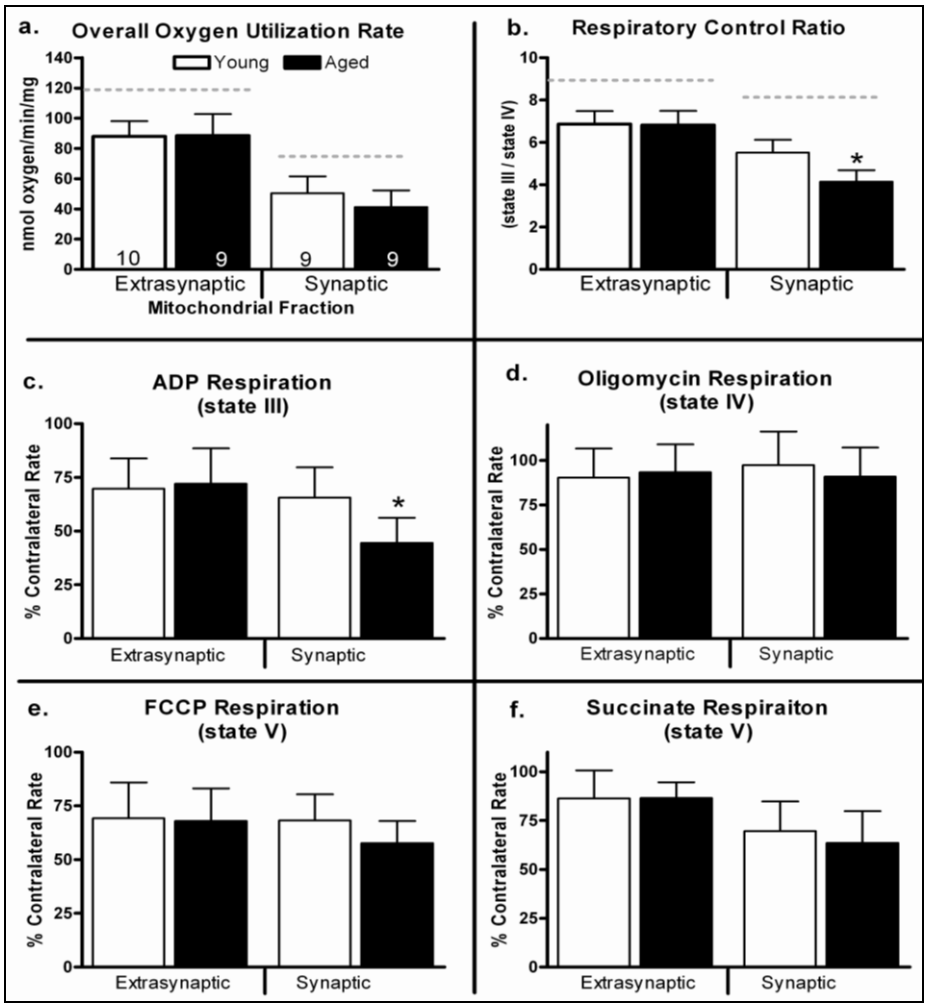

Figure 5.2. A significant difference between respiration of synaptic and extrasynaptic mitochondria exists (Figure 5.1), but is not apparent since data is reported as percent of contralateral rates. **a**. Overall oxygen utilization rate. Dashed line represents rates from the contralateral hemisphere. No significant age-related changes were apparent in either mitochondrial fraction. The subtle decline seen in the synaptic fraction of aged animals may be indicative of changes in only certain states of respiration. **b**. Respiratory Control Ratio (RCR) values serve as a general index of how coupled respiration is to ATP production. Dashed line indicates contralateral (control) values. Mitochondria are severely dysfunction and respiration becomes uncoupled from ATP production when RCR values are < 5. RCR values declined significantly as a result of the injury in both mitochondrial fractions, but only synaptic mitochondria exhibited severe dysfunction. Extrasynaptic mitochondria retained their ability to efficiently produce ATP for at least 3h post-injury, unlike the synaptic fraction. Percent of contralateral respiration rates for synaptic and extrasynaptic fractions: **c.** state III – ADP **d.** state IV – oligomycin **e.** state V – FCCP **f.** state V – Succinate. Bars represent group means ± SD. * $p < 0.05$ compared to young age group values.

proteins in the ipsilateral hemisphere [F (1, 18) = 59.111, $p < 0.0001$] but failed to demonstrate a main effect for age [F (1,18) = 1.669, $p > 0.1$] Figure 5.3a. Post hoc analysis showed significantly higher levels of PC in the injured hemisphere for both age groups. The synaptic fraction demonstrated a significant hemisphere elevation in PC [F (1, 18) = 29.842, $p < 0.001$] and also a main effect for age [F (1, 18) = 15.624, $p < 0.001$]. Post-hoc testing revealed significantly greater levels of PC in the injured hemisphere for both age groups. In the contralateral hemisphere, a paired t-test failed to demonstrate a difference in the levels of PC between synaptic and extrasynaptic fractions [$t_{19} = 1.12$, $p > 0.1$].

4-HNE. An analysis of the 4-HNE levels in the extrasynaptic fraction using a two-way repeated measure ANOVA (age X hemisphere) revealed a significant elevation in the injured hemisphere [F (1, 18) = 9.693, $p < 0.01$] but failed to demonstrate a main effect for age [F (1, 18) = 3.938, $p > 0.05$] Figure 5.3b. Post-hoc testing showed that the injured hemisphere had significantly higher 4-HNE levels. A similar analysis carried out on the synaptic fraction demonstrated a significant effect for hemisphere [F (1, 18) = 16.516, $p < 0.001$] and also a main effect for age [F (1, 19) = 10.708, $p < 0.005$]. Post-hoc testing revealed significantly higher levels in the injured hemisphere and significantly higher levels in the aged animals. In the contralateral hemisphere, a paired t-test demonstrated that the synaptic fraction displayed significantly higher levels of 4HNE than extrasynaptic fractions [$t_{19} = 2.36$, $p < 0.05$].

3-NT. 3-NT was used as a biomarker of reactive nitrogen species formation [211] specifically peroxynitrites-induced protein modification [212]. An analysis of the 3-NT levels in the extrasynaptic fraction, using a two-way repeated measure ANOVA (age X hemisphere), revealed a significant elevation in the injured hemisphere [F (1, 18) = 7.571, $p < 0.01$] but failed to demonstrate a main effect for age [F (1, 18) = 1.741, $p > 0.1$] (Figure 5.3c). Post-hoc testing revealed significantly higher levels in the injured hemisphere. A similar analysis of the 3-NT levels in the synaptic fraction demonstrated a significant hemisphere elevation in 3-NT [F (1, 18) = 6.037, $p < 0.05$] and also a main

Figure 5.3. Markers of oxidative damage associated with age and TBI.

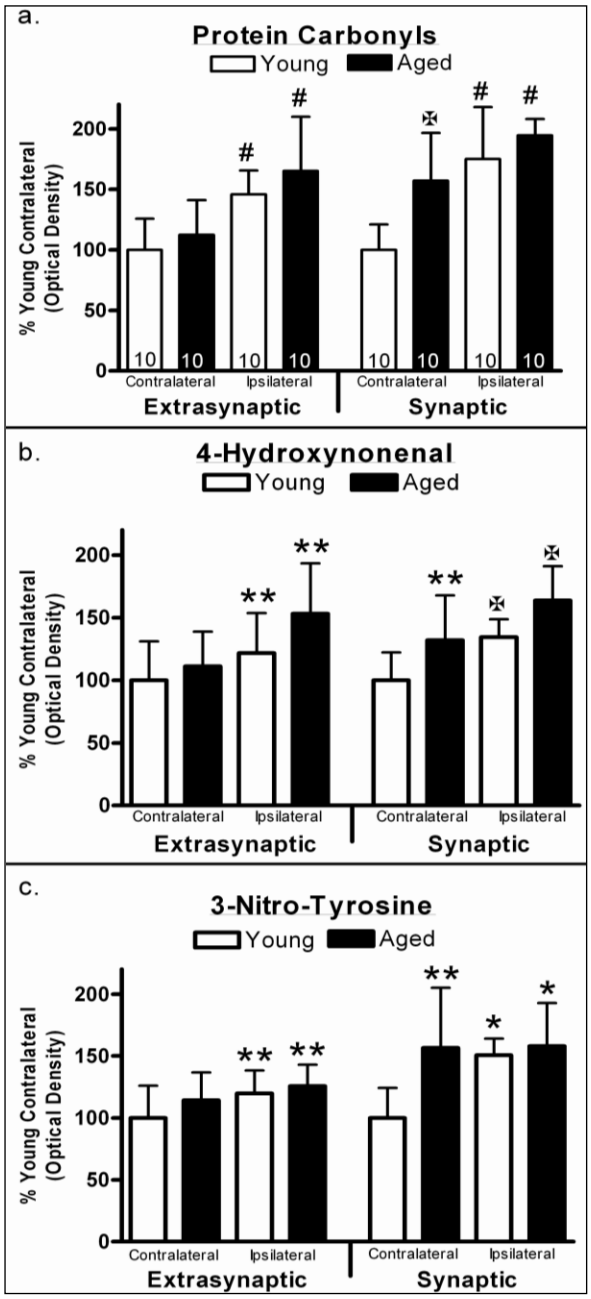

Figure 5.3. Changes in levels of oxidative stress due to age and following injury. The levels of oxidized proteins, measured by levels of protein carbonyls (**a.**), 4-hydroxynonenal (4-HNE)-protein adducts, a marker of lipid peroxidation (**b.**) and nitrotyrosine (3-NT), a biomarker of reactive nitrogen species damage (**c.**) were determined in purified synaptic and extrasynaptic mitochondrial fractions. Bars represent group means ± SD. * $p < 0.05$, ** $p < 0.01$, ✠ $p < 0.001$, and # $p < 0.0001$ compared to levels seen in the contralateral cortex of young rats.

effect for age [F (1, 18) = 9.775, $p < 0.01$]. Post-hoc testing revealed significantly higher levels in the aging animals and significantly higher levels in the injured hemisphere. In the contralateral hemisphere, synaptic mitochondria displayed significantly higher levels of 3-NT than the extrasynaptic fraction [paired $t_{19} = 2.69$, $p < 0.01$].

Discussion

This is the first study to fully characterize injury-induced age-related changes in mitochondrial bioenergetics in the cortex. Aged F344 rats subjected to a moderate TBI demonstrated a significant decline in ATP production capabilities in the synaptic mitochondrial fraction when compared to younger rats subjected to the same injury. This mitochondrial fraction also showed significant declines in RCR values, a general measurement of mitochondrial functionality. The present set of experiments differs from previous TBI studies in that synaptic and extrasynaptic mitochondria were analyzed separately. Enriched extrasynaptic fractions are primarily composed of mitochondria from neuronal cell bodies, axons and dendrites, as well as support cells (astrocytes and microglia), while synaptic fractions only contain mitochondria from pre and post synaptic terminals. These experiments also revealed an increase in oxidative damage with the greatest changes occurring in the synaptic fraction. Synaptic mitochondria from aged animals may be burdened with elevated oxidative damage to their mitochondrial machinery prior to injury resulting in an exacerbation of dysfunction when the system is stressed. Extrasynaptic mitochondria do not display elevated levels of oxidative stress with age [253] supporting previous findings indicating this fraction may be better equipped to compensate for oxidative damage.

Clinical data strongly suggest that an individual's age at the time of injury negatively correlates with positive outcome, but positively correlates with increased mortality [236]. However, there is considerable disagreement as to the critical age at which these changes begin to have a significant influence [151, 153-155, 195, 197, 241, 250, 261-263]. Initial injury severity may be a more important variable in determining the extent of recovery than age alone, with older patients showing significantly higher mortality rates compared to younger individuals when the injury was moderate to severe

[263]. It has been suggested that the aging central nervous system's ability to cope with increased physiological stress following a severe insult is significantly diminished [264, 265].

Similar to the clinical data, age-related deficits are observed following experimental neurotrauma with results varying by the type of injury administered, the severity of the insult, the age of animal, and outcome measurements collected. There is ongoing research in experimental TBI to define the age threshold and injury severity needed that coincides with clinical data of higher morbidity and mortality. Nevertheless, age-related declines have been previously noted in experimental brain injury [75, 246, 266-268].

Deterioration of mitochondrial function has been proposed as a contributor to the aging process [33, 54, 269, 270] and has been well-characterized in pathological conditions [199, 200, 271, 272]. Age-related oxidative damage could accumulate in proteins responsible for importing substrates inside mitochondria, enzymes needed to start the tricarboxylic acid cycle (TCA), such as pyruvate dehydrogenase (PDH) [182], various complexes of the ETC [40, 96], or exchanging ADP for ATP across the inner mitochondrial membrane by the adenine nucleotide translocator (ANT)[90, 273]. Any alterations to these structures required for energy production could hinder normal respiration capacities or their ability to recover when system is challenged. Recently, we showed that naïve F344 rats display no age-related deficits in mitochondrial respiration [253]; therefore deficits may not be apparent until the mitochondria are challenged. Other data supports these findings in Fisher 344 rats, showing mitochondrial membrane potential and susceptibility to permeability transition was stable out to 24 mo, suggesting that subtle changes in mitochondrial bioenergetics may affect their overall function and render them more prone to damage and dysfunction[110]. Several groups report that cortical synaptic and extrasynaptic mitochondria have different respiration capacities [111, 134, 194, 253]. Therefore, it is important to separate these two fractions because they may have different tolerances and responses to perturbation. Total mitochondria would be a less sensitive measurement of damage because one fraction of mitochondria may mask changes seen in the other.

We have previously reported early mitochondrial bioenergetic changes following a moderate cortical contusion [183]. Significantly more mitochondrial protein was needed in the injured hemisphere to obtain state II respiration rates equivalent to sham operated controls in that study. The present study confirmed these previous findings, but further defines respiration alterations in specific mitochondrial fractions following TBI. A significant injury-induced decline in respiration was observed in both extrasynaptic and synaptic fractions. Extrasynaptic mitochondria displayed significant injury-induced respiration deficits, but failed to show any age-related alterations compared to the contralateral hemisphere. The contralateral hemisphere was utilized as an internal control due to the fact that at 3 h post-injury respiration values are equivalent to naïve animals [183]. Perhaps the decline in respiration in the synaptic fraction would have more serious cellular consequences due to their inherently lower respiration capacities (Figure 5.1) compared to extrasynaptic mitochondria. A decline in ATP production rates in synaptic mitochondria would make it more difficult to continue repeated synaptic transmission considering the pathological levels of intracellular calcium that would need to be sequestered. Utilization of more synaptic mitochondria isolated from the injured cortex was necessary to obtain equivalent state II respiration observed from the contralateral hemisphere, while the age of the rat had no effect on the amount of protein needed. The most notable injury-induced age-related change was observed in the assessment of state III (ADP) respiration, which in turn resulted in significantly lower RCR values. A decline in state III respiration is indicative of a decline in the rate of ATP production. Previous research has shown that increased oxidant formation and decreased proton force, is associated with ATP production, which impairs both normal cellular activities and the ability to adapt to stress [141]. Data from this study indicates that synaptic mitochondria are major contributors to the dysfunction observed following TBI in total mitochondria.

Numerous factors may have played a role in the greater dysfunction seen in synaptic mitochondria following injury. Greater membrane disruption may have occurred at the synaptic terminal at 3 hrs post-injury than in other regions of the neuron, leading to more damaged fragments that are similar in size, thus contaminating the mitochondrial preparation. Synaptic mitochondria may have simply sustained a greater

extent of damage to their ETC protein complexes due to their specialized role in the synaptic terminal and distance from the nucleus. Excitotoxicity, calcium overload, and ensuing increased oxidant productions [126, 127, 129] may be more severe in the synapses following TBI due to disrupted axoplasmic flow [123, 274-276].

The overall oxygen utilization rate monitors overt changes in respiration. A significant decline in this respiration parameter was found between the ipsilateral and contralateral cortices in both mitochondrial fractions, but age-related alterations were not observed. These data indicate that both mitochondrial fractions contribute to this measure of bioenergetic integrity. Overall oxygen utilization rate declined by 25% in the ipsilateral cortex for both age groups in extrasynaptic fraction compared to contralateral values. Synaptic mitochondria isolated from young rats displayed a 21% decline in overall oxygen utilization rates, while a 45% decline was observed in aged animal. Although this difference between age groups did not reach statistical significance, it may be the case that a 45% decline in oxygen utilization in aged rats would have serious consequences in the synaptic terminal. Previous studies have reported that synaptic mitochondria have inherently lower respiration capabilities [253] and are more sensitive to cellular perturbation [134] than extrasynaptic mitochondria. Since the differences in the overall oxygen utilization are subtle across age, it is necessary that each state of respiration to be analyzed separately to detect possible age-related differences.

Following TBI, state III respiration was reduced by approximately 30% in extrasynaptic mitochondria in both age groups, while synaptic mitochondria displayed even greater declines with aged animals demonstrating a 55% loss of function. The age-related decline in synaptic state III respiration was almost twice that seen in extrasynaptic mitochondria. This age-dependent response to TBI in the synaptic fraction was not observed in the extrasynaptic response. Previous research has shown that increased oxidant formation and decreased proton force is associated with a lowered ability to produce ATP that can impair both normal cellular activities and the ability to adapt to stress [141]. Aging appears to be affecting the rate of ATP production in synaptic mitochondria differently than in extrasynaptic. Although the state IV respiration rates were between 3-10% of contralateral values in both mitochondrial fractions, they still

contributed to the decrements in the RCR. A severe injury would likely result in an increase in state IV respiration rates [183], whereas the injuries administered in this study were less severe.

The RCR takes into consideration the rate that ATP is produced (state III) as well as the basal metabolic rate that is not associated with ATP production (state IV). In this regard, RCR is a measurement of how coupled the electron transport chain is to ATP production. Consequently, the RCR is a more realistic indication of mitochondrial "functional health." RCR values declined significantly following the injury in both extrasynaptic and synaptic mitochondria. In addition to the injury effect, synaptic mitochondria also displayed a significant aging effect. RCR values dropped from 8.5 to 5.5 (35% reduction) in young animals and to 4.1 in aged rats (53% reduction). The extrasynaptic mitochondria showed a decline in RCR values of 15% in young animals and 18.7% in the aged rats. Synaptic mitochondria appear to be not only more susceptible to damage than extrasynaptic mitochondria, but increasingly more sensitive to perturbation with age. A 35-50% reduction in mitochondrial bioenergetics would certainly have serious cellular consequences especially if that problem involved the coupling of the ETC to ATP production. The injury severity used in this study was severe enough to lead to significant reduction in ATP production rates, but not to produce significant damage to the inner mitochondrial membrane as evidenced by small but non-significant changes in state IV respiration. The main determinate for the significant drop in RCR values was due to the large decline in state III respiration rates. Regardless of age, both populations of mitochondria display functional impairments from various complexes of the ETC, but were able to maintain inner membrane integrity at this early time point post-injury. There were apparent age-related functional deficits to synaptic mitochondria when the system was challenged, while the extrasynaptic mitochondria appeared to be less sensitive to aging. Our data suggests that synapses may be the first region of the neuron to be lost after a TBI, followed by Wallerian degeneration of the rest of the neuron. The disruption of axoplasmic flow to the neuron terminal that occurs following a neurological insult impedes the neuron's ability to replace cellular components needed at the synapse. Others support the idea that synaptic mitochondria

not only have different respiration capabilities than extrasynaptic mitochondria but are more fragile as well [109, 111, 134, 187, 194, 210].

Most tissue types produce distinct amounts of free radicals, and therefore accumulate and respond to oxidative damage differently. With age, the production of oxidants increases, there is a decrease in antioxidant protection, and/or the damage oxidants inflict begins to exceed the cell's ability to repair the resulting damage [277]. When the production of oxidants exceeds antioxidant defenses, the condition is known as oxidative stress and has been implicated in playing a substantial role in accelerated aging and following TBI [165]. Accumulation of damage to various complexes, proteins, and membranes may explain this increased vulnerable to perturbations, supported by age-related differences in cortical tissue sparing after TBI [75].

Aging alone can cause several problems with molecular machinery. Age-related increases in ROS can damage any structure within close proximity (e.g. proteins, lipids, and DNA). Oxidative damage accumulates with age and damage to mitochondrial membrane proteins can accelerate the production of ROS in aged animal in the presence of Ca^{2+}[68].These detrimental factors culminate to produce a feasible theory on what drives the process of aging. Although it is commonly accepted that mitochondria play a major role in the aging process, the relative importance or extent of mitochondrial involvement for mammalian aging remains to be determined [270].

In this study, there were no significant increases in PC in extrasynaptic mitochondria with age (12%), but the injury resulted in a significant increase in PC in extrasynaptic mitochondria of both young (46%) and aged animals (65%). There was a significant age-related increase in PC, marker for oxidized proteins, in synaptic mitochondria isolated from contralateral tissue (57%). The injury also resulted in significant increases in PC in synaptic mitochondria of young (75%) and again in aged rats (95%) compared to levels seen in young uninjured mitochondria. Data comparing synaptic and extrasynaptic fractions, from young tissue, indicate that they carry similar loads of oxidized proteins.

4-HNE is one of the most prevalent and toxic products generated during lipid peroxidation (LP) formed during oxidative stress [229]. 4-HNE then exerts cytotoxic

effects primarily by modifying intracellular proteins [230]. There was no significant increase in 4-HNE in extrasynaptic mitochondria isolated from the contralateral hemisphere with age (11%) compared to values observed in the same hemisphere for the young age group. There was a significant age-related increase in 4-HNE in synaptic mitochondria isolated from contralateral tissue of aged animals (32%) compared to values observed in the same hemisphere for the young age group. The injury resulted in significant increases in 4-HNE levels in the synaptic fraction in young animals (35%) and again in aged rats (55%) compared to levels seen in contralateral mitochondria from young animals. Synaptic mitochondria from contralateral tissue of young rats maintain higher levels of lipid peroxidation and may be more prone to this damage than extrasynaptic mitochondria. This increased susceptibility may arise from a number of feasible explanations including increased oxidant production, decreased antioxidant capacity, or greater exposure to calcium influx.

Several reactive nitrogen species are derived from nitric oxide (NO), one of which is peroxynitrite ($ONOO^-$). $ONOO^-$ is chemically unstable, has significant biological activity [233] and is formed by the biradical reaction of NO and $O_2^{-\cdot}$. A number of oxidation and nitration products are produced from the reaction of $ONOO^-$ with cellular macromolecules. One such product, 3-nitrotyrosine, can be used as a marker of $ONOO^-$ formation *in vivo* [234]. There was no significant increase in 3-NT in extrasynaptic mitochondria with age (14%), but a significant elevation was noticed in the ipsilateral hemisphere of young (20%) and aged rats (26%) following the trauma when compared to values from the contralateral hemisphere of young rats. The age-related increase in synaptic 3-NT levels (57%) was identical to what we previously found [253] and essentially shifted the baseline, resulting in a failure to observe an injury effect such as that in the young animals (51%). These data may signify a possible upper limit for the production of 3-NT. Higher levels of protein nitration in the synaptic fraction may underlie their greater susceptibility to this type of damage than extrasynaptic mitochondria.

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Chapter 6: General Discussion

Mitochondrial dysfunction has been implicated in aging, and secondary damage after traumatic brain injury (TBI), as well as several neurodegenerative diseases. All eukaryotic organisms rely heavily on mitochondria for cellular energy (ATP) and regulation of cellular levels of calcium. Mitochondria can sequester high levels of calcium or maintain high levels of ATP production, but both tasks utilize the mitochondrial proton gradient. Mitochondria operate close to their physiological peaks and are very sensitive to cellular perturbations. Mitochondria are unable to maintain both tasks effectively under pathological levels of influx of calcium and extremely high energy demands [132, 133], which have been shown to occur following TBI [127-129]. For the first week following TBI, brain mitochondria become dysfunctional and can no longer keep up with energy demands resulting in extensive neuronal tissue loss by either necrosis or apoptosis. Immediately following the injury, the affected neuronal tissue is subjected to various damaging secondary cascade events that play out over the next several days [278]. There is experimental evidence for the potential to have significant tissue loss following a cortical contusion injury in the core (mainly necrotic) and in penumbral tissue (susceptible to both necrosis and apoptosis) as a result of prolonged mitochondrial dysfunction [121, 122, 188, 279].

Effects of Traumatic Brain Injury on mitochondrial respiration

Mitochondria produce a majority of the ATP needed to carry out a majority of cellular processes since other energy sources produce a limited number of ATP (e.g. glycolysis, beta-oxidation, or anaerobic respiration). Restoring cellular homeostasis is necessary to minimize secondary injury cascades, promote neuronal cell survival, and ultimately improve functional recovery following trauma. Data from Chapter 2 investigated mitochondrial bioenergetic changes in the rat neocortex at 1 and 3 h after mild, moderate, and severe injuries. Samples from young adult Sprague-Dawley rats were harvested from the injured and contralateral cortex to assess possible changes in mitochondrial respiration abilities following a unilateral cortical contusion injury.

Differential centrifugation was used to isolate synaptic and extrasynaptic mitochondria from cortical tissue. Bioenergetics were assessed using a Clark-type electrode and results were graphed as a function of injury severity and time post-injury. Respiration was significantly affected by all injury severity levels compared to uninjured tissue. Complex 1 and 2 driven respiration was affected proportionally to the severity of the injury, indicating damage to mitochondria may occur on a gradient. The overall oxygen utilization rate, RCR values, state III (ATP production), and state V (maximal respiration capabilities) via complex 1 and 2 were all significantly decreased in the injured cortex at both 1 and 3 h post-trauma. State IV respiration (inner membrane integrity), on the other hand, increased with injury severity indicating greater proton loss across the inner membrane back into the matrix. A very early time frame for mitochondria dysfunction was established. Although mitochondria displayed bioenergetic deficits at 1 h following injury, damage was not exacerbated by 3 h. This study demonstrated the importance of early therapeutic intervention and suggests a window of approximately 1 to 3 h before greater dysfunction occurs.

Mitochondrial targeted therapeutic interventions for TBI

Several different compounds have been shown to offer neuroprotection following experimental TBI by reducing mitochondrial dysfunction. Impaired mitochondria produce higher levels of ROS and free radicals which damage lipids, proteins, and DNA [63, 121, 141, 142]. Damage to mitochondrial membranes and component of the ETC slow the flow of electrons which results in an even higher production of oxidants. Promising therapies have targeted controlling cellular dysregulation of calcium, attempted to increase energy production, or utilize antioxidants to decrease oxidative damage. Restoring cellular homeostasis reduces the extent of mitochondrial dysfunction and has been shown to enhance neurological outcome following TBI [121, 141, 148, 149, 159, 188, 280].

Effects of age on mitochondrial respiration

Data from Chapter 3 demonstrate the importance of looking at synaptic and extrasynaptic mitochondria separately due to their inherent differences in respiration. Analyzing mitochondrial fractions separately enabled a better understanding of how certain populations of mitochondria age in specific brain regions. Analyzing mitochondria separately also revealed that they respond differently to TBI due to their different respiration profiles. In this dissertation, it was also revealed that synaptic and extrasynaptic mitochondria of Sprague-Dawley (SD) and Fischer 344 (F344) rats display very similar respiration profiles. This was a key finding since we wanted to look at the effects of age on TBI and there was a disconnect in the literature with most TBI studies being performed on SD, while aging studies utilize F344 rats. Since mitochondrial respiration of these two animals were not different from each other, at two distinct ages of development (3 - 5 and 12-14 mos), there is a high probability that these two strains of rat age and respond to TBI similarly. In terms of mitochondrial respiration, a lack of strain differences indicates that the responses seen with age and following injury may be similar in other species.

Chapter 4 probed possible age-related changes in mitochondrial bioenergetics in naïve Fischer 344 rats. This was the first study to test the effects of aging on respiration capabilities from intact cortical synaptic and extrasynaptic mitochondria, instead of measuring enzymatic activity of certain subunits of the ETC, across three different age groups. Several bioenergetic parameters were quantified to determine if neuronal (synaptic) and extrasynaptic mitochondria, originating from neurons (soma and axons) and support cells (e.g. astrocytes and glia), could retain their capacities with age. Synaptic and extrasynaptic mitochondria were isolated from the cortex of one hemisphere of young (3-5 mos), middle (12-14 mos), or aged (22-24 mos) rats and purified on a Ficoll gradient. Several respiration parameters were obtained using a Clarke-type electrode. Aged rats displayed no significant alterations in respiration; indicating mitochondria must be more resilient to the aging process with deficits possibly occurring much later than previously thought. Synaptic mitochondria again displayed lower respiration capacities than the extrasynaptic fraction, as previously suggested. Aged F344

rats were capable of normal mitochondrial function and failed to show significant age-related declines in ability to produce ATP.

Markers of oxidative damage were quantified from the same mitochondrial samples used for respiration analysis to determine if higher levels of damage associates with lower respiration capabilities. Levels of oxidative damage were also quantified from cortical homogenates to gain a more holistic idea of the extent of overall damage occurring with age. Markers of cortical oxidative damage (3-nitrotyrosine [3-NT], 4-hydroxynonenal [4-HNE], and protein carbonyls [PC]) were collected from the post mitochondrial supernatant (PMS) from the contralateral hemisphere and from the remaining mitochondrial samples following respiration analysis. All markers were significantly elevated in the PMS of aged animals. Increases in PC and 3-NT levels were observed in synaptic mitochondria of aged rats (22-24 mos), whereas significant elevations were only found in middle aged rats in the extrasynaptic fraction. These findings support an age-related increase in oxidative damage in the cortex, while proposing the two fractions of mitochondria are affected by the aging process differently. Levels of oxidative damage that accumulates in the cortex with age does not appear to significantly impair cortical mitochondrial respiration of F344 rats.

Mitochondrial targeted therapeutic interventions for aging

The brain is continually exposed to oxidative stress throughout aging. Oxidative damage to lipids, proteins and DNA have been evident especially in brains of elderly subjects, suggesting that oxidative injury may directly cause the aging process [56]. Oxidative damage is believed to play a causal role in many diseases specific to the CNS or at least represent a consequence of the disease's progression. Since the most reliable risk factor for neurodegenerative diseases is age, lowering oxidative damage may delay the onset of these diseases. Free radical scavenging enzymes have been shown to decrease lipid peroxidation, protein oxidation, and oxidized glutathione levels [228, 281, 282]. A diet rich in antioxidants was shown to retard age-related declines in motor

behavior and motor learning, indicating that accumulation of oxidative damage play a key role in producing functional deficits [283-285].

Other groups have taken a different approach to lowering oxidative stress. Instead of increasing antioxidant defenses, some have shown that modulating the rate at which ROS are produced also has some promising therapeutic potential. Caloric restriction is thought to lower oxidative stress by reducing the basal rates of ROS production and evidence has been shown to increase in synaptic mitochondria with age [286]. Caloric restriction [287] and mild mitochondrial uncoupling [288] have been shown to lower oxidative damage and increase longevity, probably due to lowering oxidant production.

Effects of age and Traumatic Brain Injury on mitochondrial respiration

There were no significant age-related declines in cortical mitochondrial bioenergetics in naive Fischer 344 rats. Before this dissertation work, it was unclear if age-related deficits in mitochondrial respiration would be apparent if the system were stressed. Chapter 5 attempted to determine if there is an age-related increased mitochondrial dysfunction early following TBI to give a possible cellular mechanism to why many have reported age-related differences in several animal models [59, 242-249]. In this chapter, possible age-related changes in the cortical mitochondrial bioenergetics following TBI were assessed. Three hours following a moderate TBI, tissue from the ipsilateral hemisphere (site of impact and penumbra) and corresponding contralateral region were harvested from young (3-5 mos) and aged (22-24 mos) Fischer 344 rats. Synaptic and extrasynaptic mitochondria were isolated using a Ficoll gradient and several bioenergetic parameters examined using a Clark-type electrode. Injury-related respiration deficits were observed in both young and aged rats. Synaptic mitochondria showed an age-related decline in the rate of ATP production and a decline in respiratory control ratios (RCR), which were not apparent in the extrasynaptic fraction. Following respiration analysis, mitochondrial samples were probed for oxidative damage (3-nitrotyrosine [3-NT], 4-hydroxynonenal [4-HNE], and protein carbonyls [PC]). All markers of oxidative damage were elevated with injury and age in the synaptic fraction,

but only with injury in the extrasynaptic fraction. Synaptic mitochondria displayed the highest levels of oxidative damage and may contribute to the synaptic bioenergetic deficits seen following injury. The data indicates cortical synaptic mitochondria appear to have an increase susceptibility to perturbation with age, suggesting that the increased mitochondrial dysfunction observed following injury may impede recovery in aged animals.

Mitochondrial targeted therapeutic interventions for aged individuals sustaining a TBI

This thesis served as a step forward into understanding one possible mechanism underlying the exacerbated response of aged individuals to TBI (increased mitochondrial dysfunction). Mitochondrial dysfunction is not the only problem, and defining several additional mechanisms underlying age-related decrements will be necessary before effective therapeutics can be developed.

Future Directions

This thesis project focused on mitochondrial damage that occurs in vivo following a TBI within the first three hours. Following this time period, mitochondria were isolated in buffers that mimic a cellular environment within normal physiological ranges. All data collected was in vitro leaving many questions left unanswered. Determining whether or not there are differences in respiration among different cell types (neurons and astroglia) are still warranted. Using tissue culture could facilitate comparing respiration capabilities of mitochondria from specific cell types. Tissue culture would enable isolation of mitochondria from specific cell types, but patch clamping or microdialysis would have to be utilized to determine how they respire in the intact cell. Even though I have data that supports that mitochondria from the synapse have different respiration capabilities, it may be that they do not respire differently when actually in the cell. To definitely answer whether or not mitochondria respire differently in microdomains of the cell (e.g. axonal, somal, or synaptic) could also be accomplished using cell culture. In vivo work is still needed to determine how mitochondria behave when supraphysiological conditions that are produced following an insult occur. This could be accomplished by harvesting

mitochondria isolated from naïve animals or tissue culture and hitting them with an insult, such as high calcium levels or an oxidant that could compromise the structural integrity of mitochondria.

Data from this thesis project has shown that respiration is very similar in Sprague-Dawley and Fischer 344 rats at young and middle aged animals, but the comparison also needs to be made in aged animals as well. Furthermore, it would be quite useful to determine how mitochondria from these two strains of rats respond to TBI both in the acute phase following the insult as well as chronically. These studies demonstrate an age-related increase in mitochondrial dysfunction in the synaptic fraction very early following TBI. It is imperative to determine if this dysfunction persists and if it does for how long. Also we need to determine if this increased dysfunction is apparent in middle aged animals, since we know that this particular age groups displays an increased tissue loss, similar to aged rats [75]. Regardless, this thesis work has shown that we can administer a moderate head injury to an aged animal and produce greater mitochondrial dysfunction within 3 hours of the insult. This could be a great research tool to implement different therapeutic interventions to determine if we can modulate this age-related increase in mitochondrial dysfunction. Mitochondria sustain both structural and functional damage following TBI that could be modulated with antioxidants, mild uncouplers, regaining calcium homeostasis, and restoring ATP levels.

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Glossary of Important Terms

$\Delta\Psi_m$	mitochondrial membrane potential
ΔpH	proton motive force
3-NT	3-nitrotyrosine
4-HNE	4-hydroxynonenal
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
ATP	adenosine triphosphate
Ca^{2+}	calcium
CCI	controlled cortical impact
CNS	central nervous system
CypD	cyclophilin D
Cyt *c*	cytochrome *c*
e^-	electrons
ETC	electron transport chain
$FADH_2$	reduced flavin adenine dinucleotide
FCCP	*p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone
FRTA	free radical theory of aging
FR	free radicals
H^+	protons
IMM	inner mitochondrial membrane
LP	lipid peroxidation
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
mDNA	mitochondrial DNA
MTA	mitochondrial theory of aging
mPTP	mitochondrial permeability transition pore
mtNOS	mitochondrial nitric oxide synthase
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NO	nitric oxide
NOS	nitric oxide synthase
$O_2^{\cdot-}$	super oxide anion
$ONOO^{\bullet}$	peroxynitrite anion
ONOOH	peroxynitrite acid
$ONOOCO_2$	nitrosoperoxocarbonate
PC	protein carbonyls
PN	peroxynitrite
RCR	respiratory control ratio
ROS	reactive oxygen species
TBI	traumatic brain injury

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Vita

Lesley Knight Gilmer

Nationality:	U.S. Citizen
Date of Birth:	October 13, 1981
Born:	Atlanta, Georgia
Education:	B.A. Berea College, Berea, KY (Cellular and Molecular Biology) 2004
Research:	Mitochondrial bioenergetic changes associated with aging and following traumatic brain injury Therapeutic intervention strategies following injury to minimize tissue loss and behavioral deficits
Teaching:	Certified Instructor of Anatomical Sciences
Societies:	Society for Neuroscience Neurotrauma Society

Research and Professional Experience

2004-9 **Doctoral candidate** in Anatomy and Neurobiology

- Thesis mentor: Dr. Stephen W. Scheff
- University of Kentucky (UK) College of Medicine.
- Thesis topic: "*Mitochondrial aging and their role in Traumatic Brain Injury*"

2005-8 **Graduate school representative** for Anatomy and Neurobiology

- Host current student entering the Integrated Biomedical Sciences program
- Speaker at annual departmental retreat

2006-7 **Organizer** for the Lexington Conference Translational Neuroscience

- *Trophic Factor Therapy for Parkinson's Disease*. 2007
- *siRNA symposium*. 2006

2005-6 **Host** for prospective graduate students

- UK College of Medicine

2004-5 **Graduate student** in the Integrated Biomedical Sciences Program

- UK College of Medicine

2004 **Summer research assistant**

- Sanders-Brown Center on Aging, Lexington, KY
- Dr. Stephen Scheff

Invited Seminars

2009 "Mitochondria: The Problem and the Solution to Traumatic Brain Injury." Athens Technical College, Athens, GA.

2009 "Age and Injury Go Together Like Peanut Butter and Mayonnaise." Western States Chiropractic College, Portland, OR.

2009 "Minor Age-related Changes in Mitochondrial Respiration May Cause Major Problems Following Injury." Chicago College of Osteopathic Medicine, Downers Grove, IL.

2009 "Aging May be Hazardous to Recovery from Traumatic Brain Injury: the Mitochondrial Connection." The Commonwealth Medical College, Scranton, PA.

2009 "Mitochondria, Aging, and Injury." West Virginia School of Osteopathic Medicine, Lewisburg, WV.

Teaching experience

Morehead State University (January-May)

2009 **Course Director** for dissection-based gross anatomy (ANA611-201: *Regional Human Anatomy*)

- Physician assistant students
- 28 lectures (2 hour/each) and 28 dissection-based gross laboratory sessions (2 hour/each)
- 116 instruction hours (lecture/lab – 50/50)
- Class size: 15
- Review sessions
- Evaluation of lecture and laboratory material
- Office hours

University of Kentucky

2009 **Literature review instructor** for the Health Researchers Youth Academy

- High school kids (seniors) across Kentucky desiring a career in the health professions
- 8 lectures (1.5 hours/each)
- 12 instructional hours
- Established a brief curriculum of human anatomy
- Class size: 27
- 2 week summer camp led by the UK Area Health Education Center

2009 **Guest lecturer** for Anatomy and Physiology (ANA 109)

- Human anatomy course for first year nursing students
- "Female reproductive system"
- Course instructor: Dr. Pam Stein
- Class size: 200

2008 **Literature review instructor** for the Health Researchers Youth Academy

- High school kids (juniors and seniors) across Kentucky desiring a career in the health professions
- 10 lectures (1 hour/each) and 10 literature review sessions for poster presentation (1 hour/each)
- 20 instructional hours (lecture/literature review)
- Class size: 16
- 2 week summer camp led by the UK Area Health Education Center

2008 **Teaching Assistant** for Dissection-based Gross Anatomy (ANA611/ANA811).

- Physician assistant/physical therapist students
- Course instructor: Dr. Brian MacPherson

Teaching experience (continued)

- Class size: 90
- Laboratory instructor
- Setting up the laboratory practical portion for examinations
- Proctoring examinations

2009 **Guest lecturer** for Anatomy and Physiology (ANA 109)

- Human anatomy course for first year nursing students
- "Female reproductive system"
- Course instructor: Dr. Pam Stein
- Class size: 120

2007 **Research instructor** for the Health Researchers Youth Academy

- High school kids (juniors and seniors) across Kentucky desiring a career in the health professions
- Class size: 20
- Established a brief curriculum to cover the scientific method, research techniques, and potential careers
- 2 week summer camp led by the UK AREA Health Education Center

2007 **Anatomy instructor** for the Health Researchers Youth Academy

- High school (freshman and sophomores) from across Kentucky desiring a career in the health professions
- Class size: 25
- Established a condensed curriculum of human anatomy
- 12 lectures (1.5 hour/each) and 12 gross laboratory sessions (0.5 hour/each)
- 24 instruction hours (lecture/lab – 75/25)
- 6 week summer camp led by the UK Area Health Education Center
-

2007 **Teaching Assistant** for Anatomy and Physiology (ANA 110)

- First year nursing students
- Course instructor: Dr. Pam Stein
- Class size: 160
- Review sessions for examinations
- Proctor examinations

2007 **Guest lecturer** for Anatomy and Physiology (ANA 209)

- Human anatomy course open to undergraduate students
- "Bone development"
- Course instructor: Dr. Pam Stein
- Class size: 200

Teaching experience (continued)

2006 **Teaching Assistant** for Anatomy and Physiology (ANA 109)

- First year nursing students
- Course instructor: Dr. Pam Stein
- Class size: 200
- Lecturer
- Ensuring grades uploaded on Blackboard
- Review sessions for examinations
- Proctor examinations

Murray State University

2002 **Teaching Assistant** for Field Botany

- Biology course open to undergraduate students
- Class size: 20
- Collection/Organization of specimens needed for examinations

Berea College

2002-3 **Host ambassador** for Berea College

- Prospective high school students interested in Berea College
- Approximately 10-15 potential students/year

Fellowships / Funding

2007-8 NIH Blueprint Translational Neuroscience Training Grant "*Therapeutic Strategies for Neurodegeneration*"
2006 RCTF Travel Fund for Neuroscience meeting
2005 RCTF Travel Fund for KSCHIRT meeting

Publications

Gilmer, L.K., Ansari, M.A., Roberts, K.N., and Scheff, S.W. Early age-related changes in synaptic and extrasynaptic mitochondrial respiration Fischer 344 rats following traumatic brain injury. Submitted in November 2009.

Gilmer, L.K., M.A. Ansari, M.A., Roberts, K.N., and Scheff, S.W. Age-related changes in mitochondrial respiration and levels of oxidative damage in Fischer 344 rats. Submitted in August 2009.

Gilmer, L.K., Roberts, K.N., Joy, K.M, Sullivan, P.G., and Scheff, S.W. Early mitochondrial dysfunction following cortical contusion injury. J. Neurotrauma 2009, Aug; 26 (8): 1271-80.

Publications (continued)

Anderson, K.J., Scheff, S.W., Miller, K.M., Roberts, K.N., **Gilmer, L.K**., Yang, C., Shaw, G. The phosphorylated axonal form of the neurofilament subunit NF-H (pNF-H) as a blood biomarker of traumatic brain injury. J. Neurotrauma 2008, Sept; 25 (9):1079-1085.

Gilmer, L.K., Roberts, K.N., Scheff, S.W. Efficacy of progesterone following a moderate unilateral cortical contusion injury. J. Neurotrauma 2008, Jun; 25 (6): 593-602.

Abstracts / Posters

Gilmer, L.K., Roberts, K.N., Ansari, M.A., and Scheff, S.W. Aging May be Hazardous to Recovery from Traumatic Brain Injury: the Mitochondrial Connection. Neurotrauma Symposium: Santa Barbara, CA. September 2009.

Gould, D.J., Hartsfeld, A., Ballard, J., Norton, J., Davis, **L., Gilmer**, L., Brueckner, J.K. The effectiveness of the anatomy component of a summer program for disadvantaged kids desiring a career in the health professions. American Association for Clinical Anatomists conference: Toronto, ON. July 2008.

Gilmer, L.K., Roberts, K.N., Rock, P.J., and Scheff, S.W. Edema and cavity formation following a moderate cortical contusion injury in Sprague Dawley rats. Neurotrauma Symposium: Orlando, FL. July 2008.

Gilmer, L.K., Roberts, K.N., Ansari, M.A., and Scheff, S.W. Age-related synaptic and extrasynaptic mitochondrial bioenergetic changes in the Fischer 344 rat. Neuroscience Convention: San Diego, CA. November 2007.

Gilmer, L.K., Roberts, K.N., and Scheff, S.W. Efficacy of progesterone following a moderate unilateral cortical contusion injury. Neurotrauma Symposium: Kansas City, MO. July 2007.

Gilmer, L.K., Roberts, K.N., Joy, K.M, and Scheff, S.W. Analysis of mitochondrial bioenergetics one and three hours after cortical contusion injury in Sprague Dawley rats. Neuroscience Conviention: Atlanta, GA. October 2006.

Gilmer, L.K., Roberts, K.N., Joy, K.M, and Scheff, S.W. The hippocampus shows no age-related differences to focal cortical contusion in the Fisher 344 rat. Neurotrauma Symposium: St. Louis, MO. July 2006.

Gilmer, L.K., Roberts, K.N., Sullivan, P.G., and Scheff, S.W. Injury severity associated changes in mitochondrial respiration. Neurotrauma Symposium: Washington, D.C. November 2005.

Abstracts / Posters (continued)

Scheff, S.W., Roberts, K.N., **Gilmer, L.K.**, and Miller, K.M. Is depth of cortical compression or impact velocity more predictive of cortical and hippocampal pathology? J. Neurotrauma 2004, 21: 1295.

Conventions / Symposiums

2005-9 The 23-27th Annual Society for Neurotrauma Symposium
2006-7 The 36-37th Annual Society for Neuroscience Meeting
2006-7 Lexington Conference on Translational Neuroscience
2005 Eleventh Annual Kentucky Spinal Cord and Head Injury Trust