The Effect of Gonadal Steroids Following Repeat Mild Traumatic Brain Injury on Anxiety-Like Behaviors in Rats

Trevor Nykamp

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THE EFFECT OF GONADAL STEROIDS FOLLOWING REPEAT MILD TRAUMATIC BRAIN INJURY ON ANXIETY-LIKE BEHAVIORS IN RATS

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN NEUROSCIENCE

BY TREVOR NYKAMP CHICAGO, ILLINOIS DECEMBER 2021
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For my parents
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LIST OF ABBREVIATIONS

TBI – Traumatic Brain Injury
mTBI – Mild Traumatic Brain Injury
rmTBI – Repeat Mild Traumatic Brain Injury
CDC – Centers for Disease Control and Prevention
CCI – Controlled Cortical Impactor
SEM – Standard Error of the Mean
PTSD – Post-Traumatic Stress Disorder
BNST – Bed Nucleus of the Stria Terminalis
DHT – Dihydroxytestosterone
AR – Androgen Receptor
TH – Tyrosine Hydroxylase
DBH – Dopamine Beta Hydroxylase
COX4i1 – Cytochrome C Oxidase Subunit 4 Isoform 1
IHC – Immunohistochemistry
PFA – Paraformaldehyde
ROI – Region of Interest
CHAPTER ONE
INTRODUCTION

Each year, approximately 69 million individuals across the world sustain a traumatic brain injury (TBI)\(^1\). The incidence of TBI around the world is often referred to as a 'silent epidemic', as the injury occurs internally and remains invisible to everyone aside from the victim\(^{1,2}\). TBIs can occur in any age group, ethnicity, geographical region, gender, or other major demographic. Everyone is at risk of sustaining a TBI, so precaution and prevention are crucial. TBIs result in debilitating symptoms and poor health outcomes that can worsen and persist over time. Regardless of severity, even a single mild TBI (mTBI) can lead to a multitude of physical, cognitive, and behavioral symptoms. Even more, repeat mild TBI (rmTBI) can lead to a host of symptoms that worsen with each injury. The exacerbation of symptoms after each mTBI can occur non-linearly. Each subsequent mTBI can result in symptoms that exponentially worsen. For example, a third mTBI can result in much more severe vestibular deficits than the occurrence of symptoms between the first and second mTBI. It is for this reason that immediate diagnosis and treatment is paramount to obtain the best possible health outcome and to prevent further injury.

One prevalent emotional symptom following rmTBI is anxiety\(^3\). The development of anxiety can be crippling for the individual; however, it is also a symptom that often goes undiagnosed due to a lack of desire to seek treatment\(^4\). The primary goal of this thesis was to identify whether anxiety-like behaviors increased after rmTBI. Anxiety has
long been associated with the amygdala, as a brain region involved in fear and emotional processing\(^5\). Less studied, however, is the role that TBI and, specifically, rmTBI plays in the development of anxiety. For this reason, the medial amygdaloid nucleus was chosen to study in this project as a key region involved within the anxiety neurocircuitry. In order to study the effects of rmTBI on anxiety, a five-hit rmTBI experimental rat model was utilized. Through this model, anxiety-like behaviors could be observed and then the brains of rats that have endured repetitive hits could be investigated. The results of this thesis could provide more insight into the development and persistence of anxiety, as well as could help pinpoint a brain region that could be involved in anxiety progression.

After anxiety-like behaviors were identified following rmTBI, the next step in this thesis was to determine whether a therapy could be utilized to reduce or even eliminate acute and/or persistent anxiety-like behaviors. The second goal of this thesis was to identify whether a gonadal steroid treatment after rmTBI could effectively reduce anxiety-like behaviors or be neurotherapeutic. The gonadal steroid utilized within this treatment was the androgen, testosterone. Testosterone has been shown to be suppressed after even a single TBI\(^6\), so restoring testosterone to a physiological level within that individual could be critical. Since testosterone has been shown to be anxiolytic, it was an obvious choice as a potential neurotherapeutic or treatment\(^6\text{-}^8\).

Another impactful effect that testosterone has within the body is in its ability to alleviate oxidative stress within various cell types\(^9\text{-}^{10}\). The medial amygdaloid nucleus, as a subpopulation of cells within the amygdala, may be exposed to oxidative stress which could lead to mitochondrial dysfunction. Oxidative stress and mitochondrial
dysfunction can eventually lead to cell death when the cell becomes overwhelmed by the oxidative load. rmTBI could cause mitochondrial dysfunction within this brain region which could lead to anxiety. Therefore, the hypothesis tested in this thesis is that treatment with testosterone could reduce oxidative stress that occurs within the medial amygdaloid nucleus after rmTBI and subsequently reduce anxiety-like behaviors as a result.

The prevalence of rmTBI across the world demands attention and calls for further preventative measures to be taken. Data collected, analyzed, and disseminated from TBI research not only provides more awareness of this epidemic, but it encourages individuals to seek medical attention by a licensed medical professional following injury. This information also fosters the development of new screening methods, therapies, and even potential treatments.

**Significance Statement**

Individuals sustaining rmTBIs could subsequently suffer from worsening emotional deficits and psychiatric disorders due to persistently low levels of gonadal steroids. These disorders include generalized anxiety, Post-traumatic stress disorder (PTSD), depression, and many others that lower quality of life and lead to a variety of health complications. These complications, such as heart disease, can even lead to death. This thesis proposal addresses the TBI public health concern and provides further insight into how anxiety-like behaviors are exacerbated by hypogonadism after rmTBI as a result of mitochondrial dysfunction in the medial amygdaloid nucleus.
CHAPTER TWO
LITERATURE REVIEW AND BACKGROUND

Traumatic Brain Injury

The Centers for Disease Control and Prevention (CDC) reported nearly 2.7 million TBI-related emergency room visits in the US in 2014 alone\textsuperscript{11}. Over 56,000 deaths were attributed to TBI that year. Despite millions of TBIs that are treated annually, there are likely many more cases that go unreported by individuals who chose not to seek medical diagnosis or treatment\textsuperscript{12}. A mTBI, also known as a “concussion”, is the result of a bump, blow, or jolt to the head that disrupts normal brain function and can result in a temporary loss or alternation of consciousness\textsuperscript{13}. The vast majority, between 70-90\%, of TBIs that occur annually are of mild severity\textsuperscript{14,15}, and while many patients recover fully, some will continue to experience lasting, debilitating symptoms. mTBIs lead to physical, cognitive, emotional, and/or behavioral symptoms that can last for days, months, or years beyond the time of injury. There are two major components at play after an individual endures a mTBI and the subsequent physiological response, known as the primary injury and secondary injury. The primary injury, or the direct, immediate injury is the result of the initial impact by an external force on brain tissue through direct mechanical forces\textsuperscript{16}. The secondary injury, or the indirect, delayed injury is the result of physiological stress in the brain tissue triggered by the primary injury after a period of time, after seconds, minutes, days, or weeks have passed since the initial blow.
Examples of secondary injuries are hematomas, contusion, edema, chronic inflammation, etc.\textsuperscript{11}

**Populations at Risk of mTBI**

mTBIs are a significant public health concern and can affect any individual or population in the United States. Some have called mTBIs a ‘silent epidemic’ since many symptoms and impairments that result (i.e. memory impairments) are not visible.\textsuperscript{17} Several vulnerable populations at increased risk of sustaining mTBIs include athletes that endure sports-related injuries, the elderly, children, active military service members and veterans, and victims of domestic abuse. Risk is referred to as the chance or likelihood that an event will occur within a given amount of time. In terms of overall numbers of TBIs that occur annually, it was reported that between 1.6 to 3.8 million concussions occur in sports and other recreational activities annually.\textsuperscript{15} The greatest incidence of sports-related concussions is those sustained in football.\textsuperscript{18} Incidence is defined as the number of first occurrences of an event in a population within a given amount of time, whereas prevalence would refer to the total number of occurrences of an event in a population within a given amount of time, usually given as a percentage of that population.\textsuperscript{19} Risk and prevalence are similar in that they can each provide information about the likelihood of an event occurring within the overall population, such as the United States. The statistic provided for sports and recreational activities demonstrates that, of all sports-related TBIs, football accounts for the greatest incidence of TBIs within that category each year. The CDC identified falls as the leading cause of TBIs in 2014.\textsuperscript{13} Falls accounted for nearly half of all TBI-related emergency room visits that year. Children and older adults experience a disproportionate number of falls.
compared to other populations with high risk. Nearly half of TBI-related emergency room visits were from falls in children, and four out of five emergency room visits were from falls in adults ages 65 and older\textsuperscript{13,20}. Another population that has largely been overlooked in mTBI research include victims of domestic abuse. Physical injuries, such as repeated blows to the head by domestic partners or falls as a result of these blows can lead to mTBIs and increased negative health outcomes in those affected\textsuperscript{21}. One study reported that between 44-75\% of women experiencing intimate-partner violence have sustained repetitive mTBIs from their domestic partners, or abusers\textsuperscript{22}.

**Repeat mTBI**

Sustaining a single mTBI increases the risk of incurring another by 200-500\% and is associated with higher likelihood of persistent symptoms and long-term dysfunction\textsuperscript{23}. Thus, athletes or active military members who ‘return-to-play’ or ‘return-to-duty’, respectively, after sustaining a mTBI without proper recovery time are at greater risk of subsequent TBI\textsuperscript{15}. Both clinical studies and experimental animal models of rmTBI have pointed to an increased vulnerability for behavioral, cognitive, metabolic, and motor impairments, as well as increased risk of developing long-term neurodegenerative diseases\textsuperscript{24}. Sustained rmTBIs have also been associated with the development of chronic traumatic encephalopathy, which is a neurodegenerative disease diagnosed by detection of tauopathy and atrophy of the cerebrum due to accumulation of hyper-phosphorylated tau protein and aggregated neurofibrillary tangles\textsuperscript{25}.

When a TBI is sustained, there are two major sites where primary injury occurs on the brain in respect to the skull. The brain is positioned within the skull where it is
suspended in cerebral spinal fluid. The first site of injury, also known as the coup injury, is the initial site after impact. For example, in a direct impact injury, an individual falls into or is struck by a fixed object. The brain is forced into the skull, ipsilateral to site of injury. After this coup injury, if the head can rotate freely in space, the brain then recoils away from the side of the skull ipsilateral to injury and impacts the contralateral side of the skull. This is referred to as the contrecoup injury. Each of these injuries, ipsilateral and contralateral to site of initial impact, are considered the primary injury.

Various animal models have been developed to examine the mechanistic links between rmTBI and psychological and neurodegenerative pathology. These models of rmTBI utilized in the field include fluid percussion\textsuperscript{26}, closed-headed closed cortical impactor (CCI) device\textsuperscript{25,26}, open-headed CCI\textsuperscript{25}, weight drop\textsuperscript{26}, and blast injury\textsuperscript{26}. Studies in this thesis utilized the closed-headed CCI device, as it best represented rmTBI clinically and allowed for free rotation of the head and subsequent contrecoup of the brain from impact. Other models require a craniotomy, metal bars that keep the head in position, an energy wave, or a weight dropped on the head, while the closed-headed CCI model more accurately represents individuals sustaining a mTBI in regular, day-to-day life.

**Hypogonadism following mTBI**

It has been reported that 15-68\% of TBI survivors experience deficiencies in pituitary hormones, with 35-50\% meeting criteria for at least one neuroendocrine dysfunction\textsuperscript{27}. Hypogonadism, or depressed endogenous testosterone levels, occurs acutely in just about every patient following a TBI\textsuperscript{28}. As many as 80\% of men experienced hypogonadism following a severe TBI\textsuperscript{29}. While many patients recover their
gonadal status within days or weeks following a mTBI, as many as 41% continue to suffer from chronic hypogonadism, with endogenous testosterone levels that do not return to baseline, pre-injury levels even months or years post-injury. Gonadotropin-releasing hormone released by the hypothalamus, as well as follicle-stimulating hormone and luteinizing hormone released by the anterior pituitary, are altered in humans after mTBI. Women also undergo changes in gonadal steroid production following TBI. As many as 46% experience amenorrhea for up to 60 months post-TBI. Several studies have shown that, while chronic hypogonadism in the general population likely lies between 25-40%, prevalence is likely between 40-80% in military populations that have received a TBI. However, the exact location of dysfunction that results in altered hormone levels remains unknown. This dysfunction due to TBI could occur at the level of the hypothalamus, the pituitary gland, or it could occur somewhere else along the hypophyseal portal vasculature that connects the hypothalamus to the anterior pituitary gland. Another possibility is diffuse edema in the brain that could affect multiple brain regions.

Anxiety Disorders and TBI

Anxiety disorders are common in the general population of the United States and are divided into different categories based on presentation of symptoms. PTSD, generalized anxiety disorder, panic disorder, obsessive compulsive disorder, and other phobia-specific anxiety disorders make up common diagnoses and are even more prevalent following TBI. The prevalence of TBI-related psychiatric disorders, in accordance with the DSM-IV, is listed in Table 1 below. In this table, PTSD is the second most common anxiety disorder diagnosed post-TBI, behind ‘anxiety disorders,
not otherwise specified"\textsuperscript{35}. The ‘anxiety disorders, not otherwise specified' diagnostic determination in this table was used to represent symptoms that fail to meet essential criteria to be considered a specific anxiety disorder.

Although anxiety is one of the most common and prominent deficits that develops post-TBI\textsuperscript{35}, two-thirds of Americans will fail to seek treatment for their anxiety disorder\textsuperscript{36}. Approximately 10-34\% of the U.S. population is diagnosed with anxiety following a TBI\textsuperscript{35}. It was also reported that 44\% of U.S. soldiers that returned from Iraq and Afghanistan after they sustained a TBI met criteria for a positive PTSD diagnosis\textsuperscript{37}. Proper diagnosis of PTSD remains a challenge due to considerable overlap of symptoms or non-specific symptomatology between PTSD and TBI, which include mood and anxiety symptoms\textsuperscript{38}. Patients with PTSD or TBI often present with nonspecific symptoms that include generalized anxiety, memory issues, irritability, etc., further complicating accurate diagnosis and treatment\textsuperscript{39}. 
Table 1. Prevalence of TBI-related Psychiatric Disorders. DSM-IV psychiatric disorders of interest, diagnosed pre- and 12 months post-TBI, have been listed in this table. Anxiety disorders have been identified and include the first and second most common 12-month post-TBI diagnoses, ‘Anxiety disorder not otherwise specified’ and ‘PTSD’. (Table modified from Ponsford et al., 2018)

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Pre-TBI (%)</th>
<th>Post-TBI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any disorder</td>
<td>52.9</td>
<td>60.9</td>
</tr>
<tr>
<td>Any mood disorder</td>
<td>23.5</td>
<td>42.2</td>
</tr>
<tr>
<td>Any anxiety disorder</td>
<td>21.6</td>
<td>44.1</td>
</tr>
<tr>
<td>Major depressive</td>
<td>13.7</td>
<td>29.4</td>
</tr>
<tr>
<td>PTSD</td>
<td>2.0</td>
<td>12.7</td>
</tr>
<tr>
<td>Generalized anxiety disorder</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Anxiety disorder, not otherwise specified</td>
<td>5.9</td>
<td>35.3</td>
</tr>
<tr>
<td>Substance use disorder</td>
<td>34.3</td>
<td>11.8</td>
</tr>
</tbody>
</table>

Brain Regions Involved in Anxiety

Dysfunction within the amygdala, prefrontal cortex, hippocampus, and the hypothalamus have been linked to the pathophysiology of anxiety disorders and development of PTSD⁴⁰. Upon receiving sensory input from the environment through the medial prefrontal cortex, the basolateral amygdala integrates and sends this information to the central amygdala for further processing before it leaves the amygdala⁴¹. One particular region of interest for this thesis is the medial amygdaloid nucleus. This area has been shown to contribute to various social behaviors, including approach-avoidance behaviors⁴², stress responsivity⁴³, and other behaviors related to
fear and anxiety. However, this area has not been well studied within individuals that have suffered rmTBIs.

The amygdala is known to trigger defensive responses through neuronal projections to regions such as the bed nucleus of the stria terminalis (BNST), ventral tegmental area, hypothalamus, hippocampus, and orbitofrontal cortex. Brain structures such as the amygdala and hippocampus have been well studied in anxiety research and are linked to fear learning processes. Another key region that remains central to the anxiety circuitry in the brain is the BNST. The BNST has been implicated in the more sustained, longer-term responses to threats perceived as more unpredictable. While the amygdala response lessens when the immediate threat is absent, the BNST continues sustained activation and demonstrates the hypervigilant state observed in some anxiety disorders. The BNST is centrally located within the anxiety neurocircuitry, with connections to other limbic and brainstem regions such as the amygdala, anterior insula, and hippocampus.

**Testosterone and Anxiety**

Testosterone has been shown to be anxiolytic across many studies, particularly through action of its metabolites, dihydrotestosterone (DHT) and 3-alpha-androstanediol, on androgen receptors (AR). Androgens can be converted into estrogens by aromatase enzymes and act on estrogen receptors, that are also distributed throughout the CNS. Testosterone has been demonstrated to act within the cell through several different pathways. First, classical testosterone signaling occurs within the cell through a slower, genomic pathway where testosterone binds the nuclear associated AR. Upon AR binding, testosterone then enters the cell, binds DNA, and
activates gene transcription\textsuperscript{48}. Another mechanism in which testosterone acts is through a non-classical signaling pathway, where testosterone undergoes a faster, pulsative release. This non-classical, more rapid response to testosterone occurs has been demonstrated, for example, in courtship and reproductive behaviors\textsuperscript{48}. However, in terms of anxiety, a faster, pulsative release of testosterone is advantageous, as this action results in a reduction of anxiety-like behaviors in a shorter period of time. The mechanism of choice is dependent on which brain region testosterone is acting in, or whether testosterone will be converted into estrogen by aromatase to act on estrogen receptors within that region. Reward pathways, including the nucleus accumbens and the preoptic area in the brain, and pathways that demonstrate anxiolytic effects are mediated by similar mechanisms involving testosterone\textsuperscript{48}. Interestingly, after gonadectomy, dopamine release was impaired in the mesolimbic system but was recovered following DHT supplementation\textsuperscript{6}. Likewise, testosterone administration enhanced dopamine release in the neostriatum and nucleus accumbens\textsuperscript{49}. Sex steroids appear to have some level of hormonal control of dopamine release within dopaminergic pathways in the brain.

Since testosterone is known to be anxiolytic, depressed levels of testosterone can result in increased anxiety-like behaviors. Individuals with depressed endogenous testosterone, such as those experiencing the deleterious effects of hypogonadism, have shown worsened anxiety-like symptoms. Hypogonadism and anxiety have been further associated in individuals experiencing sexual dysfunction. Anxiety and sexual dysfunction are positively associated following TBI in men, demonstrated by decreased intercourse frequency, decreased arousal, and increased erectile dysfunction\textsuperscript{50}. A 70%
increased risk of sexual dysfunction is also observed among female patients after TBI as well\textsuperscript{51}. Low-dose testosterone administration was found to reduce anxiety-like behaviors and improve depression ratings in both males and females\textsuperscript{6}. Efficacy of testosterone administration on anxiety is dependent on length of androgen deficiency and age of the patient\textsuperscript{52}. The mechanism by which testosterone supplementation reduces anxiety in those who are hypogonadal, either acutely or chronically, remains unknown.

**Oxidative Stress and Neurodegeneration**

Mitochondrial dysfunction has been implicated in neuronal degeneration and subsequent neuronal cell death in the brain. Mitochondria are key regulators of cell death, as they control rates of reactive oxygen species generation and detoxification, control release of proapoptotic factors, and regulate the respiration rate of the cell\textsuperscript{53,54}. Mitochondrial dysfunction has been identified after mTBI through a reduction in $N$-acetyl aspartate, ATP, and acetyl-CoA levels\textsuperscript{55}. These molecules are markers of proper mitochondrial function, so a reduction in any or all of these markers after rmTBI would demonstrate mitochondrial dysfunction. Similarly, a reduction in an important marker for proper mitochondrial function, cytochrome c oxidase subunit 4 isoform 1 (COX4i1) within complex IV of the electron transport chain, has been shown within the brains of animals with neurodegenerative diseases, such as Alzheimer’s disease\textsuperscript{56,57}. In particular, COX4i1 expression was reduced in Alzheimer’s disease transgenic mice, which demonstrated mitochondrial dysfunction through impaired mitochondrial metabolism\textsuperscript{56}. As the rate-limiting enzyme and terminal node in the mitochondrial electron transport chain, dysfunction of the COX complex is associated with oxidative
stress in mitochondria\textsuperscript{58}. Changes in COX4i1 subunit expression could impact how a cell is producing ATP and its overall function. Genetic variants of COX4i1 have been associated with severe epilepsy, intellectual disability, and developmental regressions\textsuperscript{57}.

Together, these findings suggest that COX4i1 expression, as a marker of proper mitochondrial function, could be reduced within the mitochondria of neurons within the regions affected by secondary effects of TBI, including the amygdala. This reduction is associated with mitochondrial oxidative stress. Neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, and multiple sclerosis, further point to associations between psychiatric disorders and the involvement of mitochondrial dysfunction.

**Oxidative Stress and the Amygdala**

Oxidative stress has been associated in anxiety, depression, and other mood disorders through pro-inflammatory processes that result in an increase in reactive oxygen species and reactive nitrogen species\textsuperscript{59}. Increased production of reactive oxygen species and reactive nitrogen species can result in stress at a cellular level, which can lead to cell dysfunction and even cell death. On a behavioral level, these processes can further exacerbate the detrimental behavioral effects of disorders associated with mental health and wellness. The brain is sensitive and vulnerable to oxidative stress due to the amount of oxygen the brain requires to function when compared to many other organs in the body\textsuperscript{60}. The amygdala is particularly susceptible to the effects of oxidative stress and imbalances of reactive oxygen species and antioxidants, as dysfunction in the amygdala due to increased oxidative stress is associated with an increased chance of developing the mental health disorders
previously mentioned. One study demonstrated susceptibility of the amygdala to oxidative stress through induction of xanthine and xanthine oxidase treatment followed by measurement of two different indices of oxidative stress, 8-isoprostane and malondialdehyde, present within the amygdala brain tissue. 8-isoprostane was significantly increased in urine and serum, and malondialdehyde was specifically increased in the amygdala and the hippocampus, which demonstrated the effect of oxidative stress that can occur within the amygdala. Alternatively, others have shown that oxidative stress within the amygdala can be decreased, or even prevented, in part, due to moderate physical exercise, which resulted in decreased anxiety-like behaviors.
CHAPTER THREE
HYPOTHESIS AND SPECIFIC AIMS

Overall Hypothesis

Androgens play a neurotherapeutic role after rmTBI through a reduction in anxiety-like behaviors and their therapeutic effects are associated with markers of improved mitochondrial function in dopaminergic neurons in the medial amygdaloid nucleus.

Specific Aims

Specific Aim 1: To determine the effect hypogonadal status has on anxiety-like behaviors after rmTBI and to determine whether there is an optimal therapeutic time window for hormone replacement.

Specific Aim 1a.) To determine whether persistently reduced levels of gonadal steroids result in prolonged anxiety-like behaviors after rmTBI and whether testosterone replacement started at a timepoint associated with persistent anxiety reduces anxiety-like behaviors.

Specific Aim 1b.) To determine whether reduced levels of gonadal steroids result in early anxiety-like behaviors following rmTBI and whether testosterone replacement started at an early timepoint, either immediately or 7 days after rmTBIs, reduces anxiety-like behaviors.
**Specific Aim 2:** To determine whether gonadal steroid status affects a marker of mitochondrial function in dopaminergic neurons in the medial amygdaloid nucleus after rmTBI.

**Specific Aim 2a.)** To determine whether rmTBI alters a marker of mitochondrial function in dopaminergic neurons in the medial amygdaloid nucleus.

**Specific Aim 2b.)** To determine if testosterone replacement, either immediately or 7 days after rmTBIs, increases a marker of mitochondrial function in dopaminergic neurons in the medial amygdaloid nucleus.
CHAPTER FOUR
RESEARCH DESIGN AND METHODS

Specific Aim 1

To determine the effect changes in gonadal steroid status have on anxiety-like behaviors after rmTBI and to determine whether there is an optimal therapeutic time window for hormone replacement.

Experimental Question #1

Will rmTBI lead to anxiety-like behaviors that are not further exacerbated by castration?

Rationale

Virtually all male patients who have been tested for testosterone levels show suppressed levels of the hormone immediately after a mTBI. While many recover gonadal status, a subset of individuals remain chronically hypogonadal. Since testosterone is known to be anxiolytic, increased anxiety-like behaviors would be expected in rats that have suppressed testosterone levels due to sustaining rmTBIs. In addition, this reduction in testosterone can exacerbate anxiety-like behavior that persists and/or worsens over time. Prolonged anxiety, therefore, could be linked to gonadal status following rmTBI and must be investigated in order to better understand how it originates and progresses. Even a single mTBI can lead to anxiety-like symptoms, so sustaining multiple mTBIs further increases the risk of developing
anxiety-like symptoms that become persistent and long lasting. A goal of this thesis is to identify the effect impaired gonadal status and testosterone have on anxiety-like behaviors following rmTBI. The following experiment will allow for observation of possible exacerbated anxiety-like behaviors and help identify the effect that testosterone has on anxiety-like behaviors following rmTBI.

**Methods**

**Animals**

8-week old, male Long-Evans Hooded rats were utilized for all experiments. After arrival to the Edward Hines Jr. VA Veterinary Medical Unit, rats were acclimated to new housing for three days prior to any handling. Rats were group-housed in controlled temperature and humidity room with a 12-hour light-dark cycle. Lights were turned on at 6am and turned off at 6pm. Rats were fed standard chow and provided water *ad libitum*. All experimental animal procedures were approved by the Edward Hines Jr. VA Institutional Animal Care and Use Committee (IACUC approval number: 1593048-3). Animals were randomly assigned into experimental groups as follows: Sham-intact, Sham-castrate, rmTBI-intact, and rmTBI-castrate, as demonstrated in Figure 1.
Figure 1. Diagram of Experimental Groups – Aim 1, Experimental Question 1. Four groups will be utilized in this experiment. Groups include Sham-intact, Sham-castrate, Sham-castrate, rmTBI-intact, and rmTBI-castrate.

The experimental timeline used to address the first experimental question is presented in Figure 2. On the first day of the experiment, the first rmTBI was given and animals were either castrated to represent hypogonadism assumed after the first mTBI or had undergone sham castration. After the first rmTBI, four subsequent mTBIs occurred, each spaced 48 hours apart. The 48-hour window between each of the mTBIs was chosen based on previous similar models of rmTBI. The largest amount of cellular disruption after a mTBI occurs beings to subside within the first 48-hour period. Therefore, an adequate period of 48-hours was used to allow cellular disruption to subside before another mTBI was delivered. The open field behavioral test was conducted on PTD 28 to observe anxiety-like behaviors.
Figure 2. Timeline for Experiments – Aim 1, Experimental Question 1. The timeline for experiments proposed in Aim 1, Experimental question 1 is illustrated in this figure.

**rmTBI Model using CCI**

A closed-headed CCI electromagnetic device was used to provide mTBI to male Long-Evans Hooded rats. Figure 3A shows the closed-headed CCI device. The animal was anesthetized with isoflurane and was placed on a foam pad during impact to ensure there was free range of head movement, which allowed not only for the coup injury from impact, but the contrecoup of the brain on the side of the skull opposite from impact due to acceleration and deceleration forces. The CCI was positioned over the animal’s skull approximately 20 degrees from vertical and halfway between the eye and the tip of the ear. This impactor tip was located over the right sensorimotor cortex region of the brain, shown in Figure 3B. These landmarks have been determined by a previous group and are used to produce a TBI consistently placed, as verified by measurements compared to bregma. This region was chosen so that motor function would not be
impaired following rmTBI. Just prior to injury, the nose cone providing isoflurane was removed to allow for impact and free range of head motion. After the nose cone was removed, the impactor, with a 5 mm steel impactor tip, traveled at approximately 6.5 m/s at a depth of 10mm with a dwell time of 300ms. Immediately after impact, the skull was checked to ensure there were no signs of fracture after rmTBI. Half of the animals either received a mTBI by the CCI device (rmTBI group) or a sham impact in which they receive isoflurane and were placed on the foam pad under impactor without injury (sham group). The first mTBI was then followed by four consecutive mTBIs, each spaced 48 hours apart. The fifth and final mTBI was defined as post-injury day (PTD) 0.

Figure 3. Experimental Repeat Mild TBI Rat Model. A) CCI device setup including stereotaxic frame with electromagnetically-driven piston, 5 cm thick foam pad, plexiglass shield along left side of foam, CCI control box, electromagnetic actuator, and nose cone providing isoflurane. B) Location and orientation of impactor tip on rat skull over right hemisphere sensorimotor cortex (Pictures from Segismundo, 2019).
Castration

Animals were further divided into castration or sham castration (intact) groups. Animals were placed under 4% isoflurane and maintained at 2% isoflurane for duration of mTBI induction and surgery. Animals under anesthesia received rmTBI or sham injury and were immediately castrated or received sham castration surgery. Castrated groups received an approximate 1-cm incision made in the midline of the scrotum using surgical scissors. The left cremaster muscle and tunica vaginalis were then opened to expose the testis and spermatic cord. The testis was then pulled out of the surrounding sac and a thin silk suture ligated the spermatic cord. A distal cut was then made to remove the testis and associated spermatic cord. The remaining spermatic cord was placed back into the surrounding sac and then back into the scrotum. This procedure was then repeated on the right testis using the same midline scrotal incision. Following the removal of the other testis, the incision was closed using an 8-mm staple. The other half of the animals received an incision along the midline of the scrotum but the testes were not removed, representing the sham group controlling for the incision itself known as the intact group. The intact group then received the staple to close the incision.

Behavioral Test for Anxiety-like Behavior

Open field behavioral test. Rats were placed in the open field arena for 10 min and their movement was tracked while they freely explored the arena. The floor of the arena was marked with blue grid lines that separates the arena into 25 smaller squares, as well as a red square in the center of the arena comprised of 9 squares that comprise the “center square”. The 16 outer squares that touch the four walls comprise the periphery of the arena. An image of the open field apparatus is presented in Figure 4.
Rats were placed within the center of the red center square at the beginning of the 10 min session. After test, the rats were removed from the open field arena and placed back into their respective cage.

Observational variables included:

a) Latency in time to leave center square: time in seconds from placement in the center red square to time when all four limbs are outside of the red center square.

b) Total number of red lines crossed

c) Total time spent in red center square in seconds

d) Time spent freezing, which excluded small movements of the head or movement required for breathing, in seconds

e) Exploring: time spent in the arena doing anything other than grooming or freezing in seconds

f) The number of wall explorations: number of rears or "high--leaning" behavior, when the animal is actively leaning on the walls or looking above the wall and outside of the arena with two front limbs on the wall.
Figure 4. Open Field Apparatus. Image of the open field apparatus utilized in all behavioral experiments. Single frame image taken using video recording device.

Power Analysis

Power analysis was conducted using the statistical program, G*Power 3.1. For Specific Aim 1, experimental question 1, an a priori ANOVA: between factors, F test was chosen in order to determine sufficient sample size required for an effect to be detected within the behavioral tests. Four groups were utilized for this behavioral test, which include: sham-intact, sham-castrate, rmTBI-intact, and rmTBI-castrate. Therefore, the two main factors tested were rmTBI and castration. An effect size of 0.8 was chosen as the smallest effect size to be considered meaningful for the behavioral experiments. Previous rodent mTBI models that utilized behavioral tests like the open field arena
demonstrated a meaningful difference when there was a ± 20% change in anxiety-like behaviors\textsuperscript{66,67}. This change in anxiety-like behaviors reflects a 0.8 effect size, and a meaningfully significant change in the anxiety-like behaviors in this study comparable to the previous studies. The $\alpha$ level is $p=0.05$, the probability of $\beta$ error is 0.05, and the corresponding power is 0.95 (1-0.05). Using these parameters in G*Power 3.1, the non-centrality parameter value produced was 14.72 and the critical F was 4.38. The total sample size required was 23 for the four groups, which is 5.75, rounded up to 6 animals per group, which results in an actual power of 0.953. This value represents a 95.3% chance of seeing a difference at the given effect size of 0.8. It must be noted that the total sample size utilized in this first experiment was larger than the sample size acquired through the power analysis. The total sample size for the four groups was 47, or about 12 per group. The reason more samples were utilized was because of further manipulations made in a later experiment. Animals from the four groups were to be further split into groups that did or did not receive treatment. Therefore, the sample size used for this first experiment was approximately double the calculated sample size. A large enough number was needed at the end of the study, which required more animals to be utilized since all animals would be included in the open field behavioral test at PTD 28.

**Statistical Analysis**

Statistically significant changes in the behavioral observational variables in the open field test at PTD 28 were analyzed using a two-way ANOVA statistical test. The main effects measured were rmTBI and castration. A p-value of <0.05 was used to determine statistical significance.
Experimental Question #2: Do anxiety-like behaviors in rats persist after rmTBI, castration, or combination of the two?

Rationale

Anxiety-like behaviors that persist at PTD 28 as a result of rmTBI and/or castration could continue to worsen due to persistently suppressed serum testosterone levels in animals with hypogonadal status. Many studies report increased anxiety-like behavior up to one month after mTBI\textsuperscript{66,67}, but some also report that anxiety-like behaviors begin to fade after one month\textsuperscript{68}. For this reason, the next step was to determine if increased anxiety-like behaviors would persist, or become exacerbated, at PTD 42 in animals that endured rmTBI, castration, or both. Alternatively, this experiment would also demonstrate whether anxiety-like behaviors waned over time without treatment or intervention. The experimental timeline utilized for this experimental question is shown in Figure 5.

Figure 5. Timeline for Experiment – Aim 1, Experimental Question 2. The timeline for experiment proposed in Aim 1, experimental question 2 are illustrated in this figure.
Methods

Statistical Analysis

Statistically significant changes in the behavioral observational variables in the open field test at PTD 42 were analyzed by a two-way ANOVA statistical test. The main effects measured were rmTBI and castration. A p-value of <0.05 was utilized to determine statistical significance.

Experiment Question #3: Will testosterone supplementation reduce persistent anxiety-like behaviors in rats?

Rationale

Since testosterone has been shown to be anxiolytic, and testosterone suppression has been demonstrated in individuals after rmTBI, it was determined whether replacement of testosterone could reduce anxiety-like behaviors. Supplementation with testosterone brought circulating levels to physiological levels. If testosterone loss that follows rmTBI contributes to increased anxiety-like behaviors, then it would be expected that animals supplemented with testosterone would show less anxiety-like behaviors in the open field behavioral test. Individuals that experience persistent anxiety after rmTBI could greatly benefit from the neurotherapeutic and anxiolytic effects of testosterone supplementation if provided with the most effective dosage and at the most effective timepoint after rmTBI. Testosterone supplementation has the potential to substantially impact recovery following rmTBI and requires further investigation so that it can become a more therapeutic intervention following TBI. The experimental timeline for the second experimental question is presented in Figure 6. This figure depicts the mTBI given and castration surgery that occurred on the first day,
followed by four subsequent mTBIs spaced 48 hours apart. The open field behavioral test was then conducted at PTD 28. Testosterone supplementation was then provided at PTD 35 and the open field behavioral test was conducted once again at PTD 42, one week following supplementation. A change score was calculated for the frequency or duration of anxiety-like behaviors from PTD 28 to PTD 42. These scores were calculated by taking the value from each individual animal at PTD 42 for a particular observational variable and subtracting the value from that same animal at PTD 28.

**Figure 6. Timeline for Experiment – Aim 1, Experimental Question 3.** The timeline for experiment proposed in Aim 1, experimental question 2 are illustrated in this figure.

### Methods

**Testosterone Supplementation**

Testosterone was administered to rats through silastic implants of crystalline testosterone delivered subcutaneously in the dorsal nape of the neck. Testosterone was packed into a 1.5 cm long silastic tube with a diameter of 1.57 mm and was sealed with wooden stoppers and silicone-based medical adhesive at each end. The testosterone
packed into the capsule was 1.0 cm in length, with each stopper approximately 0.25 cm in length. Sealing both ends of the capsule with a silicone-based medical adhesive prevents levels of testosterone rising to supraphysiologic levels in the serum.

Testosterone capsules were placed into phosphate buffered saline overnight prior to administration in order to prime the testosterone within the capsule before placement into the subcutaneous space in the nape of the neck. Testosterone capsules were placed 35 days after the final rmTBI. The subcutaneous implantation of the testosterone capsule produces an average serum testosterone level of $4.49 \pm 1.40$ ng/ml in rats.

**Power Analysis**

For Specific Aim 1, experimental questions 3, an a priori ANOVA: between factors, F test was chosen in order to determine sufficient sample size in behavioral tests with 4 groups, including rmTBI-intact + testosterone supplementation, rmTBI-intact without testosterone supplementation, rmTBI-castrate + testosterone supplementation, and rmTBI-castrate without testosterone supplementation. An a priori decision was made to only utilize the rmTBI groups for this analysis, since they were the groups of greatest interest to study the effects of testosterone supplementation. An effect size of 0.8 was chosen, as in the previous experiments, to obtain a meaningful difference in changes in anxiety-like behavior. The $\alpha$ level is $p=0.05$, the probability of $\beta$ error is 0.05, and the corresponding power is 0.95. Using these parameters in G*Power 3.1, the non-centrality parameter value produced was 14.72 and the critical F was 4.38. The total sample size required was 23 for the 4 groups, which is 5.75 and rounds up to 6 animals per group, which results in an actual power of 0.953. This value represents a 95.3% chance of seeing a difference at the given effect size of 0.8.
**Statistical Analysis**

Statistical significance in the change score of anxiety-like behavior variables in the open field test between PTD 28 and PTD 42 was analyzed using a two-way ANOVA statistical test. The main factors were castration and testosterone treatment. A p-value of <0.05 was used to determine statistical significance.

**Experiment Question #4:** Will testosterone supplementation at a more acute timepoint, either immediately or 7 days after the final rmTBI, reduce anxiety-like behaviors in rats at PTD 14?

**Rationale**

After observing increased and prolonged anxiety-like behavior in animals following rmTBI, the next step was to identify whether this increased anxiety-like behavior was normalized, or even eliminated, at an earlier timepoint through testosterone supplementation. Testosterone supplementation either immediately or 7 days after the final rmTBI was used to characterize effects of testosterone on anxiety-like behaviors. The experimental groups were presented in Figure 7. This figure shows the sham-intact, sham-castrate, rmTBI-intact, and rmTBI-castrate, further separated into testosterone treatment groups. The sham-castrate group was separated into sham-castrate without testosterone or with 7-day testosterone. The immediate testosterone treatment group for sham-castrate was not included since testosterone levels would be approximately the same as the sham-intact once testosterone was removed through castration and immediately replaced. In addition, rmTBI-castrate was split into rmTBI-castrate without testosterone, with immediate testosterone, or with 7-day testosterone. Since a critical window for testosterone supplementation has not yet been determined,
this experiment was designed to explore if testosterone is neurotherapeutic when reintroduced at the earlier timepoints described above. The experimental timeline was presented in Figure 8. This timeline follows the previously used timeline in Figure 6 with the same TBI induction and castration setup. However, the immediate testosterone supplementation timepoint has been added on the first day of rmTBI induction and a 7-day testosterone supplementation timepoint on PTD 7. The open field behavioral test was conducted on PTD 14 and animals were sacrificed on PTD 15. This experiment was designed to help determine if supplementation with testosterone at an earlier time point (immediately or at PTD 7) would reduce anxiety-like behaviors at PTD 14 in this experimental rmTBI rat model. There could be a critical window of time in which testosterone is needed following a TBI, potentially at a time when hypogonadal individuals will not have testosterone. If testosterone can be given back to the individual at an earlier timepoint following injury, before development of anxiety and other deleterious symptoms, it could have significant effect on the individual and not only improve symptoms but could prevent new symptoms from developing.
Methods

Figure 7. Diagram of Experimental Groups – Aim 1, Experimental Question 4. There will be 7 groups utilized in this experiment. Groups include Sham-intact, Sham-castrate + T at 7 days, Sham-castrate + No T, TBI-intact, TBI-castrate + immediate T, TBI-castrate + T at 7 days, and TBI-castrate + no T.

Figure 8. Timeline for Experiments – Aim 1, Experimental Question 4. The timeline for experiments proposed in Aim 1, Experimental Question 4 are illustrated in this figure.
**Statistical Analysis**

Statistically significant changes in the behavioral observational variables in the open field test at PTD 14 were determined using a multiple linear regression statistical model (Linear regression factors = castration, rmTBI, immediate treatment, and 7-day treatment). A p-value of <0.05 was used to determine statistical significance.

**Specific Aim 2**

To determine whether gonadal steroid status affects a marker of mitochondrial function in dopaminergic neurons in the medial amygdaloid nucleus after rmTBI.

**Experimental Question #5:** How does rmTBI affect a marker of mitochondrial function in dopaminergic neurons in the medial amygdaloid nucleus?

**Rationale**

The rate-limiting enzyme in catecholamine synthesis, tyrosine hydroxylase (TH), has been shown to be highly expressed on neurons in key areas related to anxiety. Specifically, the posterior medial amygdaloid nucleus, an area associated with social and sexual behaviors, contains high expression of both TH and androgen receptors\(^7\). Since TH is the rate-limiting enzyme in both dopamine and norepinephrine biosynthesis pathways, it is critical to explore presence of dopamine beta hydroxylase (DBH), as the enzyme that converts dopamine into norepinephrine, used as a proxy to distinguish cell type within the medial amygdaloid nucleus.

Dopaminergic neurons in the medial amygdaloid nucleus could experience a reduction in the level of mitochondrial function as a result of sustained rmTBIs. COX4i1 is the cytochrome c oxygenase subunit associated with complex IV of the mitochondrial respiratory chain. Reduced COX4i1 expression, then, is associated with impaired
mitochondrial function. Mitochondrial dysfunction can lead to increased oxidative load and cellular stress in dopaminergic neurons. Oxidative stress, if left unchecked, can then lead to dopaminergic neuronal cell death. The level of mitochondrial dysfunction after rmTBI could be a key component in the development and exacerbation of anxiety.

In order to experimentally test for mitochondrial dysfunction in dopaminergic neurons in the medial amygdaloid nucleus, COX4i1, DBH, and TH mRNA expression markers were identified within that region. Both dopaminergic and noradrenergic neurons require the catecholamine rate-limiting enzyme, TH, in order to synthesize neurotransmitter, but DBH further converts dopamine into norepinephrine in noradrenergic neurons. Therefore, TH-positive, DBH-negative neurons were identified through fluorescent in situ hybridization in order to visualize and quantify changes in COX4i1 expression levels within dopaminergic neurons in the medial amygdaloid nucleus.

**Methods**

*In Situ Hybridization – Fluorescent Multiplex Assay*

Animals were sacrificed and brains were quickly extracted and placed into a beaker of isopentane on dry ice for rapid freezing. Frozen brain tissue was then stored at -80°C until use. Frozen brain tissue was blocked to obtain the medial amygdaloid nucleus. Cuts of the frozen brain tissue were made to obtain a tissue block that contained the medial amygdaloid nucleus, but also regions of the hippocampus and other subregions of the amygdala. The location of each cut was determined by first identifying nearby anatomical landmarks and by consulting the Paxinos and Watson Rat Brain Atlas, 6th edition. The frozen brain tissue blocks were sectioned into 25µm-thick
tissue slices at -20°C using a cryostat (Leica CM3050 S Cryostat, Leica Biosystems, Buffalo Grove, IL 60089) and were mounted onto 1mm thick slides coated with 3-Aminopropyltriethoxysilane for adhesion (Electron Microscopy Sciences, Cat. No. 63734-01). Each slide contained two sections of brain tissue. Slides to be utilized for in situ hybridization were selected through cresyl violet staining and through consulting the Paxinos and Watson Rat Brain Atlas, 6th edition in order to select slides demonstrating the same spatial proximity to key anatomical landmarks. The location and spatial orientation of the optic nerve, internal capsule, and lateral ventricle were each used to indicate a consistent position within the medial amygdaloid nucleus between each brain for in situ experiments.

In situ hybridization was conducted using an RNAscope Fluorescent Multiplex Assay Kit (acdbio, Cat. No. 320850). COX4i1, DBH, and TH mRNA expression levels were measured with RNAscope target probes in order to mark differences in mRNA expression between groups with fluorescent tags. These tags were used to determine localization of each mRNA within the medial amygdaloid nucleus. Slides were removed from -80°C and placed onto dry ice. Slides were next submerged and fixed with 4% paraformaldehyde at 4°C for 15 minutes. After tissue fixation, tissue sections underwent dehydration, which included 5 minutes in 50% ethanol, 5 minutes in 70% ethanol, 5 minutes in 100% ethanol, and another 5 minutes in fresh 100% ethanol, all at room temperature. After this dehydration step, slides were removed from ethanol and allowed to air dry for 5 minutes prior to application of hydrophobic barrier with ImmEdge hydrophobic barrier pen around each section on the slide. Next, Protease IV reagent was applied to dried slides and incubated at room temperature for 30 minutes. Protease
IV was then removed and slides were washed in 1X PBS, followed by another wash in fresh 1X PBS. Target probes were supplied in two different dilutions. The first target probe, COX4i1 (acdbio, Cat. No. 425921), was supplied in a diluted, 1X form. The two other target probes, DBH (acdbio, Cat. No. 316421–C2) and TH (acdbio, Cat. No. 314651-C3), were supplied in a 50X concentration. Therefore, the target probe mixture required a 50:1:1 dilution ratio for COX4i1, DBH, and TH, respectively. COX4i1, DBH, and TH target probes were each designed to be exposed to a different fluorescent tag, selected to optimize detection and quantification. The target probe mixture was applied in sufficient quantity to completely cover each brain section, at approximately 50 µL. Slides were incubated at 40°C for 2 hours to hybridize to tissue. Following probe incubation, probes were removed and slides were washed twice with 1X wash buffer. The first amplifier, Amp 1, was applied and incubated again at 40°C for 30 minutes to hybridize. After first amplifier incubation, Amp 1 was removed and slides were washed twice with 1X wash buffer prior to Amp 2 application. Slides were incubated at 40°C for 15 minutes. Next, Amp 2 was removed, slides were washed twice with 1X wash buffer, Amp 3 was applied, and then another 30 minutes incubation at 40°C followed to hybridize. Lastly, Amp 3 was removed, slides were washed twice with 1X wash buffer, and Amp Alt B (selected based on preferred coloration for each probe to optimize visualization and quantification) was applied and incubated at 40°C for a final 15 minutes. Following Amp Alt B incubation, slides were washed twice more with 1X wash buffer and wash buffer remained on slides until mounting media was applied. Next, wash buffer was removed and DAPI was added to each section and incubated at room temperature for 30 seconds. Following incubation, DAPI was removed and slides were
immediately coverslipped using fluorescent mounting media and kept in the dark at 4°C to preserve fluorescent label for further visualization and quantification. Slides were visualized through widefield fluorescent microscopy (Olympus IX-81, motorized inverted system microscope, Olympus Life Science).

**In Situ Hybridization – Quantification**

COX4i1, DBH, and TH-label in the medial amygdaloid nucleus was observed using widefield fluorescence microscopy. The Olympus CellSens program was utilized to make and process images. A multi-image array with z-stacks of the medial amygdaloid nucleus was taken of the three combined channels for each tissue section at 20X magnification. Twenty-one Z-slices were taken in each channel, each with a 0.5 micron step size. Images with 21 Z-slices were processed using extended focus image processing in order to generate a single quantifiable image containing the brightest pixels from each Z-slice. ROIs could then be drawn using the extended focus processed images, shown in Figure 9. This image shows an ROI drawn as a red line around a TH+ cell, represented through clusters of red puncta, and DBH- cell, demonstrated by a lack of green puncta clusters, using the freeline drawing tool. TH+, DBH- cells were deemed positive based on pre-selected criteria, including number and shape of puncta, signal strength of label, and colocalization pattern within each cell body.
Figure 9. Example of ROI Drawn Around +TH Cell Using CellSens Dimension Program. This 20X magnified image depicts the medial amygdaloid nucleus from the CellSens Dimension program demonstrating a TH+ cell with an ROI drawn around the cell body with the red line. Red puncta represent TH, Blue-DAPI, White-COX4i1

The criteria utilized to select positive cells also took potential channel overlap resulting in cloudy, unclear puncta shape into account, as well as signs of general autofluorescence. Only clear, round, and bright puncta that did not demonstrate overlap with another channel were included in the criterion for number of puncta to be considered a positive cell as depicted in Figure 10. The red channel, depicted in Figure 10A, and the white channel, depicted in Figure 10B, were separated in order to determine any overlap between channels or to detect autofluorescence. The overlapped channel, presented in Figure 10C, demonstrates several areas where white and red puncta overlap. These areas of overlap are not true signal as they are not clear or bright as puncta visualized with this technique should be. The amount of COX4i1 expression
was then quantified within TH+, DBH- ROIs through an automated process on the software. This was done by setting a threshold for the COX4i1 channel, segmenting the puncta, and counting the number of puncta present within each of the ROIs.

Figure 10. Example of Channel Fluorescence Overlap and Autofluorescence in Process of Identifying TH+ Cells. This 20X magnified image demonstrates an example of fluorescent signal overlap between channels which results in unclear, cloudy puncta shape that is not true fluorescent signal. Examples of overlap are represented in the combined image by pink color, indicated by white arrows, compared to separated red and white channel images. The red channel (A) contains red puncta that represent TH, the white channel (B) contains white puncta that represent COX4i1, and the red and white channels overlapping (C) contain pink puncta that represent nonspecific label.

Power Analysis

For Specific Aim 2, experimental questions 5 and 6, an a priori ANOVA: between factors, F test was chosen in order to determine the sufficient sample size for fluorescent in situ hybridization with five groups, including Sham-intact, rmTBI-intact, rmTBI-intact + immediate T, rmTBI-castrate + 7 day T, and rmTBI-castrate + empty capsule. The standard deviation was set to 1 for all five groups. An effect size of 1.5 was chosen. The α level is p=0.05, the probability of β error is 0.05, and the corresponding power is 0.95. Using these parameters in G*Power 3.1, the non-centrality parameter value produced was 42.75 and the critical F was 2.54. The total sample size
required was 23 for the five groups, which is 4.6, rounded up to 5 animals per group, which results in an actual power of 0.953. This value represents a 95.3% chance of seeing a difference at the given effect size of 1.5.

**Experimental Question #6:** Does testosterone supplementation at a more acute timepoint, either immediately or 7 days following last rmTBI, result in reduced mitochondrial dysfunction in dopaminergic neurons in the medial amygdaloid nucleus?

**Rationale**

Gonads are the major source of gonadal steroid production, so performing a gonadectomy in animal models will reflect suppressed testosterone levels. Removal of endogenous testosterone resulted in increased anxiety-like behaviors. Some studies have reported a neurotoxic role of testosterone when presented to the cellular microenvironment in states of oxidative stress or neuroinflammation. These studies often utilize in vitro models or present testosterone early to the microenvironment following injury. For this reason, identifying a critical window of time in which testosterone could either be deleterious or beneficial if provided at an earlier timepoint was essential within the experimental rat rmTBI model.

The effect of testosterone given at an earlier timepoint on mitochondrial function was measured by COX4i1 expression levels in dopaminergic neurons in the medial amygdaloid nucleus. If the level of mitochondrial dysfunction is too high in dopaminergic neurons in the medial amygdaloid nucleus, then supplementing earlier with testosterone might serve as a way to regulate or help restore proper mitochondrial function.
Methods

Animals

Sham and TBI groups were further divided into categories based on testosterone supplementation, as diagramed above in Figure 7. An a priori decision was made to utilize the five most important groups in order to identify key changes in effect of injury, castration, and/or treatment on the expression of COX4i1 within the medial amygdaloid nucleus. For the sham groups, these included a sham castrate with empty capsule group and a sham castrate with testosterone given 7 days following final injury group. For the TBI groups, these included a TBI castrate with empty capsule group, a TBI castrate with immediate testosterone group, and a TBI castrate with testosterone given at 7 days following final injury group.

Statistical Analysis

Significant changes in the amount of COX4i1 expression in PTD 14 brains were observed using a two-way ANOVA statistical test. The main factors measured were rmTBI and testosterone treatment. Since data from all previous groups could not be applied in this statistical analysis to utilize a full effects model, a main-effects-only model was required for this analysis. A Dunnett post-hoc multiple comparisons analysis was utilized to determine significant differences between each rmTBI group compared to the sham group. A p-value of <0.05 was used to determine statistical significance.
CHAPTER FIVE

RESULTS

Specific Aim 1, Experimental Question 1

Will rmTBI lead to anxiety-like behaviors that are not further exacerbated by castration?

In the first study, it was determined whether rmTBI would cause anxiety to the same degree as the loss of testosterone itself. The open field behavioral test, a standard test used to identify anxiety-like behaviors in rodents, was used in this study. Animals in the rmTBI group have been hypothesized to demonstrate significantly increased frequency and duration of anxiety-like behaviors. In addition, castrated groups should also demonstrate a similar increase in anxiety-like behaviors since testosterone is absent or suppressed. Summary data are shown in Figures 11 and 12 and demonstrate the results of the open field behavioral test at one month after the final rmTBI. The number of red lines crossed and the number of wall explorations, also known as high-leaning or rearing behavior, were assessed in Figure 11A and 11B, respectively. Anxiety-like behavior is expressed by less rearing along the outer walls of the apparatus. A reduced number of red lines crossed is also a measure of increased anxiety-like behavior. These red lines surround the innermost nine squares in the arena and outline the center of the arena.
Figure 11. rmTBI Resulted in Decreased Number of Wall Explorations. (A) Number of wall explorations and (B) the number of red lines crossed in male Sham and TBI intact or castrated rats 28 days after rmTBI. The two-way ANOVA did not demonstrate a statistically significant interaction between rmTBI and castration ($F(1,90) = 3.24$, $p=0.075$) on number of wall explorations. The test did demonstrate a significant main effect of rmTBI ($p=0.005$) on the number of wall explorations observed in the open field test 28 days after the last rmTBI. The two-way ANOVA for number of red lines crossed did not demonstrate a statistically significant interaction between rmTBI and castration ($F(1,91) = 1.682$, $p=0.20$). The test did not demonstrate significant main effects of rmTBI or castration on mean number of red lines crossed in the open field arena. However, there was a trend for the main effect of rmTBI on mean number of red lines crossed ($p=0.058$).

Latency time to leave the center, red-lined square was observed to detect changes in anxiety-like behavior, represented in Figure 12A. The greater the latency time correlates to less anxiety-like behavior and vice versa. Time spent along the periphery and outside of the center of the area was also observed as a marker of anxiety-like behavior. The overall amount of time spent in the inner, red-lined square was assessed, where less time spent in the inner square equates to greater anxiety-like behavior. This is represented in Figure 12B. Time spent freezing during the open field trial indicates anxiety-like behavior. More time spent immobile within the arena is associated with greater anxiety-like behavior. The mean time spent freezing in the
arena was shown in Figure 12C. The amount of time spent moving in the open field was shown in Figure 12D, where more time spent moving equates to reduced anxiety-like behavior.

Figure 12. rmTBI and Castration Resulted in Decreased Latency and Increased Time Freezing at PTD 28, Castration Resulted in Decreased Time in Inner Square. Behaviors depicted in these graphs include (A) Latency in time to leave the center square, (B) Time in the inner square, (C) Time spent freezing in open field, and (D) Time spent exploring, or moving, in the open field at PTD 28. The two-way ANOVA statistical test did not demonstrate a statistically significant interaction between rmTBI and castration ($F(1,88) = 1.839, p=0.18$) on mean latency time to leave center square. However, the test
did demonstrate significant main effects of rmTBI (p=0.020) and castration (p=0.030) on the mean latency time in leaving the center square at PTD 28. The two-way ANOVA statistical test did not demonstrate a statistically significant interaction between rmTBI and castration (F(1,88) = 0.019, p=0.89) on time spent in the inner, red-lined square. However, the test did demonstrate a significant main effect of castration on time spent in the inner square (p=0.048). The two-way ANOVA statistical test did not demonstrate a statistically significant interaction between rmTBI and castration (F(1,89) = 0.70, p=0.41) on the time spent freezing in the open field arena. However, the test did demonstrate statistically significant main effects of rmTBI (p=0.014) and castration (p=0.011) on the amount of time spent freezing in the arena. The two-way ANOVA statistical test did not demonstrate a statistically significant interaction between rmTBI and castration (F(1,90) = 0.92, p=0.34) on the amount of time spent exploring in the open field arena. Likewise, the test did not demonstrate statistically significant main effects of rmTBI (p=0.32) or castration (p=0.37) on amount of time spent exploring in the arena. n=23-24 per experimental group. Bars represent mean ± SEM.

Specific Aim 1, Experimental Question 2

Do anxiety-like behaviors in rats persist after rmTBI, castration, or combination of both?

This 5-hit closed-headed CCI rmTBI experimental animal model has been previously shown to lead to significantly reduced serum testosterone levels in the rmTBI groups compared to the sham groups for at least one month after rmTBI. The next experiment was designed to determine whether anxiety-like behaviors in rats persisted in the open field behavioral test at PTD 42, or if the anxiety-like behaviors would begin to wane over time. The 4 groups from the previous experiment, which were sham-intact, sham-castrate, rmTBI-intact, and rmTBI-castrate, were utilized again for this experiment, but included the PTD 42 timepoint. Similar to the last experiment, none of the anxiety-like behaviors observed at PTD 42 in Figures 13 and 14 demonstrated a significant interaction between rmTBI and castration on anxiety-like behavior. However, there again were multiple significant main effects present at this timepoint.
Figure 13. rmTBI Resulted in Fewer Red Lines Crossed at PTD 42. Graphs include behavioral data from open field behavioral test 42 days after final rmTBI. The two-way ANOVA for number of red lines crossed did not demonstrate a statistically significant interaction between rmTBI and castration (F(1,43) = 0.0035, p=0.95). The test did demonstrate a significant main effect of rmTBI on mean number of red lines crossed in the open field arena (p=0.02). The test did not demonstrate a significant main effect of castration on mean number of red lines crossed (p=0.11). The two-way ANOVA for number of wall explorations did not demonstrate a statistically significant interaction between rmTBI and castration (F(1,43) = 0.032, p=0.86). The test also did not demonstrate significant main effects of rmTBI (p=0.11) or castration (p=0.55) on number of wall explorations in the open field arena. n=11-12 per experimental group. Error bars represent mean ± standard error of the mean (SEM).
Figure 14. rmTBI Resulted in Decreased Latency Time and Time in Inner Square at PTD 42. These graphs depict (A) the mean latency to leave center square, (B) time spent in the inner square, (C) time spent freezing, and (D) time spent exploring the open field 42 days after final rmTBI. The two-way ANOVA test did not demonstrate a statistically significant interaction between rmTBI and castration ($F(1,41) = 1.20$, $p=0.28$) for the mean latency time to leave the inner square. The test did demonstrate a significant main effect of rmTBI on latency time to leave the inner square of the open field arena ($p=0.02$). The test did not demonstrate a significant main effect of castration on latency time to leave the inner square ($p=0.93$). The two-way ANOVA test did not demonstrate a statistically significant interaction between rmTBI and castration ($F(1,43) = 0.002$, $p=0.97$) for the time spent in the inner square of the open field arena. The test did demonstrate a significant main effect of rmTBI on time spent in the inner square of the open field arena ($p=0.04$). The test did not demonstrate a significant main effect of castration on time spent in the inner square ($p=0.35$). The two-way ANOVA test did not demonstrate a statistically significant interaction between rmTBI and castration ($F(1,21) = 0.33$, $p=0.57$) for the mean time spent freezing in the open field arena. The test did not demonstrate significant main
effects of rmTBI (p=0.64) or castration (p=0.44) on time spent freezing in the open field arena. The two-way ANOVA test did not demonstrate a statistically significant interaction between rmTBI and castration (F(1,21) = 1.16, p=0.29) for the mean time spent exploring in the open field arena. The test did not demonstrate significant main effects of rmTBI (p=0.37) or castration (p=0.84) on time spent exploring in the open field arena. Error bars represent mean ± SEM. n=11-12 per experimental group for 14A and 14B. n=4-7 per experimental group for 14C and 14D. INT = intact, CAS = castrated.

Specific Aim 1, Experimental Question 3

Will testosterone supplementation reduce persistent anxiety-like behaviors in rats?

Since anxiety-like behaviors persisted 42 days after the final rmTBI, and were significantly increased due to rmTBI, testosterone supplementation was given to the rmTBI groups to reduce persistent anxiety-like behaviors. Since the effect of testosterone supplementation on sham groups was not as relevant in the next experiment, only rmTBI groups given testosterone supplementation were analyzed in this statistical analysis. Also, a repeat measures four-way ANOVA is difficult to interpret and would not provide as much insight to help and answer the question posed in this experiment. In order to distinguish if testosterone supplementation could reduce persistent anxiety-like behaviors, testosterone capsules were implanted in rats at PTD 35 and the open field behavioral test was conducted again one week later, at PTD 42. The animals in this experiment were tested in the open field arena twice, at PTD 28 and PTD 42. A change score was calculated to determine the change in frequency or duration of previously identified anxiety-like behaviors. These scores were calculated by taking the value from each individual animal at PTD 42 for a particular observational variable and subtracting the value from that same animal at PTD 28. A positive change
score means that the animal exhibited a higher frequency or a longer duration of that event on PTD 42 than at PTD 28. Interestingly, there were no significant interactions or main effects of rmTBI or testosterone treatment on any of the anxiety-like behaviors observed from PTD 28 to PTD 42. The testosterone treatment did not result in a significant decrease in anxiety-like behavior at PTD 42, one week after testosterone was given systemically. Summary data for change scores for anxiety-like behaviors are represented in Figures 15 and 16. These anxiety-like behaviors persisted at PTD 42 and did not improve with treatment given at PTD 35.
Figure 15. No Change in Number of Red Lines or Wall Explorations from PTD 28 to PTD 42. Graph depicts average change score from PTD 28 to PTD 42, 7 days post testosterone treatment, for (A) number of red lines crossed and (B) number of wall explorations. Change score is calculated by taking the value at PTD 42 – value at PTD 28 for each animal in each group, represented as individual points on graph. The two-way ANOVA analysis for change in number of red lines crossed did not demonstrate a statistically significant interaction between castration and testosterone treatment ($F(1,24) = 0.90, p=0.35$). The test did not demonstrate a significant main effect of castration on mean change in number of red lines crossed in the open field arena ($p=0.17$). The test did not demonstrate a significant main effect of testosterone treatment on mean change in number of red lines crossed ($p=0.59$). The two-way ANOVA analysis for change in number of wall explorations did not demonstrate a statistically significant interaction between castration and testosterone treatment ($F(1,24) = 1.28, p=0.27$). The test did not demonstrate a significant main effect of castration on mean change in number of wall explorations in the open field arena ($p=0.16$). The test did not demonstrate a significant main effect of testosterone treatment on mean change in number of wall explorations ($p=0.10$). n=7 per experimental group. Error bars represent mean ± SEM.
Figure 16. No Change in Time Spent in Inner Square, Latency Time, Time Freezing, or Time Exploring from PTD 28 to PTD 42. Graph depicts average change score from PTD 28 to PTD 42, 7 days post testosterone treatment, for (A) time in the inner square, (B) latency time to leave inner square, (C) time spent freezing, and (D) time spent exploring. Change score is calculated by taking the value at PTD 42 – value at PTD 28 for each animal in each group, represented as individual points on graph. The two-way ANOVA analysis for change in time spent in inner square did not demonstrate a statistically significant interaction between castration and testosterone treatment (F(1,22) = 0.84, p=0.37). The test did not demonstrate a significant main effect of castration on mean change in time spent in inner square of the open field arena (p=0.53). The test did not demonstrate a significant main effect of testosterone treatment on mean change in time spent in inner square (p=0.53). The two-way ANOVA analysis for change in latency time to leave inner square did not demonstrate a statistically significant interaction between castration and testosterone treatment (F(1,24) = 0.04, p=0.84). The test did not demonstrate a significant main effect of castration on mean change in latency time to leave inner square of the open field arena (p=0.62). The test did not demonstrate a significant main effect of testosterone treatment on mean change in latency time to leave inner square (p=0.50). The two-way ANOVA analysis for change in time spent freezing in open field arena did not demonstrate a statistically significant interaction between castration and testosterone treatment (F(1,24) = 0.06, p=0.80). The test did not demonstrate a significant main effect of castration on mean change in time spent freezing in the open field arena (p=0.58). The test did not demonstrate a significant main effect of testosterone treatment on mean change in time spent freezing (p=0.51). The two-way
ANOVA analysis for change in time spent exploring in open field arena did not demonstrate a statistically significant interaction between castration and testosterone treatment (F(1,21) = 0.006, p=0.94). The test did not demonstrate a significant main effect of castration on mean change in time spent exploring in the open field arena (p=0.15). The test did not demonstrate a significant main effect of testosterone treatment on mean change in time spent exploring (p=0.84). n= 6-7 per experimental group. Error bars represent mean ± SEM.

**Specific Aim 1, Experimental Question 4**

Will supplementing with testosterone at a more acute timepoint, either immediately or 7 days following the last rmTBI, reduce anxiety-like behaviors in rats?

Throughout the previous experiments, ANOVA statistical tests were utilized to analyze the behavioral data. However, once the experimental design included the testosterone supplementation factor, 3-way ANOVA tests were required and could not be accurately analyzed with this experimental design. This was due to an uneven number of samples within each group, as well as some groups being absent from this analysis. For example, the sham-castrate plus immediate testosterone treatment group was excluded from this analysis since it was deemed unnecessary, as this group would be no different physiologically than the sham-intact group. Therefore, multiple linear regression models were used in this experiment and all subsequent experiments in this thesis to effectively analyze and interpret the behavioral data. The factors included in this regression included rmTBI, castration, and testosterone supplementation. Figure 17 demonstrates the mean time spent in the inner square during the 10 minute trial in the open field arena amongst all experimental groups. Anxiety-like behavior was significantly reduced following immediate testosterone, but not 7-day testosterone, in the rmTBI-intact group.
Figure 17. Injured Rats Demonstrated Reduced Time in Inner Square, Reversed by Immediate Testosterone Treatment. Graph includes rats from each experimental group at PTD 14, either one week (7-day T) or two weeks (Immediate T) after testosterone treatment. The multiple linear regression model was used to test if rmTBI, castration, and testosterone treatment significantly predicted the time spent in the inner square. The fitted regression model was time spent in the inner square (sec) = 33.51 – 12.36*(rmTBI) + 18.03*(immediate testosterone treatment). The overall regression for the data was statistically significant ($R^2=0.1536$, $F(4,60) = 2.723$, $p=0.04$). It was found that rmTBI predicted the time spent in the inner square ($β=-12.36$, $p=0.008$). It was also found that immediate testosterone treatment predicted the time spent in the inner square ($β=18.03$, $p=0.012$). Castration was not a predictor of time spent in the inner square ($p=0.72$), nor was 7-day testosterone treatment a predictor of time spent in the inner square at PTD 14 ($p=0.54$). $p<0.05 = *$. $n=8-10$ per experimental group. Error bars represent mean ± SEM.

Specific Aim 2, Experimental Question 5

How does rmTBI affect a marker of mitochondrial function in dopaminergic neurons in the medial amygdaloid nucleus?

The medial amygdaloid nucleus contains TH+ cells that also highly express ARs in several species of rodents, which include the Syrian hamster$^{70}$ and the prairie vole$^{76}$. Since testosterone is suppressed after rmTBI and the medial amygdaloid nucleus,
involved in defensive and anxiety-related social behaviors, contains a high level of AR expression, the effect of androgens within this brain region are important to explore after rmTBI. This thesis is one of the first academic works to demonstrate the presence of TH+ cells within rat medial amygdaloid nucleus. The data collected and analyzed from the in situ experiments in this thesis demonstrated clusters of TH mRNA through in situ hybridization that represent TH+ cells in rat medial amygdaloid nucleus. The animals utilized in specific aim 2 are those from the early anxiety timepoint behavioral data at PTD 14. Animals tested in the open field arena at PTD 14 were sacrificed at PTD 15 and brains were quickly extracted. These animals had been tested in the open field only once the day prior to sacrifice. In order to identify TH+ cells within the medial amygdaloid nucleus, fluorescence in situ hybridization was utilized to detect TH mRNA expression. TH+ cells were selected and quantified by drawing regions of interest (ROI) around each TH positive cell, deemed positive based on pre-selected criteria and depicted by a red line, using a freehand drawing tool in the CellSens Dimension computer program. Criteria utilized to determine a TH+ cell included: at least five nuclear-associated puncta, puncta were circular in shape, puncta were bright, and puncta still met all previous criteria after eliminating autofluorescence or bleed through between channels. An example of a TH+ cell, depicted by the presence of clusters of red TH puncta within blue-colored cell bodies stained with DAPI, are presented in Figure 18 in a rmTBI-intact rat.

TH+ cells were identified in both sham and rmTBI animals. However, rmTBI animals demonstrated clearer, more distinct TH+ signal compared to sham animals. This was determined through a qualitative assessment as clusters of TH puncta appeared brighter and more concentrated within the nucleus in the rmTBI group (Figure
18B) compared to the sham group (Figure 18A). Once TH+ cells were identified, ROIs were drawn around TH mRNA puncta with red lines using a freehand drawing tool. These ROIs depicted regions of the nucleus with a high density of TH mRNA puncta, where the mRNA was likely being transported outside of the nucleus and followed the previously defined criteria to be a TH+ cell. Representative images for mean number of ROIs per experimental group is demonstrated in Figure 18. The representative image for mean number of ROIs in sham animals is presented in Figure 18A. Likewise, the representative image for mean number of ROIs in rmTBI animals is presented in Figure 18B. TH puncta, regardless of ROI designation, appear more diffusely spread across the medial amygdaloid nucleus within the sham group, demonstrated in Figure 18A, compared to more concentrated TH puncta present within ROIs in the medial amygdaloid nucleus in the rmTBI group, demonstrated in Figure 18B.
Figure 18. Representative Examples of Number of ROIs Between Sham and rmTBI Groups. These images provide representative examples of the number of ROIs taken at 20X magnification in the medial amygdaloid nucleus. Images are presented as follows: (A) sham and (B) rmTBI. DAPI = blue staining, TH = red puncta. Red circles indicate TH+ cells.
Specific Aim 2, Experimental Question 6

Does testosterone supplementation at a more acute timepoint, either immediately or 7 days following last rmTBI, result in a reduced marker of mitochondrial dysfunction in dopaminergic neurons in the medial amygdaloid nucleus?

In order to visualize and quantify differences in the amount of COX4i1 mRNA expression, the ROIs previously drawn around TH+ cells were used to evaluate the amount of COX4i1 puncta within each ROI. Qualitative differences prior to quantification between sham, rmTBI without testosterone treatment, rmTBI with immediate testosterone treatment, and rmTBI with 7-day testosterone treatment are depicted in Figure 19.
Figure 19. Representative Examples of COX4i1 mRNA Expression in Sham, rmTBI, rmTBI + Immediate Testosterone, and rmTBI + 7-day Testosterone. These images provide representative examples of the number of COX4i1 puncta present within TH+ ROIs taken at 20X magnification in the medial amygdaloid nucleus. The sham group, represented in Figure 11A, portrays a 20X magnified image and depicts the white COX4i1 puncta present within both the entire section and within each ROI drawn with a red circle. The rmTBI group, represented in Figure 11B, similarly demonstrates the same magnification and white COX4i1 puncta present in each ROI as within the sham intact group. Both rmTBI with immediate testosterone (Figure 11C) and rmTBI with 7-day testosterone (Figure 11D) demonstrate the same medial amygdaloid nucleus region, but after respective treatment with testosterone. Red circles indicate TH+ cells. White puncta – COX4i1, Blue – DAPI staining.

Summary data for mean number of TH+ ROIs, mean number of COX4i1 puncta, and mean number of COX4i1 puncta per ROI are presented in Figure 20. These variables were used to identify any significant changes to TH+ cells within the medial amygdaloid nucleus after rmTBI. The main factors analyzed were rmTBI and testosterone supplementation. These data were statistically analyzed using a two-way ANOVA statistical test, followed by a post-hoc multiple comparisons test. Since not all experimental groups were present within the test (i.e. Sham-intact with immediate testosterone supplementation), an interaction between the two main factors could not be obtained.
Figure 20. rmTBI Resulted in Increased Number of TH+ ROIs. This image demonstrates (A) the mean number of TH+ ROIs, (B) COX4i1 puncta, and (C) number of COX4i1 puncta per TH+ ROI within each experimental group. TH+ ROI represents TH+ mRNA clusters associated with the TH+ cell soma. rmTBI Cas I (immediate testosterone treatment), rmTBI Cas 7 (7-day testosterone treatment). The two-way ANOVA statistical test used for the mean number of TH+ ROIs demonstrated a significant main effect of rmTBI (p=0.031) but did not demonstrate a main effect of testosterone supplementation (p=0.24). The multiple comparisons analysis demonstrated a significant difference between rmTBI with immediate testosterone treatment and Sham (p=0.046), as well as a trend for significance between rmTBI with immediate testosterone treatment and Sham (p=0.09), but did not demonstrate a difference between rmTBI with 7-day testosterone treatment and Sham (p=0.90). The mean number of COX4i1 puncta were analyzed using a two-way ANOVA statistical test, followed by a post-hoc multiple comparisons test. The two-way ANOVA did not demonstrate a significant main effect of rmTBI or main effect of testosterone treatment on the number of COX4i1 puncta present within the medial amygdaloid nucleus. Multiple comparison analysis did not find any statistically significant differences between experimental groups. Lastly, the mean number of COX4i1 puncta per ROI within the medial amygdaloid nucleus was statistically analyzed using two-way ANOVA statistical test, followed by a post-hoc multiple comparisons test. The two-way ANOVA statistical test did not demonstrate a statistically significant main effect of rmTBI or main effect of testosterone treatment on the mean number of COX4i1 puncta per ROI present within the medial amygdaloid nucleus. The multiple comparisons analysis did not demonstrate a significant difference between any experimental group. p= <0.05 = *. n= 3-6 per experimental group. Error bars represent means ± SEM.
CHAPTER SIX
DISCUSSION

The results from the behavioral experiments in the first specific aim of this thesis identified both acute and persistent anxiety-like behaviors that resulted after rmTBI. The effects of androgens on rmTBI through the removal and replacement of testosterone were also determined in order to identify an optimal therapeutic time window for testosterone replacement. The second specific aim of this thesis identified the medial amygdaloid nucleus, a region with TH+ cells that also highly express androgen receptors and looked at changes in expression of a marker of mitochondrial function to determine how it was affected by rmTBI. This brain region is known to be involved in the modulation of various fear and defensive behaviors, so it was of interest in the study of anxiety-like behaviors after rmTBI.

The open field behavioral test utilized in the first specific aim is a well-known assay used to assess anxiety-like behaviors in rats. When placed into a new environment, rats will explore the novel area. Rats that exhibit more anxiety-like behavior tend to stay out of the center of the open field due to increased risk of predation and will spend more time near the walls on the outside of the arena. This wall-hugging is known as 'thigmotaxis' behavior. Anxiety-like behaviors are identified in the open field through the amount of time spent in the center of the arena, the latency in leaving the center of the field after initial placement, behaviors such as freezing, exploring, or grooming, as well as the number of times an animal explores the
Anxiety is a common behavioral symptom that develops due to rmTBI and can be exacerbated. This thesis utilized a closed-headed CCI rmTBI model that has not before been used to study anxiety-like behaviors at both early and persistent anxiety timepoints. The effect of closed-headed CCI rmTBI was used in this study to model how rmTBI affects the development of anxiety in individuals that sustain multiple mTBIs. This model was used to characterize the effect of testosterone supplementation on anxiety that develops after rmTBI.

Since suppression of circulating testosterone occurs after rmTBI, this thesis addressed how hypogonadism could affect development of anxiety-like behaviors, or even exacerbate these behaviors. One region of concern implicated in the development of anxiety after rmTBI was the medial amygdaloid nucleus. This region contains dopaminergic neurons that could be affected by rmTBI and hypogonadism. Gonadal steroids are known to influence dopamine levels within various regions of the brain, either through testosterone or aromatase conversion into estrogen, following injury or induced neurotoxicity. The connectivity between regions involved in the anxiety neurocircuitry, such as the amygdala and the BNST, could be impaired functionally due to rmTBI. Overactivation, or an inability to attenuate activation, in the BNST after rmTBI could lead to deleterious anxiety symptoms if left untreated. Current studies often include only single mTBI and utilize different animal models. Rodents have been used in TBI research for decades now, in part due to less ethical concerns and better economic advantages. Even more, rats have been increasingly utilized as animal models in research due to the number of genetic tools available and the ease of working with the animal. Several species of rats have also been widely used in rmTBI.
research\textsuperscript{12,14,15}. This study utilizes the repeat mTBI rat model to determine how multiple mTBIs affect the development of anxiety that persists.

In order to better model the hypogonadal status of a rmTBI patient, castration groups were used to reflect persistently suppressed testosterone levels for at least a month after rmTBI. Additionally, the sham intact group was used to control for any effects of anesthesia on gonadal steroid levels in each experimental group. Since it was uncertain whether rmTBI animals would remain hypogonadal for at least one month after rmTBI, castration was used to ensure persistent hypogonadism through the length of this study. Testosterone can be suppressed in certain individuals for months or years post-TBI, even after a single mTBI\textsuperscript{79}. It is possible that testosterone will never return to baseline levels in some of these individuals. The amount of testosterone in circulation after subsequent testosterone supplementation can then be controlled and variability is greatly reduced.

Animals that were exposed to rmTBI and castration exhibited increased anxiety-like behaviors at PTD 28, although there were not any significant interactions between rmTBI and castration for the anxiety-like behaviors observed at this timepoint. This means that the separately calculated mean for rmTBI or the calculated mean for castrated groups demonstrated a significant main effect on an anxiety-like behavior, but there were no interactions found between rmTBI and castration. For example, both rmTBI and castration demonstrated significant main effects separately on the time spent freezing in the open field; however, there was not a significant interaction of the two factors on time spent freezing. This means that rmTBI caused an increase in freezing time and that castration caused an increase in freezing time when the groups were
averaged, independently. In addition, both rmTBI and castration demonstrated significant main effects separately on latency time to leave the inner square in the open field. There was also a significant main effect of castration on time spent in the inner, red-lined square. As far as time spent moving, there was not any significant interaction or main effects of rmTBI or castration on amount of time spent moving in the open field arena at PTD 28.

Expansive environments that without cover or refuge, such as a field without trees or vegetation, are associated with increased risk of predation compared to an area with cover or shelter. Experimentally, an open field behavioral test is utilized to assess anxiety-like behaviors associated with this risk of predation, such as avoidance behavior. In the open field arena, for example, increased anxiety-like behavior is represented by an animal that leaves the center red-lined square more quickly upon initial placement. When the animal remains closer to the walls and periphery of the arena, this exhibits thigmotaxis behavior. Some animals in the rmTBI groups could have had reduced levels of testosterone at PTD 28, which could then have resulted in exacerbated anxiety-like behavior demonstrated by a reduced latency to leave the center square.

The data presented in Figures 13 and 14 represent animals that were observed for anxiety-like behaviors in the open field behavioral test at PTD 42. These figures provide insight into the presence of anxiety-like behaviors beyond one month and the effect of rmTBI as anxiety-like behaviors are maintained or could even worsen. This experiment helped determine whether anxiety-like behaviors persisted, or were exacerbated, beyond one-month post-rmTBI. The analysis for the anxiety-like behaviors
observed at PTD 42 did not demonstrate any significant interactions between rmTBI and castration. However, there were several significant main effects for the anxiety-like behaviors. First, there was a significant main effect of rmTBI on mean number of red lines crossed at PTD 42. There was also a significant main effect of rmTBI on time spent in the inner square at PTD 42. However, there were no significant interactions or main effects for number of wall explorations, time spent freezing, or time spent moving at PTD 42 in the open field arena.

Each of these significantly reduced observational variables due to a main effect of rmTBI demonstrated persistent anxiety-like behaviors that lasted for over a month after the final rmTBI. Therefore, the anxiety-like behaviors measured by the open field behavioral test in this experiment did not wane over time, or improve without treatment or intervention, as suggested in previous studies. Since the persistent anxiety-like behaviors at PTD 42 did not improve without treatment, the next step was to determine if testosterone supplementation could effectively reduce persistent anxiety-like behaviors at this same timepoint.

The sample size for the first experimental design included a group number that was approximately double the number calculated in the power analysis when effect size was determined. Since a larger number was required for the subsequent testosterone supplementation experiment, the group number had to be twice the size of the first experiment to obtain sufficiently sized experimental groups. The problem with this large sample size and an overpowered experiment is an increased risk of finding significance for something that would not be meaningful clinically. In other words, overpowering an experiment can lead to significant findings for a miniscule effect that does not mean
anything. In addition, over-powered studies can mean wasted resources when it is not necessary to include that many samples. However, in the context of this study, it was necessary to add more animals to the experiment due to the combined groups that were not separated until a later experiment. It is important to make note of these possible risks when the data is interpreted for significant and meaningful results.

In order to study the effects of testosterone supplementation on persistent anxiety-like behaviors, crystalized testosterone-filled capsules were implanted in rats at PTD 35 and the open field behavioral test was utilized once again to determine changes in anxiety-like behavior. Since the rats tested in the open field at PTD 42 had already been subjected to the open field arena at PTD 28, a change score was calculated in order to determine the change in frequency or duration of anxiety-like behaviors between the two timepoints. A positive change score meant that the animal demonstrated a larger number or longer duration of a behavior in the open field on PTD 42 compared to the first open field test on PTD 28. For example, a positive change score of 5 in number of wall explorations for an animal meant that the animal demonstrated 5 more wall explorations in the open field on PTD 42 than in the open field on PTD 28. The results from this experiment, interestingly, did not demonstrate any significant interactions or main effects of rmTBI, castration, or treatment on anxiety-like behavior. This means that the one week of testosterone treatment did not effectively reduce anxiety-like behaviors in animals at PTD 42. The change scores for all anxiety-like behavioral variables were insignificant, which demonstrated that anxiety-like behaviors were similar at PTD 42 compared to PTD 28.
It is possible, however, that the testosterone supplemented only one week prior to the open field experiment at PTD 42 did not allow the supplemented testosterone enough time to initiate the intrinsic gonadal steroid-producing machinery within the body. Testosterone treatment has been utilized for a longer duration and has proven to be more therapeutic in some gonadectomized rodent models that study anxiety-like behavior\textsuperscript{80}. Other papers have reported that testosterone supplementation presented to the circulation demonstrates more benefits after it has been present for greater than one week\textsuperscript{81,82}, or several months in older, hypogonadal men\textsuperscript{83}. Another paper reported a reduction in depressive symptoms and an improvement in overall mood in hypogonadal men after testosterone supplementation at least one week after first treatment. This improvement persisted for at least eight weeks with consistent treatment\textsuperscript{81}. For these reasons, it was important to see what testosterone supplementation introduced earlier and for longer duration would do to these anxiety-like behaviors.

Alternatively, testosterone treatment given at PTD 35 could be too late to completely eliminate persistent anxiety-like behaviors. Suppressed testosterone levels within circulation of these individuals over time could increasingly exacerbate anxiety. Individuals that experience persistently suppressed testosterone levels after rmTBI could exhibit an increase in anxiety that is not effectively reversed by testosterone provided after prolonged deprivation\textsuperscript{52}.

Increased risk-taking behavior is a potential confound for the anxiety data at the more persistent anxiety timepoint, which includes open field tests at PTD 28 and 42. There is some debate within the literature on when this behavior begins after mTBI. Some investigators suggest that anxiety-like behaviors begin to turn into more risk-
taking behaviors as early as 14 days or one month in mice\textsuperscript{84}, while others speculate that risk-taking behavior isn’t observed until at least several months following injury\textsuperscript{85}. In order to identify risk-taking behavior within this experimental rmTBI model, more specific behavioral tests would be required to separate fear-related behaviors from risk-taking behaviors.

The action of testosterone in male Long-Evans hooded rats could also be affected due to pubertal changes that occur around the period in which animals receive rmTBIs. If testosterone is required during puberty but is suppressed in animals after rmTBI, then the body may not be as receptive to testosterone later in life. This provides a plausible explanation for the lack of response to testosterone in the reduction of anxiety-like behaviors at PTD 42. However, this explanation is unlikely for the experiments conducted in this thesis due to the specific experimental design chosen. Male Long-Evans hooded rats were approximately 60 days old when received and were acclimated to their new environment for at least 3-7 days before they received any rmTBI. Studies on male Long-Evans hooded rats have demonstrated that puberty ends at approximately 42 days after birth, measured by preputial separation and copulation behavior\textsuperscript{101,102}. Therefore, the male Long-Evans hooded rats in this study are more likely to have already gone through puberty, demonstrate proper receptiveness to testosterone, and have normal, physiologic testosterone levels prior to rmTBI.

Immediate testosterone supplementation provided after the final rmTBI was found more effective in reducing anxiety-like behaviors, compared to 7-day supplementation. Since immediate supplementation reduced anxiety-like behaviors, the next step was to determine whether immediate supplementation also facilitated proper
function at a cellular level after rmTBI. Specifically, the goal of the second aim was to determine whether immediate supplementation reduced mitochondrial dysfunction in the medial amygdaloid nucleus, a brain region of interest.

The medial amygdaloid nucleus is an important region of interest for rmTBI and anxiety, not only due to its potential link with anxiety-like symptomology, but for the localization of ARs\textsuperscript{86,87} and respective response to testosterone after rmTBI. Proper mitochondrial function is a requirement for all cells and must be regulated. Without proper mitochondrial function within the cells, cell death can occur. The second specific aim of this thesis was designed to identify changes in mitochondrial functioning as a response to injury, as well as in response to testosterone treatment. The goal was to measure COX4i1 expression within the medial amygdaloid nucleus as a marker of mitochondrial dysfunction following injury, reflected by a decrease in COX4i1 mRNA expression.

It was demonstrated that rmTBI caused an increase in the number of TH mRNA expressed by the number of isolated cells in the medial amygdaloid nucleus, based on visualization of ROIs in that brain region. If there was to be any change in the amount of TH mRNA present within injured animals, the expectation would have been a decrease in TH mRNA; however, what occurred was an increase in the amount of TH gene expression after rmTBI, and specifically through multiple comparison analysis in the rmTBI with immediate supplementation group. This study was one of the first within the literature to clearly demonstrate the presence of TH+ cells in the medial amygdaloid nucleus in rat through fluorescence \textit{in situ} hybridization.
This finding was particularly interesting due to the amount of controversy around presence of TH+ cells in the medial amygdaloid nucleus in rat. The literature has suggested that the presence of TH+ cells in the medial amygdaloid nucleus may be species-specific. Some papers have reported that TH+ cells do not exist in larger numbers, or in distinct populations, in the rat\textsuperscript{70,76}. Instead, investigators have shown TH+ cells in larger numbers in the male Syrian hamster\textsuperscript{70} and the prairie vole\textsuperscript{76}, but have claimed that TH+ cells are non-existent in the male rat. Studies in this thesis have demonstrated a small number of TH+ ROIs within the medial amygdaloid nucleus through fluorescence \textit{in situ} hybridization. In the sham group, TH positive cells were not obvious and the TH mRNA puncta could have been present more in the axons. However, after rmTBI, there was a visible increase in the number of TH mRNA puncta observed within the cell compared to TH mRNA puncta around the cells. This could be, in part, why other investigators have not report distinct, clear TH+ cells within the medial amygdaloid nucleus of rat. Clusters of red TH puncta were also denser in rmTBI animals through the \textit{in situ} experiments. This was determined through a qualitative assessment of images taken of the rmTBI group compared to the sham group. Despite not being as densely clustered, TH+ cells were still identifiable by TH mRNA puncta within sham animals. Examples of TH+ cells within rmTBI and sham groups were depicted in Figure 18. In order to corroborate these findings, the medial amygdaloid nucleus was also investigated through the Allen Institute Brain Atlas\textsuperscript{72}, which is an online database that includes chromogenic \textit{in situ} hybridization experiments and provides images for many different mRNA sequences, including TH. This database allowed for the identification of what appeared to be TH+ cells within the coronal
sections of the medial amygdaloid nucleus of non-injured animals. This was, however, in the mouse brain, as they do not have this specific atlas available in rat. This provides more support for the idea that TH+ cells are present within other species besides the Syrian hamster or the prairie vole. It is important to note that, while investigators have claimed to not find positive cells in the rat, much of the literature is older and the sensitivity of techniques like the modern-day in situ hybridization experiments are much more advanced than at that time.

As previously mentioned, the number of TH+ mRNA clusters were significantly increased after rmTBI regardless of testosterone replacement. While the immediate testosterone supplementation results show high variability and would require further experiments to increase the group n, these data could oppose the behavioral findings from specific aim 1. Immediate testosterone treatment in the first aim appeared to be more effective in reducing anxiety-like behaviors compared to the 7-day treatment. There are several possibilities regarding what is occurring within the medial amygdaloid nucleus. First, it could be that immediate testosterone is not neurotherapeutic within the medial amygdaloid nucleus at this PTD 14 timepoint. Since there was an increase in the number of TH mRNA clusters after rmTBI, this poses the possibility that an increase in TH mRNA clusters occurs as a pathophysiologic response to rmTBI. In this case, the 7-day testosterone treatment could allow more time for the brain to adequately recover before testosterone can act neurotherapeutically. The original hypothesis was that an increase in COX4i1 mRNA clusters would be therapeutic. However, that is not what resulted through this in situ hybridization experiment after rmTBI and testosterone supplementation; there was no significant change in COX4i1 mRNA expression. It is
currently unknown whether an increase in TH+ cells in the medial amygdaloid nucleus is
detrimental or therapeutic. Some studies have reported increased dopamine levels one
month after TBI in the frontal cortex and the striatum due to dysregulation in
dopaminergic neurotransmission\textsuperscript{89,90}. This response is in stark contrast with the
decrease in dopamine expression that occurs in other brain regions, such as the
substantia nigra, after TBI\textsuperscript{68,91}. Changes in dopamine expression after rmTBI are region-
specific and require further studies to help identify the mechanism in which
dopaminergic neurotransmission is affected within each brain region. However, for the
medial amygdaloid nucleus, this study demonstrates a significant increase in TH mRNA
expression that suggests increased dopamine expression after rmTBI. Secondly, it
could be that testosterone treatment does not affect the number of TH mRNA clusters
within this brain region at all, and the only difference seen is by rmTBI. In that scenario,
the compensatory increase in TH mRNA clusters could be declining by the PTD 14
timepoint and is not affected by treatment.
Figure 21. TH+ Cell in the Medial Amygdaloid Nucleus. This image depicts a TH+ cell within the medial amygdaloid nucleus of a rmTBI-intact male rat at 20X magnification. Red puncta represent TH, DAPI-blue.

These apparent “cells” may not actually be cell bodies but could instead be axonal clusters or axon terminals surrounding a particular cell. Each of these possibilities were contemplated after examination of previous immunohistochemistry work on medial amygdala brain sections from a collaborator in the department and within previous literature on the presence of mRNA trafficking down axons and toward axon terminals. TH mRNA has been found within the axons of sympathetic neurons and trafficking of this mRNA can be disrupted, which leads to impaired synthesis of catecholamines, such as dopamine\textsuperscript{92,93}. One downside to using \textit{in situ} hybridization is that only the mRNA within cells is fluorescently labeled and you are unable to see the cell itself, so you cannot effectively localize where the mRNA is present within the cell. The mRNA could then be within the nucleus, the cytoplasm, in the axon, or at the axon
terminal of the pre-synaptic neuron. Further experiments are necessary to better visualize where the TH mRNA is localized; however, the fact that this thesis may be one of the first to detect TH mRNA in the rat at all is already novel in and of itself. The localization of TH mRNA will help better determine the location of dopaminergic neurons within the medial amygdaloid nucleus. TH is required to synthesize dopamine, and rmTBI resulted in an increase in TH expression within this region. An increase in TH mRNA within the cell body could be due to disruption in TH mRNA trafficking down the axons after rmTBI, or it could be that TH gene expression is increased in dopaminergic neurons after rmTBI. It is also possible that dopamine needs to be synthesized more rapidly and secreted at the axon terminals surrounding a particular neuronal synapse after rmTBI.

One hypothesis of this thesis was that TH expression in the medial amygdaloid nucleus would be decreased following injury, as a result of mitochondrial dysfunction and potential cell degeneration and death. Interestingly, TH expression levels, qualitatively measured through number of TH+ ROIs, was increased in the medial amygdaloid nucleus as a result of rmTBI. Changes in TH expression may be more region-specific, where TH expression levels are increased after rmTBI in areas like the frontal cortex\textsuperscript{94} and the substantia nigra\textsuperscript{95}. Alternatively, other brain regions have demonstrated decreased TH expression levels after rmTBI, such as the ventral tegmental area\textsuperscript{96} and the median eminence\textsuperscript{97}. The medial amygdaloid nucleus could be an area that exhibits an increase in TH expression after rmTBI.

Rat brains in this study were collected 14 days after final rmTBI. It is possible that compensatory responses are occurring rather than degeneration at this timepoint.
Neuronal degeneration could take months to occur in certain brain regions after TBI or within neurodegenerative disease, so there may be compensation that occurs earlier which could then contribute to degenerative processes if left unchecked. There were previous cohorts of rats that had gone for one or more months after rmTBI prior to sacrifice and brain collection. The likelihood of observing degeneration is higher in those brains compared to brains in this study that were collected at an earlier timepoint in this study. In addition, increases in TH expression have been shown in other animal models including hypothermal stress\textsuperscript{98}, immobilization stress\textsuperscript{99}, and foot shock stress\textsuperscript{100}.

Contrary to the hypothesis, rmTBI did not affect COX4i1 expression in cells that showed increased TH mRNA and suggests at this early timepoint there was no degeneration due to mitochondrial dysfunction. There did not appear to be any significance when comparing the five groups of interest for any of the three variables investigated. The amount of time following injury, however, may not be long enough to see true degeneration in the dopaminergic cells within the medial amygdaloid nucleus. Instead, there could also be compensatory responses occurring within this region following injury. It is possible that the TH+ cells within this area are not experiencing mitochondrial dysfunction, or that any dysfunction is not yet evident due to compensatory responses within the cells of this brain region. In addition, the n for a few of the groups is not large and, with a few more animals added to each of those groups, there may be more significant results for each of the COX4i1 \textit{in situ} hybridization variables.

Throughout the \textit{in situ} hybridization process, optimization of the method was required in order to obtain the best possible results from the tissue and to accurately
detect the fluorescent label within that tissue. However, due to technical difficulties with the \textit{in situ} hybridization procedure and vulnerability with the amygdala brain region, many samples could not be processed. This reduced the total number of viable samples for the \textit{in situ} hybridization experiments.
CHAPTER SEVEN

CONCLUSIONS

The goal of this thesis was to find answers to several important questions surrounding rmTBI, changes in gonadal status, and anxiety. These goals included identifying the effect of rmTBI on anxiety-like behaviors, determining if androgen supplementation reduces anxiety-like behaviors after rmTBI, and testing whether mitochondrial dysfunction within the medial amygdaloid nucleus is associated with rmTBI and subsequent reduction of normal, physiologic testosterone levels.

The first specific aim focused on identifying anxiety-like behaviors in rats through the open field behavioral test. Indeed, there were significant increases in various anxiety-like behaviors at PTD 28 following the five-hit rmTBI experimental rat model. This information helps provide insight into individuals experiencing multiple mTBIs and experiencing anxiety as a prevalent symptom and could encourage seeking medical attention sooner rather than later. The development of anxiety-like behaviors can persist without proper diagnosis and treatment.

Animals were observed in the open field at PTD 42 to determine if anxiety-like behaviors were reduced one week after testosterone supplementation began. The results of this experiment were less clear. Significant changes in change score between PTD 28 and PTD 42 were not seen in any of the anxiety-like behaviors observed, even in animals that were given testosterone supplementation. Ideally, testosterone supplementation would be neurotherapeutic and reduce anxiety-like behavior long after
the final rmTBI. However, testosterone supplementation could require more than one week before it demonstrates an effect, as the gonadal machinery in animals deficient for an extended period of time. Testosterone supplementation provided immediately after the final rmTBI reduced anxiety-like behavior, as shown by an increase in latency in time to leave the center square, compared to the 7-day testosterone supplementation. This further supports the idea that individuals could continue to experience persistent anxiety and should be diagnosed as soon as possible. It also stresses the need for continued development of neurotherapeutic therapies and treatments. The earlier those therapies and treatments are provided to the individual, the better they will fare and have a better health outcome.

The second specific aim was designed to identify whether the medial amygdaloid nucleus, implicated in the anxiety neurocircuitry, could be exposed to oxidative stress following rmTBI and result in increased anxiety-like behaviors. Interestingly, mitochondrial function in this region was not impacted after rmTBI, as measured by COX4i1 expression, at this particular timepoint. This could be because COX4i1 was the only marker of mitochondrial function utilized in these experiments. It could also be that COX4i1 expression levels measured by fluorescent in situ hybridization follow a more complex pattern after rmTBI. The number of TH+ cells in the medial amygdaloid was increased as a result of rmTBI. This result still requires further study, specifically looking further into the presence of TH+ cells and TH expression within the medial amygdaloid nucleus in the rat. Whether it be disrupted TH mRNA trafficking away from the cell body after rmTBI that results in higher density of TH label in the cell body, or increased
transcription of TH at the axon terminal that results in increased dopamine synthesis and secretion, this novel finding generates even more questions.

The goal of this thesis was to test the effect of systemic testosterone supplementation on markers of mitochondrial function in the medial amygdaloid nucleus. Therefore, testing whether immediate or 7-day testosterone supplementation could be neurotherapeutic within this brain region was important to future studies. However, there did not appear to be any significant findings when comparing groups in terms of COX4i1 mRNA expression levels. It is possible then, that testosterone acts in another brain region and does not affect mitochondrial function in the medial amygdaloid nucleus after rmTBI. Many other parts of the brain are being affected and could contribute to the detrimental outcomes of increased anxiety. In addition, this study looked at COX4i1 expression as a marker of mitochondrial function, but there may be other markers that could provide more information about what occurs in the mitochondria and in the cell that require further study.

Overall, it appears that androgens are neurotherapeutic in reducing multiple anxiety-like behaviors expressed by rats within the open field behavioral test. The androgens given systemically, however, did not cause a change in COX4i1 expression levels within the medial amygdaloid nucleus at the earlier anxiety timepoint. Alternatively, the behavioral data in this thesis suggests that the earlier the testosterone supplementation, the better. While the immediate testosterone supplementation was not as neurotherapeutic in the medial amygdaloid nucleus, as far as COX4i1 expression levels and proper cell function, it likely acts within other regions important for emotional regulation and processing in the brain.
CHAPTER EIGHT

FUTURE DIRECTIONS

The work in this thesis has resulted in several novel findings that promote individuals with TBIs to seek medical attention sooner. It also encourages medical professionals to use broader screening methods, such as testing for androgen levels in individuals that sustained a single mTBI or rmTBI. Beyond the scope of this thesis, many of these findings pose even more questions that require further experiments. First, immediate testosterone treatment appeared to have the most significant effect on anxiety-like behaviors, as demonstrated by time spent in the center square of the open field at PTD 14. The next step would be to determine if immediate testosterone treatment also helps reduce anxiety-like behaviors following injury at later timepoints, either at one month or several months beyond injury. While immediate testosterone treatment did not appear to have the same effect on mitochondrial function and TH expression in the medial amygdaloid nucleus, it could still be acting in another brain region to help reduce anxiety-like behaviors in rat. Therefore, further experiments would be necessary to better grasp the location immediate testosterone treatment is most neurotherapeutic.

Second, while the use of the open field behavioral test is a standard test to measure anxiety-like behaviors, there are a variety of other behavioral tests that could provide further insight into various behaviors related to anxiety. Behavioral tests such as the elevated plus maze and the light-dark box have also been used within the anxiety
Literature. Each of these tests can identify other behavioral phenomena that occur as a result of rmTBI and could be essential to gain a better understanding of the symptomology and potential for neurotherapeutic and/or neuroprotective gonadal steroid treatments.

Third, further experiments are necessary to determine accurate localization of TH mRNA in the medial amygdaloid nucleus after rmTBI. One such experiment could be to utilize both fluorescence *in situ* hybridization + IHC on medial amygdaloid brain sections to better visualize whether the TH mRNA is located in the cell body, cytoplasm, axon, or another location in the cell. This would provide more information on where the TH expression is after rmTBI compared to a sham animal, whether that be through increased dopamine secretion as a compensatory response in this region or stuck within the axon of a neuron due to disruption of trafficking after rmTBI.

Lastly, while the effect of testosterone treatment on proper mitochondrial function in the medial amygdaloid nucleus did not appear to be significant, further *in situ* hybridization experiments to detect AR expression within the medial amygdaloid nucleus of rats could provide more insight into how testosterone interacts in this region. Identifying where/if androgens bind in rat medial amygdaloid nucleus would allow for greater understanding of changes in gonadal status following injury. Other papers reported high levels of AR and TH colocalization in cells in the medial amygdaloid nucleus of the Syrian hamster\textsuperscript{70}. However, it has been reported that this population of TH+ cells is species-specific, so it would be important to gain a better understanding of AR colocalization in TH+ cells in the rat as well.
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