INVESTIGATING THE ROLE OF PREMORBID CHRONIC STRESS IN TBI RECOVERY IN MICE: A PILOT STUDY

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ABSTRACT

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Traumatic brain injury (TBI) is a prominent cause of premature death, disability, and financial burden worldwide. Prolonged exposure to stress serves as a precursor to several mental health conditions that are known to complicate the process of recovery from TBI. Animal models offer unique utility to the study of this intersection of affective disorders and TBI. Thus, the purpose of this study was to ascertain the significance of premorbid stress exposure as a risk factor for functional and psychological deficits following TBI in mice. A Chronic Unpredictable Mild Stress protocol was employed to induce stress, then both the stressed and non-stressed mice were then randomly assigned to receive either an impact or sham surgery procedure. All mice were assessed for signs of functional recovery and underwent comprehensive behavior testing. Findings from this study indicated that exposure to stress contributed to variable behavior responses in both the acute (2 weeks) and post-acute (1 month) stages of TBI recovery. Further, differences in anxiety and depression-like behaviors were more pronounced among mice that sustained a moderate TBI, compared to a mild injury. Future research should continue to refine both the procedure for inducing concussion and mild TBI in mice, as well as the procedures for assessing the nuanced functional and behavioral recovery in this population to better understand vulnerability factors that contribute to prolonged recovery.

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CHAPTER I

INTRODUCTION

Traumatic brain injury (TBI) occurs when a sudden external or accelerating force causes disruption in normal brain function (Maas et al., 2017). The severity and duration of subsequent impairment depends on the impact on the skull and underlying tissue, as well as the focalized or global location of injury and presenting neurological symptoms (Belanger et al., 2017). While many individuals who suffer TBIs experience a full recovery, some experience persistent neurological, cognitive, and emotional symptoms, and varying degrees of associated functional impairment (Roebuck-Spencer & Sherer, 2017). Several pre-morbid mechanisms have been studied for their contribution to poor recovery outcomes, including comorbid psychiatric and physical illness, pain, substance use, poor sleep, and stress (Belanger et al., 2017). This research aimed to elucidate the role of pre-injury stress, depression, and anxiety in TBI recovery using an animal model.

Background

TBIs represent a significant source of global disability and burden for individuals across the life span (Dewan et al., 2018; Nguyen et al., 2016). Causal mechanisms of head injury are vast, with the most common sources including transportation-related events, sports, and military combat (Belanger et al., 2017). Due to the unpredictable and often unpreventable nature of many TBI events, pathology and treatment remain the focus of research. Animal models provide significant utility in understanding TBI in humans. Various methods of injury have been developed and validated in mice to observe TBI pathology and outcomes in a safe and cost-efficient manner (Ma et al., 2019). Additionally, animal models of TBI are particularly advantageous in their capacity to control for and observe pre-injury factors, which are otherwise studied through self-report in clinical populations. Questions regarding the contribution of pre-existing conditions in TBI, therefore, may be addressed using animal models.

Research Problem

Recent research indicates that any cognitive, functional, and behavioral deficits as a result of TBI are expected to resolve through rehabilitation and spontaneously, apart from some severe, focal injuries (Belanger et al., 2017). However, a prolonged recovery sequence marked by general malaise, neurological complaints, and psychiatric distress, previously referred to as postconcussive syndrome (PCS), has been uniquely observed among individuals sustaining concussion, mild TBI, and some moderate injuries (American Psychiatric Association [APA], 2013; Carroll et al., 2014; McMahon et al., 2014). The heterogeneity of this prolonged recovery trajectory suggests that some individuals may possess a predisposed vulnerability to poor TBI outcomes.

Aims and Objectives

The purpose of this study was to examine the role of chronic, unpredictable stress in functional and behavioral recovery from TBI in mice. A chronic unpredictable mild stress (CUMS; Katz & Wykes, 1985) protocol was employed to induce stress in the experimental group. Stress-exposed and control mice were then randomly assigned to receive either an impact or sham surgery procedure. A measure of functional recovery and tests of behavior were used to investigate the overall impact of pre-injury stress on TBI outcomes. We hypothesized that mice exposed to stress and impact surgery would demonstrate poorer recovery on standardized functional and behavioral measures.

Significance

A more thorough understanding of the role of chronic stress in TBI outcomes has the potential to provide valuable insight into areas of intervention in rehabilitation. Importantly, individuals who are young or of advanced age, victims of assault, possess low socioeconomic status, and veterans are among the most vulnerable to both TBI and affective disorders, and thus have the most to gain from advancements in recovery research (Belanger et al., 2017).

The use of animal models allows for further exploration of the relationship between stress and TBI while protecting vulnerable populations. The CUMS model in particular has been established as an effective tool with translational utility to human models of anhedonia and apathy and has the potential to demonstrate the significance of chronic emotional distress in the recovery process (Antoniuk et al., 2019). Findings from this study may contribute to understanding the pathophysiology of TBI and the effects of stress, as well as identifying effective treatments for those who suffer from TBIs.

Limitations of Animal Models

Despite the advantages, animal models also possess shortcomings. Psychiatric conditions in humans are highly complex, and often manifest in a wide range of clinical presentations. It is because of this complexity that animal models will undoubtedly fall short of fully capturing the human experience (Planchez et al., 2019). Similarly, heterogeneity of outcomes following TBI is common in both human and animal models (Belanger et al., 2017; Fehily et al., 2019). As such, this study aimed to focus on specific, reproducible symptoms of anhedonia shared among several psychiatric disorders and pursue uniformity in impact administration in hopes of minimizing investigator-induced variability.

CHAPTER II

LITERATURE REVIEW

Introduction

The increasing incidence of TBI produces significant and cumulative economic and social cost (Jassam et al., 2017; Maas et al., 2017). Certain environmental factors, such as stress, may indirectly exacerbate the impact of these injuries (Belanger et al., 2017). In order to treat and rehabilitate individuals who sustain TBIs, a thorough understanding of pre-injury risk factors and vulnerabilities is needed. The purpose of this study was to explore the relationship between premorbid exposure to stress and TBI recovery outcomes. First, current relevant literature related to TBI is reviewed, including a discussion of the prevalence, classification, treatments, and areas of future research. Next, literature related to chronic stress in humans and animals is discussed, focusing on the role of stress in TBI, as well as methods for inducing stress using animal models. The literature review concludes with a rationale for the proposed investigation.

Translational Utility of Animal Models

The following investigation utilized an animal model with the aim of understanding the relationship between stress and traumatic brain injury in humans. As such, a review of relevant literature related to the human experience of stress-induced psychopathology and TBI is covered in this section. The existing gaps in the literature concerning the human experiences of both stress and TBI serve as rationale for present and future exploration of this relationship in animal models. Further, animal models provide robust utility in the study of mechanisms, impairment, and rehabilitation of TBIs, as well as in the process of validating methods of clinical treatment prior to use in humans (Malkesman et al., 2013).

The present study involved comparison of controls and a stress-induced phenotype in response to a single impact injury. The chosen method of stress-exposure was designed to produce behavioral manifestations of anhedonia, or anxiety, which are observable using several

established, face-valid behavior tests (Belzung & Lemoine, 2011; Katz & Baldrighi, 1982). These symptoms are prominent among diagnostic criteria for several mental health syndromes and disorders and provide this study with translational significance (Kotov et al., 2017, Latzman et al., 2020; Perkins et al., 2020). Additionally, because human and animal models of stress involve similar physiological and biological processes, animal models of stress possess mechanistic validity in their ability to consistently demonstrate the effects of a stress condition (Antoniuk et al., 2019). Additionally, the stress-induction method utilized in this study maintains predictive validity, as the protocol consistently reproduces similar responses using different animal models (Belzung & Lemoine, 2011). Thus, the proposed model of observing stress-induced changes in TBI recovery in mice offers a unique contribution to TBI research in humans.

Traumatic Brain Injury

TBI is a prominent cause of premature death, disability, and financial burden worldwide (Dewan et al., 2018). The heterogeneous nature of injury pathology and clinical presentation creates challenges for establishing best practices in identification, diagnosis, and treatment. Review of the current literature related to TBI reveals significant gaps involving pre-injury risk and protective factors that may impact treatment success.

Defining the Problem

TBI involves a disruption in brain function or pathology due to insult or force (Maas et al., 2017). Injuries are classified by type (i.e., penetrating or closed-head injury), length of alteration in consciousness, presence of pre- or post-traumatic amnesia, findings on imaging, and functional impairment (Belanger et al., 2017).

Prevalence and Cost

The global incidence rate of TBI is estimated at 69 million occurrences annually (Dewan et al., 2018). TBIs incur an estimated annual cost of $400 billion and represent a substantial

burden for families and health care systems globally (Jassam et al., 2017; Maas et al., 2017). In the United States alone, TBI-related events lead to approximately 56,000 deaths annually, with the highest rates of injury occurring among individuals over the age of 75 and under the age of 24 (Taylor et al., 2017). Estimates of the cost of comprehensive medical services associated with a single TBI in the U.S. range from $9,000 to $103,667 (Dismuke et al., 2015). Thus, further understanding of the variation in TBI recovery trajectories may reduce the financial burden associated with TBI among individuals and healthcare systems.

Severity Classification

TBI severity is determined at the time of injury by evaluation of the following factors: alterations of consciousness (AOC) or loss of consciousness (LOC) measured by the Glasgow Coma Scale (GCS; Teasdale & Jennett, 1974), presence and severity of post-traumatic amnesia (PTA), presence of skull fracture, or evidence of intracranial lesion on neurological examination or neuroimaging (Belanger et al., 2017; Roebuck-Spencer & Sherer, 2017). Changes in consciousness are often assessed by neurological exam and may be quantified by the amount of time it takes for an individual to become responsive to the point of following simple commands (Roebuck-Spencer & Sherer, 2017). The GCS is a standardized instrument for classifying responsiveness in the first 24 hours following a TBI that can provide useful predictions related to recovery outcomes (Teasdale & Jennett, 1974). The GCS has a score range of 0 to 15, with 15 representing the mildest changes in consciousness. A score between 3 and 5 on the GCS indicates a severe TBI, while a score between 9 and 12 classifies a moderate TBI (Roebuck-Spencer & Sherer, 2017). Individuals who score between 13 and 15 on the GCS are diagnosed with a mild TBI. Individuals may experience PTA, which involves memory loss due to an acute period of confusion or disorientation following a TBI wherein new memories cannot be formed (Roebuck-Spencer & Sherer, 2017). The length of PTA is another indicator of

injury severity and may last between 1 and 7 days in severe TBI (Roebuck-Spencer & Sherer, 2017).

The mechanism and location of injury can also contribute to TBI severity classification. Contact injuries, wherein the head is struck by a force, or the brain otherwise comes into contact with the skull, may cause scalp lacerations, skull fractures, contusions, and hematomas (Roebuck-Spencer & Sherer, 2017). Acceleration/deceleration injuries incurred from abrupt and forceful movement often contribute to more global strain on the brain and cause vascular injury or tearing, hematomas, and diffuse axonal injury (Roebuck-Spencer & Sherer, 2017). Some injuries may be classified as focal, or contained to a particular region of the brain, while others may demonstrate a more diffuse pattern through downstream neuropathological processes such as swelling, hypoxia, pressure, and infection (Roebuck-Spencer & Sherer, 2017).

Although not fully understood, additional research suggests that TBI may provoke an underlying neurometabolic cascade wherein neuronal membrane regulators require increased metabolic fuel in the form of adenosine triphosphate (ATP), which subsequently increases glucose metabolism, decreases blood flow, and ultimately creates an energy crisis that renders the brain vulnerable to further injury or lasting symptoms (Giza & Hovda, 2001). Evidence of this pathophysiological response to injury can be seen in neuroimaging scans post-injury but may manifest in a wide array of clinical symptoms across injury type and severity (McCrea, 2008). This study sought to understand the role of environmental factors in combination with underlying pathophysiological recovery processes in mild and moderate head injuries.

Moderate and Severe TBI

Moderate and severe TBIs were estimated to compose approximately 15% of military service related TBIs in 2020 and between 10-30% of all TBIs in the United States annually (Belanger et al., 2017; U.S. Department of Defense, 2016). Classification of moderate TBI requires a GCS score between 9 and 12, 1 to 24 hours of PTA, and the individual must be able

to follow basic commands within 6 hours of the injury (Roebuck-Spencer & Sherer, 2017). The most common pathologies of moderate and severe head injuries involve intracranial tissue damage, either from skull penetration or secondary injury processes. Diffuse axonal injury (DAI) is the most severe form of TBI and involves a pattern of axon shearing, tissue tearing, and hemorrhage throughout the brain. The trajectory of recovery for individuals who experience the more severe forms of TBI often includes a period of coma or a vegetative state, followed by progress through a minimally conscious state and posttraumatic confusion before regaining full consciousness. Previous data indicate that people with severe TBIs may continue to recover, through rehabilitation and spontaneously, for up to 2 years (Roebuck-Spencer & Sherer, 2017). Persisting cognitive, motor, and psychological impairments may occur dependent on the primary location, severity, and mechanism of injury.

Mild TBI and Concussion

Mild TBI represents approximately 70-90% of all TBIs that occur in the United States annually, or nearly 1.36 million (Belanger et al., 2017). Mild TBI is often used synonymously with the term concussion in the literature; however, a concussion may also refer to an injury considered less serious than a mild TBI. Additionally, when an individual presents to a medical facility with a vague report of head injury or no one witnessed the event, the injury may be referred to as a possible mild TBI (Belanger et al., 2017). Diagnosis of a mild TBI is predicated on a GCS score above 13, LOC or AOC lasting 30 minutes or less, or a period of PTA that does not exceed 24 hours post-injury (American Congress of Rehabilitation Medicine, 1993). A mild TBI can be further classified as a complicated mild TBI when an individual satisfies the criteria for a mild TBI, but also demonstrates positive signs on a neurological exam or abnormal findings on neuroimaging. An estimated 80-99% of people who sustain mild TBIs are expected to progress to a full recovery within a few days, with a small number of injuries that require up to a year to recover (Belanger et al., 2017; Pundlik et al., 2020). Physical injuries that accompany

a TBI, as well as pre-injury chronic pain, sleep disorders, mood disorders, substance use disorders, and overall poor health status may complicate or prolong an individuals' return to baseline function (Belanger et al., 2017). As mild forms of TBI are the most prominent and most often linked to prolonged symptoms, this study utilized an animal model of TBI that is generally consistent with mild, complicated mild, or moderate injuries.

Populations of Interest

Leading causes of TBI in the United States include falls, transportation-related accidents, assault, and other blows to the head by or against various objects (McCrea, 2008). Individuals who are of young or advanced age, male gender, black and indigenous person of color (BIPOC) identity, low socioeconomic status, or have a history of substance use disorders are more vulnerable to experiencing TBIs (Belanger et al., 2017). Research on the topic of mild TBI specifically has focused on injuries in sport and military contexts.

Sport-related head injuries are most often referred to as concussions but share clinical presentation characteristics with mild TBI. Many athletes depend on their ability to return to play in order to pay for their education or earn a living and may therefore be less motivated to address a suspected injury with coaches and trainers (Belanger et al., 2017). Due to the high rates of under-reporting in athlete populations, the published annual incidence rate, between 1.6-3.8 million, is likely an underestimate (Belanger et al., 2017; King et al., 2014). American football, ice hockey, and soccer players are at the highest risk for concussion. Progress in the research related to TBI and risk factors is needed to better advocate for athletes involved in ongoing and future litigation for sport concussion.

Literature documenting the incidence of concussion and mild TBI in military populations is vast, earning classification as the "signature injury" among veterans (Belanger et al., 2017). Progress in medicine and protective equipment have contributed to fewer fatal TBIs and an increasing number of veterans living with head injuries sustained during combat (Boyle et al.,

2014). A significant majority of mild TBIs acquired among members of the Army are due to blast exposure, which can be further classified by blast severity. While mild TBIs sustained by civilians are expected to recover fully in a short time with minimal persisting symptoms, similar injuries experienced by veterans may be complicated by environmental factors and exposure to forms of trauma that are unique to deployment (Carroll et al., 2004). A more thorough understanding of the interplay between emotional and physical trauma is needed to provide the highest standard of care to this uniquely vulnerable population of individuals.

Factors Involved in Recovery and Treatment

Interdisciplinary collaboration in treatment is common in TBI rehabilitation, and most often includes physicians, physical therapists, occupational therapists, speech therapists, and psychologists. The main goals in the acute stages of recovery are achieving medical stability and treating co-occurring physical injuries. Inpatient and outpatient rehabilitation services may follow medical stability and include therapies administered in a graded manner according to injury severity and individual patient needs (Pundlik et al., 2020).

Physical Recovery

Many events that result in head injury, such as motor vehicle accidents and assault, also pose a risk for other forms of physical injury. While injuries associated with TBI-causing events have not been extensively studied, severe comorbid physical injuries are associated with poorer outcomes and higher rates of disability (Carroll et al., 2004; Dunn et al., 2000). Additionally, comorbid pain is a significant predictor of persisting cognitive complaints and PCS symptoms among individuals with concussion and mild TBI (Belanger et al., 2017).

Apart from the diagnostic indicators described, several other acute symptoms may follow injury and resolve proportionally to TBI severity. These symptoms include seizures, slurred speech, poor coordination, lethargy, vomiting, headache, dizziness, irritability or uncharacteristic emotionality, light sensitivity, tinnitus, and changes in concentration (McCrea, 2008).

Cognitive Recovery

Cognitive deficits, particularly in attention, memory recall, executive functioning, and processing speed, are expected within the first few days following TBI (Carroll et al., 2004; Roebuck-Spencer & Sherer, 2017). Neuropsychological test performance generally indicates a full recovery of cognitive function between 3- and 12-months post-injury, with rare and more severe TBI cases requiring up to 24 months. Various focal injuries may cause persisting impairments in isolated functional areas such as vision perception or language. There is also literature to suggest that a history of moderate to severe TBI increases vulnerability for developing various dementias later in life (Roebuck-Spencer & Sherer, 2017). Multiple mechanisms have been proposed to explain the relationship between TBI and dementia, largely related to long-term effects of the neurometabolic cascade and the impact of injury on cognitive reserve (McCrea, 2008; Stern, 2009).

Psychological Recovery

An abundance of empirical support suggests that TBI is a risk factor for development of several psychiatric disorders (Belanger et al., 2017). Depressive disorders are the most common psychiatric comorbidity with TBI, particularly among those who sustain mild injuries. Individuals with pre-existing depressive symptoms prior to injury demonstrate poorer outcomes on measures of functional recovery and general mental health (Kumar et al., 2014). Prominent depressive symptoms in this population include somatic symptoms and stress, which may exacerbate post-injury anxiety and prolong recovery (Bay & Covassin, 2012; Belanger et al., 2017). Further, co-occurring hormonal and inflammatory responses complicate the task of differentiating physical from emotional consequences of trauma (Shulman, 2020). Thus, animal models of TBI provide unique utility in research because objective measures of recovery are less impacted by this parallel physical and psychological experience of trauma.

Gaps in the Literature

Recent media attention on TBIs has contributed to widespread misinformation about the expected course of recovery. More specifically, misconceptions about the expected course of concussion and mild TBI pose a unique risk affecting individuals, families, and communities. Individuals sustaining mild injuries showed a significant increase in PCS symptoms over time in a 3–12-month period (McMahon et al., 2014). Previous studies have addressed iatrogenic effects of TBI diagnosis: the observable changes in cognition, emotion, and behavior, which cannot be attributed to the injury itself, preceded by medical attention or diagnosis. Recent findings indicate that the specific terminology used to characterize the injury, such as using the label of concussion rather than mild TBI, can contribute to dramatically different recovery outcomes (Belanger et al., 2017). The impact of this slight change in language is consistent with additional research supporting a strong relationship between measured outcomes and patient beliefs about head injuries, self-concept, outcome expectations, and perceived injustice related to injury (Iverson et al., 2018; McMahon et al., 2014; Snell et al., 2013). Attribution errors may partially explain this relationship, as individuals may associate mild and common physiological symptoms, such as headaches or fatigue, to a previous head injury rather than to benign causes (Belanger et al., 2017). Taken together, the factors that may contribute to individual vulnerability to prolonged recovery following TBI have yet to be fully understood. This study aimed to provide supporting evidence to the literature that points to behavioral and emotional factors as a primary contributor to prolonged recovery from most mild and moderate TBIs.

Chronic Stress

Premorbid stress exposure is one area of particular interest in investigations of individual vulnerability to prolonged TBI recovery. The experience of chronic stress provokes similar neuropathological consequences to that of head injuries and may therefore represent a substantial risk factor for complications.

Defining the Problem

Stress can be generally conceptualized as a physiological and behavioral response to an environmental threat (McEwan et al., 2012). An acute stressor, such as detection of life-threatening danger, causes activation of the hypothalamic-pituitary-adrenal axis (HPA) and subsequent release of the primary stress hormone, cortisol (Campbell & Ehlert, 2012). Release of cortisol has downstream effects on the skeletomuscular system, preparing for "flight" via lung constriction, increased heart rate, muscle contraction, and increased cognitive vigilance (Campbell & Ehlert, 2012). This adaptive response to the environment and restoration of homeostasis is referred to as allostasis (McEwen, 2006). The immediacy of the body's physiological response, which is seen in humans and animals, serves as a protective factor in adaptation (Sapolsky et al., 2000). However, forms of emotional trauma, abuse, significant life events, and other non-life-threatening circumstances in the environment can trigger the same physiological stress response (McEwen, 2006).

Over time, recurrent events that are perceived as threatening may contribute to more chronic forms of stress and may lead to over-activation and inflammation of peripheral systems that maintain allostasis (McEwen, 2006). Chronic stress has been implicated in several debilitating illnesses such as depressive disorders, cancers, cardiovascular disease, and Alzheimer's disease (Pavlides et al., 2002). Prolonged excitation of the stress response induces physiological and subsequent behavior changes such as sleep disturbance, appetite changes, reductions in physical activity, and substance use (McEwen, 2006). These symptoms trigger a behavioral cascade that increases and maintains overall negative affect and serves as a precursor to anxiety and depression (Watson & Clark, 1984).

Prevalence, Cost, and Populations of Interest

A survey of stress-related symptoms published in 2017 indicated that over 70% of people in the U.S. experience physical or psychological symptoms of stress, including fatigue,

headaches, GI distress, tension, irritability/anger, nervousness, and tearfulness. Estimates of the annual cost of stress-related health care and productivity in the U.S. approached nearly $300 billion in 2014 (American Psychological Association, 2017). Despite increasing levels of stress, about half of Americans surveyed reported using strategies for stress management, which included both healthy coping skills, such as exercise or meditation, as well as unhealthy coping skills, such as smoking (American Psychological Association, 2017). Reduced ability to cope with chronic stress contributes to rising levels of depression and anxiety disorders (Watson & Clark, 1984).

In 2015, global estimates of the number of individuals living with a depressive disorder was over 300 million, serving as the largest source of disability around the world (World Health Organization, 2017). Depression is a cause of premature mortality due to suicide as well as its direct and indirect contribution to various comorbid illnesses (Üstün et al., 2004). The World Health Organization (2017) estimates the number of people with anxiety disorders to be close to 264 million worldwide. Anxiety disorders may present differently across cultures, but often involve a recurrent course with associated impairment in education, role, income, and relationships (Baxter et al., 2013; Baxter et al., 2014; World Health Organization, 2017).

Individuals in highly developed countries may be more vulnerable to anxiety and depressive disorders; however, attempts to compare incidence rates of anxiety and depression across cultures have been complicated by variation in symptom manifestation and diagnostic tools. In the United States, increased vulnerability for mental health-related disability and utilization of welfare services is most prominent among individuals who experience heightened distress and disparities in access to resources due to various and intersecting marginalized identities. Some of these contributing identity factors include sexual orientation and gender identity (Cambron et al., 2014; Cochran et al., 2003), race and ethnicity (Neighbors et al., 2007; Randle, 2021; Riolo et al., 2005), and socioeconomic status (Bassuk et al., 1998; Baum et al.,

1999; Reiss, 2013). As such, understanding more about chronic stress, particularly that related to the experience of discrimination, can provide insight into preventative treatment and recovery from stress-induced psychiatric conditions among vulnerable populations.

Stress and TBI

The potentially detrimental effects of stress are particularly salient in recovery from TBI. As previously mentioned, many individuals report post-concussive symptoms and deficits long after the expected recovery trajectory (Kennedy et al., 2007). Comorbid stress, anxiety, and depressive disorders are a commonly studied mechanism for this prolonged period of deficit (Iverson, 2005). Studies of the relationship between chronic stress and TBI have shown higher levels of perceived stress and hypocortisolemia among individuals with poor psychological outcomes following mild-moderate TBI (Bay et al., 2009). Evidence suggests that greater pre- and post-injury psychological stress may account for functional impairment following mild TBI (Bay & Liberzon, 2009). Individuals presenting with persistent impairment beyond expected trajectories, previously diagnosed as PCS, often report vague cognitive, neurological, and emotional complaints. This impairment profile may progress to continued decline from baseline, under- and unemployment, and rumination on the injury as a part of one's identity (Bay & Liberzon, 2009; Belanger et al., 2017). Recent research has also examined the phenomenon of cogniphobia following mild TBI, which is conceptualized as a stress-related avoidance response to cognitively stimulating activity (Silverberg et al., 2017). Cogniphobia may be a product of conditioning during acute TBI recovery phases, when individuals may see a drastic change in function and develop an aversion to activities that may further expose areas of weakness (Silverberg et al., 2017). Such self-imposed limitations of ability may contribute to overall disability following TBI. The degree of stress, both before and after injury undoubtedly plays a role in the recovery process.

Chronic Unpredictable Mild Stress Model (Katz & Wykes, 1985)

While animal models necessarily fall short of providing robust generalizable data to human populations regarding complex psychological syndromes, they provide invaluable utility in exploring the mechanisms of behavioral sequelae and specific symptoms. CUMS (Katz & Baldrighi, 1982) is one of the most widely used methodologies for understanding how the effects of stress contribute to many mental health disorders.

Background and Theoretical Framework

The stress-diathesis hypothesis of depression suggests that, in addition to genetic predisposition, recurrent exposure to traumatic experiences helps to determine an individuals' vulnerability to developing an affective disorder (Willner, 2017). Early versions of the CUMS protocol were developed using the stress-diathesis hypothesis and sought to recreate a chronic stress phenotype in rats and mice by administering a series of mild stress conditions over several weeks. Examples of such stress conditions included disturbance of the animals' food and water schedule, introduction of unpleasant sounds or cage conditions, and exposure to the scent of a socially threatening animal (Antoniuk et al., 2019). Stressors are administered in an unstructured and variable manner to prevent the animal from adaptation or learning to predict incoming stimuli.

The CUMS method offers a relevant proxy for the continuous pattern of distress and diminished stress management often endorsed by individuals who are diagnosed with affective disorders (Antoniuk et al., 2019; Nollet et al., 2013). The CUMS model has also been proven effective to induce comparable changes in atrophy of the cortex and limbic region, hippocampal neurogenesis, serotonin, noradrenergic activation, inflammation, gliosis, and HPA axis activity to clinical manifestations of major depressive disorder (MDD), as well as diagnostic indicators of behavioral apathy and anhedonia (Antoniuk et al., 2019; APA, 2013).

Validity and Reliability

Recent meta-analysis of studies utilizing a CUMS protocol acknowledge its robust association with depression-like anhedonia, with marked heterogeneity observed with alterations in protocol (Antoniuk et al., 2019). Compared to other animal models of depression, including physical pain, learned helplessness, chronic social defeat, and chronic restraint, CUMS outcomes most effectively induce symptoms like anhedonia, reduced reward response, and changes in sleep (Song & Kim, 2021; Zhu et al., 2014).

Several studies have validated the CUMS method using established animal behavior tests. The sucrose consumption test, forced swim test (FST), and tail suspension test (TST) are well-validated measures of depression-like behaviors in rodents, such as anhedonia and despair, which have repeatedly demonstrated the efficacy of the CUMS model for inducing a state of affective distress (Monleon et al., 1995; Steru et al., 1985; Willner et al., 1987; Zhu et al., 2014). The sucrose consumption test acclimates the animals to the taste of sucrose, a sweet additive to water, to test the animals' pleasure-seeking behavior (Willner et al., 1987). The FST involves placing the animal in a small beaker of water to observe behavioral indications of despair (Porsolt et al., 1977; Porsolt et al., 1978). The TST provides similar data to the FST, wherein the animal is suspended by the tail to a rod and observed for attempts to right itself (Steru et al., 1985).

Chronic unpredictable mild stress protocols have also elicited commensurate anxiety-like phenotypes to established behavior tests, such as the open field or dark-light box test, novelty-suppressed feeding, and elevated plus maze (EPM; Katz et al., 1981; Zhu et al., 2014). The open field or dark-light box (DLB) test involves placing the animal in a contained area and observing the animals' willingness to explore the open or well-lit areas compared to time spent in darker areas (Katz et al., 1981). Novelty-suppressed feeding utilizes a similar technique, but first deprives the animal of food for a short period to test the motivational impact of hunger in

exploring the open area (Li et al., 2010). The EPM involves placing the animal in a device with open and closed arms and observing the animals' willingness to explore (Katz et al., 1981). The expected outcome of the CUMS protocol includes behavioral manifestations of symptoms such as despair, anhedonia, or anxiety.

The Relationship Between Chronic Stress and TBI

The impact of chronic unpredictable mild stress on functional and psychological recovery from TBI is not well understood. On occasion, stress induction protocols have been utilized concurrently with TBI or repeated mild TBI surgeries in rodents. Findings from these studies indicate that post-injury HPA axis abnormalities contribute to hyperactivity, which may serve as a protective factor against manifestations of affective disorders (Algamal et al., 2019; Patricia et al., 2021). Yet, other studies have observed greater anxiety and social behavior impairment, as well as increased corticosterone, axonal injury, and inflammation among mice who were exposed to both a stress condition and mild TBI compared to controls (Ojo et al., 2014). Impaired fear-inhibition circuitry due to stress exposure within the ventral medial prefrontal cortex has been studied as a potential mechanism in the development of post-injury PTSD, depression, and anxiety symptoms (Bryant, 2011). The present study aimed to contribute to the literature related to chronic stress and brain injury, with particular interest in the role of pre-injury stress in TBI recovery and post-injury anxiety- and depression-like behaviors.

Gaps in the Literature

Given the prominence of stress-related functional and psychological impairment following TBI, understanding factors of individual vulnerability or resilience was a priority of this study. Aspects of neurogenesis, glucocorticoid receptor availability, high concentration of corticosterone (CORT), mitochondrial dynamics, HPA-axis function, fatty acid metabolism, and inflammation have been studied as potential risk factors for vulnerability to stress in animals and humans (Aliev et al., 2020; King et al., 2001; Van Zuiden et al., 2011). Similar processes have

been implicated in TBI sequelae, including the proposed neurometabolic cascade and subsequent neuroinflammation, cell damage and axonal shearing (Belanger et al., 2017). Thus, chronic premorbid stress may pose a unique source of risk in TBI outcomes.

Summary and Rationale for the Proposed Investigation

TBIs represent a significant source of burden for individuals, families, and medical systems around the world. Previous history of TBI, particularly of a mild-moderate severity, increases individual vulnerability to persistent psychological and functional symptoms and subsequent development of a range of affective disorders (Belanger et al., 2017; Iverson, 2005; Kennedy et al., 2007). Chronic exposure to stress, manifested in increased allostatic load, is also a precursor to several mental health conditions, and may produce compounded complications in the process of TBI recovery (Bay & Liberzon, 2009; McEwen, 2006). Animal models offer unique utility to the study of this intersection of affective disorders and TBI (Antoniuk et al., 2019; Ma et al., 2019). Thus, the purpose of this study was to ascertain the significance of premorbid stress exposure as a risk factor for functional and psychological deficits following TBI in mice

CHAPTER III

METHODS

Research Design

This study involved a 2x2 repeated measures factorial design investigating the impact of pre-injury exposure to chronic stress on functional and behavioral outcomes following mild traumatic brain injury. Thus, the independent variables of this inquiry included two experimental conditions: 1) implementation of a chronic unpredictable mild stress protocol to induce a mixed anxiety- and depression-like phenotype (Antoniuk et al., 2019; Katz & Baldrighi, 1982; Song & Kim, 2021; Willner, 2017); and 2) controlled administration of a TBI, induced by impact surgery. The dependent variables included standardized measures of functional recovery, including the Neuro Severity Score (NSS; Flierl et al., 2009), in addition to established behavior tests to assess anxiety- and depression-like symptoms, including DLB, EPM, TST, and FST. The animals exposed to an extensive stress protocol were hypothesized to exhibit overall poorer outcomes following surgical procedures. Further, the compounding interaction of stress exposure and TBI was expected to produce the poorest recovery outcomes. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas Woman's University, which complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (see Appendix A).

Animals

A total of 66, 8-week-old C57bl6 mice were used in all experiments. The mice were housed in cages of five and acclimated for 1 week prior to experimentation. The animals were maintained in an animal housing unit at a constant temperature with a 12-hour light/12-hour dark cycle and unlimited access to food and water. Following acclimation, an adjusted 4-week CUMS protocol was administered to half of the mice to delineate the healthy and stress condition groups. Following the CUMS protocol, the two groups were randomly assigned to one

of four groups: Non-CUMS Sham (NS [*n* = 15]; control with sham surgical procedures), CUMS Sham (CS [*n* = 15]; stress-exposed with sham surgical procedures), Non-CUMS TBI (NT [*n* = 14]; control with impact surgery), and CUMS TBI (CT [*n* = 22]; stress-exposed with impact surgery; see Table 1).

Table 1

Experimental Design

Variable	Sham procedure (S)	TBI procedure (T)
Non-CUMS (N)	NS (*n* = 15)	NT (*n* = 14)
CUMS (C)	CS (*n* = 15)	CT (*n* = 22)

Independent Variables

Stress Procedure

Prolonged exposure to stress is implicated in anxiety- and depression-related syndromes due to the development of apathy and anhedonia (McEwen & Sapolsky, 1995; Nestler et al., 2002). Animal models of chronic exposure to unpredictable mild stressors remain a prominent and effective method of inducing and investigating the role of stress in affective disorder development (Antoniuk et al., 2019; Katz & Baldrighi, 1982; Song & Kim, 2021; Willner, 2017). Past data suggest that rotating different stress-inducing procedures may be the most effective method of preventing habituation to a stressful environment thereby producing a chronic state of distress (Antoniuk et al., 2019).

The CUMS protocol for this experiment incorporated the most common and effective components indicated in the literature, including the following: food and water deprivation, light

cycle modification, soiled bedding exposure, cage tilting, social stress, and noise (Antoniuk et al., 2019). Chronic and unpredictable stress procedures were conducted in randomized order for 4 weeks prior to surgical procedures (Zhu et al., 2014). Outcomes of CUMS administration have been validated using several behavior tests including DLB, EPM, TST, and FST (Deng et al., 2015; Katz et al., 1981; Zhu et al., 2014). Results from this study were expected to replicate previous findings by eliciting symptoms of apathy and anhedonia predominantly among stress-exposed mice (Nollet et al., 2013).

Surgery Procedure

Aseptic techniques were used, and animals were treated with necessary postoperative analgesics, oxygen, and wound care. Animals were placed on a homeothermic pad throughout surgical procedures to maintain body temperature. Mice were initially anesthetized (3% isoflurane) and maintained via vaporizer (1-3% isoflurane). Pre-operative analgesia was administered (bupivacaine and lidocaine 50/50 drip; 0.5 mL 2% lidocaine, 1 mL 0.5% bupivacaine, 0.5 mL sterile saline), and ointment applied to protect the eyes. The head of each animal was shaved and disinfected prior to transfer to a stereotaxic frame fixed with the controlled cortical impact device.

Once the animals were anesthetized, an incision was performed on the scalp to expose the skull. Approximately half of the mice, composed partly of stress-exposed and non-stressed animals, were exposed to a single impact injury. Injury was induced by a mechanical impact device (Impact One from Leica) using a blunt, impactor tip 5 mm in diameter according to previously described animal models of head injury (Chen et al., 1996; Flierl et al., 2009; Hylin et al., 2013; Laurer et al., 2001; Mouzon et al., 2012). The center of the impactor tip was positioned over the sagittal suture midway between lambda and bregma, and driven at a velocity of 5 m/sec to a depth of 1.0 mm. This procedure was intended to mimic a mild to moderate level of injury, which is not localized but rather causes a mild, global effect (Belanger

et al., 2017). Following procedures, incisions were cleaned and closed with sutures or tissue glue, and animals were transferred to a heated chamber. Animals were monitored for righting reflex immediately following surgery. Approximately 1-hour post-surgery, mice were evaluated for baseline functional impairment using a modified version of the Neuro Severity Scale (Flierl et al., 2009), prior to returning to home cages. Following baseline assessment, mice were returned to clean home cages and monitored closely for signs of pain and distress. Sham animals, including stress-exposed and non-stressed animals, were anesthetized, incised, and tested for subsequent recovery measures, but did not receive an impact injury. Any animals sustaining a skull fracture were excluded from the study (n = 6).

Dependent Variables

Assessment of Functional Outcomes

One aim of this investigation was to explore potential variance in the trajectory of functional recovery between stressed and non-stressed mice. Functional recovery measures were used to compare the speed and quality of restoration to fundamental pre-injury motor abilities. Aspects of recovery were observed for 14 days immediately following sham or impact surgeries.

Return-of-Righting Reflex (To & Nasrallah, 2021)

Return-of-righting reflex (RRR; To & Nasrallah, 2021) refers to a one-time measurement of the amount of time each animal requires to recover from pre-surgery sedation. Time was measured from the moment impact or sham procedures conclude to the first sign of 'righting,' or attempts to return to a prone position. Extended latency to righting may indicate variation in responses to anesthesia or injury.

Neurological Severity Score (Flierl et al., 2009)

The Neurological Severity Scale (NSS; Flierl et al., 2009) is a standardized tool for evaluating recovery of ten specific areas of function following impact or sham procedures. The

current study utilized a modified version of NSS (mNSS), which typically includes a brief assessment of the following abilities or attributes on a binary (present/absent) scale: exit circle; monoparesis/hemiparesis; straight walk; startle reflex; seeking behavior; beam balancing; round stick balancing; beam walk (3 cm); beam walk (2 cm); beam walk (1 cm; see Appendix B for a sample mNSS protocol record form). The mNSS was administered to all groups at the time points *t* = 1, 24, 72 hours, and 7 days post-injury, with *t* = 1 hour serving as a baseline.

Assessment of Behavior Outcomes

Behavioral measures were used to further investigate the effects of pre-injury stress on post-injury recovery. All behavioral tests were administered 14- and 30-days post-injury and quantified by Ethovision software (Noldus et al., 2001).

EPM (Pellow et al., 1985)

Mice are placed on an elevated maze with two open and two closed arms. Mice are then observed for exploration and activity within the arms of the maze for 10 minutes. Increased time spent in the closed arms of the maze is an indicator of an anxious phenotype.

DLB (Hall, 1934; Walsh & Cummins, 1976)

Mice are placed in a contained space for 10 minutes. Animals are observed for activity in the lit portion of the field relative to the dark portion of the container. Increased time spent and activity within the dark portion of the field reflects anxiety-like symptoms.

TST (Steru et al., 1985)

Mice are secured to a suspension rod by adhesive tape and are suspended for 6 minutes. Time spent immobile is considered an indicator of helpless behavior, similar to apathy or anhedonia.

FST (Porsolt et al., 1977; Porsolt et al., 1978)

Mice are placed in a cylinder of room temperature water for up to 6 minutes. Immobility or minimal activity reflects a sense of helplessness or despair.

Hypotheses and Statistical Analyses

All hypotheses predicted that exposure to stress and TBI would lead to poorer outcomes (i.e., more neurological dysfunction, more behavioral anxiety or learned helplessness) on functional and behavioral measures, and better outcomes on these measures (i.e., minimal to no neurological dysfunction, minimal anxiety-like or learned helplessness behavior) would be observed among the no stress and sham conditions. Specifically, the stressed-TBI group was expected to exhibit the most significant functional and behavioral impairments, followed by the non-stressed TBI group, the stressed-sham group, and finally the non-stressed-sham group. All hypotheses were tested with two-way analysis of variance (ANOVA) tests to compare scores among stressed, non-stressed groups, sham, and injury condition groups over time. Tukey's or Fisher LSD post-hoc tests were used to identify the time point at which the experimental groups differ and to maintain a conservative familywise error rate. Additionally, differences in performance between experimental groups on all measures were analyzed by one-way ANOVAs. A significance level of $p = 0.05$ was used for all analyses.

Data Collection

CUMS protocol (see sample in Table 2) was administered by three lab technicians. Impact surgery preparation and administration were performed by a graduate student and a principal investigator across 2 days. Functional and behavioral tests were administered by 2 graduate and 2 undergraduate students, and data were collected both manually and using Ethovision software (Noldus et al., 2001).

Table 2

Sample CUMS Stressor Schedule

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	Water deprive	Light cycle	Wet bedding	Cage tilt	Social stress	Strobe light	White noise
Week 2	Light cycle	Social stress	Food deprive	Strobe light	Wet bedding	White noise	Cage tilt
Week 3	Wet bedding	Strobe light	Light cycle	White noise	Cage tilt	Water deprive	Social stress
Week 4	Cage Tilt	White noise	Wet bedding	Food deprive	Social stress	Light cycle	Strobe light

CHAPTER IV

RESULTS

The overall goal of the study was to understand the impact of stress on recovery from mild-moderate TBI using an animal model. The independent variables included the following conditions: no stress or chronic stress as well as a sham or TBI procedure. The dependent variables included functional (RRR, mNSS) and behavioral measures including the EPM (Pellow et al., 1985), DLB (Hall, 1934; Walsh et al., 1976), TST (Steru et al., 1985), and FST (Porsolt et al., 1977; Porsolt et al., 1978) to determine the effect of the independent variables.

Adjustments to Procedure

At the conclusion of the first round of experiments, tissue from the brains of all mice were harvested for further analysis of stress- and injury-related changes. Based on post-mortem analysis, seven sham surgery mice sustained unintended lesions as a result of the craniotomy surgery. Given the literature concerning the functional and behavioral impact of lesions this size (Siebold et al., 2018), the injuries among these mice were considered more severe, and were therefore removed from further analysis. In order to more effectively characterize the outcomes of a mild TBI, a second cohort of mice was given a closed-skull impact surgery, as outlined in previous studies of mild TBI (Flierl et al., 2009). A third cohort of mice was given a “moderate TBI” using craniotomy or open-skull surgery to increase the sample size from the first cohort of mice. Head injuries were characterized as “moderate TBI” if exposed to the open-skull procedure, and “mild TBI” if conducted using the closed-skull impact surgery. Thus, mice were separated into two classifications of TBI injury severity, which is reflected in the cohort summaries contained in Table 3.

Subjects

A total of 66, 8-week-old C57bl6 mice were involved in all experiments. The mice were housed in cages of five and acclimated for 1 week prior to experimentation. The animals were

maintained in the vivarium at a constant temperature with a 12-hour light/12-hour dark cycle and had *ad libitum* access to food and water.

The first round of surgeries included 20 mice, the second round included a total of 25 mice, and the third round included a total of 21 mice. In total, 38 of the 66 mice were randomly assigned to a 4-week CUMS protocol, wherein a mild stressor was administered once per 12-hour period, or once during light hours and once during dark hours. Age-matched littermate control mice were randomly assigned to the non-stressed group in which mice were housed in standard ventilated cages and were left unperturbed during the duration of the stress procedure.

The first and third round of surgeries involved an open-skull TBI induction method in which all mice underwent isoflurane anesthesia and a craniotomy, wherein a small drill was used to remove a small portion of the skull, exposing the motor cortex. Across the first and third rounds, 18 mice received the sham procedure in which the skull flap was immediately replaced, while 23 mice receiving the impact procedure sustained a moderate-level injury using a controlled cortical impact device. Injuries were induced at a depth of 1 mm at a velocity of 5 mm/sec. The second round of surgeries involved a closed-skull TBI induction method in which isoflurane-anesthetized mice received an incision to expose the skull. Fifteen mice received a mild TBI, in which the controlled cortical impact device delivered an impact at a depth of 1 mm at a velocity of 5 mm/sec using a rubberized, blunt impactor, while 10 sham mice were immediately sutured. Two mice died during surgery or within the first 14 days following and were excluded from analyses. Another two mice died within 14 days of the surgery, and thus are included in analyses of functional recovery, but for which behavioral data are not available. Upon autopsy, six mice sustained significant lesions, and are excluded from further analyses. Table 3 includes the final count among each comparison group.

Table 3

Experimental Group Sizes

	Mild (*N* = 25)		Moderate (*N* = 33*)	
	Sham (S)	TBI (T)	Sham (S)	TBI (T)
Non-CUMS (N)	5	5	6	6
CUMS (C)	5	10	10[a]	11[a]

Note. CUMS = Chronic Unpredictable Mild Stress.

[a]Includes two mice that died after functional assessment but prior to behavior testing.

Measures

As indicated, a one-way ANOVA was performed to compare performances among the four experimental groups on measures of functional and behavioral outcomes at 2 weeks and 1-month post-surgery. When statistically significant differences were found, Fisher's LSD Test for multiple comparisons was used to maintain a conservative familywise error rate while exploring specific group differences. A two-way ANOVA was used to analyze the contribution of stress exposure, TBI, and the interaction of stress and injury in performance on functional and behavioral outcome measures.

Functional Recovery Measures

RRR

Mild TBI Cohort. In the mild TBI cohort, NS mice had the lowest mean RRR latency time (*M* = 186.80 sec), while the CT mice had the highest average RRR latency (*M* = 321.50 sec). Both TBI groups (NT, CT) had a higher average return-to-righting reflex latency time than either sham groups (see Table 4; Figure 1). A one-way ANOVA did not reveal significant group differences (see Table 5; $F_{(3, 21)} = 1.66$, $p = .210$).

Table 4

Mild TBI RRR Descriptive Statistics

					95% Confidence interval for mean	
	N	Mean	*SD*	*SE*	Lower bound	Upper bound
NS	5	186.80	33.97	15.19	144.62	228.98
CS	5	237.00	50.62	22.64	174.15	299.85
NT	5	282.80	82.90	37.07	179.87	385.73
CT	10	321.50	164.13	51.90	204.09	438.91

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI;

CT = CUMS + TBI

Table 5

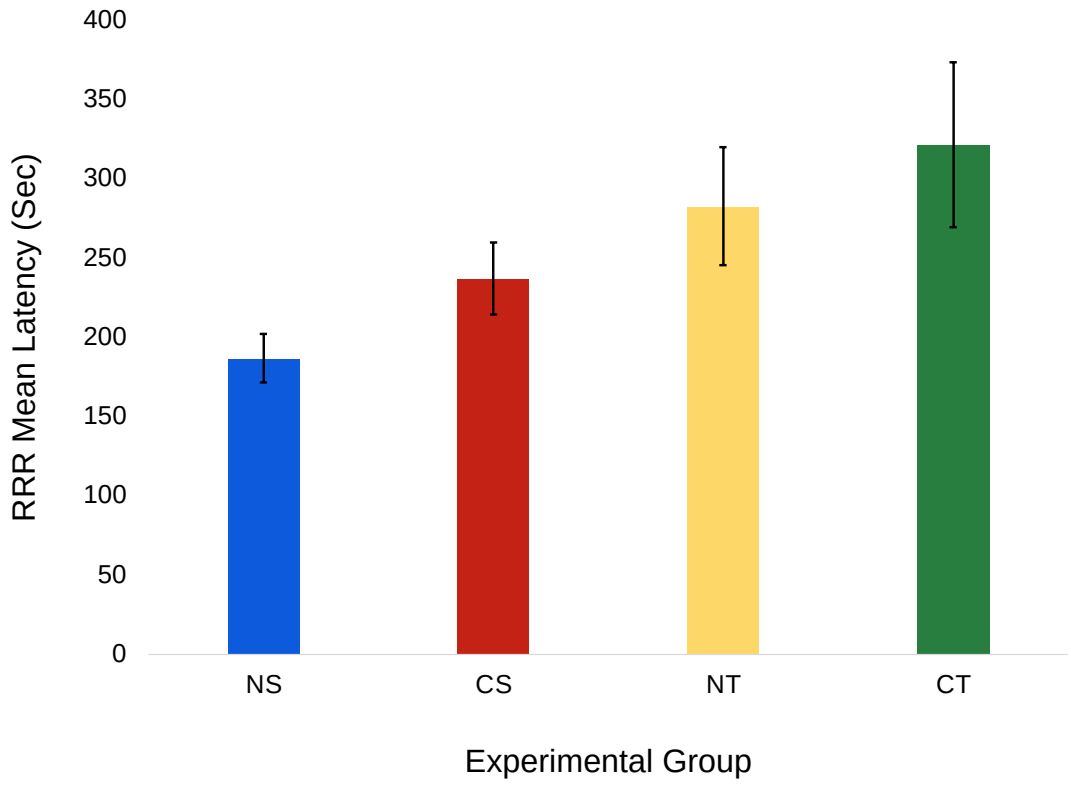
Mild TBI RRR One-Way Analysis of Variance

	Sum of squares	*df*	Mean square	*F*	Sig.
Between Groups	67397.74	3	22465.91	1.66	.210
Within Groups	284806.10	21	13562.20		
Total	352203.84	24			

*$p < .05$.

Figure 1

Mild TBI RRR Latency



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Moderate TBI Cohort. Similarly, NS mice in the moderate TBI cohort demonstrated the lowest average RRR latency (M = 581.00 sec), while CT mice had the highest mean RRR latency (M = 1210.33 sec). Both CUMS groups (CS, CT) demonstrated higher average latency to righting response relative to non-stressed groups (NS, NT; see Table 6, Figure 2). Group differences were not statistically significant based on a one-way ANOVA (see Table 7; $F_{(3, 11)}$ = 1.49, p = .290).

Table 6

Moderate TBI RRR Descriptive Statistics

					95% Confidence interval for mean	
	N	Mean	*SD*	*SE*	Lower bound	Upper bound
NS	3	581.00	7.81	4.51	561.60	600.40
CS	5	810.60	223.80	100.08	532.72	1088.48
NT	1	769.00	.	.	.	.
CT	3	1210.33	675.53	390.02	-467.78	2888.44

Note. Several descriptives unavailable due to group size. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Table 7

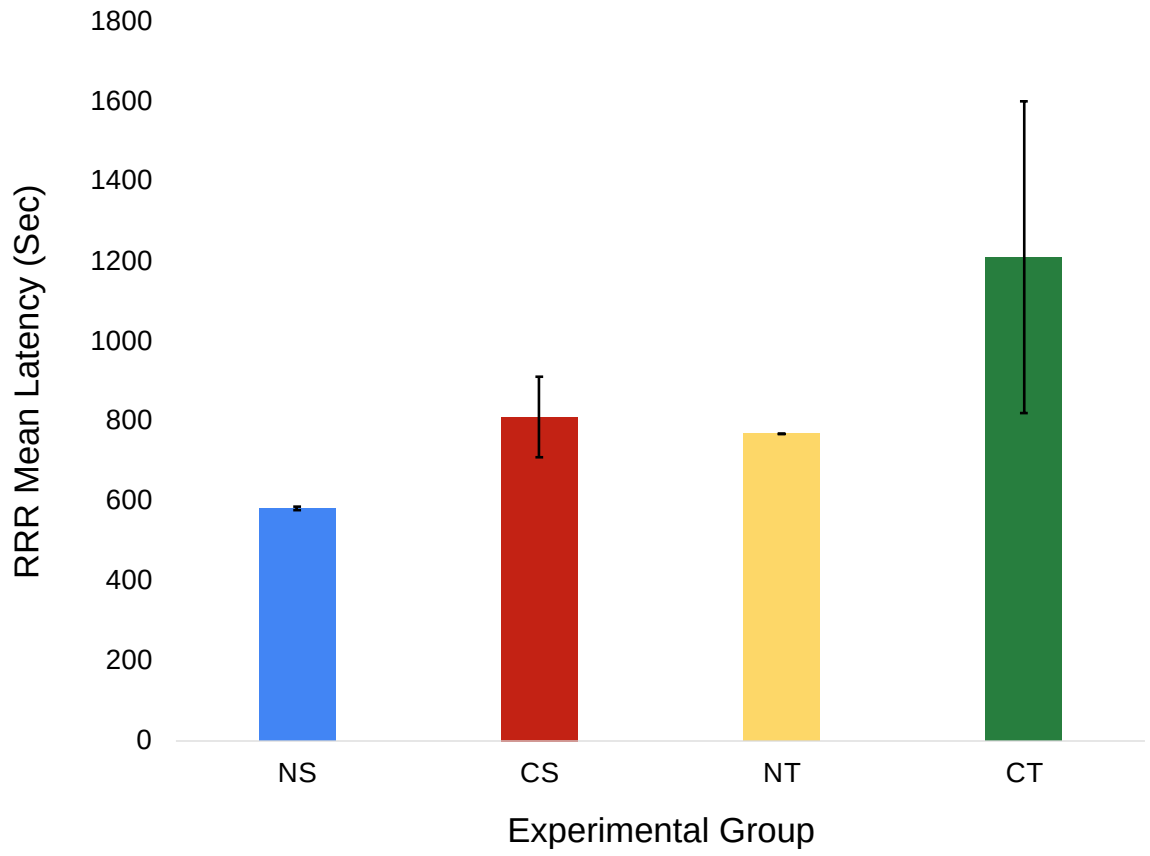
Moderate TBI RRR One-Way Analysis of Variance

	Sum of squares	*df*	Mean square	*F*	Sig.
Between Groups	620924.80	3	206974.93	1.49	.290
Within Groups	1113141.87	8	139142.73		
Total	1734066.67	11			

*$p < .05$.

Figure 2

Moderate TBI RRR Latency



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Overall, outcomes of RRR measurement did not yield statistically significant differences between experimental groups. Importantly, as the findings from RRR measurement were insignificant and did not contribute uniquely to measurement of independent variables, RRR latency was not measured during the third round of surgeries.

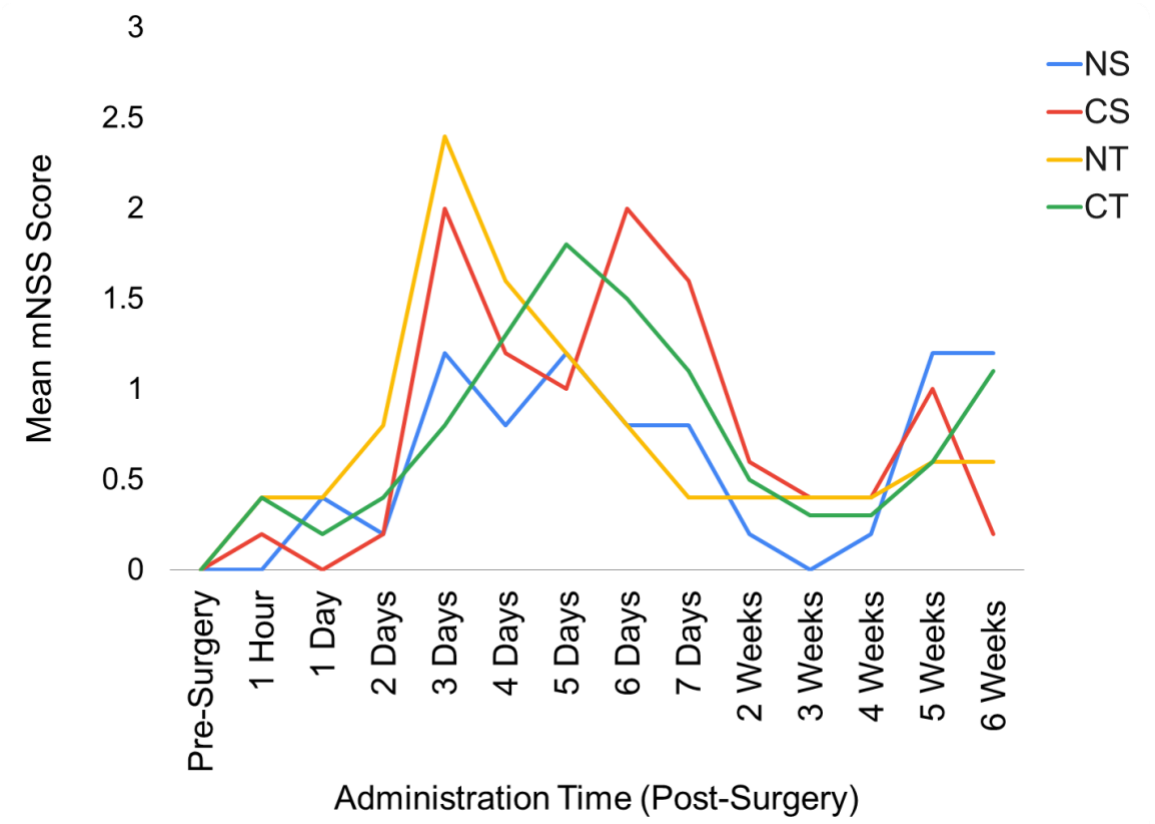
mNSS

All mice that survived surgery were evaluated using the mNSS to indicate areas of functional impairment at baseline, 1-hour post-surgery, daily for 7 days, then once weekly for 6 weeks. Two-way ANOVAs were used to understand interaction effects and the contribution of independent variables to performance on mNSS, and one-way ANOVAs were used to determine group differences. Then, repeated measures ANOVA helped to identify any differences in performance across time points of administration.

Mild TBI Cohort. No significant differences were found between experimental groups on mNSS scores (see Figure 3). This finding was consistent across 14 assessment time points.

Figure 3

Mild TBI mNSS Scores



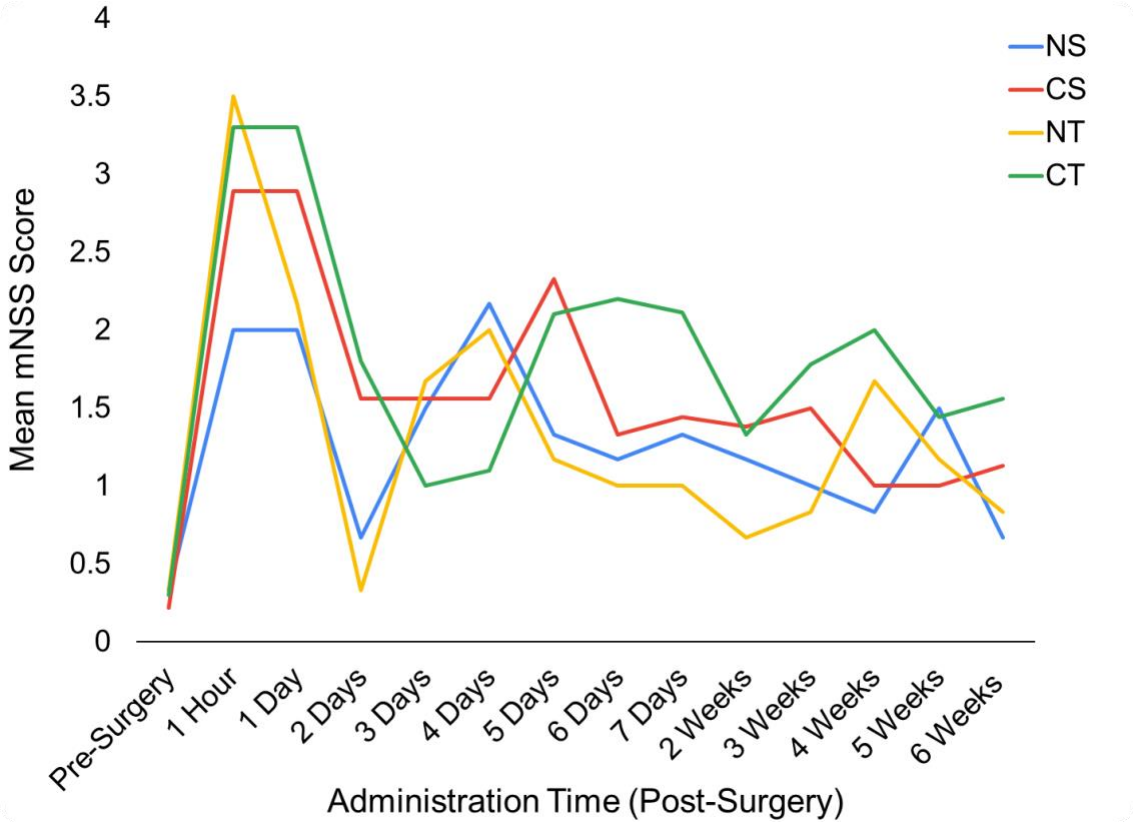
Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

A repeated measures ANOVA was performed to compare the effect of time on mNSS scores. Significant differences in mNSS scores across administrations were not observed, indicating that mice performance on mNSS did not worsen over time ($F_{(1,3)} = 0.09$, $p = .965$).

Moderate TBI Cohort. Similarly, no significant differences were found between experimental groups on mNSS scores in the Moderate TBI cohort (see Figure 4). This finding was consistent across 14 assessment time points.

Figure 4

Moderate TBI mNSS Scores



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

A repeated measures ANOVA was performed to compare the effect of group membership on mNSS scores. There were no statistically significant differences in mNSS mean score over time ($F_{(1,3)} = 0.46$, $p = .710$).

Behavioral Recovery Measures

Fourteen days after surgery, mice underwent behavioral testing. One-month post-surgery, behavior tests were then repeated in the same order and conducted by the same test experimenter. Two-way ANOVAs were performed to determine the interaction effects of stress

and TBI on behavior measures. Then, one-way ANOVAs were performed to compare group differences in behavior tests. When statistically significant differences were found, Tukeys and/or Fisher LSD post-hoc tests were used to identify specific group differences.

TST

Mild TBI Cohort. At 2 weeks post-surgery, there were no significant interaction effects between stress and TBI on TST as determined by a two-way ANOVA ($F_{(1, 21)} = .13$ $p = .719$). Simple main effects analysis showed that neither stress nor TBI had a statistically significant effect on time spent immobile ($p = .396$, $p = .719$, respectively). Of the experimental variables, exposure to TBI contributed the most to variance in TST performance, though the effect was small ($\eta^2 = .04$). There were no significant differences between experimental groups in time spent immobile on TST (see Table 8, Figure 5; $F_{(1,21)} = .35$, $p = .790$).

One month after surgeries, there was a significant interaction effect between stress and TBI (see Table 9; $F_{(3, 21)} = 10.61$, $p = .004$) with a large effect size ($\eta^2 = .34$) on time spent immobile. Simple main effects analysis showed that CUMS and TBI had small-medium effects on time spent immobile ($\eta^2 = .08$, $\eta^2 = .10$, respectively), though this finding was not statistically significant ($p = .193$, $p = .152$, respectively). Time spent immobile was significantly different between groups one month post-surgery (see Table 8, Figure 5; $F_{(1, 21)} = 4.28$, $p = .017$). Fisher's LSD post-hoc analyses test revealed significant differences between mice in the NS group and all other experimental groups, including CS ($p = 006$, 95% C.I. = -48.66, -9.16), NT ($p = .005$, 95% C.I. = -49.54, -10.04), and CT ($p = .042$, 95% C.I. = -34.89 -.68).

Table 8

Mild TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent Immobile on TST

	NS	CS	NT	CT	*F* (1,21)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	311.18 (21.12)	296.83 (81.62)	320.34 (24.90)	319.27 (31.43)	.35	.790
1 Month Post-Surgery	318.40 (18.90)	347.31 (3.94)	348.19 (10.77)	336.18 (17.58)	4.28	.017*

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Table 9

Mild TBI TST - Time Spent Immobile 1 Month Post-Surgery Two-Way Analysis of Variance

	Sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Stress	408.21	1	408.21	1.81	.193	.08
Injury	497.64	1	497.64	2.21	.152	.10
Stress * Injury	2392.07	1	2392.07	10.61	.004*	.34
Error	4735.02	21	225.48			

Note. Effect Size = Partial η^2

*$p < .05$.

A two-way repeated measures ANOVA was used to compare simple main effects of stress and TBI exposure over time between two administrations of the TST. Results revealed a statistically significant main effect of time spent immobile between TST trials (see Table 10, Figure 5; $F_{(1,21)} = 9.65$, $p = .006$, $\eta^2 = .31$), in which all groups spent more time immobile during the second administration. Simple main effects analysis indicated that the difference in TST performance was not attributable to statistically significant interaction between experimental variables.

Table 10

Mild TBI Two-Way Repeated Measures Analyses of Variance in Time Spent Immobile on TST

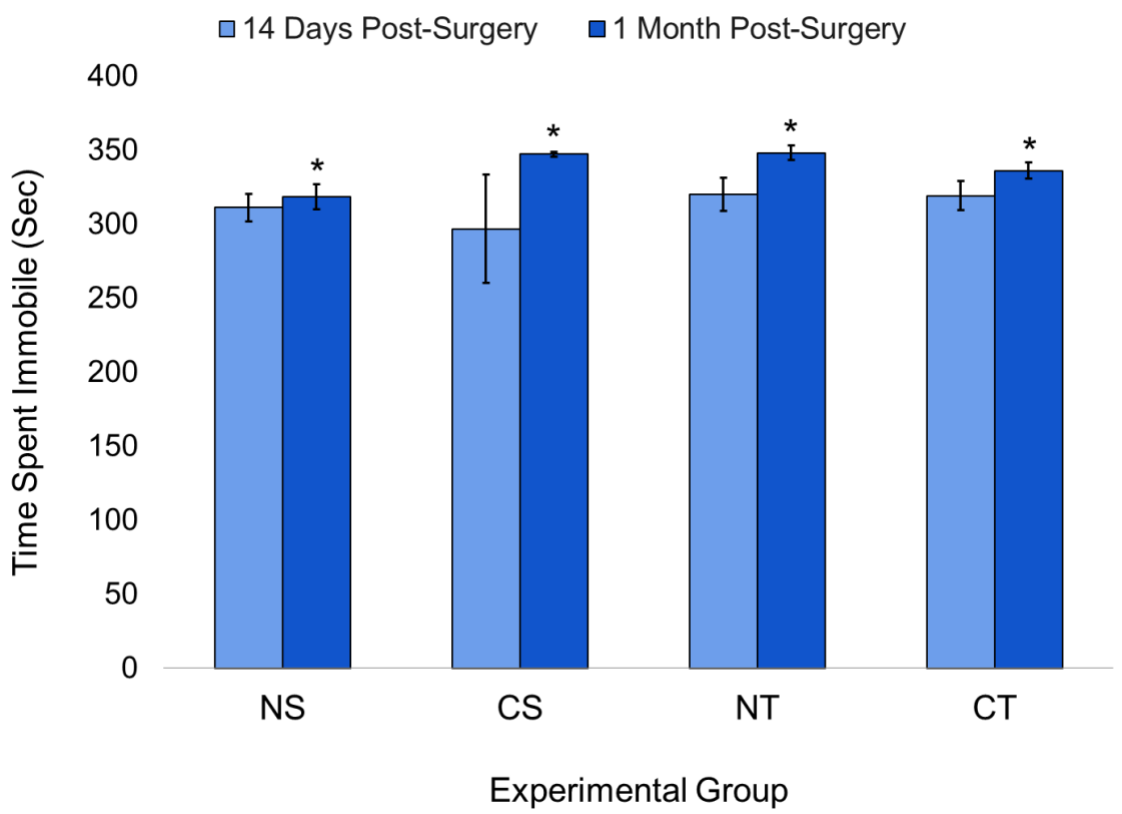
	Type III sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Time	7499.19	1	7499.19	9.56	.006*	.31
Time * Stress	746.13	1	746.13	.95	.340	.04
Time * Injury	119.38	1	119.38	.15	.700	.01
Time * Stress * Injury	2098.93	1	2098.93	2.68	.117	.11
Error	14645.81	21	784.09			

Note. Effect Size = Partial η^2

*$p < .05$.

Figure 5

Mild TBI TST



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Moderate TBI Cohort. At 14 days post-surgery, there was not a significant stress by TBI interaction effect ($F_{(3,25)} = .92$, $p = .347$), and a one-way ANOVA did not reveal significant differences in behavioral despair as assessed by TST (see Table 11, Figure 6; $F_{(1,25)} = 1.03$, $p = .398$). Simple main effects analysis showed that stress and TBI ($p = .948$; $p = .347$, respectively) had no significant impacts on immobility time.

At 1-month post-surgery, there were no significant interaction effects between stress and TBI contributing to time spent immobile on TST, nor were group differences statistically

significant as assessed by two-way ($F_{(3,25)} = 1.59$, $p = .219$) and one-way ANOVAs (see Table 11, Figure 6; $F_{(1,25)} = 1.68$, $p = .196$). Neither stress ($p = .146$) nor TBI ($p = .207$) had a significant effect on time spent immobile as determined by a simple main effects test.

Table 11

Moderate TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent Immobile on TST

	NS	CS	NT	CT	*F* (1,25)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	281.47 (51.17)	261.25 (62.85)	230.79 (51.63)	248.44 (42.32)	1.03	.398
1 Month Post-Surgery	337.74 (26.85)	351.27 (5.52)	350.27 (7.69)	351.44 (3.56)	1.68	.196

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

A two-way repeated measures ANOVA was used to compare simple main effects of stress and TBI exposure over time between two administrations of the tail suspension test. Results revealed a main effect of time spent immobile between TST trials (see Table 12; $F_{(1,25)} = 4.97$, $p = .034$, $\eta^2 = .16$), with mice in all groups more immobile during the second trial. Simple main effects analysis indicated that the difference in TST performance was not attributable to statistically significant interaction between experimental variables.

Table 12

Moderate TBI Two-Way Repeated Measures Analyses of Variance in Time Spent Immobile on TST

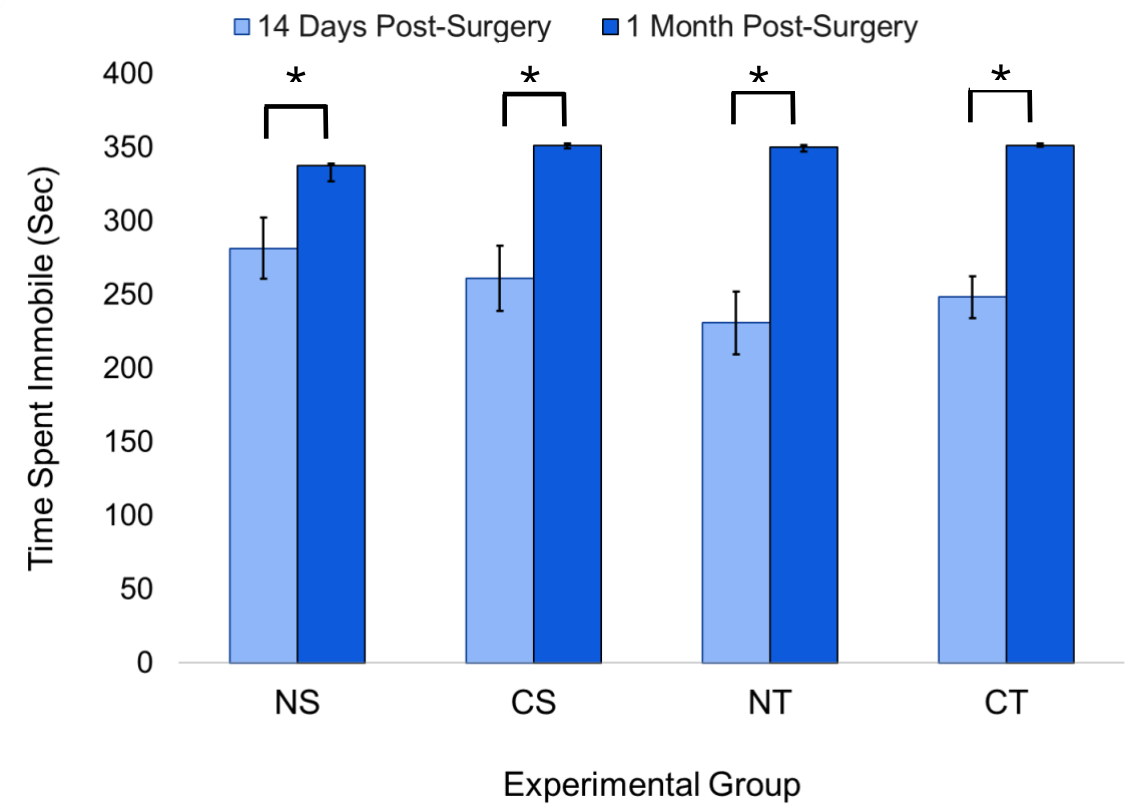
	Type III sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Time	238795.31	1	238795.31	77.31	.000*	.76
Time * Stress	524.10	1	524.10	.17	.684	.01
Time * Injury	10191.92	1	10191.92	3.30	.081	.12
Time * Stress * Injury	4430.92	1	4430.92	1.43	.242	.05
Error	77222.01	25	3088.88			

Note. Effect Size = Partial η^2

*$p < .05$.

Figure 6

Moderate TBI TST



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

FST

Mild TBI Cohort. At 2 weeks post-surgery, there were no significant differences in time spent immobile on FST attributable to stress, TBI, or interaction effects ($F_{(3,21)} = 2.17$, $p = .155$), nor were there significant differences between experimental groups (see Table 13, Figure 7; $F_{(1,21)} = 1.37$, $p = .278$). Neither stress ($p = .223$) nor TBI ($p = .155$) had significant effects on time spent immobile as assessed by a simple main effects analysis.

Immobility time in the FST 1 month post-surgery was not impacted in either the CUMS or TBI groups as determined by one-way (see Table 13, Figure 7; $F_{(1,21)} = .45$, $p = .718$) and two-way ANOVAs ($F_{(3,21)} = .26$, $p = .618$). Simple main effects analysis showed that neither stress ($p = .635$) nor TBI ($p = .315$) significantly affects behavioral despair using FST.

Table 13

Mild TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent Immobile on FST

	NS	CS	NT	CT	*F* (1,21)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	215.28 (72.05)	256.48 (10.73)	251.47 (8.50)	248.17 (25.36)	1.37	.287
1 Month Post-Surgery	265.92 (29.41)	266.18 (21.712	281.98 (17.55)	271.65 (26.93)	.45	.718

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

A two-way repeated measures ANOVA was used to compare stress and TBI exposure over time between two administrations of FST. Results revealed a main effect of time spent immobile between administrations of FST (see Table 14, Figure7; $F_{(1,21)} = 8.59$, $p = .008$, $\eta^2 = .29$), in which all groups spent more time immobile during the second trial. Simple main effects analysis indicated that the difference in FST performance was not attributable to statistically significant interaction between experimental variables.

Table 14

Mild TBI Two-Way Repeated Measures Analyses of Variance in Time Spent Immobile on FST

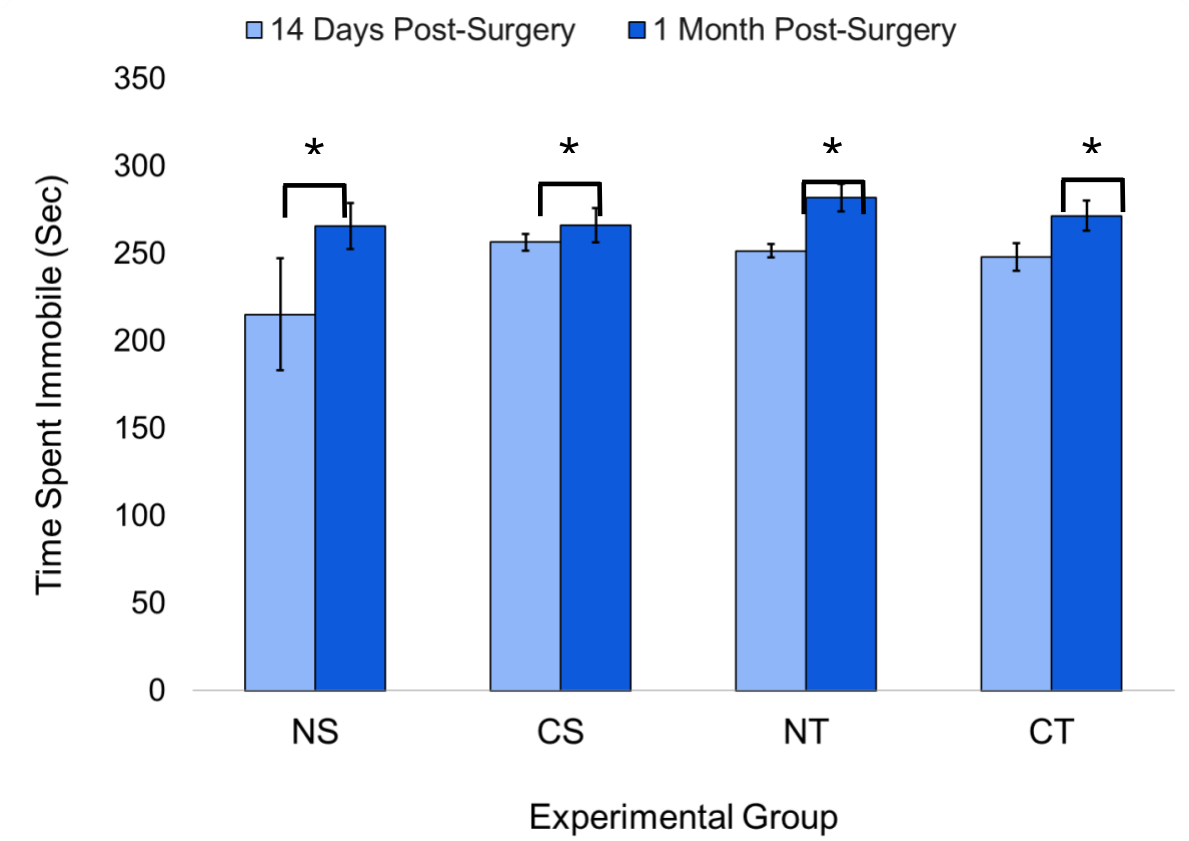
	Type III Sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Time	9336.35	1	9336.35	8.59	.008*	.29
Time * Stress	1644.70	1	1644.07	1.51	.232	.07
Time * Injury	28.75	1	28.75	.03	.872	.00
Time * Stress * Injury	821.45	1	821.45	.76	.395	.04
Error	22832.90	21	1087.28			

Note. Effect Size = Partial η^2

*$p < .05$.

Figure 7

Mild TBI FST



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Moderate TBI Cohort. At 2 weeks post-surgery, neither stress ($p = .103$) nor TBI ($p = .103$) had a significant effect on immobility time, nor were there any significant interaction effects between stress and TBI exposure ($F_{(3,25)} = .09$, $p = .768$). Additionally, no significant differences were found in immobility time on FST using one-way ANOVA (see Table 15, Figure 8; $F_{(1,25)} = 1.96$, $p = .146$).

Table 15

Moderate TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent Immobile on FST

	NS	CS	NT	CT	*F* (1,25)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	196.08 (90.98)	131.63 (102.69)	131.71 (92.89)	86.55 (56.52)	1.96	.146
1 Month Post-Surgery	264.07 (20.50)	245.90 (42.63)	245.47 (37.88)	206.54 (41.39)	3.22	.040*

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Although there were no significant interaction effects between stress and TBI (see Table 16; $F_{(3,25)} = 0.53$, $p = .473$), TBI ($p = .053$; large effect [$\eta^2 = .14$]) and stress ($p = .056$; large effect [$\eta^2 = .14$]) each had an impact on immobility time on FST 1-month post-surgery with large effect size; however, these contributions were not statistically significant. Overall, experimental factors contributed to 27.9% of the variance in immobility on FST 1-month post-surgery.

Immobility time in FST 1-month post-surgery was significantly different between experimental groups (see Table 15; $F_{(1,25)} = 3.22$, $p = .040$). Post-hoc analyses Fisher's LSD test revealed differences between the CT groups and both the NS ($p = .008$, 95% C.I. = -98.55, -16.50) and CS groups ($p = .042$, 95% C.I. = -77.18, -1.54). There was no statistically significant difference between CT and the NT groups ($p = .062$).

Table 16

Moderate TBI FST - One Month Post-Surgery Two-Way Analysis of Variance

	Sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Stress	5723.82	1	5723.82	4.01	.056	.14
Injury	5898.91	1	5898.91	4.13	.053	.14
Stress * Injury	756.68	1	756.68	.53	.473	.02
Error	35706.06	25	1428.24			

Note. Effect Size = Partial η^2

*$p < .05$.

A two-way repeated measures ANOVA was used to compare interaction effects of stress and TBI exposure over time between two administrations of the FST. Results revealed a statistically main effect of time spent immobile between administrations of FST (see Table 17, Figure 8; $F_{(1,25)} = 79.60$, $p = .000$, $\eta^2 = .76$), with mice in all groups spending more time immobile during the second trial. Simple main effects analysis indicated that the difference in FST performance was not attributable to statistically significant interaction between experimental variables.

Table 17

Moderate TBI Two-Way Repeated Measures Analyses of Variance in Time Spent Immobile on FST

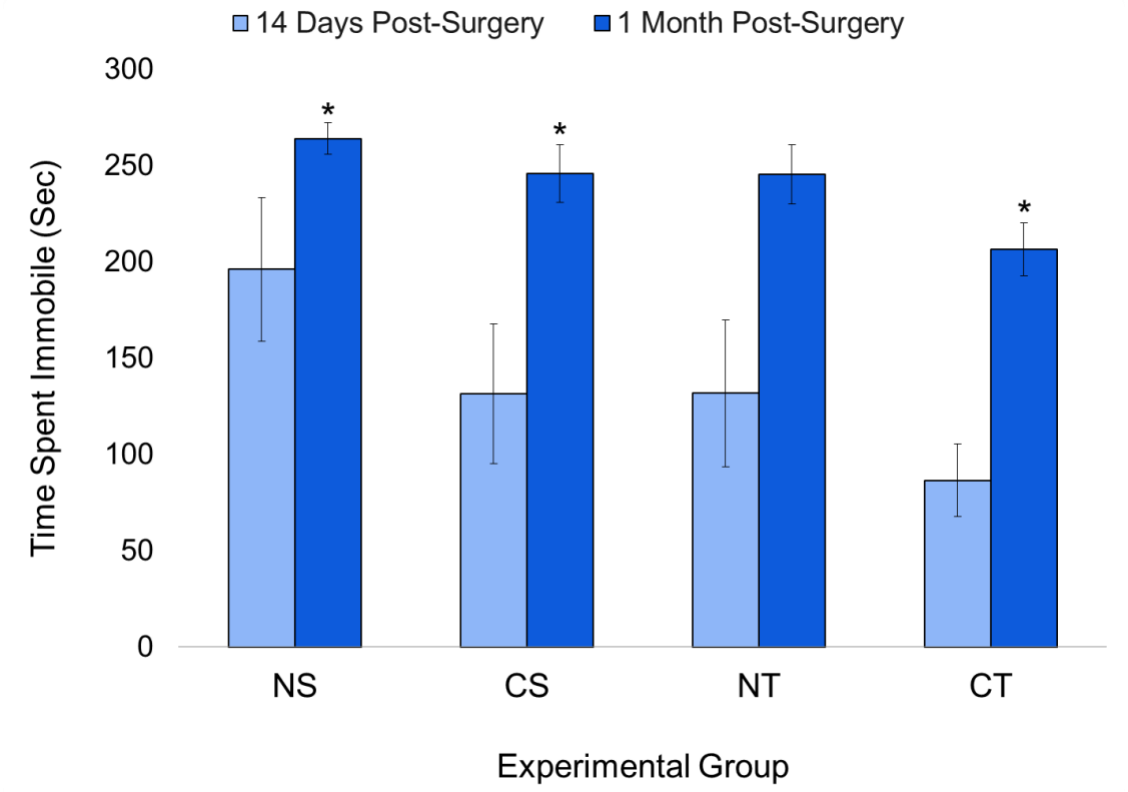
	Type III sum of squares	*df*	Mean square	*F*	*Sig.*	Effect size
Time	303914.58	1	303914.58	79.60	.000*	.76
Time * Stress	4843.91	1	4843.91	1.27	.271	.05
Time * Injury	4656.61	1	4656.61	1.22	.280	.05
Time * Stress * Injury	2817.10	1	2817.10	.74	.399	.03
Error	95456.52	25	3818.26			

Note. Effect Size = Partial η^2

*$p < .05$.

Figure 8

Moderate TBI FST



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

EPM

Mild TBI Cohort. Two weeks post-surgery, a significant interaction effect between stress and TBI was found on the EPM test using a two-way ANOVA (see Table 19; $F_{(3,21)} = 7.53$, $p = .012$) with modest effect size ($\eta^2 = .26$). Simple main effects analysis indicated that TBI had a significant effect on time spent in open arms on EPM ($p = .032$; modest effect size $\eta^2 = .20$), but stress did not impact time spent in open arms ($p = .197$).

Additionally, significant group differences were found in time spent in open arms on EPM using a one-way ANOVA (see Table 18, Figure 9; $F_{(1,21)} = 5.06$, $p = .009$). Fisher's LSD Test showed that CS mice spent significantly more time in open arms than all other experimental groups including NS ($p = .014$, 95% C.I. = 6.12, 47.44), NT ($p = .026$, 95% C.I. = 3.19, 44.51), and CT ($p = .001$, 95% C.I. = 15.24, 51.03).

Table 18

Mild TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent in Open Arms on EPM

	NS	CS	NT	CT	*F* (1,21)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	17.65 (11.39)	44.43 (24.00)	20.58 (19.55)	11.30 (9.60)	5.06	.009*
1 Month Post-Surgery	9.6 (9.50)	14.56 (6.87)	10.26 (9.88)	8.75 (6.14)	.64	.598

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Table 19

Mild TBI EPM - 14 Days Post-Surgery Two-Way Analysis of Variance

	Sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Stress	437.30	1	437.30	1.77	.197	.08
Injury	1302.91	1	1302.91	5.28	.032*	.20
Stress * Injury	1858.84	1	1858.84	7.53	.012*	.26
Error	5181.46	21	246.74			

Note. Effect Size = Partial η^2

*$p < .05$.

One-month post-surgery, time spent in open arms was no longer significantly different between groups, nor was there a significant interaction effect (see Table 18, Figure 9). Based on simple main effects analysis, neither stress ($p = .603$) nor TBI ($p = .440$) impacted time spent in open arms on EPM.

A two-way repeated measures ANOVA was used to assess interaction and main effects of stress and TBI exposure over time between two administrations of the elevated plus maze test. Results revealed a statistically significant main effect of time spent in open arms between administrations of EPM (see Table 20, Figure 9; $F_{(1,21)} = 28.78$, $p = .000$, $\eta^2 = .58$), in which all groups spent less time in the open arms during the second trial. There was a significant time by TBI interaction effect in time spent in the open arm ($p = .015$, $\eta^2 = .25$). A significant three-way interaction effect was also found between time, stress, and TBI on time spent in the open arms of the EPM ($p = .005$, $\eta^2 = .32$).

Table 20

Mild TBI Two-Way Repeated Measures Analyses of Variance in Time Spent in Open Arms on EPM

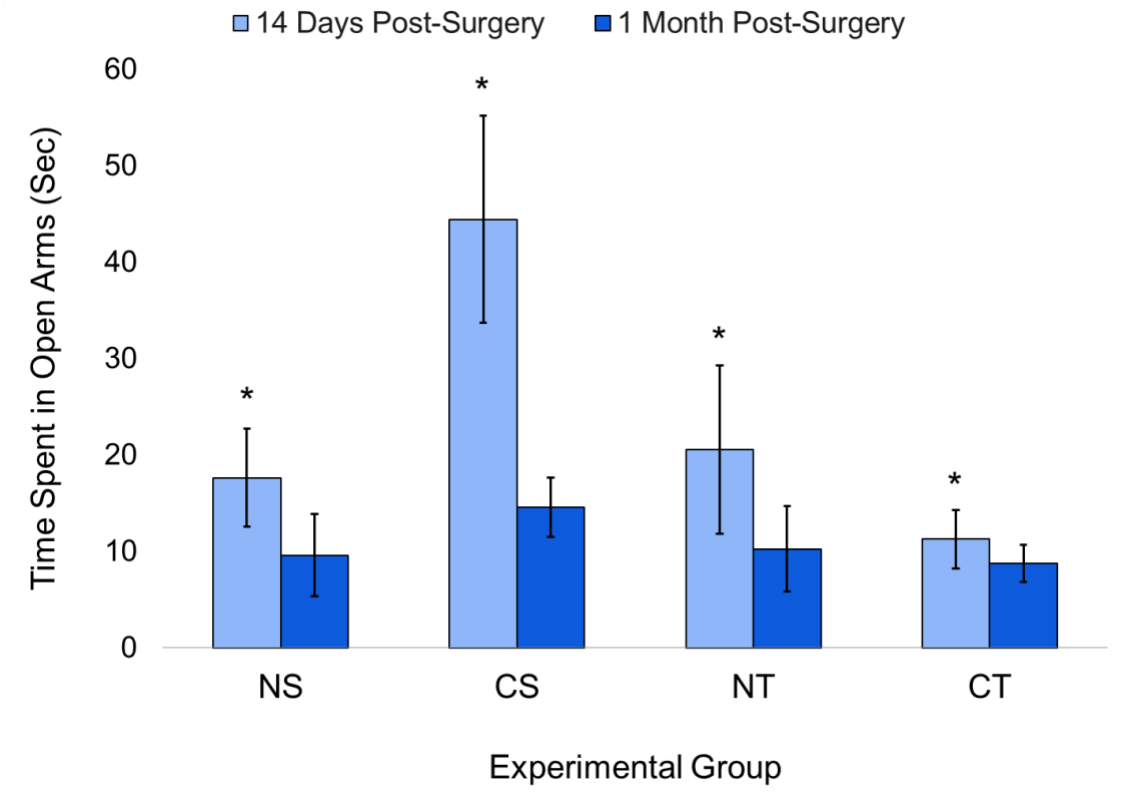
	Type III sum of squares	df	Mean square	*F*	Sig.	Effect size
Time	1842.15	1	1842.15	28.78	.000*	.59
Time * Stress	140.96	1	140.96	2.20	.153	.10
Time * Injury	448.43	1	448.43	7.01	.015*	.25
Time * Stress * Injury	625.83	1	625.83	9.78	.005*	.32
Error	1344.33	21	64.02			

Note. Effect Size = Partial η^2

*$p < .05$.

Figure 9

Mild TBI EPM



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Moderate TBI Cohort. There were no significant interaction effects of stress and TBI (see Table 21). Stress significantly affected time spent in open arms on EPM as assessed by simple main effects analysis ($p = .036$; large effect size [$\eta^2 = .15$]). However, TBI did not impact anxiety-like behavior ($p = .057$). Overall, experimental variables contributed to 28.9% of the variance in time spent in open arms on EPM.

A one-way ANOVA revealed significant differences in time spent in open arms on EPM 2 weeks post-surgery (see Table 21, Figure 10; $F_{(1,25)} = 3.65$, $p = .025$). Post-hoc analyses revealed that mean time spent in open arms was significantly different between mice in the NS

group and all other experimental groups (CS: p = .008, 95% C.I. = 5.39, 32.99; CT: p = .020, 95% C.I. = 3.02, 33.26; ST: p = 006, 95% C.I. = 6.21, 33.25).

Table 21

Moderate TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent in Open Arms on EPM

	NS	CS	NT	CT	*F* (1,25)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	28.80 (22.00)	9.61 (9.82)	10.66 (8.69)	9.07 (9.59)	3.65	.025*
1 Month Post-Surgery	15.20 (15.03)	5.35 (9.68)	6.33 (5.40)	5.88 (9.94)	1.34	.284

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*p < .05.

Table 22

Moderate TBI EPM - 14 Days Post-Surgery Two-Way Analysis of Variance

	Sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Stress	793.012	1	793.02	4.87	.036*	.15
Injury	641.28	1	641.28	3.94	.057	.13
Stress * Injury	568.60	1	568.60	3.49	.073	.12
Error	4396.03	27	162.82			

Note. Effect Size = Partial η^2

a. *$p < .05$.

There were no significant differences in anxiety-like behavior 1 month after surgery (see Table 21, Figure 10; $F_{(1,25)} = 1.34$, $p = .284$). There were also no significant interaction effects between stress and TBI. Neither stress nor TBI ($p = .190$; $p = .286$, respectively) had significant simple main effects on time spent in open arms on EPM.

A two-way repeated measures ANOVA was used to compare simple main effects of stress and TBI exposure over time between two administrations of the EPM test. Results revealed a statistically significant main effect of time spent in open arms between administrations of EPM (see Table 23, Figure 10; $F_{(1,25)} = 7.41$, $p = .011$, $\eta^2 = .22$), as all groups spent less time in open arms during the second administration. There were no significant interaction effects between time by stress, time by injury, or time by stress by injury.

Table 23

Moderate TBI Two-Way Repeated Measures Analyses of Variance in Time Spent in Open Arms on EPM

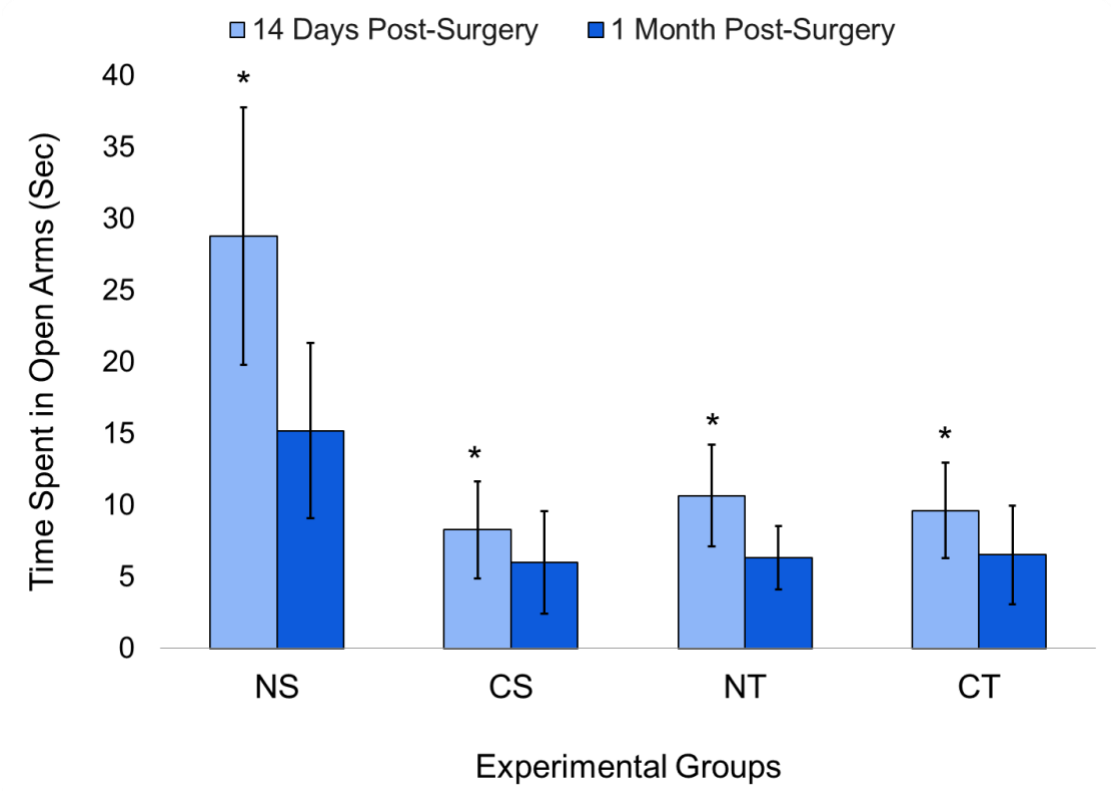
	Type III sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Time	591.29	1	591.29	7.41	.011*	.22
Time * Stress	100.59	1	100.59	1.26	.271	.05
Time * Injury	98.13	1	98.13	1.23	.277	.04
Time * Stress * Injury	61.70	1	61.70	.77	.387	.03
Error	2155.05	25	79.82			

Note. Effect Size = Partial η^2

*$p < .05$.

Figure 10

Moderate TBI EPM



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

DLB

Mild TBI Cohort. At 2 weeks post-surgery, there was no significant difference in time spent in the lighted box on the DLB between groups (see Table 24, Figure 11; $F_{(1,21)} = 1.04$, $p = .389$). There were also no significant interaction effects between stress and TBI. Simple main effects analysis showed that neither stress nor TBI ($p = .226$; $p = .950$, respectively) impacted time spent in the lighted compartment on DLB.

There were no differences in time spent in the lighted box on DLB (see Table 24, Figure 11) and no significant interaction effects between stress and TBI. Neither stress nor TBI ($p =$

.122; p = .347, respectively) had a significant impact on anxiety-like behavior in DLB according to simple main effects tests.

Table 24

Mild TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent in Light Side on DLB

	NS	CS	NT	CT	F (1,21)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	90.06 (52.87)	185.89 (109.77)	138.54 (54.91)	132.86 (97.20)	1.04	.398
1 Month Post-Surgery	120.14 (61.00)	179.60 (74.11)	124.54 (36.12)	134.02 (37.75)	1.44	.259

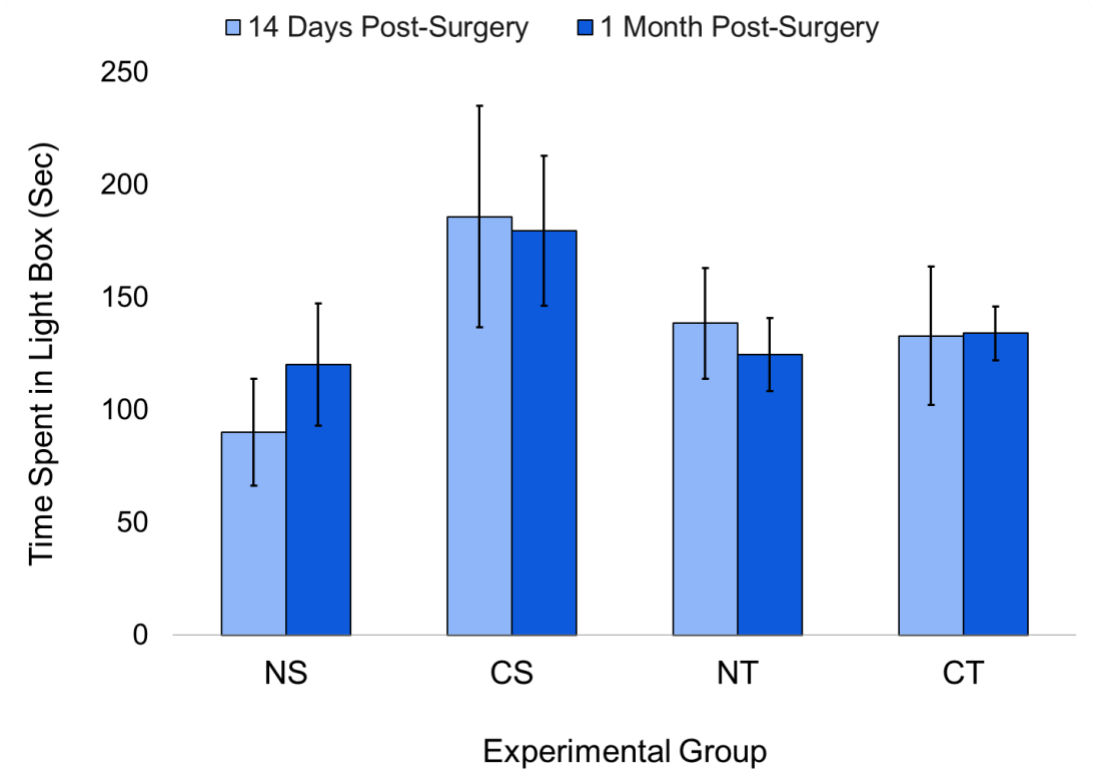
Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*p < .05.

Results of a two-way repeated measures ANOVA indicated that there was not a statistically significant difference in time spent in the light box between administrations of the DLB test ($F_{(1,21)}$ = .03, p = .875).

Figure 11

Mild TBI DLB



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Moderate TBI Cohort. Moderate TBI also did not impact anxiety-like behavior as determined by DLB 2-weeks post-surgery (see Table 25, Figure 12). Simple main effects analysis showed that neither stress ($p = .892$), or TBI ($p = .491$) had a statistically significant effect on time spent in the lighted box on DLB.

One month post-surgery, anxiety-like behavior in DLB did not change (see Table 25, Figure 12; $F_{(1,21)} = .77$, $p = .519$), nor were there significant interaction effects between stress and TBI. Simple main effects analysis showed that neither stress nor TBI ($p = .370$; $p = .760$, respectively) had a statistically significant effect on time spent in the lighted box on DLB.

Table 25

Moderate TBI Means, Standard Deviations, and One-Way Analyses of Variance in Time Spent in Light Side on DLB

	NS	CS	NT	CT	*F* (1,25)	Sig.
	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*	Mean *(SD)*		
2 Weeks Post-Surgery	59.41 (65.74)	49.65 (48.62)	62.91 (47.35)	79.18 (81.11)	.35	.791
1 Month Post-Surgery	125.37 (94.92)	72.00 (32.01)	104.24 (76.39)	109.47 (78.93)	.77	.519

Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

A two-way repeated measures ANOVA was used to compare simple main effects of stress and TBI exposure over time between two administrations of the dark-light box test. Results revealed a statistically significant main effect of time between administrations of DLB (see Table 26, Figure 12; $F_{(1,25)} = 14.65$, $p = .001$, $\eta^2 = .35$), as all groups spent more time on the light side during the second administration. There were no significant interaction effects in any of the other variables examined.

Table 26

Moderate TBI Two-Way Repeated Measures Analyses of Variance in Time Spent in the Light Box on DLB

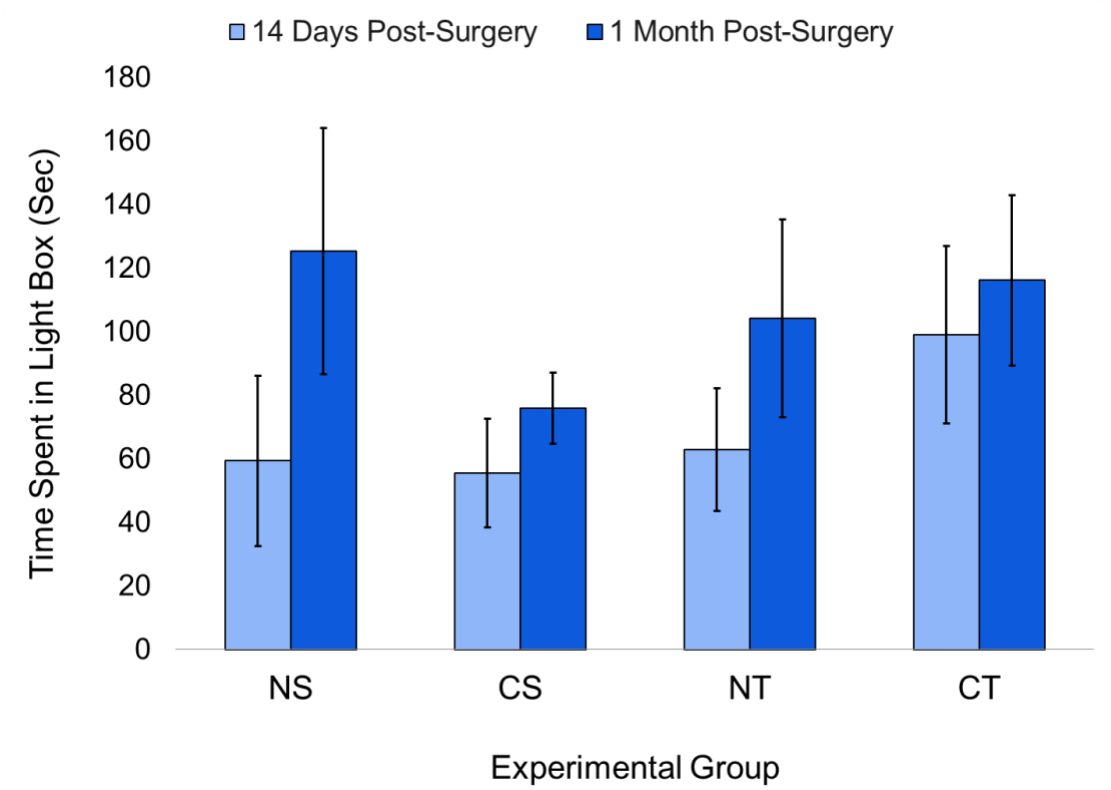
	Type III sum of squares	*df*	Mean square	*F*	Sig.	Effect size
Time	23492.84	1	23492.84	14.65	.001*	.35
Time * Stress	2742.39	1	2742.39	1.71	.202	.06
Time * Injury	255.82	1	255.82	.16	.693	.01
Time * Stress * Injury	973.80	1	973.80	.61	.443	.02
Error	43299.34	25	1603.68			

Note. Effect Size = Partial η^2

*$p < .05$.

Figure 12

Moderate TBI DLB



Note. NS = Non-CUMS + Sham; CS = CUMS + Sham; NT = Non-CUMS + TBI; CT = CUMS + TBI

*$p < .05$.

Summary of Findings

The primary objectives of the present study included the measurement of functional and behavioral recovery exposure to stress or a TBI, ranging in severity from mild to moderate. Regarding measures of functional recovery, significant group differences were not found in RRR latency time or performance on mNSS. Further, groups did not demonstrate statistically significant differences in functional recovery trajectory, as demonstrated by findings from repeated measures ANOVA exploring mNSS score across administrations.

On behavioral measures of hopelessness, including TST and FST, CUMS and TBI significantly contributed to performance in both mild and moderate TBI cohorts. In the mild TBI cohort, mice in the NS group spent significantly less time immobile than all other experimental groups on TST. The interaction effect between stress and TBI was significant at 1-month post-surgery, with a moderate effect size (η^2 = .336). While the average time spent immobile on FST was the lowest in the NS group, this finding was not statistically significant. In the moderate TBI cohort, exposure to stress had a significant impact on time spent immobile on TST, though subsequent group differences were not statistically significant. On FST, stress had a modest effect on time spent immobile at 2-weeks post-surgery (η^2 = .22). At 1-month post-surgery, the NT group spent significantly less time immobile than the NS and CS groups, and exposure to TBI had a significant effect on outcomes (η^2 = .14).

On behavioral measures of anxiety, including EPM and DLB, experimental variables contributed to statistically significant group differences in both the mild and moderate TBI cohorts. In the mild cohort, mice in the CS group spent significantly more time in the open arms than all other experimental groups at 2-weeks post-surgery. Further, the interaction effect between stress and TBI exposure as well as exposure to TBI alone had a significant impact on test performance with moderate effect sizes (η^2 = .26, η^2 = .20, respectively). In the moderate TBI cohort, mice in the NS group spent significantly more time in the open arms than all other experimental groups. Further, stress exposure had the most significant impact on scores (η^2 = .15). Overall, findings from the DLB test were not significant between groups in either cohort.

Taken together, although premorbid stress did not have a significant impact on functional measures of recovery following TBI ranging from mild to moderate, it contributed to variable performance on measures of behavioral anxiety and despair. Specifically, mice in the non-CUMS group that sustained a mild TBI spent the least amount of time immobile on measures of behavioral despair on average, though this finding was only statistically significant

on TST 1-month post-surgery. In the moderate cohort, non-CUMS mice with a TBI generally spent less time immobile on TST, though this finding did not reach significance. In contrast, mice in the CUMS + TBI (moderate) group spent significantly less time immobile than all other groups at one-month post-surgery. Regarding findings from anxiety behavior assessment, mice in the mild cohort exposed to CUMS and a sham surgery appeared significantly less anxious than those in other groups; a finding primarily attributable to the effects of TBI exposure and the interaction effect of TBI and stress 2-weeks post-surgery. In the moderate cohort, non-CUMS + Sham mice spent significantly more time in open arms, exhibiting the unique contribution of CUMS on performance. This finding, however, was no longer significant one-month post-surgery. Overall, behavior assessment revealed significantly more indications of anxiety and learned helplessness during the second administration of all measures.

CHAPTER V

DISCUSSION

TBIs represent a prominent source of disability and economic burden worldwide (Center for Disease Control [CDC] 2023; Dewan et al., 2018). Efforts in TBI research are essential to protecting populations that are particularly vulnerable to TBI, including children, older adults, marginalized racial and ethnic groups, individuals of low socioeconomic status, and those with a history of substance use disorders (Belanger et al., 2017; Cancelliere et al., 2023). Prolonged recovery trajectories are common following mild to moderate TBI due to PCS and comorbid affective stress, anxiety disorders, and depression (Iverson, 2005). Recent studies have reported on the impact of premorbid mental health symptoms on post-injury recovery, noting that certain psychotherapeutic treatments (i.e., cognitive-behavioral therapies) may be beneficial for treating postconcussive syndrome by addressing underlying, underappreciated psychiatric symptoms (Vanderploeg et al., 2019). Further, researchers have recently emphasized the utility of characterizing TBI recovery as points on a spectrum due to the challenge of differentiating pre- and post-injury mental health symptoms (Howlett et al., 2022; Karr et al., 2020). As such, premorbid characteristics are a prominent point of inquiry in predicting recovery from TBI of varying severities (Belanger et al., 2017).

Animal models offer a unique method of inducing and measuring premorbid factors in TBI outcomes that cannot be replicated in human studies (Antoniuk et al., 2019; Ma et al., 2019). In the current study, functional and behavioral measures were used to determine the impact of chronic stress following mild and moderate TBI in mice. The goal of the study was to contribute to available literature exploring factors that prolong recovery from mild or moderate TBI and provide additional insight for treatment in populations that are more vulnerable to these injuries. Independent variables included stress or non-stress conditions prior to either a sham or TBI procedure. Dependent variables included measures of functional recovery (RRR, mNSS)

and behavior, such as the EPM (Pellow et al., 1985), DLB (Hall, 1934; Walsh et al., 1976), TST (Steru et al., 1985), and FST (Porsolt et al., 1977; Porsolt et al., 1978). It was hypothesized that exposure to stress prior to injury would contribute to poorer outcomes on functional and behavioral measures.

Key Findings

A number of key findings with implications for both human and animal TBI research emerged from this study. First, functional recovery was not impacted by exposure to stress or injury severity. Second, behavioral manifestations of affective distress, primarily those similar to despair, and anxiety, varied by experimental group and time since injury. Finally, behavioral markers exhibited changes over the course of recovery.

Functional Recovery

Estimations of time spent unconscious are an important element in classifying TBI severity in human populations, as prolonged loss of consciousness is associated with greater injury severity (Roebuck-Spencer & Sherer, 2017). In the current study, animals were monitored in acute recovery for RRR in order to parallel loss of consciousness in humans. This measurement was calculated by recording the time between initial administration of anesthesia and observed attempts by the animal to return to a normal upright position, indicating an approximate return to baseline level of consciousness (Whyte et al., 2001). Exposure to stress prior to surgery did not contribute to poorer outcomes on RRR in either the mild TBI or moderate TBI cohorts.

In addition to RRR, a modified version of the NSS was administered prior to surgery, 1 hour after, then on each subsequent day for 7 days, and once per week for 6 weeks. The NSS evaluates an animals' ability to successfully complete several basic tasks requiring neurological skills and is considered an equivalent measure to a neurologists' exam in animal model research (Siebold et al., 2018). Similar to the findings on measurements of RRR, exposure to

stress prior to surgery did not contribute to poorer outcomes on the mNSS in either the mild TBI or moderate TBI cohorts across all 6 weeks of monitoring.

Behavioral Outcomes

Overall, the role of stress pre-exposure on depressive-like behavior was variable based on injury severity (i.e., mild or moderate TBI) and recovery time. In the mild TBI cohort, the interaction between stress and TBI exposure contributed to group differences in behavioral despair as measured by the TST in the month following surgery; however, this finding was not replicated with the FST, which uses a different modality to assess similar constructs. In the moderate TBI cohort, the non-stressed + sham mice demonstrated the least behavioral despair on TST; however, CUMS + TBI mice displayed the least behavioral despair on FST, 1 month after surgery.

Similar to findings related to depression-like behavior, the contribution of CUMS and TBI to anxiety-like behaviors was inconsistent. In the mild TBI cohort, sham mice that were exposed to stress demonstrated less behavioral anxiety on EPM than TBI groups at 2-weeks post-surgery; however, this finding was not replicated using the DLB test. Similarly, in the moderate TBI cohort, mice in the non-stressed sham group demonstrated the least anxiety-like behaviors on EPM at 2-weeks post-surgery. However, the stressed TBI group showed increased anxiety-like behavior on DLB. Overall, exposure to stress appeared to have a more pronounced impact on anxiety-like behavior following moderate TBI, whereas premorbid stress primarily contributed to post-injury changes in the mild TBI cohort in combination with exposure to TBI.

Injury Severity

On TST, non-stressed sham mice in both the mild and moderate TBI cohorts exhibited more behavioral despair, although this trend was only statistically significant in the mild TBI group at 1-month post-surgery. On FST, non-stressed sham mice in the mild TBI cohort demonstrated more mobility, while in the moderate TBI cohort, the CUMS + TBI mice were more

mobile. On EPM, CUMS + sham mice in the mild TBI group showed the least anxiety, while non-stressed sham mice in the moderate TBI group showed the least anxiety, suggesting that the lasting effects of stress on post-injury anxiety may vary by injury severity. However, these findings were not corroborated by the results of DLB test, which measures similar constructs.

Recovery Time

Overall, it is notable that behavioral despair, as measured by TST and FST, increased between administrations. This finding may demonstrate genuine increases in affective distress over time or may be indicative of changes in the animals' responses to the measure itself in repeated administrations. Additionally, mice in both cohorts spent less time in open arms on EPM at 1-month post-surgery, indicating more anxiety-like behavior. As this finding was not consistent on DLB, this discrepancy could indicate that these tests may capture different constructs related to anxiety (Weiss et al., 2000). Importantly, several studies have observed similar increases in immobility or anxiety behaviors over time associated with repeated exposure to behavior tests of these constructs (Bourin, 2019; Kazavchinsky et al., 2019).

In conclusion, it is important to note that pre-exposure to stress plays a role in recovery following TBI, both independently and in conjunction with the injury itself. The exact contribution of stress in behavioral outcomes varies by exposure to TBI, injury severity, and time following the injury, or recovery time. A better understanding of the relationship between stress and recovery is necessary to illuminate potential factors in evaluation and intervention processes which may facilitate effective recovery.

Implications

Importantly, this study is among the first of its kind to develop a clinically relevant animal model of mild to moderate TBI, as well as to capture how pre-existing factors may exacerbate injury outcomes. First, the use of a stress-induction method with robust predictive validity allowed for reliable measurement of the impact of stress in post-injury outcomes (Belzung &

Lemoine, 2011). As hypothesized, exposure to stress produced behavioral differences in recovery both in isolation, and in conjunction with impact injury. Second, the methods used in the present study allowed for observation of differences between levels of severity in TBI. Milder TBIs are highly underrepresented in animal model research despite the overwhelming need for similar research in human populations (Shultz et al., 2017). Finally, the current study illuminated several avenues for future animal model research that will further our understanding of the effects stress on TBI outcomes.

Functional Outcomes

In the current study, neither stress nor TBI contributed to significant differences in measurements of RRR. This finding replicates previous studies which showed functional recovery between control and TBI experimental groups as indistinguishable during the first 72 hours post-injury (Tweedie et al., 2007). Thus, although RRR measurement is considered a useful tool to understanding injury severity, it may not be the most robust tool for predicting outcomes of more mild injuries, and accurate measurement is highly dependent on standardization in surgical procedures. Importantly, previous studies have demonstrated that CUMS induces hyperactivity in mice, suggesting that the pathophysiological consequences of stress may serve as a protective factor against functional impairments or development of affective syndromes (Aglamal et al., 2019; Patricia et al., 2021). In humans, there is not sufficient empirical evidence to support the conclusion that concussion or mild TBI contributes to similar hyperactivity in the acute stage of recovery, nor do TBIs exacerbate existing hyperactivity in the context of diagnosed attention-deficit hyperactivity disorder (Iverson et al., 2016; Stewman et al., 2018). Future directions for research may involve exploring potential mechanisms of this finding with transgenic models, which are increasingly useful for isolating various neuroprotective factors in studies of stress and recovery from brain injuries (Chan et al., 1995). For example, one such study found that mitochondrial manganese SOD and neuronal

nitric oxide (NO) synthase were involved in functional recovery from TBI, as transgenic mice with over-expressed SOD and NO synthase did not have lingering neurological deficits (Chan et al., 1995). Additional studies have explored the role of oxidative stress in TBI outcomes in mice by utilizing transgenic mouse models to demonstrate that alterations in altered oxidation and inflammation regulating properties expedite post-injury recovery (Bhowmick et al., 2021; Ismail et al., 2020; Wang et al., 2019).

The present study utilized a modified version of NSS, a measure used in animal model studies to detect the severity of functional impairment following injury (Siebold et al., 2018). Performance on this task across mild and moderate TBI cohorts was grossly intact, with the exception of several mice which, upon autopsy, sustained significant lesions during surgery. The mNSS, therefore, was sensitive to very significant injuries, but was insufficient in specifying any subtle changes due to more minor injuries. Other studies have found similar results from the NSS, noting that many of the parameters measured by the instrument are uncommon, even in the case of severe TBI, and are thus less sensitive to any changes following mild or moderate injuries (Chen et al., 2021; Siebold et al., 2018). Recent reviews of available literature indicate that the NSS and RRR were used in measuring post-injury outcomes in 31% and 10% of mTBI studies, respectively; many of which demonstrated minimal to no deficits in mild injuries, similar to the current study (Bodnar et al., 2019). These findings parallel patterns in human TBI literature, in which any neurological or cognitive deficits occur in the acute stage of mTBI, then are expected to recover fully to baseline. Future studies may benefit from using more comprehensive protocols for assessing neurological functioning and behavior in animal models of milder TBIs. For example, future studies could use the SHIRPA procedure, which provides examiners with a comprehensive phenotypic picture using a three-stage assessment process (Rogers et al., 1997). In the SHIRPA procedure, stage one involves observational assessment of aspects of behavior and functioning, such as gait, coordination, excitability, and temperature.

The second stage of screening includes a comprehensive battery of assessments to measure locomotor activity and autonomic functioning. The final stage includes similar measures to the present study, gathering data regarding neurological functioning and behavioral phenotype. Additionally, various itemized behaviors in the current protocol could be expanded to involve a graded approach, which may illuminate minor differences between experimental groups. For example, previous studies have utilized the rotarod test, in which the speed of a rotating rod slowly increases until the animal can no longer sustain its position (Curzon et al., 2011). The current study utilized only one beam; however, other studies have tested the animal on several beams that progressively decrease in width to test the limits of the animals' balance (Curzon et al., 2011). Use of the SHIRPA procedure is gaining popularity in research studies due to the unique phenotyping capabilities and ability to observe areas of vulnerability and resilience (Lalonde et al., 2021; McCarson, 2020).

Behavioral Outcomes

Given the variability in outcomes between TBI severity cohorts, as well as changes observed over time post-surgery, the findings of this study are consistent with an overwhelming majority of the animal model literature, which suggests that behavioral recovery following TBI is highly dependent on injury severity, both in the symptom manifestation and symptom significance (An et al., 2016). Several questions emerge from the findings of the current study regarding the measurements of various constructs related to anxiety and depression behaviors, as well as the role of activity or mobility in stress and TBI outcomes.

Although different patterns of behavioral despair were observed across mild and moderate cohorts in performance on TST and FST, these instruments are still considered highly valid measures of depression-like behaviors (Kraeuter et al., 2019b; Stukalin et al., 2020). Future studies using different types of depressive-like tests such as sucrose consumption test or lateral hypothalamic self-stimulation may be more informative as these other behavioral tests

are able to specifically isolate different dimensions of depression (i.e., anhedonia; Liebman, 1983). Tests of pleasure-seeking behavior have been used in previous animal model studies of depression and substance use behaviors, which commonly co-occur in TBI populations and thus may be more applicable for use in future studies (Cryan & Holmes, 2005; Lowes et al., 2021; Taylor et al., 2003).

Stressed mice in the moderate TBI cohort demonstrated more mobility on one measure (DLB) of behavioral anxiety. This group also demonstrated the least behavioral despair on FST, which may provide additional support for an increase in activity levels due to stress, TBI, or both in moderate TBI. In the mild TBI cohort, however, the interaction of stress and injury contributed to more behavioral despair on TST. Similar findings have been observed in studies using the open field test, similar to DLB, and FST, where stress and injury contributed to variable activity levels (Heldt et al., 2014; Jones et al., 2008). Still, other studies have seen a specific delineation between phenotypes following either stress alone or stress and TBI, wherein stress alone contributed to a mixed anxious-depressed phenotype, while the combination of stress and TBI produced a primarily anxious phenotype (Algamal et al., 2019; Jones et al., 2008). These findings may parallel the human TBI literature, which indicates that many individuals who experience TBI develop a variable constellation of affective symptoms and syndromes after injury. In a review of 34 studies within the human TBI literature, approximately 21% of individuals across studies were diagnosed with anxiety disorders and 17% with depressive disorders within the first year after experiencing a TBI (Scholten et al., 2016). Monitoring of symptoms in the acute phase may be beneficial to identifying individuals who may be vulnerable to prolonged recovery, as one study found that certain anxiety-related symptoms reported 3-months post-injury helped to predict PCS severity at 12-months post-injury (Sigurdardottir et al., 2009).

Of note, the present study primarily focused on time spent in low-, moderate-, and high-mobility states, as derived from settings using Ethovision software during DLB and EPM (Noldus et al., 2001). Previous studies have demonstrated the utility of DLB and EPM to differentiate anxiety-like behavior by comparing performance across mice bred for low-, medium-, and high-activity levels (Booher et al., 2021). Additional data points or calculations derived from Ethovision during administration of DLB and EPM, such as the number of crossings, distance traveled, or ratios of time spent in different areas of the box, could provide helpful insight into the relationship between stress, TBI, and locomotor activity levels. DLB and EPM have repeatedly proven valid measures of anxiety-like behavior, particularly in sample sizes as large as that of the current study (Booher et al., 2021; Kraeuter et al., 2019a; Smalheiser et al., 2021). However, recent studies have noted variable reliability and test-retest variability using these measures in anxiolytic treatment trials (Bourin, 2019; Rosso et al., 2022). As such, additional measures such as Y-maze activity, startle reactivity, and inhibition assessments may be useful for characterizing psychomotor mobility and could be implemented in future experiments (Paulus et al., 1999).

In addition to exploring changes in mobility, future studies could assess behaviors mediated by the prefrontal cortex, such as impulsivity, to determine the impact of stress and TBI on higher-order cognitive functions (Adriani et al., 2003). Tests that assess spatial memory (e.g., T-maze, radial maze, Morris water maze, Barnes maze) and problem-solving (puzzle box tests) could characterize the impact of stress in TBI outcomes and provide additional data to inform factors of individual ability or resilience (Galsworthy et al., 2005; Sharma et al., 2010).

Additionally, both animal and human models of PTSD and mild TBI may be an important area of study, given the significant overlap of symptoms between PCS and PTSD in human populations. One study designed to asses this overlap determined that several symptoms reported in association with PCS are better characterized as hyperarousal symptoms

associated with trauma and stress-related disorders (Lagarde et al., 2014). The interconnected nature of emotional and physical trauma involved in several instances of TBI is yet to be fully understood. A recent literature review of psychological contributors to PCS indicated that prolonged recovery from mild TBI may not only be attributable to emotional trauma experienced simultaneously with the injury, but with previous trauma exposure or diagnosis of PTSD (Williams et al., 2010). Recently developed treatments targeting the overlap between these syndromes have proven effective in both promoting PTSD recovery and preventing PCS (Ragsdale et al., 2022).

Limitations

It is important to acknowledge the limitations of this study and, therefore, its generalizability to the body of research on stress and TBI. As with many animal model studies, several aspects of the environment have the potential to impact results of animal model studies. For example, studies have shown that inter-rater variability is possible in animal model research, as animal behavior can be conditioned by human factors, such as body odor (Nigri et al., 2022). Additionally, the mice were individually housed with minimal enriching items, which is known to contribute to anxiety and depression (Duman, 2005; Liu et al., 2020). Additional studies have explored the impact of completing behavior assessments individually compared to group administrations, demonstrating some potential for variability in outcomes (Ueno et al., 2022). The animals in this study were maintained in single-housing with limited enrichment in their environment, which have both been correlated to anxiety- and depression-like behaviors in mice (Berry et al., 2012; Kazlauckas et al., 2011; Kempermann et al., 1997). Further, studies have explored the role of sleep and sleep deprivation as potential mechanisms for prolonged affective distress following TBI, and no intentional efforts were made in this study to monitor the length or quality of sleep (Portillo et al., 2022).

As previously stated, animal models are limited in their ability to fully characterize the human emotional experience (Planchez et al., 2019). Given the importance of self-concept, beliefs, and meaning-making to TBI recovery outcomes in humans, measuring anxiety-like behaviors and behavioral despair captures a portion of the full clinical picture (Bay & Liberzon, 2009; Belanger et al., 2017; Iverson et al., 2018; McMahon et al., 2014; Silverberg et al., 2017; Snell et al., 2013).

Although data from this study may provide some insight into the pathology of chronic exposure to psychological stressors in mice, it offers an incomplete picture of the complex etiology and pathology of anxious and depressed states in humans. Not only are human presentations of affective disorders highly variable in reported symptoms, but presentations also vary in the pattern of progression or resolution of symptoms over time (Nandi et al., 2009). Furthermore, the significant overlap between anxious and depressive syndromes provides an obstacle to both measurement and treatment (Kaiser et al., 2021; Konstantopoulou et al., 2020; Unick et al., 2009). As a result, there is increasing interest in research studies such as this one that focus on specific symptoms that are characteristic of affective disorders (Kotov et al., 2017).

The present pilot study represents a model for exploring the contribution of stress in mild to moderate TBIs, which is fairly underrepresented in animal model research. In this uncharted territory, the surgical protocols resulted in tissue damage in several mice following skull penetration, which would necessitate classification as moderate to severe TBI in human research (Roebuck-Spencer & Sherer, 2017). Adjustments to the protocol ensured a milder injury for the mild TBI cohort, which did not include exposing cortical tissues. Due to the need for relevant research in the area of concussion and mild injuries, future studies might employ additional models with less risk of tissue damage or more robust empirical support for reliably producing a mild injury that more adequately mimics a concussion or mild TBI (Main et al., 2017). Similar to research in human populations, some of the mice may have been more

resistant or resilient to the stressors that were employed than others, and thus the specific CUMS model may not have been a viable animal model of stress (Russo et al., 2012). Previously published data indicated that mice can be separated into two stress response categories: susceptible or resilient (Russo et al., 2012). Mice who are more resilient to stress do not display the same types of depressive-or anxiety-like phenotypes as mice who are considered susceptible (Dudek et al., 2021; Willmore et al., 2022). The current study did not include assessment of susceptibility or resilience in the experimental process, which could contribute to some of the observed variability in behavioral outcomes. Thus, efforts to delineate between these 2 groups prior to introducing CUMS and TBIs may be necessary in future studies using this animal model.

The heterogeneity of both human and animal model research on TBI outcomes represents a challenge to any study in these fields. Apart from the substantial difference in brain mass, humans also possess a far superior cerebral cortex to mice, which has been studied for its capacity to process some aspects of affective disorders that cannot be replicated in animal models (Cryan & Holmes, 2005; Woodcock & Morganti-Kossmann, 2013). For example, diagnostic criteria for both anxious and depressive syndromes in humans include items related specifically to thought content (e.g., suicidal thoughts, self-esteem), which cannot be measured in mice. However, animal models represent a way to explore individual vulnerabilities, such as those broached in the present study, which could allow for more individualized approaches to recovery and treatment in the future (Woodcock & Morganti-Kossmann, 2013).

Implications for Counseling Psychology

Importantly, the implications of individual factors of vulnerability and resilience in TBI research are particularly salient to the field of counseling psychology due to the discipline's traditional focus on individual differences and orientation to avenues of growth (Scheel et al., 2018). The observed variability in this study parallels that of the greater body of TBI research,

wherein prolonged TBI recovery can often be attributable to identity and environmental factors of the individuals in research samples (Gonçalves & Perrone-McGovern, 2014). Collaboration between the fields of neuroscience and counseling psychology are therefore imperative to address gaps in the current literature. Two important areas of focus from counseling psychology with increasing interest in TBI include the roles of culture and subjective experience in injury and recovery (Caplan et al., 2021; Goss, 2016). For example, a recent study examining incidence rates of TBI among various racial and ethnic groups determined that POC experience more TBIs and less follow-up medical care than White individuals and are significantly under-represented in TBI research (Brenner et al., 2020). Additionally, one study compared case examples from retired athletes and found that changes in identity, subjectivity of experience, self-awareness, and perceived levels of social support were implicated in the degree of somatic and psychological symptoms reported after TBI (Senecal & Whitehead, 2021). Improvements in neuroscience research to incorporate values of counseling psychology by observing and characterizing the role of individual vulnerability and protective factors may contribute to better means of diagnosis and treatment for individuals who experience TBI and reduce the potential for prolonged symptoms or stunted recovery (Taylor & Seebeck, 2020).

The findings of this study provide several considerations for counseling psychologists working with populations who experience TBIs. First, psychologists who are knowledgeable of the expected recovery trajectory of TBIs are uniquely positioned to provide supportive psychoeducation to address areas of misinformation surrounding head injuries. Research indicates that several misconceptions regarding recovery are shared among the general public, particularly on the topic of concussion and mild TBI, which can contribute to stigma and poor recovery expectations (Ralph & Derbyshire, 2013). Counseling psychologists may provide helpful insight that is more digestible in the context of the therapeutic relationship. Second, psychologists are highly equipped to provide helpful conceptualizations of TBI and recovery to

treatment teams, family members, and individuals. A recent literature review indicated that the most effective method for conceptualizing the experience of PCS, and therefore identifying vulnerable individuals, requires consideration of predisposing, precipitating, and perpetuating factors (Rickards et al., 2022). Predisposing factors include premorbid psychiatric factors, such as chronic stress, as well as pre-injury intelligence, personality, perceptions, behaviors, and neurological history. Precipitating factors include simultaneous diagnosis of a psychiatric condition related to the injurious event, such as posttraumatic stress disorder, and misdiagnosis of injury severity. Finally, perpetuating factors include persistent psychiatric conditions, pessimism or low expectations of recovery, hindsight bias, and potential motivation for disability compensation. Considering this model, counseling psychologists possess a unique set of skills to identify, understand, and provide treatment to individuals who demonstrate prolonged recovery from concussion and mild TBI.

Recommendations

The goal of a pilot study, such as the present study, is to add to the body of research and provide examples of the efficacy of a specific protocol. Additional research is needed to further clarify modes of classification for TBI severity in animal models, establish more effective means of standardizing the injury and subsequent monitoring, as well as gathering the most relevant measures of behavior to mirror clinical postconcussive presentations. Though the present study included large sample sizes in the context of animal model research, future studies should seek to examine the impact of stress on TBI outcomes on a larger scale to improve the consistency of findings and generalizability of results. Finally, as with all animal model research, confounding factors related to experimenter variability and animal housing environment should be considered in the planning, execution, and analysis process.

To conclude, future TBI research is needed in order to identify individual sources of vulnerability, such as stress, contribute to recovery outcomes. Given the global burden of TBI

and subsequent treatment needs, elucidating factors that contribute to the heterogeneity of responses to injury is essential for developing effective and innovative means of recovery. The present study explored the role of pre-injury stress in functional and behavioral outcomes of TBI, and future research should focus on the interaction of stress and TBI as a mechanism for symptomatology, differences in recovery time by injury severity, and further standardization of the research protocol for animal models of mild and moderate TBI.

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APPENDIX A

APPROVED IACUC PROTOCOL

ANIMAL USE PROTOCOL APPLICATION

Texas Woman's University

Institutional Animal Care and Use Committee (IACUC)

Submit completed protocol electronically in Word document format to LByford@twu.edu at least one week prior to the next IACUC meeting. Attach addendum for Non-TWU investigators.

1. ADMINISTRATIVE DATA

☒ New ☐ Renewal: Previous Protocol # (if applicable):		☐ Protocol for Breeding Only ☐ USDA covered species included (species other than rats, mice, birds, fish, reptiles, amphibians) ☐ Non-TWU investigators included
Title of Project: **The effects of chronic mild stress on TBI outcomes.**		
Principal Investigator: Zane Lybrand		
Department: Biology		**Office Building and Room: SRC304J**
Office Phone: 940-898-2192	**Cell Phone: 817-688-3057**	**E-Mail: Zlybrand@twu.edu**
Co-Principal Investigator (if applicable; attach additional table for multiple Co-PIs): Dr. Elisa Na		
Department: Psychology		Office Building and Room: 204E
Office Phone: 940-898-2307	Cell Phone: 319-621-1565	E-Mail: ena@twu.edu
Funding Source: TBD If federally funded, provide a copy of the vertebrate section of the grant.		
Application Date: 7/28/2021		

2. PEER REVIEW

Has or will this protocol undergo peer review and be evaluated for scientific merit and experimental design? ☒ Yes ☐ No

If yes, who provides peer review (e.g. NIH/NIA, or the name of an individual if no review committee)?
Department of Defense. This application was originally submitted as an amendment to #2020-07, but after consultation with Dr. Averitt and comments from the IACUC, it was changed to a new parent protocol.

If no, and the project is USDA category D or E or a USDA covered species outside review of scientific and/or educational content is required before IACUC review. Has this protocol undergone outside peer review? ☐ Yes ☐ No

If yes, who provided the review?_____

Filled out by IACUC office only - signatures will be collected by ORSP electronically following approval

IACUC REVIEW *(for committee use only)* **Protocol Number: 2021-08**

Date Protocol Application was Received by IACUC: *(MM/DD/YY)* 07/30/21

Date Protocol Approved: *(MM/DD/YY)* _____

Date of Protocol Expiration: *(MM/DD/YY)* 08/05/2024

Institutional Animal Care and Use Committee Approval: _____ Date: _____
Committee Chair

Radiation Safety Committee Approval: _____ Date: _____
Committee Chair

Institutional Biosafety Committee (IBC) Approval: _____ Date: _____

Committee Chair

3. PI/COLLABORATORS/STUDENTS/OTHER PERSONNEL (Training & Qualifications)

Indicate by completing the following table the qualifications of investigators, professional, technical, or student personnel who will be overseeing or actually performing experimental procedure(s) with animals including all personnel who may have direct contact with animals and animal tissues. If non-TWU personnel, complete addendum form. Indicate the individual's involvement in surgical procedures or euthanasia.

Name Degree, Certification, or Licensure	On Campus/ Office Phone Number	Emergency Contact Phone Number	**Date of most recent CITI Training (IACUC use only)**	**Experience with Species (years)**	**Experience with Procedure(s) (years)**	**Role *, **** List Expected Role on Protocol (animal handling, behavior testing, injections, surgery, euthanasia)
Zane Lybrand, PhD	940-898-2192	8176883057		10+	3+	PI; Animal handling, surgery, euthanasia, training, behavior
Elisa Na, PhD	940-898-2307	396-621-1565		10+	10+	PI; animal handling, behavior, euthanasia
Myles Gladen	940-765-0213	469-232-1561		<1	<1	Graduate Student; Animal handling, surgery, behavior, euthanasia
Abbie Baird	512-744-5499	817-929-0282		<1	<1	Graduate Student; Animal handling, surgery, behavior, euthanasia
Monica Ruiz	940-898-2307	940-442-9321		<1	<1	Undergraduate Student; Animal handling, behavior

*** If an investigator, student, or technician listed in this protocol application is performing the procedure for the first time, describe the type of training (below) he/she will receive, the person(s) who will provide that training, and the qualifications of that person to provide such training.**

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

Dr. Lybrand has many years of experience with rodent stereotaxic surgeries and has trained and supervised Myles Gladen on the TBI surgical procedure and aseptic technique. Dr. Na has 10+ years experience with animal behavior tests and will be training and supervising Abbie Baird and Monica Ruiz. All students have completed all required CITI training and supervised hands-on training. They will continue to be supervised by Dr. Lybrand and Dr. Na for this project.

**** Surgical and euthanasia technique training or refresher sessions are available to TWU investigators by the Attending Veterinarian and professional veterinary technicians. Does the PI request either training or a refresher in any techniques listed on the protocol?** *A refresher session is required for any techniques listed on the protocol that key personnel have not performed within the last 5 years. If yes, please provide the request in the box below.*

☐ Yes, training or a refresher session is requested.
☒ No, at least one of the key personnel has performed each listed procedure within the last 5 years.

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

4. FUNDING INFORMATION

Is this Application associated with an internal/external grant? ☒ Yes ☐ No; Departmental Funding

***If yes**, list the title used on the Grant application to assure proper notification of the approval status to the funding agency. The grantee must be either the Principal Investigator or the Co-Investigator on the IACUC protocol. For federally funded projects, the PI must submit a copy of the vertebrate animal section of the grant with the IACUC application.*

Principal Investigator on Grant: Lybrand and Na (Co-PIs)		
Funding Agency or Fund Source: Department of Defense		
Grant Title: The effects of chronic mild stress on TBI outcomes.		
Grant or Project Duration Dates:	Beginning: TBD	Ending: TBD
Contract/Grant Number (if external):		

5. Non-technical (Lay) Summary of Project

In the space below, please provide a brief nontechnical (lay) description of this project. The language used should be understandable to a non-scientist with a 9th grade education. Please avoid using medical/scientific terminology. This summary should include (1) an introductory statement of the purpose of and need for the studies, (2) descriptions of the animal use from start to endpoint (with a statement explaining that the animals will be humanely euthanized by approved methods), and (3) brief explanations of procedures, measures, and time courses of the experiments involving animals. The summary should include how discomfort, pain, or distress to the animals will be minimized. The summary should be limited to 300 words and be succinct, informative, and complete to facilitate review by a broad audience.

[Type text in the text box — Spacing will adjust to accommodate the length of the narrative]

(1) The goal of this project is to understand how environmental factors alter the course of traumatic brain injury (TBI) recovery. Following TBI, outcomes vary from individual to individual and our overall goal is to determine how chronic mild stress prior to TBI changes the brain to alter outcomes. This is particularly relevant to the military and Department of Defense as many combat veterans who report care for TBIs are under chronic stress conditions.
(2) To address this, we will be exposing mice to a classic chronic mild stress (CMS) paradigm prior to giving TBI. CMS is a well-validated and widely used animal model of depression where mice are randomly presented with environmental stressors from 2-6 weeks. Following CMS, mice will be given a moderate TBI with the controlled-cortical impact model and monitored for neurological changes for the next 2 months. At 1 month following TBI mice will undergo a battery of behavior tests to assess changes to anxiety, depression, learning and memory. Behavior testing will span 2 weeks. One group of mice will be euthanized for slice electrophysiology prior to behavior. A second group will be euthanized after behavior tests for molecular analysis. Both groups will be humanely euthanized by IACUC approved methods.

This project involves some discomfort, pain, and distress in mice and we will minimize in all possible ways by using pain medication following all surgical procedures (e.g. CCI) as well as daily wellness checks that involve a modified Neurological Severity Score (mNSS) that evaluates neurological function including motor, sensory, reflex, balance, and pain evaluation.

6. Animal Numbers & USDA Classification of Animal Use

In the chart, provide animal numbers per year, per animal use classification. This includes any pups bred or acquired in-house used in this study. These numbers must be consistent with the number of animals described in the justification. Please see below a description of each USDA pain category (please note there is no Cat A).

BREEDING: It is understood that the number of pups bred for studies can only be estimated. Please provide your best estimate based on the approximate number of pups needed for the study, pups that cannot be used, and breeders needed for cross-breeding or repopulating. These numbers should not include any pups used in the study. Pups must be counted as an individual the day of birth.

Species: Mouse *If have additional species, attach additional pages as necessary.*

Project Period * (1 year per line)			**Number of Animals by Category:**				**Total number of animals**	
Year	**From (mo/yr)**	**To (mo/yr)**	**B**	**C**	**D**	**E**	Total of animals per category/year	Total of animals per category/year
1	8/2021	8/2022			20	60	80	
2								
3								
4								
5								
Total number of animals for procedures *(should be consistent with the number of animals described in the justification)*:							**Study Total 3yrs: 80**	*Study Total *5yrs:*
Total number of animals used exclusively for breeding (not used for study). Including breeders and unusable pups:							**Breeding Total 3yrs: 0**	*Breeding Total *5yrs:*
TOTAL NUMBER OF ANIMALS:							**Total 3yrs: 80**	*Total *5yrs:*

Include total anticipated period of project funding (for grants beyond 3 years) and animal use. PHS policy stipulates that anticipated use of animals more than three years beyond approval date should be included, even though new IACUC approval will be required after three years.

USDA Classification

Classification B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes (e.g. breeding or observation protocols).

Classification C: Animals upon which testing, research, experiments, or tests will be conducted involving only momentary or slight pain or distress and do not require pain-relieving drugs (e.g. routine injections, feed studies, tail snips).

Classification D: Animals upon which experiments, teaching, research, tumor bearing experiments, surgery, or tests will be conducted which have the potential to cause pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used to prevent this pain and distress (e.g. surgery, UV exposure, biopsies).

Classification E: Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests (pain analysis, food/fluid deprivation, toxicology).

6a. Justification for Classification E Animals (Required):

If you have Classification E animals, provide a justification below. Otherwise, skip to 6b.

An explanation of procedures producing pain or distress and the justification for not using appropriate anesthetic, analgesic or tranquilizing drugs must be provided. This information is required to be reported to the USDA, will be available from the USDA under the Freedom of Information Act, and may be publicly available through the internet via USDA's website. (NOTE: You do not need to provide this justification if you do not have Classification E animals.)

[Type text in the text box -- Spacing will adjust to accommodate the length of the narrative]

All 60 mice in experiment 2 will undergo behavior test. One test will be tail suspension test which is rated as a USDA Category E. This test is a robust assay for learned helplessness and depression-like behaviors.

6b. Procedures to be Performed in Live Animals:

Check the box next to each procedure that will be performed.

Procedures	Mark an X below to indicate the procedure will be performed.
1. Administration of experimental agents	☐ Chemical ☐ Biological ☐ Other
2. Antibody production/collection (including hybridomas)	☐
3. Breeding	☐
4. Blood/tissue collection in live animals	☐
5. Behavioral tests	☒
6. Cardiac perfusion *terminal procedure involving replacement of blood with fixative while the animal's heart is still beating*	☒
7. Euthanasia followed by tissue harvest	☒
8. Invasive field study *study of animals in natural habitat involving an invasive procedure or one that harms or alters normal behavior*	☐
9. Any variations in standard housing or husbandry *e.g. food/water restriction, special diet/water, alteration of cage environment, withholding standard environmental enrichment, etc.*	☒
10. Use of Adult or Embryonic Stem Cells (including iPS cells)	☐
12. Potentially stressful techniques *such as gavage, bronchial lavage, restraint*	☒
11. Minor survival surgery *any incision that does not penetrate a body cavity but requires aseptic techniques*	☐
12. Major survival surgery *surgical procedures which either penetrate a body cavity or result in permanent impairment of normal functions*	☒
13. Multiple major survival surgeries	☐
14. Terminal surgery *animal is not recovered from anesthesia*	☐

7. DOCUMENTATION / LITERATURE SEARCH

A literature search must be performed to prevent unnecessarily duplication of research projects/courses performed at this and/or other institutions, and to demonstrate that there are no alternatives (such as computer models, tissue culture, etc.) to the use of live animals.

Please complete both an AWIC search (www.nal.usda.gov/awic/) for alternatives AND complete a MEDLINE search (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed) to rule out unnecessary duplication.

7a. Date of Literature Search: 7/29/2021

7b. Databases, Indexes or other sources used for review of literature: PubMed

7c. Years covered in review: 2013-2021

7d. Keywords: Chronic Mild Stress, TBI, hippocampus, and/or BDNF

Results of the Literature Search (Required of all protocols):
Provide a narrative description of the result of the literature search. **Include a Statement of Assurance that the literature was reviewed for non-animal or less sentient animal species to partially or fully replace animals (such as tissue culture, or insect model), and that this project is not unnecessarily duplicative of research projects/courses performed at this or other institutions.** This narrative should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternatives or alternative methods. If the database search or other source identifies a valid alternative method (one that could be used to accomplish the goals of the animal use proposal), the written narrative should justify why this alternative was not used.

More Detailed Documentation Required for Classification D & E: If any procedures fall into USDA's Classification D or E, causing more than momentary or slight pain or distress to the animals, describe your consideration of alternatives and your determination that alternatives are not available. Delineate fully the methods and sources (7a-d above) used in the search. Alternatives include methods that (a) refine existing tests by minimizing animal distress, (b) reduce the number of animals necessary for an experiment, or (c) replace whole-animal use with *in vitro* or other tests. **When ascites production is used to produce antibodies, justification needs to be given** as to why *in vitro* systems cannot be used. You must certify that no valid alternative was identified to any described procedures which may cause more than momentary pain or distress, whether relieved or not.

[Type text in the text box — Spacing will adjust to accommodate the length of the narrative]

When "Chronic mild stress AND TBI and hippocampus" were searched on PubMed 7 recent papers came up from 2013-2021. When I included BDNF it only returned 1, "Impact of Repetitive Mild Traumatic Brain Injury on Behavioral and Hippocampal Deficits in a Mouse Model of Chronic Stress" DOI: 10.1089/neu.2018.6314. This paper used a more intense chronic stress paradigm than our planned mild chronic stress and characterized behavior and cortisol levels. We plan to investigate specific changes to molecular and neurophysiology pathways of the hippocampus (e.g entorhinal cortex to dentate gyrus). Additional search results focus on a variety of TBI models (e.g. blast). We will use an impact model TBI.

Alternatives to Category D. Our TBI surgery includes craniotomy and impact to the brain. For pain, we are using a slow-release opioid analgesic for pain management (Buprenorphine SR).

Alternatives to Category E. We are including tail suspension test as a measure of depression-like behaviors. Alternatives include Forced Swim Test, which we are also including. Together, both tests will give us a robust assay for changes in learned helplessness, depression-like behaviors, and behavioral despair – all prominent comorbidities of TBI.

To estimate the number of animals to include for behavior, 22 For slice physiology, we chose an n=5/group based on previous experience with these types of experiments. Typically, 12-15 recordings from 3-5 mice are acceptable replicates.

Note: *Answer items 8 -22 separately for each species of animal to be used.*

8. What species will you be using? Mouse

8a. List the strain name(s): C57/bl6

8b. List the source(s) of animals: Jackson; Envigo

8c. List the age and/or weight of animals at acquisition (if appropriate): 5-6 weeks old; 12-15g

8d. What is the maximum number of this species to be housed at one time? 80

8e. What sex is requested? ☐ Male ☐ Female ☒ Both

9. Special requirements for maintaining animals: ☒ **Yes** ☐ **No**

If yes, indicate the requirements below, such as enrichment, caging type, bedding, type of water and dietary requirements. If the PI will conduct any feeding, refer to TWU IACUC Policy 003. If no, animals will be maintained according to the standard operating procedure of the animal facility (skip to 10).

[Type text in the text box — Spacing will adjust to accommodate the length of the narrative]

As part of the CMS protocol, mice will be food deprived or water deprived for 10-12 hours. Special cage cards will be used to identify these mice.

9a. Other special instructions for animal care staff:

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

10. Will animals be individually identified (ear tag, microchip, etc.)**?** ☒ **Yes** ☐ **No**

If yes, describe method: ear punch or ear tag

11. Wild or exotic species? ☐ **Yes** ☒ **No**

If, yes, Permits? ☐ **Yes** ☐ **No**

12. Restraint (other than while under surgical plane of anesthesia):

12a. Will manual restraint be used? ☐ **Yes** ☒ **No**

If yes, for how long? Duration: _____ Frequency: _____

12b. Will a restraint device be used? (chairs, slings, tethers, stanchions, metabolism cages or other devices) ☐ **Yes** ☐ **No** If yes, answer 12b-i – b-vii. If no, skip to 13.

12b-i. Method: _____

12b-ii. Duration: _____

12b-iii. Frequency: _____

12b-iv. Frequency of observation during restraining: _____

12b-v. Person(s) responsible for observation: _____

13. Anesthesia and Analgesia:

Will any procedures require anesthesia, analgesia, or neuromuscular blocking agents? If Avertin is used for anesthesia in mice, refer to TWU IACUC Policy 001.

☒ **Yes** ☐ **No** If yes, answer 13a-b. If no, skip to 14.

13a. Person(s) administering agents: Zane Lybrand and Myles Gladen

All personnel administering anesthesia and giving post-anesthesia care must have received appropriate hands on training.

13b. If yes list method, dose, route, frequency, and duration:

	Drug	Dose mg/kg	Route	Frequency/Duration

Non-Surgical Procedure	2,2,2-Tribromoethanol (TBE; Avertin) from Sigma-Aldrich T48402	250mg/kg	IP	Once for transcardial perfusion
Preoperative	50/50 bupivacain/lidocaine drip	Dilute 2% (20 mg/ml) Lidocaine 1:4 and 0.5% (5mg/mL) Bupivacaine 1:2 in the same vial	topical	Prior to incision
	Buprenorphine SR	1mg/kg	SQ	Once; Prior to survival surgery
Intraoperative	Isoflurane Gas	5% isoflurane with oxygen at 4L/min to induce; 1-3% isoflurane and oxygen at 0.5-2L/min	Vaporizer	Constant, during surgery
Postoperative				
Neuromuscular blocking agents*				

** If neuromuscular blocking agents are used during the surgical procedure, a narrative for the justification of its use must be included below.*

14. Surgery: ☒ Survival ☐ Multiple Survival ☐ Terminal ☐ None (If none, skip to 15)

14a. Person(s) performing the surgery: Zane Lybrand and Myles Gladen

14b. Describe the surgical procedure(s) in the space below (refer to TWU IACUC Policy 007 on Aseptic Rodent Surgery as needed):

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

I. For TBI (Controlled Cortical Impact) model (stereotaxic surgery):

1. Sterile surgical gloves, mask, and hair coverings will be used. All surgical instruments and drill burr are autoclaved and sterile surgical techniques will be used. A hot bead sterilizer will be used in between animals within a given surgery. TWU Policy 007 regarding Aseptic Surgery will be followed.
2. Mice will be anesthetized.
3. Pre-operative analgesia (e.g Buprenorphine SR; 1mg/kg; 23G, 25G, or 26G needle SQ) and saline are given.
4. Animal preparation: Animals will be provided with a heat source (heating pad) during the pre-, intra-, and post-operative periods. After mice are fully sedated, Puralube ointment will be applied to eyes to prevent dryness. Animal(s) heads will be shaved and betadine SCRUB followed by 70% alcohol is applied to the skin (repeat process 3 times), before placing in the stereotaxic frame. Sterile

draping (Glad Press' n Seal) is applied and is cut to expose the surgical field above the animal's head. Press n' seal is applied to stereotaxis knobs, vaporizer, and additional surgical devices.
5. For the craniotomy, a 4mm diameter circle is cut to remove the skull in the target region. Bone is removed to expose dura mater.
6. Induce TBI with pneumatic piston (Impact One from Leica). See Romine et al., 2014 DOI: 10.3791/5178. Sham mice will receive no impact.
7. Wound site is cleaned of blood and the wound is closed back up once bleeding has stopped by suture or tissue glue.
8. Mouse is returned to warm pad until awake and upright walking around.

II. Transcardial Perfusion (terminal):
1. Mice will be injected with 0.5mL of Avertin (250mg/kg; 23G, 25G, or 26G needle). TWU IACUC policy 001 regarding the use of Avertin will be followed.
2. Once unresponsive to tail/toe pinch reflex mice thoracic cavity will be dissected to expose heart.
3. PBS will be perfused through until all blood is removed.
4. 4% PFA will be perfused through until all tissues are fixed.

III. Brain dissection for slice preparation (terminal):
1. Mice will be anesthetized with isoflurane.
2. Once unresponsive to tail/to punch reflex, mouse will be cervically dislocated and decapitated.
3. Mouse brain will quickly be dissected and prepped for live slice preparation.

IV. Decapitation for tissue collection* (terminal):
*Because administration of pharmacological agents (Isoflurane, Avertin, etc.) may interfere with the tissue preparation and collection of blood plasma, it is necessary to euthanize without agents (Junuzovic et al 2011, Acta Inform Med). Only certain trained approved individuals (e.g. Dr. Lybrand or Dr. Na) will perform decapitation for tissue collection.
1. Mice will be decapitated with sharpened scissors.
2. Mouse brain will quickly be dissected and prepped for RNA/DNA extraction or ELISA.

14c. Describe the post-operative care. (For survival procedures only. If terminal, skip to 14d.)

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

For postoperative care of TBI, mice coming out of anesthesia will be placed on a heating pad. Once upright and moving freely the mouse will be returned to a clean home cage. TBI mice will be monitored daily for 7 days for pain and distress using the TWU Pain and Distress Assessment Form and evaluated with the modified Neurological Severity Score (mNSS) (see mNSS chart in 18b). All mice that experience severe weight loss (up to 20%) will be given post-operative care that includes moist chow, fluids, and placed on a heating pad. Incision sites will be monitored for infection and integrity until closed. When sutures are used they will be removed 14 days after surgery.

14d. Will neuromuscular blocking agents be used? ☐ Yes ☒ No

If yes, describe below how and by whom animals will be monitored. Also, if neuromuscular blocking agents are used without general anesthesia, provide justification.

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

15. Other invasive procedures (other than surgery, blood collection, catheterization, intubation)?
☐ **Yes** ☒ **No**

15a. If yes, please describe procedure below:

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

16. Blood/fluid/biopsy collection:
Will blood, fluid, and/or a biopsy need to be collected on live animals before euthanasia or before a terminal procedure? ☐ **Yes** ☒ **No** (If no, skip to 17.)
If yes, answer the questions below:

16a. Tissue collected: _____

16b. Method: _____

16c. Volume collected at one time: _____

16d. Frequency: _____

If necessary, describe the method in the text box below (for Tail Biopsies refer to TWU IACUC Policy 004):

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

17. Food/water restriction:
Will food or water be restricted during the study? ☒ **Yes** ☐ **No**
If yes, explain what is restricted, how long the restriction will last, and a justification in the text box below:

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

For CMS procedure (See 22 for more details), a brief water or food restriction. This will occur overnight as a mild stressor and returned the next morning. Food and water will not be restricted simultaneously. We do not anticipate any weight loss or changes to health status due to this brief <12 hr period.

18. Monitoring and Managing Unanticipated Pain and Distress:
Intervention for pain or distress can only be withheld for scientific reasons. Interventions may be needed for painful study procedures or for accidental injuries and infections. Please specify which interventions can and cannot be given. If one type is preferred over others, please explain in the text box below. ***Refer to the TWU Quantitative Rodent Pain/Distress Assessment as needed****.*

18a. Describe signs, symptoms, and species-specific behaviors that will be monitored to identify pain, distress, and discomfort in animals outside of the expectations of the experiment that would require intervention.

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

Signs of pain or distress such as changes in grooming, loss of body weight, mobility/gait, responsiveness, or open wounds will be checked to ensure proper health and wellbeing of mice. These behaviors will be quantified using the TWU Pain and Distress Form.

18b. Describe the frequency of monitoring of the animals throughout the experiment, in additional to the daily general observations made by the TWU vivarium staff, and any interventions that will be performed to relieve unexpected pain, distress, and discomfort in the animals.

Type text in the text box –- Spacing will adjust to accommodate the length of the narrative]

Following TBI, mice will be monitored daily for 7 days for signs of pain, discomfort, and distress and additionally evaluated with the modified Neurological Severity Score (mNSS) to monitor neurological changes (see mNSS chart below). Briefly, mice are evaluated and scored out of a total of 10 points. The higher the score the more severe. The mNSS evaluates motor, sensory, balance, and reflex function. Following that initial 7 days post-TBI, mice will be monitored weekly with the mNSS for the remainder of the study (6 weeks post-TBI)

Moist chow, extra fluids, warming pad will be administered to relieve any pain or discomfort. Mice will be euthanized if they exhibit moribund conditions.

18c. Monitoring of animals must be documented (e.g. daily observations, weights, treatments, etc.). Monitoring records must be made available to the TWU Attending Veterinarian and IACUC members upon request. Describe how monitoring will be documented (e.g. lab notebook, log placed in animal room, electronic records, etc.).

[Type text in the text box –- Spacing will adjust to accommodate the length of the narrative]

The TWU Pain and Distress Forms and the mNSS will be maintained in a binder on the back of the housing door while the animal is being housed. Forms will then be stored in the PIs lab/office. Further details will be recorded in lab notebooks and electronic records in the PIs lab/office.

19. Study Endpoints and Planned Disposition of Animals:

19a. Identify and explain the planned study endpoint that is both humane and scientifically sound (i.e. planned euthanasia, adoption, protocol transfer, etc.). Adoption requires a health assessment. Include endpoint assessment criteria used (refer to TWU IACUC Policy 002 on Humane Endpoints for Euthanasia as an Alternative to Death as needed):

[Type text in the text box –- Spacing will adjust to accommodate the length of the narrative]

All planned experiments end in terminal procedures; either transcardial perfusion for immunohistochemistry or euthanasia for slice physiology and tissue collection. Experiments will be terminated and the animal euthanized if the mouse appears to be losing significant weight (over 20% body weight) and/or unresponsive to external stimuli or lack of grooming.

19b. Describe the frequency of the animal observations: Daily for the first 7 days with mNSS/Pain and Distress then monitored once per week using mNSS.

19c. Person(s) responsible for the observations: Zane Lybrand, Myles Gladden,

NOTE: The individuals listed above must be trained to assess and recognize the humane endpoints.

19d. What response is required when the endpoint is reached:

☒ Euthanasia ☐other:

19e. In the box below, describe method(s) for euthanasia; for drugs, give name, route and dose. Refer to the TWU IACUC Policy 008 for Guillotine Maintenance as needed.

19f. Person(s) performing the euthanasia: Zane Lybrand, Myles Gladden, Abbie Baird, Elisa Na

[Type text in the text box –- Spacing will adjust to accommodate the length of the narrative]

Transcardial Perfusion (terminal):

5. Mice will be injected with 0.5mL of Avertin (250mg/kg; 23G, 25G, or 26G needle). TWU IACUC policy 001 regarding the use of Avertin will be followed.
6. Once unresponsive to tail/toe pinch reflex mice thoracic cavity will be dissected to expose heart.
7. PBS will be perfused through until all blood is removed.
8. 4% PFA will be perfused through until all tissues are fixed.

Brain dissection for slice preparation (terminal):
1. Mice will be anesthetized with isoflurane.
2. Once unresponsive to tail/to punch reflex, mouse will be cervically dislocated and decapitated.
3. Mouse brain will quickly be dissected and prepped for live slice preparation.

Decapitation for tissue collection* (terminal):
*Because administration of pharmacological agents (Isoflurane, Avertin, etc.) may interfere with the tissue preparation and collection of blood plasma, it is necessary to euthanize without agents (Junuzovic et al 2011, Acta Inform Med). Only certain trained approved individuals (e.g. Dr. Lybrand or Dr. Na) will perform decapitation for tissue collection.
1. Mice will be decapitated with sharpened scissors.
2. Mouse brain will quickly be dissected and prepped for RNA/DNA extraction or ELISA.

20. Hazards to personnel: ☐ **Yes** ☒ **No**

Mark each applicable hazard and describe. If any are checked, please proceed to 20a. Otherwise, skip to 21.

☐ Radioisotope _____
☐ Carcinogen _____
☐ Biohazard _____
☐ Other _____

TWU IBC Protocol # (as appropriate):

The IACUC Chair and the Office of Risk Management (for radioactive and carcinogenic materials)/ IBC Chair (for biohazards) must be consulted regarding the use of hazards before approval of the protocol. Recommendations may be submitted in the text box below or as a separate document.

20a. Who did you consult with and on what date? Please provide any written correspondence concerning consultation.
Name of official(s) consulted: _____ Date(s) of consultation: _____

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

21. May body fluids or tissue from these animals be utilized by other investigators?

☒ **Yes** ☐ **No** (If yes, describe below.)

[Type text in the text box -– Spacing will adjust to accommodate the length of the narrative]

We will only collect brain tissue. The rest of the tissues or fluids are available.

22. Summary and Judicious use of Animals: In the box below or in a separate document, give a detailed summary to describe your work to the IACUC. Please include and label (e.g., 22a.) each of the following:

22a. A brief description of the objective and significance of the proposed work, including the probable benefits of this work to human and/or animal health, the advancement of knowledge, or the good of society. **For renewals, please provide a brief update on the progress made in achieving the specific aims of the previous protocol.**

22b. A detailed description of all the procedures to which animals will be subjected. A flow chart which illustrates experimental design and required animal numbers is extremely helpful to reviewers. If using transgenics, please indicate mode of introduction, conditional vs constitutive expression, and the gene to be introduced or altered.

22c. The reason for selecting the species and justification of the number of animals proposed for use. The specific aims of the project should be described in sufficient enough detail to justify the number of animals requested even if animals are only used as a source of tissue for experiments *in vitro*. If transgenic animals are to be used, any expected effects of genetic manipulation should be described. If no effects are expected, this should be stated.

NOTE: Insufficient justification for the number of animals requested is one of the principal reasons that proposals require revision. It is the responsibility of the PI to clearly describe all experimental groups and to justify why the number of animals to be used in each group is required. To accomplish this, the results of statistical analyses (power analyses) and/or references to previous work need to be presented.

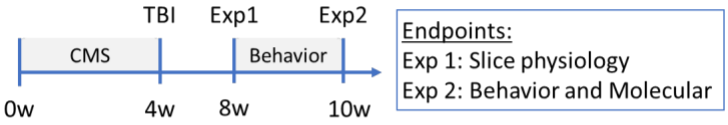
22d. Describe your experience with the proposed animal model and manipulation.

Please note, this *summary should not be a copy of a grant proposal, abstract, teaching syllabus, or reprint. In this summary you should use language such that a scientist outside your field can understand it. Although not required, the use of graphics may be very helpful to the members of the IACUC in understanding your project.*

[Type text in the text box --- Spacing will adjust to accommodate the length of the narrative]

22a. The overall goal of this research is to understand how chronic stress affects TBI outcomes with specific focus on depression and anxiety related co-morbidities. The interaction between stress and brain-derived neurotrophic factor (BDNF)expression is well established as a link to depression, anxiety and neuropsychiatric disorders (Notaras and van den Buuse, et al., 2020; https://doi.org/10.1038/s41380-019-0639-2). However little is known about how TBI affects the brain during chronic stress conditions. The overarching hypothesis is that chronic stress disrupts BDNF signaling in the cortical-hippocampus circuit to exacerbate and interfere with the brain's ability to recover from chronic stress conditions. The goal of this IACUC protocol is to investigate how chronic stress affects anxiety and depression-like behavior, the neurophysiology of the entorhinal cortex-hippocampus circuit (a putative depression circuit), and BDNF. This work has direct significance to military health, sports medicine, and the general population. Understanding how chronic stress

Timeline of experiments



Exp 1:	Exp 2:	Total:
+CMS –TBI (n=5)	+CMS –TBI (n=15)	80 mice
+CMS +TBI (n=5)	+CMS +TBI (n=15)	
-CMS –TBI (n=5)	-CMS –TBI (n=15)	
-CMS+TBI (n=5)	-CMS+TBI (n=15)	

changes the brain will advance our knowledge of brain and mental health to develop potential treatments for individuals with brain injury.

22b. The proposed IACUC protocol will have 4 groups: 1) Chronic Mild Stress (CMS) + sham surgery, 2) CMS + TBI, 3) No CMS + sham surgery, 4) No CMS + TBI. Following TBI, mice will have 4 weeks to recover. At this time, mice will be euthanized for slice physiology following the quick decapitation protocol (14. Surgery III). For slice physiology, we estimated an n=5/group based on previous experience with these types of experiments. Typically, 12-15 recordings from 3-5 mice are acceptable replicates. The second experiment will have mice undergo a battery of behavioral tests. Behavior tests will be performed over the course of 2 weeks and mice will euthanized by transcardial perfusion for immunohistochemistry preparation (14. Surgery II) or decapitation for tissue collection (14. Surgery IV). For behavior, G-Power Analysis software was used to estimate a sample size of 13-15 mice per group (Effect size = 0.6; α = 0.05; number of groups 4). We believe the effect size will be modest given that TBI can be variable in mice and included a sample size at the upper end of the range.

Chronic mild stress. Chronic mild stress is a model for stress used to evaluate depression-like behaviors homologous to psychiatric disorders where mice are exposed randomly to unpredictable micro-stressor that develop clinic related behaviors of depression and anhedonia (Willner, 2017; https://doi.org/10.1016/j.ynstr.2016.08.002). For this model, mice will be exposed to one or two stressors for a period of 4 hours to 12 hours during each 24-hour period over 4 weeks. Mice will not be stressed within 8 hours of behavioral testing. Stressors will consist of the following and will be presented in random order: food or water deprivation, periods of overnight illumination, 45° cage tilt, single housing, exposure to strobe light, bedding soiled with water or rat feces, or placed in a foreign mouse cage. These stressors will be presented randomly over the course of 4 weeks (Please see table below for an example of 1 week of the 4 week protocol) and then mice will undergo sham or traumatic brain injury (TBI). After recovery, mice will undergo the behavioral tests. Below is a detailed description of the behavior test and an example of the order in which they may be tested. Some tests (Morris water maze and Sucrose consumption) require training days that will occur throughout the behavior testing phase.

Day	Light Phase	Dark Phase
Monday	Strobe light (2 hours)	Water deprivation (10-12 hours)
Tuesday	Wet cage	Overnight illumination
Wednesday	Cage tilt	Rat feces
Thursday	White noise	Foreign cage
Friday	Rat feces	White noise
Saturday	Foreign cage	Food deprivation (10-12 hours)
Sunday	Wet cage	Cage tilt

Novel object recognition - 2 days
Social interaction test - 1 day
Elevated plus maze - 1 day
Open field test - 1 day
Morris water maze - 10 days
Tail suspension test - 1 day
Forced swim test - 1 day
Sucrose consumption test - 14 days

Novel object recognition. Novel object recognition is a test of hippocampal episodic memory. In this test, mice will be habituated to testing conditions for 10 min in an open field box. Four hours later, mice will be exposed to two of the same object (A) in the familiarization stage for 10 min. Twenty-four hours after the familiarization stage, mice will be exposed to 1 familiar object (A) and 1 novel object (B) for 10 min. Time spent interacting with objects will be scored using Ethovision (Noldus), a videotracking software program. A discrimination index in which time spent with the novel object (B) is subtracted from time spent with the familiar object (A) will be used to determine differences between groups.

Social interaction test. The social interaction test will assess the amount of time experimental and control mice spend with a novel target mouse. Mice will be placed in a large open field box with a novel target conspecific placed in a cage that allows for interaction and olfactory cues. Time spent interacting with the conspecific will be determined by Ethovision software and is the dependent variable.

Elevated plus maze. Mice will be placed on an elevated plus maze that contains 2 open arms (without walls) and 2 closed arms (with walls). The amount of time the animal spends in the open arms is indicative of anxiety, thus the less time an animal spends in the open arm, the greater the anxiety-like phenotype. Time spent in either the closed or open arms will be quantified by Ethovision software (Noldus).

Open field test. The open field test consists of placing mice in a large open field for 10 min and assessing the amount of time the mouse spends in the periphery relative to the center. Less time in the center is indicative of an anxiety-like phenotype.

Morris water maze. Morris water maze is a test of hippocampal spatial memory. In this test, mice will be placed in a room temperature pool of opaque water and will be required to swim to a hidden platform. Mice will undergo 4 trials a day for 10 days starting at a different quadrant of the pool. Spatial cues such as pictures will be placed around the room as reference points for the mice. After the tenth day of training, time spent in the target quadrant, or the quadrant in which the hidden platform was placed, will be quantified using Ethovision software. In addition, during each day of training, we will assess the amount of time it takes for mice to reach the hidden platform and plot the latency as a measure of learning. Following testing, dried off prior to returning to dry home come conditions.

Tail suspension test. The Tail Suspension test is a behavior test for behavioral despair or depression-like behavior and learned helplessness. It is useful for measuring the severity and effect of treatments on depression-related behaviors. Mice will be suspended by their tails using adhesive tape onto a wooden dowel, with a height of 24 cm. Immobility will be assessed for 6 min after which time mice will be placed back into home cages. The total amount of immobility time (defined as the time during which the animal is hanging passively and motionless) is measured for each animal and considered as an index of “depression-like” behavior. It is one of the most widely used models for depression in mice (Steru et al., 1985; DOI: 10.1007/BF00428203). All mice are carefully monitored for any adverse effects during the test and are quickly removed if they display signs of unusual distress (i.e. constant vocalization or damage to their tails).

Forced swim test. The forced swim test is an additional test for depression-like behavior. Mice will be placed in a 4 liter beakerful of room temperature water (approximately 21-23 degree Celsius) for 6 min at which time mice will be removed and dried with paper towels. The amount of time the mouse spends struggling is indicative of depression-like behavior. It is unclear if depressive-like behaviors will be present immediately following TBI. Forced swim will be tested for 1 day to determine if depressive-like behaviors are present.

Sucrose consumption test. The sucrose consumption test is a test for anhedonia. Because mice typically will prefer water sweetened with sucrose, decreases in this preference indicates a change in perceived pleasure. This is a primary symptom in mood disorders, psychosis, and neurodegenerative diseases. For this test, mice will receive a three-phase procedure: 1.) pre-training (4 days), ‘limited access’ training (10 days), and post-training. During all three phases, the mice will receive access to 0.5 M sucrose, water and chow every day from 9:00–13:00. The left/right position of sucrose and water bottles will be changed daily. During the pre- and post-training phases, mice will receive nocturnal access (13:00–9:00 the next morning) to chow and water but not sucrose. During the limited-access training phase, only water will be available through the night, without chow access in order to motivate mice to drink sucrose. Starting at 13:00 on day 10 (the last training day), chow and water will be available ad libitum until the next sucrose access. Consumption of sucrose, water, and chow during the first, second, and fourth hours will be measured throughout the three phases by weight (Yamaguchi, Yasoshima, &

Shimura 2017). For the limited access days, mice will be food deprived overnight for no more than 14 hrs (Please see below). Mice will be housed in cages that can accommodate 2 bottles of fluid (0.5 M sucrose and water) in order to weigh the amount mice are drinking during these tests.

- Pretraining (4 days)
  - 9 am – 1 pm Sucrose, water, and food available
  - 1 pm to 9 am Water and food available
- Limited access (10 days)
  - 9 am – 1 pm Sucrose, water, and food available
  - 7 pm to 9 am Food deprived for 14 hours
  - 9 am Food ad libitum
- Pretraining (4 days)
  - 9 am – 1 pm Sucrose, water, and food available
  - 1 pm to 9 am Water and food available

22c. For these studies, C57bl6 mice will be used. For slice physiology, we estimated an n=5/group based on previous experience with these types of experiments. Typically, 12-15 recordings from 3-5 mice are acceptable replicates. The second experiment will have mice undergo a battery of behavioral test. Behavior tests will be performed over the course of 2 weeks and mice will euthanized by transcardial perfusion for immunohistochemistry preparation (14. Surgery II) or decapitation for tissue collection (14. Surgery IV). For behavior, G-Power Analysis software was used to estimate a sample size of 13-15 mice per group (Effect size = 0.6; alpha = 0.05; number of groups 4). We believe the effect size will be modest given that TBI can be variable in mice and included a sample size at the upper end of the range.

22d. For the TBI surgery, Dr. Lybrand has previous experience performing the impact procedure. He also has 10+ years of rodent aseptic surgical experience. Myles Gladen has been trained and supervised by Dr. Lybrand. For behavior studies, Dr. Elisa Na has greater than 10+ years running behavior test and running the Chronic Mild Stress paradigm used in this protocol.

PRINCIPAL INVESTIGATOR ASSURANCES

ALL ASSURANCES MUST BE CHECKED BY PRINCIPAL INVESTIGATOR BEFORE SUBMISSION TO IACUC

1. I have a working knowledge of the PHS "Guide for the Care and Use of Laboratory Animals" (and the USDA "Title 9 Animal Welfare Act" if applicable) and its revisions. ☒
2. The proposed work does not unnecessarily duplicate previous experiments, based upon search results described in question 7. ☒
3. All personnel involved in this project have been trained in the procedure to be used or will be trained before performing procedures. ☒
4. I and all personnel on the project have read the Disaster Preparedness Plan for the vivarium and the applicable IACUC policies ☒
5. I shall be responsible for maintaining records of all animals used and the procedures carried out ☒
6. I am aware that per diem will be charged (see TWU IACUC Policy 004) to an account that I provide on the Per Diem Authorization Form to be submitted to the Director of the Vivarium. Alternatively, I may apply for a per diem waiver by submitting a Bridge Waiver or an Extended Waiver to the TWU IACUC for approval ☒
7. Any discomfort, distress or pain that may be associated with this research will be held to the absolute minimum. ☒
8. Alternatives to any procedures that may cause pain or discomfort have been considered ☒
9. **Controlled Substances** Yes☒ No☐

   If yes, please check: I am responsible for procurement, storage, administration, and record keeping for all controlled substances ☒
10. **Non-pharmaceutical Grade Compounds** Yes☒ No☐

    If yes, please check: I have read and understand the IACUC's policy regarding the use of NPGC's in animals. NPGC's will only be used for projects with scientific justification, when acceptable pharmaceutical compounds are unavailable, and with prior IACUC approval ☒

As Principal Investigator and/or Co-Principal Investigator, I am aware that I have the ultimate responsibility, on a day-to-day basis, for the proper care and treatment of the laboratory animals. I agree to adhere to all federal, state and local laws and regulations governing the use of animals in teaching and research. I further assure the Texas Woman's University IACUC that the minimal number of animals will be used for the project and that every possible step will be taken to minimize stress or pain to the animals.

I will submit appropriate annual review forms for this project, and obtain formal approval of the Committee prior to implementation of any changes in this protocol.

Signatures will be collected by ORSP electronically following approval. *Submit completed protocol electronically in Word document format to LByford@twu.edu at least one week prior to the next IACUC meeting.*

DocuSigned by:

Principal Investigator _____ Date _____

DocuSigned by:

Co-Principal Investigator (*if appropriate*) _____ Date _____

APPENDIX B

SAMPLE MNSS SCORING SHEET

Date					
Mouse ID					
Task	**Description**	**Score**			
Startle Reflex	Pop a clipboard; if the mouse fails to respond give 1 point	0	1		
Straight Walk	Place on flat surface and watch walking; if fails to use one or two paws, or walk in a straight line 1 point	0	1		
Grip Reflex	Suspend by the tail and touch the paw with a pen to assess grip; if mouse fails to grip, give 1 point	0	1		
Beam Balance	Place on beam of 7mmX7mm to assess balance; if mouse fails to perch, give 1 point.	0	1		
Beam Walk	Place on 3 cm beam for a maximum of 3 minutes; if the mouse reaches opposite side of beam with minimal to no difficulty, give 0 points; if mouse shows difficulty but reaches the opposite side within time limit, give 1 point; if mouse shows difficulty and does not reach the opposite side within time limit, give 2 points; if mouse falls off the beam, give 3 points.	0	1	2	3
Seeking behavior	Place mouse in new environment and watch for exploration behavior (e.g., sniffing, time in perimeter and center, movement); if mouse fails to explore, give 1 point.	0	1		
Round stick balance	Place mouse on round stick (5mm in diameter) to assess balance; if mouse fails to perch within 3 trials, give 1 point.	0	1		
Exit Box	Place mouse in a box with a hole for a maximum of 3 minutes; if mouse fails to exit within the time limit, give 1 point.	0	1		
Total score *(out of 10 maximum points)*					