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FUNCTIONAL CHANGES IN MOUSE BEHAVIOR FOLLOWING THREE DIFFERENT MODELS OF TRAUMATIC BRAIN INJURY

by

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ABSTRACT

Functional Changes in Mouse Behavior Following Three Different Models of Traumatic Brain Injury

Patricia Anh Thu Vu, MD, PhD, 2020

Thesis Advisor: Dr. Joseph T. McCabe, PhD

Mild traumatic brain injury (mTBI) remains a challenge for military and veteran health systems as well as global health. Pre-clinical models that better represent the mechanism of different types of mTBI are needed for elucidating the physiological consequences of injury and their behavioral outcomes. Thus, three closed-head mTBI mouse models were utilized to assess the effects of common types of mTBI seen in service members: blast-induced traumatic brain injury (BTBI), single concussive brain injury (CBI), and frontal repetitive CBI (frCBI). After BTBI and CBI, mice showed transient hypoactivity in home-cage activities 24 hours post-injury (PI), which returned to baseline by 48 to 72 hours PI. BTBI mice demonstrated hypoactivity in the open field (OF) and decreased anxiety at 24 hours PI. CBI mice, in contrast, demonstrated decreased anxiety at 24 and 72 hours PI in the OF. frCBI resulted in decreased freezing behavior in response to the fear conditioning context and cue. Sex differences were noted with male mice showing less freezing behavior than female mice. Hyperactivity in the OF and elevated zero maze (EZM) was seen in all frCBI mice. In the EZM, uninjured female mice that were exposed to foot-shocks showed increased anxiety relative to non-shocked

mice; however, shocked injured female frCBI mice did not demonstrate the same behavioral change. In summary, a single BTBI or CBI resulted in transient hypoactivity that may indicate a window of increased need to rest and vulnerability to repeat injury. Some differences were observed between BTBI and CBI mice; however, direct comparisons were not possible. Hyperactivity in frCBI mice may be impulsive in nature and may be mediated by neuroinflammatory changes. There was increased inflammation in cortical regions underlying the locations of direct impact in frCBI mice. Pre-clinical models continue to provide valuable insight into the mechanisms by which debilitating symptoms occur as well as provide evidence for a window of time during which repeated injury can result in worse outcomes. Data from such models have huge implications in improving return-to-duty policies to promote recovery in service members who have sustained mTBI and to prevent further injury.

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CHAPTER 1: Introduction

MILD TRAUMATIC BRAIN INJURY AS A BURGEONING GLOBAL HEALTH CHALLENGE

Traumatic brain injury (TBI) is a global health problem and has been predicted by the World Health Organization (WHO) to surpass many diseases as a leading cause of mortality and morbidity in 2020 (84). In developed countries, motor vehicle accidents (MVAs), falls, assault, workplace accidents, and sports-related injuries account for the majority of TBI cases (198). In the United States alone, over 1.7 million TBIs occur annually (62), with an estimated 1-2% of TBI survivors living with long-term disability (198; 224). Studies suggest 70 to 90% of TBI cases are mild (29), however, the incidence may be greater since milder forms of TBI are often underdiagnosed due to the heterogeneous nature of presenting symptoms and lack of unified diagnostic criteria (4; 17; 33). Although their injuries have been classified as mild, up to 48% of people who sustain mild TBI (mTBI) report at least one disability in the year following injury (184), and similar to HIV and diabetes, TBI resulting in prolonged symptom presentation meets the definition of a chronic disease (117).

TBI IS A CHALLENGE FOR THE MILITARY AND VETERAN HEALTH SYSTEMS

As a leading cause of disability in the civilian world (62), TBI has also become recognized as a major challenge to the military and veteran health systems. It is frequently referred to as the signature wound of modern-day warfare. The two most common causes of TBI in service members were exposure to blast, accounting for 45.7%

of TBI cases, and motor vehicle accidents (MVAs), accounting for 18.9% of TBI cases (5). It is important to also note that even prior to combat deployments, service members can sustain concussive brain injury (CBI) from their involvement in military training exercises, falls, MVAs, and the occasional injury from service-related participation in contact sports (121; 178). Likewise, military personnel are prone to experiencing multiple concussive events and even a combination of CBI- and blast-related TBIs (56; 156; 178). Brain regions that are most vulnerable to repetitive concussive impacts are the frontal and temporal lobes (104; 119; 189).

As a significant source of TBI, blast exposure is a relatively new challenge. Although blast injury effects have been witnessed since World War I, modern warfare has shifted towards the increased use of explosive weaponry (142). As seen in recent wars in Iraq and Afghanistan, certain terrorist groups favor the use of improvised explosive devices (IEDs) to achieve their political objectives. The use of such weaponry and the resulting wounds have driven advances in both protective armor and trauma care, resulting in higher survival rates of warfighters and civilian casualties who are exposed to blast forces that have historically been deadly (107). Thus, the prevalence of combat-related TBI has increased relative to past wars (107; 178).

The mechanism by which exposure to blast affects the brain is most likely different than direct impact to the head as in the case of CBI (58; 107; 178). Based on post-mortem analysis of brain tissue from service members, blast exposure results in distinct patterns of astroglial scarring that were not seen in civilian cases of pure impact TBI (178). An explosion can cause TBI in several biomechanically distinct ways. Travelling at supersonic velocities, a shock wave front causes an almost instantaneous

change in ambient pressure. The impact of the shock wave front has a duration in the nanosecond range (208) and its intense impact with the body, including the head, reflects off these surfaces resulting in a compressive shock wave that passes through the target as a *primary* shock wave effect, causing movement, deformation, and displacement of tissue (42; 43). The wave front is then followed by a "blast wind" force that can propel objects into a person or propel the person into an object, causing *secondary* or *tertiary* CBI effects, respectively.

Finally, an especial particular challenge for the care of service members, who may be exposed to different types of TBI, is the fact that they also experience life-threatening conditions that predispose them to developing post-traumatic stress disorder (PTSD) (95). In addition to the physical trauma, there are psychological and physiological consequences following exposure to the stress and violence of warfare. In a 2012 report, 8,127 service members returning from deployment screened positive for having sustained some form of TBI (5).Thus, mitigating neuropsychiatric sequelae after injury has been a major challenge in the military and veteran health systems.

OVERVIEW OF TBI

TBIs result from a range of forces that affect the physiological function of the central nervous system. In the past, Ommaya and earlier investigators have classified the biomechanical forces that give rise to TBI (136). More recently, Cernak has expanded on this framework to include the sources of brain injury that are common in military populations during times of global conflict (32). Briefly, TBIs can occur from "slow" *static* impacts that produce trauma associated with crushing forces, or they can be caused

by *dynamic*, "rapid" acceleration events that result in swift rotational head or brain motion and deformation of neural tissue (30). The nature of dynamic injuries can be further subdivided into direct trauma and indirect trauma. While direct trauma can arise from a penetrating wound, they more frequently occur as a result of non-penetrating impact acceleration forces, which are often associated with concussion (30). Unlike direct injuries, indirect injuries arise from dynamic forces that affect the entire body, such as forces generated by an explosive blast. The kinetic energy of such a force is imparted on tissues throughout the body resulting in trauma to various organ systems (41). For military personnel in the 21st century, TBI most commonly arises from a variety of "mild" and direct dynamic impact forces, indirect dynamic forces from exposure to explosive blast, or a combination of these factors.

RANGE OF CLINICAL SYMPTOMS

Although approximately 80% of TBI cases in the military are classified as mild (63) and there is, reportedly, full recovery in the majority of cases, a significant number of warfighters report persistent symptoms. Following mTBI, patients may present with multiple and varying symptoms that impede daily function. The impact of such symptoms on patients and their ability to fulfill societal roles and responsibilities is profound (82; 83; 119). In addition, the subtle and heterogeneous nature of the neuropsychiatric sequelae following mTBI have made clinical detection and treatment difficult (83; 189). While there can be additional sequelae with motor and sensory complaints, several themes are particular to service members. These include disturbances in overall physical activity level, disruptions of sleep-wake cycles, neuropsychiatric

complaints of anxiety, depression and PTSD, and symptoms related to frontal lobe damage such as poor executive function and impulsivity. These symptoms are briefly reviewed along with data from pre-clinical studies that provide pathological insight into development of such symptoms.

Alterations in General Activity and Sleep

In addition to the neuropsychological sequelae following TBI as discussed below, an overall decrease in physical and social activities has been reported (70; 74). This change can arise from a number of factors including the need to re-learn physical and cognitive tasks through rehabilitation, as well as overall changes in daily habits and activity. The reduction of physical activity after TBI may relate to the recovery from physical injuries, but this concomitantly is often shown to be therapeutic (171). However, there are also findings that show the beneficial effects of return to physical activity (207), which may be challenging due to the development of neuropsychological symptoms that interfere with daily activities and alter motivation.

Among the most common symptoms are sleep-wake disorders (SWDs) (34; 44; 71; 139). SWDs are reported by over 50% of TBI patients (12). Excessive daytime sleepiness (EDS) and fatigue account for over 55% of SWDs (12). These symptoms present in patients with different injury severity and locations (12; 44; 71). Clinically, the symptom of EDS has been shown to correlate with a decrease in orexin-A (13; 14). Orexin-A is a hypothalamic neuropeptide involved in the regulation of arousal as pertains to wakefulness, locomotion, and feeding behaviors (168). It is thought to be a "gatekeeper," preventing abrupt transitions between wakefulness and sleep, and its

deficiency has been implicated in narcolepsy (166). With regard to TBI, orexin-A has been shown to decrease in the cerebrospinal fluid (CSF) of patients acutely following moderate to severe TBI (14). Furthermore, loss of orexin-A-expressing neurons has also been documented in post-mortem brain samples from patients with severe TBI (13).

In pre-clinical studies, both controlled cortical impact (CCI) and fluid percussion models in rodents have been utilized to address symptoms related to changes in activity and sleep. Skopin and colleagues found that a moderate injury from lateral fluid percussion causes fragmentation of the waking state in rats as well as loss of orexin-A-positive cells in the hypothalamus (181). In another study, using CCI to model both mTBI and moderate TBI in mice, Thomasy and colleagues showed that mice with moderate injuries (impact depth of 1.0 mm) spent less time awake and more time in non-rapid eye movement (NREM) sleep than mice that sustained mild CCI (impact depth of 0.5 mm) and sham mice (196) . Moderate CCI in mice resulted in loss of orexin-positive neurons 7 and 15 days following injury. Mild CCI also resulted in significant decrease in orexin-positive neurons, but only at 15 days post-injury, suggesting a delayed effect of the milder injury in this study. These two studies demonstrate that TBI can lead to EDS, and that this is in part due to the neuropathological loss of orexin neurons. However, neither of the above studies addressed the behavioral consequences of disrupted wakefulness following TBI and how these consequences affect daily function. They also do not directly address the mechanism by which orexin-A neurons are affected by TBI, although Thomasy and colleagues do discuss the potential role of neuroinflammation in the decrease of orexin-positive neurons (196), citing that orexin neurons have been shown to be sensitive to induction of systemic inflammation (145).

Anxiety, Depression, and PTSD

Other common neuropsychiatric disorders following mTBI include anxiety, depression, and PTSD. The high rates of PTSD, depression, and anxiety disorders in military personnel who have suffered from mTBI have led Stein and McAllister to postulate that mTBI may lower a person's cognitive reserve (185). Post-mortem analysis of brain tissue from service members have shown that blast exposure results in distinct patterns of astroglial scarring in structures associated with stress processing (151; 178) and depression (57; 103): the amygdala, hippocampus, hypothalamus, ventromedial prefrontal cortex, dorsolateral prefrontal cortex, anterior cingulate cortex, and anterior insular cortex (178). In addition, the majority of veterans in this study had one or more psychiatric disorders (90) in congruence with other clinical studies that have determined that TBI is a risk factor for depression, anxiety, and or PTSD (77; 78; 89; 172; 183; 185). The rate of PTSD among veterans with mTBI can be as high as 66% with its prevalence correlating with increasing numbers of mTBI as shown by one study (164).

Interestingly, neuroinflammation appears to play a role in the development of anxiety-like behaviors in rodents. In a rat model of mild CCI, researchers found changes in gene expression of inflammatory mediators in the hippocampus (2) and increased anxiety at 7 days and 30 days post-injury as tested in the open field (3). The researchers also found decreased $GABA_A$ mediated inhibition as well as increased excitatory processes in the basolateral amygdala (3). However, while CCI provides valuable insight into the physiological changes in structures involved in the development of sleep-wake disorders, anxiety, and behavioral outcomes following a single mTBI, concerns have

been raised about the relevance of models that utilize invasive procedures. Previous studies looking at orexin-A and/or sleep-wake parameters in mice following TBI have utilized open-head injury models such as CCI and fluid percussion (162; 163; 196; 216). Furthermore, traditional measures of sleep-wake in mice have required the use of surgically invasive radiotelemetry devices to monitor electroencephalogram (181; 196).

Symptoms of Frontal Lobe Damage

Injury to the frontal lobes leads to deficits that are disruptive to social integration and function including: impulse control, emotional control, cognitive flexibility, working memory, organizational skills, and planning and prioritizing (119). The prefrontal cortex (along with the amygdala) plays an important roles in processing positive and negative emotions and governing how internal emotions guide a person's interaction with his or her environment (52). Deficits in emotional regulation have been particularly troublesome in military populations with overlapping symptoms of TBI and PTSD. The high suicide rate in this population as well as their vulnerability to developing depression (113; 114) has been attributed to frontal and basal forebrain damage (24).

ANIMAL MODELS

Because of the limitations of clinical studies and post-mortem studies, animal models have been widely used to better elucidate the mechanisms by which CBI and blast-induced traumatic brain injury (BTBI) may change the brain and make it more vulnerable to neuropsychiatric disorders. Rats and mice are commonly used to model TBI because of their smaller size and cost, enabling researchers to carry out repeated

assessments for changes in behavior, physiology, and anatomy following injury (30). Furthermore, mouse models carry transgenic potential (50; 124), providing researchers with capabilities for genetic manipulation to better assess treatment and recovery after TBI (64).

BTBI

While there are many variations of rodent models of direct impact brain injury, BTBI is also of significant importance for military medicine. In modeling BTBI in animals, researchers have employed both open-field techniques (7; 111; 156; 170) as well as blast and shock tubes for the simulation of blast overpressure conditions. Studies modeling blast injury in larger animals commonly utilize open-field techniques that involve chemical explosives (67), whereas studies utilizing smaller animals simulate blast conditions within tube devices that allow better control and measurements of the blast wave (30; 132; 155; 159; 213). Tube devices that have been used to simulate blast conditions include blast tubes in which the shock wave is generated by explosive charges (132; 159), compression air-driven shock tubes (31; 60; 109; 213), and the multi-modal designs that employ explosive and compressed gasses (155). Compression air-driven shock tubes are the most commonly used in rodent modeling of BTBI, and induce injury by exposing animals to abrupt overpressure (31; 60; 109; 213).

Neuroinflammation and Behavioral Changes after BTBI

In rodent models, BTBI results in the appearance of neuroinflammation and anxiety-like behaviors (9; 100). After exposure to a single blast overpressure, rats

experience transient increased anxiety in a chronic stress paradigm and persistent deficits in spatial memory relative to naïve rats and rats exposed to stress alone (100). In addition, the authors describe an increase in astrogliosis in the prefrontal cortex (PFC) of stressed rats as well as increase apoptosis in the ventral hippocampus and dorsal hippocampus of rats exposed to both blast and stress, but not in sham injured rats exposed to stress or naïve rats that received no stress experience or injuries (100).

Single versus Repetitive Injury

Although the study of a single CBI or BTBI is crucial to our understanding of the pathological consequences of mTBI, military health providers also face challenges in treating patients with a history of multiple mTBIs. Therefore, many researchers have turned their attention to studying the effects of repetitive mTBI (rmTBI). In modeling rmTBI, most researchers utilize mice (8). Previous studies have employed a wide range of injury models including weight drop (51; 91; 122; 130), closed-head controlled cortical impact (chCCI) on the exposed skull (18; 99; 101; 177), chCCI on a helmet fitted to the mouse's head (149), and chCCI directly on the scalp (222). The number of impacts used in these models ranged from two impacts (one per day for two days) (177) to 42 impacts (six a day for seven days with two hours in between each impact) (149). When comparing a single mTBI to rmTBIs, mice receiving more than one mTBI exhibit poorer performance on cognitive tasks than those receiving a single injury (99; 122; 125; 177; 222). Furthermore, mice that sustain repetitive TBI at a rate of one impact per day over five days perform worse than mice that experience wider inter-injury intervals of one week and one month (122).

Sites of Injury

The majority of closed-head rmTBI models to date have focused on the effects of repetitive mild CBI administered unilaterally over the parietal lobe and assessment for alteration in cognitive functions such as memory (108; 177; 201; 202). There have been few studies assessing the effects of bilateral TBI in mice. One rat study utilized a craniotomy and bilateral CCI to deliver injuries anterior to the bregma suture (79). In a mouse study, hTau mice received four CBIs that were spaced 24 hours apart and alternated between the right and left sides of the skull; however, the authors did not disclose more details about the injury parameters (24). In another mouse study, modeling closed-head repetitive injury, the authors utilized parameters that result in bilateral injury over the parietal cortical areas with each impact likely targeting the underlying corpus callosum (125). As for studies that assess injury to the frontal lobe in rodents, invasive injury models including CCI with craniectomy or craniotomy (36; 39; 79; 102; 137; 182; 205), lateral fluid percussion (140; 141), aspiration of brain matter (118; 176), and cryoinjury (97) have been predominantly used. There are currently no published studies assessing closed-head frontal CBI in mice.

As mentioned earlier, injury to the frontal lobes lead to a complex array of deficits that are disruptive to social integration and function including: impulse control, emotional control, cognitive flexibility, working memory, organizational skills, and planning and prioritizing (119). After a single CCI to the medial and orbitofrontal regions of the PFC, Chou and colleagues found that injured mice did not differ from uninjured control mice in a hippocampal-mediated memory task, but they did make more errors during a rule

shift assay when they were tasked with unlearning a previous stimulus-reward association and challenged to learn a new stimulus-reward pairing; demonstrating cognitive inflexibility (39). Furthermore, injured mice in this study demonstrated social behavior that was not observed in uninjured mice during the social approach task (39). Although they did not differ from sham mice in their preference to interact with a novel mouse over an empty cage, injured mice did show less preference for a novel mouse over a familiar mouse when compared to uninjured mice (39). In contrast, CCI to the parietal lobe in mice have demonstrated deficits on memory-based tasks such as Morris Water Maze (35; 37; 225) and the novel object recognition (225). The data here suggest some differences in the symptoms that arise relative to location of injury, which may enable researchers to better assess particular symptoms following TBI. Although, one study assessing fluid percussion injury to the frontal lobe reported deficits in memory-based tasks, this study utilized methods that require a larger craniotomy (140) than Chou and colleagues (39). Unfortunately, direct comparisons between studies utilizing different injury models cannot be made, and there are currently no studies comparing behavioral outcomes from closed-head injury over the parietal lobe versus the frontal lobe.

Stress, Fear, and TBI

Of further interest to military populations are the effects of intense stress on neurocircuits that can be compromised by rmTBI. A single exposure to intense stress as induced by 15 shocks in naïve rats, for example, has been shown to enhance fear learning independent of contextual associations, causing rats to respond with an abnormal amount of fear to a subsequent single shock in a different context (154). Furthermore, this stress-

induced enhanced fear learning is resistant to extinction and disruption by APV, an N-Methyl-D-aspartic acid receptor antagonist (154).

Several studies modeling TBI in mice have assessed the effects of fear conditioning after repetitive CBI (99; 108; 202). After two closed-head CBIs, male injured mice did not show any difference in behavior during fear conditioning training nor subsequent fear conditioning testing (99). They did, however, observe decreased social interaction in injured mice that received foot-shock during fear conditioning when exposed to a novel stranger mouse as compared to non-shock injured mice, shocked sham mice, and non-shock sham mice (99). Furthermore, they observed more depressive-like behavior in injured mice during the tail suspension test that was exacerbated in the injured mice that received foot-shocks (99). After three repetitive CBIs, researchers did observe decreased freezing behavior in injured mice when tested in the fear conditioning context at one and two weeks following fear conditioning training (108; 202).

Sex Differences after TBI

Few rmTBI studies to date include female mice cohorts even though the clinical literature suggests that women are more susceptible to developing psychiatric symptoms following TBI (16; 69; 172). As seen in mice that sustained three repetitive CBIs, female and male mice showed some behavioral differences after fear conditioning (202). The authors noted that there was an effect of injury on male mice but not female mice during testing with the fear conditioning cue/tone (202).

Sex differences were also notable one year after repetitive CBIs (201). Injured male mice displayed less anxiety-like behavior than male sham mice in the EZM and

more hyperactivity in the open field one year after the last injury; these differences between injured and uninjured mice were not seen with female mice (201). However, contrary to the clinical literature regarding higher neuropsychiatric prevalence in women (96; 106), injured female mice showed superior performance in the Morris water maze when compared to injured male mice after sustaining five consecutive CBIs (209). Thus, more studies utilizing both male and female mice are necessary to determine trends in behavioral outcome after injury, and how these trends potentially change over time.

ADDRESSING RESEARCH GAPS

From the above brief summary it is evident that developing treatments for mTBI remains a global challenge. The clinical and pre-clinical data indicate that although often not recognized at the time of the insults, mTBI can result in non-reversible pathological processes that evolve over time, and in the case of clinical populations, over a person's lifetime, and may require long-term medical supervision and management if there is functional impairment. TBI, then, can affect multiple systems, reduce quality of life by impacting daily function, and cause a significant increase in mortality for those who survive the initial insult or subsequent injuries (80; 117). Unfortunately, unlike many other chronic diseases, diagnostic screening tools for mTBI are limited and do not effectively predict behavioral and psychiatric outcomes following injury and treatments have thus far proven ineffective.

In addition, exposure of service members to different types of TBI, including life-threatening conditions that predispose them to developing PTSD (95) result in a complex array of symptoms that may have overlapping features (33). Targets for prevention and

treatment of neuropsychiatric sequelae after injury have been elusive. Further evaluation of underlying mechanisms is needed to determine the pathogenesis from blast exposure, direct impact concussion, and rmTBI. In all of these scenarios, evidence for a single focal lesion is lacking.

To understand how seemingly mild injuries can lead to persistent impairments, a great deal of study is still required at the preclinical level. Preclinical research has the advantage of allowing far greater control over conditions and the ability to focus on single variables and mechanisms. Models of mTBI that better capture the symptoms experienced by warfighters are needed to understand the underlying pathological mechanisms that contribute to the development of neuropsychiatric disorders following injury. Thus, it is necessary to assess closed-head models of mTBI, particularly blast wave-induced mTBI and CBI from direct impact on daily activities and behavioral outcomes. By investigating the three common themes in military mTBI (single CBI, single BTBI, and frontal repetitive CBI) and their associative symptoms in both female and male mice, a better understanding of how different types of TBI affects the brain may unfold and provide different models from which to approach treatment and improve quality of life.

CHAPTER 2:

Transient disruption of mouse home cage activities and assessment of orexin immunoreactivity following concussive- or blast-induced brain injury

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ABSTRACT

The employment of explosive weaponry in modern warfare exposes populations to shock wave-induced and impact-related brain injuries. Among the most common clinical complaints resulting from traumatic brain injury (TBI) are sleep-wake disturbances. The current study assessed the acute effects of mild concussive brain injury (CBI) and mild blast wave-induced brain injury (BTBI) on mouse behavior and orexin-A expression. Male C57BL/6J mice were exposed to CBI, BTBI, or sham procedures. Injured animals and their shams were further divided into the following subgroups: 24-h survival in standard group (SG) housing, 72-h survival in SG housing, and 72-h survival in Any-Maze cages (AMc). AMc enabled continuous monitoring of home cage activities. BTBI caused significant but transient decreases in wheel running and ingestive behaviors 24 h post-injury (PI), while CBI transiently decreased running and water intake. BTBI resulted in general hypoactivity in the open field (OF) at both PI time points for SG-housed animals. In contrast, CBI did not cause hypoactivity. Mice subjected to CBI traveled more in the center of the OF at both time points PI, suggesting that CBI caused reduced anxiety in mice. Increased activity in the center of the OF was also seen at 24 h PI after BTBI. CBI treatment caused increased CD11b immunostaining. However, neither injury was accompanied by an alteration in the number of orexin-A hypothalamic neurons. Taken together, shock wave exposure and concussive injury transiently reduced mouse activities, but some differences between the two injuries were seen.

INTRODUCTION

Frequently referred to as the signature wound of modern day warfare, traumatic brain injury (TBI) has been recognized as a major challenge to the military and veteran health systems (32; 75). In a 2015 report, 8,127 service members returning from deployment screened positive for having sustained some form of TBI (5). The two most common causes of TBI in these service members were exposure to blast, accounting for 45.7% of TBI cases, and motor vehicle accidents; accounting for 18.9% of TBI cases (5).

As a significant source of TBI, blast exposure is an ongoing problem for the military. Since World War I, warfare has shifted toward the increased use of explosive weaponry (142) and, as seen in recent wars in Iraq and Afghanistan, certain terrorist groups favor the use of improvised explosive devices to achieve their political objectives. The use of such weaponry and the resulting wounds have driven advances in both protective armor and trauma care, resulting in higher survival rates of warfighters and civilian casualties who are exposed to blast forces that have historically been deadly (107). However, the treatments for chronic brain injury from blast exposure is a significant challenge.

The mechanism by which exposure to blast affects the brain is likely different than direct impact to the head as in the case of concussive brain injury (CBI) (58; 107; 178). Based on post-mortem analysis of brain tissue from service members, blast exposure results in distinct patterns of astroglial scarring that were not seen in civilian cases of impact TBI (178). An explosion can cause TBI in several biomechanically distinct ways. The rapid changes in air pressure created by a travelling shock wave upon detonation of an explosive device can cause primary blast-induced traumatic brain injury (BTBI) (107). Briefly, the energy from the shockwave is thought to be absorbed by the

brain and is converted into kinetic energy, causing movement, deformation, and displacement of tissue (32). In addition, the forces generated from an explosion can propel objects into a person or propel the person into objects causing secondary or tertiary CBI arising from impacts and linear and rotational insults (20; 68).

Although approximately 80% of TBI cases in the military are classified as mild (63) and there is, reportedly, a high incidence of full recovery, a significant number of warfighters sustain lasting symptomatology. Following mild TBI (mTBI), patients may present with multiple and varying symptoms that impede daily activities. The impact of such symptoms on patients and their ability to fulfill societal roles and responsibilities is profound (82; 119).

Among the most common clinical complaints are sleep-wake disorders (SWDs) (34; 44; 71; 138). SWDs are reported by over 50% of TBI patients with excessive daytime sleepiness (EDS) and fatigue accounting for over 55% of symptoms (12). These symptoms arise regardless of severity or location of trauma (12; 44; 71). More importantly, the presence of such symptoms can impede attempts at rehabilitation and result in poor clinical outcomes by contributing to the development of maladaptive behaviors, missed appointments, poor concentration, and deficits in executive function (21; 40; 66; 86; 180).

Although more than one brain region is involved in the promotion of arousal and wakefulness, clinical (13; 14) and rodent models (181; 196; 216) have demonstrated that TBI can specifically affect orexin-A (also named hypocretin) neurons of the hypothalamus. Orexin is involved in the regulation of arousal functions for wakefulness, locomotion, and feeding behaviors, and its deficiency has been previously implicated in

narcolepsy (166). In clinical TBI, the symptom of EDS was correlated with the loss of orexin-A neurons in the hypothalamus (13; 14). Orexin-A was shown to decrease in the cerebrospinal fluid (CSF) of patients acutely following moderate to severe TBI (14). Importantly, the decrease in CSF levels of orexin-A in these patients correlated with their reported symptoms of EDS (14). In support of this finding, orexin-A levels were shown to be lower in comatose patients than in patients who were awake and alert (14). Furthermore, loss of orexin-A-expressing neurons was also seen in post-mortem brain samples from patients who died following severe TBI (13).

In addition to SWDs, an overall decrease in physical and social activities has been reported following TBI (70; 74; 88). This change in activity can arise from a number of factors, it can slow the re-establishment of daily habits and activity, and impair the re-learning of physical and cognitive tasks through rehabilitation. The reduction of physical activity after TBI may also result from the need to recover from physical injuries and is often shown to be therapeutic (171). However, there are also findings that show the beneficial effects of return to physical activity (207), which may be challenging due to the comorbid development of neuropsychological symptoms that interfere with activities of daily living.

Thus, for some patients the impact of seemingly “mild” TBI on quality of life and daily function is devastating (12; 70; 211). Persistent SWDs, particularly hypersomnia, have been documented in TBI patients one to three years after injury (94; 214). One study even suggested that SWDs can evolve over time with a decrease in the prevalence of symptoms related to EDS while symptoms of fatigue increase over a three year period after TBI (94). Unfortunately, clinical treatment of SWDs following TBI has had limited

success (38; 139; 175), and the pathophysiological processes that give rise to these symptoms are poorly understood.

Because of the limitations of clinical studies and post-mortem studies, animal models have been widely used to elucidate the mechanisms by which CBI and BTBI alter brain function and vulnerability to neuropsychiatric disorders. To address symptoms related to changes in activity and wakefulness, researchers have utilized both rats and mice. Skopin and colleagues found that a moderate injury from lateral fluid percussion causes loss of orexin-A-positive cells in the hypothalamus along with fragmentation of the waking state in rats (181). Using controlled cortical impact (CCI) to model both mTBI and moderate TBI in mice, Thomasy and colleagues showed that mice with moderate injuries (impact depth of 1.0 mm) spent less time awake and more time in non-rapid eye movement sleep than mice sustaining mild CCI (impact depth of 0.5 mm) and sham mice (196). In addition to altered wakefulness, moderate CCI in mice showed a loss of orexin-positive neurons at 7 and 15 days PI. There was also a significant decrease in orexin-positive neurons 15 days PI in mild CCI mice, but not 7 days PI, suggesting a delayed effect of the milder injury in this study.

Although these two studies demonstrate that TBI can lead to reduced wakefulness, and that this is in part associated with the neuropathological loss of orexin neurons, concerns have been raised about the relevance of models that utilize invasive procedures (craniectomy, penetrating impacts to the dura) to induce TBI. Furthermore, neither study addressed the behavioral consequences of disrupted wakefulness following TBI nor how these consequences affect daily function.

To date, no pre-clinical studies have assessed the effects of mild closed-head, impact-induced TBI or mild BTBI on the orexin system and daily activity in a rodent model. These two forms of mTBI are commonly seen in military service members who train for and deploy to combat. Thus, the goal of the current study was to assess in mice the acute effects of CBI and BTBI. To do so, Any-Maze cages (AMc) were used to continuously monitor daily activity, including wheel-running and ingestive behaviors for 72 h following either a single CBI or single exposure to blast overpressure in the Advanced Blast Simulator (ABS) (157). We also examined the effects of TBI on microglial expression of CD11b as a marker for neuroinflammation, the expression of orexin-A in hypothalamic neurons, and mouse performance in the open field and y-maze. We hypothesized that both CBI and BTBI would result in hypoactivity, which would correlate with an increase in neuroinflammation and a decrease in orexin-A-positive neurons. We also predicted that CBI and BTBI would result in deficits similar to those reported by mTBI patients, such as increased anxiety and deficits in working memory.

METHODS

Animals and housing

Male C57BL/6J mice (Cat. No. 0664), 8 weeks old, from Jackson Laboratories (Bar Harbor, ME) were allowed to acclimate to housing facilities for approximately one week prior to TBI procedures or baseline AMc measurements. The housing room was on a standard 12-h light-dark cycle, and food and water were available *ad libitum*. The facilities are humidity- and temperature-controlled, and accredited by the Association for the Advancement and Accreditation of Laboratory Animal Care. The Institutional Animal

Care and Use Committee at the Uniformed Services University of the Health Sciences (Bethesda, MD) approved all procedures.

Twelve experimental groups were included in this study Table 1 refers to the number of mice/group in the open field and y-maze tests. Mice were randomly assigned to one of two injury procedures (CBI or BTBI) or respective sham procedures and were either placed in SG housing until behavioral testing at either 24 or 72 h following the injury, or they were housed in the AMc for constant activity monitoring for 72 h, followed by behavioral testing. All groups were euthanized for tissue collection immediately following behavioral testing. A total of 47 mice underwent CBI with 45 mice serving as their sham controls. One mouse that was assigned to the 72-h SG group was euthanized immediately following CBI and eliminated from the study due to bleeding from a cracked skull. A total of 52 mice underwent BTBI with 51 mice serving as their sham controls. One mouse from the 72-h AMc group was euthanized following baseline recording of daily activities because he escaped from the cage, and was replaced with a mouse originally assigned to the sham control group before BTBI or sham procedures. Eight mice (four injured mice and four sham mice) assigned to the 72-h AMc group were excluded from the final analyses due to lost data from software malfunction.

Concussive brain injury (CBI) procedures

Mice that underwent CBI and sham procedures were anesthetized with a gas mixture of isoflurane (Forane, Baxter Healthcare Corporation, Deerfield, IL) and 100% oxygen (induction: 3% isoflurane; maintenance: 1.5-2% isoflurane). The mice were placed in a stereotax and the head was fixed via a nose bar and atraumatic ear bars. A midline scalp incision was made between bregma and lambda to expose the skull. Mice

in the injured group were given single closed-skull CBI, using a Leica ImpactOne CCI device (Leica Microsystems, Buffalo Grove, IL) to strike the parietal bone with a 5-mm diameter tip at the following coordinates relative to bregma: anteroposterior -2.5 mm, mediolateral 2.5 mm, and depth 1.5 mm. A recent report indicates a single CCI device event results in multiple impacts (98). Any period of apnea following injury was recorded, and mice were placed in a cage on a heated pad in a supine position. The righting reflex (amount of time to return to a prone position) was also recorded for each mouse. Animals in the sham group were exposed to the same conditions as their injured counterparts and maintained under anesthesia for the same amount of time; however, they did not receive a CBI. Following recovery of the righting reflex, mice were returned to home cages and given drinking water supplemented with acetaminophen (1 mg/ml; ~200 mg/kg b.w. for 24 h).

Advanced Blast Simulator (ABS) procedures

The ABS (Fig. 9A) was designed by Dyn-Fx Consulting (Ambertburg, Ontario, Canada, see (157)) and constructed by ORA, Inc. (Fredericksburg, VA). It is comprised of four sections: a high pressure driver (compartment labeled *a* in Fig. 9B), transition section (*b* in Fig. 9B), test section (*c* in Fig. 9B), and end wave eliminator/muffler (*d* in Fig. 9B). The driver section (~0.0233 m^3) was pressurized to 150-160 psi with compressed air before the membrane sealing the driver burst, releasing the pressurized air into the transition and test sections. The membranes were comprised of two or three 0.254-mm thick clear acetate sheets (Grafix Plastics, Cleveland, OH) and two layers of vinyl-coated polyester mesh (Pet Screen, Hanover/New York Wire, Cat. No. 70589, mesh

size: 14.5x10 grids/in^2, wire diameter: 0.635 mm). The membranes, located at the open end of the driver, are approximately 2.9 m from the animal holder. The cross-section of the main portion of the shock tube (*c* in 9B) measured 0.64 x 0.64 m and was 1.23 m in length. At the terminus of the main section, the end-wave eliminator (*d*) received the shock wave, after it passed the animal, and served to dissipate the wave via an S-shaped passage made of 12.7-mm thick rubber conveyor belt material. The rodent holder (Fig. 9C) was made of mesh of the same specification used in the driver gas section and was held in place by stainless steel rods (diameter: 12.7 mm) in the center of the main section. The holder measured 14 cm x 15 cm for a cross-sectional areal occlusion of the shock wave of ~5.6%, allowing the simulated blast wave to smoothly diffract around the holder or pass through the mesh of the holder.

The mice received a BTBI overpressure exposure while unconscious. Mice were individually placed in an anesthesia chamber and received 3% isoflurane in 100% oxygen for 4-6 min. While unconscious, the body of the mouse was placed on a wood tongue depressor and the body was gently wrapped with strips of flexible gauze (Vet Wrap) to ensure there was minimal head or body movement when placed in the ABS. At the completion of the anesthesia period, the mouse was quickly transported to the ABS and secured in the bi-layered plastic-mesh rodent holder positioned inside the main section of the shock tube with the ventral surface of the animal facing the direction of the approaching shock wave. Incident pressure and wave velocity were measured by a series of gauges placed along the inner wall of the shock tube, as well as a pencil gauge probe (horizontal rod shown in Fig. 9C) mounted just to the side of the animal (Quartz, free-field, ICP blast pressure pencil probe, 50 psi, 104.2 mV/psi, 137B23A, PCB

Piezotronics). Before closing the hatch to the ABS, the animal was evaluated to ensure there were no signs of a lighter plane of unconsciousness. The hatch was quickly sealed to initiate the shock wave soon after placement (within ~24 s). While the animal was in the holder, an experimenter was positioned to view the animal inside the ABS through the hatch window. The animal was removed from the ABS within ~20 s following shock wave exposure and placed in a clean nesting cage that was placed on a warming pad. Instances of apnea were recorded and the mice were monitored until they regained consciousness (determined by the righting reflex) before placement in their home cage. Sham-treated animals received anesthesia and were placed in the holder at the distal end of the shock tube, but no shock wave was produced. Animals were returned to their home cages and provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ~200 mg/kg b.w. for 24 h) once they fully regained consciousness.

Any-Maze Cage (AMc) housing and activity monitoring

The AMc (Stoelting, Wood Dale, IL, Product 67000) apparatus consisted of a sound-attenuating cubicle, inside of which was a Plexiglas housing unit (24 cm H x 17.5 cm W x 12.5 cm D) for a single mouse. A running wheel was accessible adjacent to the housing cage, and a sensor on the wheel allowed the number of rotations of the wheel to be counted by Any-Maze software. Food and water were available *ad libitum* via apertures in the wall of the cage; the food and water hoppers (which collected any spillage) sat on weights that allow the software to calculate amount of food and water consumed. In addition, the chambers had built-in infra-red-sensitive cameras and infra-red and ambient light LEDs. Software controlled the light period (ambient light LEDs on)

and dark period (infra-red light LEDs on/ambient light LEDs off). A temperature sensor and ventilation fan allowed for temperature stability.

Three days prior to TBI or sham procedures, eight mice (four injury and four sham mice per cohort) were randomly placed in individual AMcs for collection of baseline levels of wheel running and eating and drinking behaviors. They were returned to their AMc units an h after recovery of the righting reflex for continuous 72-h assessment of wheel running and ingestive behaviors. For each succeeding cohort of eight mice placed in the AMc, placement was counterbalance in each cage.

Open field (OF)

On either Day 1 or Day 3 following injury or sham procedures mice were tested in an open field (OF) environment as previously described (200). The OF apparatus (Stoelting, Wood Dale, IL) was 40 cm x 40 cm with opaque walls (34 cm high). Illumination was approximately 175 Lux. Mice were placed into the center of the OF, and an overhead camera tracked movements of the mice for a 20-min session. Any-Maze software provided measures including the total distance traveled and the distance traveled in the center of the arena.

Y-maze test of spontaneous alternation

Shortly following OF testing on either Day 1 or Day 3, working memory was assessed in the y-maze test of spontaneous alternation as previously described (200). The y-maze apparatus (Stoelting, Wood Dale, IL) has three arms at a 120° angle to one another; each arm is 36 cm in length and enclosed by walls 16 cm in height. A small

visual cue was located at the distal end of each arm, and other objects in the room provided further visual cues. Mice were individually placed at the end of a randomly chosen arm and allowed to freely explore the maze for 5 min. An overhead camera linked to Any-Maze software (Stoelting) recorded all movements of the animals, and entries into each arm were scored by an individual blinded to the injury condition of the animals, with an entry considered when all four paws entered the arm. Percent correct alternation was calculated as $100 \times \frac{total\ number\ of\ alternations}{total\ arm\ entries-2}$; an alternation was counted when the mouse visited three different arms consecutively.

Histology and immunohistochemistry

Following behavioral testing, mice were anesthetized and transcardially perfused with cold phosphate buffer solution, followed by 4% paraformaldehyde in phosphate buffer. Their brains were removed, further fixed in paraformaldehyde for 24 h, and then placed in 20% sucrose for 72 h before freezing. Coronal sections, 30 microns thick, were produced using a microtome. The sections were collected in 24-well plates in a 1-in-6 serial fashion beginning approximately from the stereotaxic coordinates bregma -0.82 mm to approximately bregma -2.92 mm based on the mouse brain atlas of Franklin and Paxinos (65). Previous experiments in our lab established that orexin cells densely populated sections within this range (J. Liu, unpublished).

The sections were washed in tris-buffered saline with 0.05% Triton (TBST) before incubation at room temperature for 30 min with 0.3% hydrogen peroxide, to block endogenous peroxidase. The sections were washed again in TBST before incubation in blocking buffer (rabbit serum, TBST with 0.20% Triton, and 10% bovine serum albumin

(BSA)) for 1 h at RT. The sections were then stained with orexin-A Antibody (C-19), goat polyclonal immunoglobulin (IgG) (1:1000; Santa Cruz Biotechnology, Inc., Dallas, TX; Cat. No. sc-8070) overnight at 4°C.

The following day, the sections were washed in TBST and incubated in Biotin-SP-conjugated AffiniPure Rabbit-Anti-Goat-IgG (H+L) (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA; Cat. No. 305-065-003) in modified blocking buffer (TBST with 0.05% Triton, rabbit serum, and BSA) for 1 h at room temperature (RT). The sections were then washed with TBST before incubation in ABC solution from the Vectastain ABC HRP Kit (Vector Laboratories, Inc., Burlingame, CA; Cat. No. PK-4000) for 45 min at RT. A final 2 min wash with TBST preceded development with DAB from the DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA; Cat. No. SK-4100). The free-floating sections were mounted onto glass slides for analysis.

Images of the hypothalamic areas from sections containing orexin-positive cells were randomly selected from six mice per treatment group, were captured at 10x magnification, and Image J software (version 1.50i) was utilized to determine the number of cells present in each section (45). The background was subtracted from each image with a rolling ball radius of 12.0 pixels and converted to a 16-bit black and white image. The threshold was adjusted using the Yen method (221), and the particle analysis feature was employed to count cells with a minimum size of 75 pixel units.

Additional sections were immunostained for CD11b (1:500; rat anti-CD11b, ThermoScientific, Cat. No. MA1-80091), a marker for activated microglia, or for hematoxylin-eosin (H & E) for the evaluation of tissue injury. For evaluation of CD11b

immunostaining, sections were randomly obtained from six mice from each of the treatment groups and processed using the procedure described for orexin immunohistochemistry. Images of six brain regions were captured at 10x magnification, including the dorsal lateral geniculate nuclei, the optic tracts, the dorsal hippocampi, the dorsal cerebral cortex, the hypothalami, and the corpus callosum. Densitometry was performed using a Zeiss Axioskop microscope with an attached AxioCam MR.5 camera and ImageJ software (45). The brain regions of interest were traced via freehand selection and the mean grey density was determined using the measurement feature. Average density values were calculated using 3-4 sections from each animal by subtracting the background from the mean grey density. The background of each image was calculated by measuring a selected area that lacked immunostaining. All measurements were taken by a microscopist blinded to the treatment conditions of mice from which histological samples were obtained.

For H&E staining, samples were randomly selected from 10 mice from each of the treatment groups. Sections were mounted on glass slides and allowed to dry prior to staining. The sections were rinsed in distilled water three times for 1 min each and then submerged in 100% ethanol for 2 min. The sections were stained with hematoxylin (Sigma-Aldrich; Cat. No. GHS132-1L) for 3 min and immediately rinsed under running tap water for 5 min before being quickly submerged in acid ethanol (0.125 mL of 37% hydrochloric in 50 mL of 70% ethanol solution) 12 times. The sections were then rinsed under running tap water for 3 min followed by a 2-min rinse in distilled water. The sections were stained with a 1:2 eosin solution (50% eosin solution, Sigma-Aldrich; Cat. No. HT110316 and 50% deionized water) for 30 seconds, and then placed in running tap

water for 3 min. The sections were placed in a 95% ethanol solution for 2 min twice and then in 100% ethanol for 2 min twice before being moved to xylene for 2 min twice. Glass cover slips were applied for microscopy, and the samples were grossly assessed for the presence of bleeding or cellular injury at 2.5x magnification and 20x magnification.

Statistical analyses

With the exception of the data for apnea measures, analyses were performed separately for CBI and BTBI groups. Apnea was compared with just the injured groups using Fisher's exact test. Righting reflex data did not pass the homogeneity of variance (HOV) test as assessed by Levene's test of the equality of variances; SPSS (version 21; IBM SPSS Statistics, Armonk, NY) was employed to evaluate these data using Mann-Whitney *U* tests. A natural logarithmic transformation minimized violations of HOV in the CD11 staining density data. Statistical analyses for all behavioral and immunohistochemical data were performed with SAS 9.3 (SAS Institute Inc., Cary, NC, USA). AMc data (eating, drinking, wheel running) were analyzed with a two-way mixed linear model, with injury as a fixed factor and day as a repeated measure (Kenward-Rogers degrees of freedom approximation, auto-regressive (Lag-1) covariance structure). Significant time by injury interaction effects reported in the AMc data (wheel running, eating, drinking) were followed up by one-way ANOVAs at each time-point. The amount of water consumed for the BTBI groups on Day 1 did not pass the HOV test as assessed by Levene's Test of Equality of Error Variances; squared values passed this test and the one-way ANOVA was performed on the squared values.

OF, y-maze, and orexin data for the animals that were in AMc housing were analyzed with a one-way ANOVA, with injury as a fixed factor. Two-way ANOVAs were performed on all data (OF, y-maze, immunohistochemistry) collected from animals in standard housing; both injury and time following injury were treated as fixed factors. Results were considered significant when the *p*-value was less than 0.05, and data summarized in figures represent the mean $\pm$ standard error of the mean, except where noted (Fig. 2).

RESULTS

Shock wave exposure

The shock wave generated by the ABS (Fig. 1) mimicked a Friedlander wave; the waveform conventionally used to model an ideal shock wave (10). The mean peak incident overpressure of the air shock wave for the 48 exposed mice was 15.02 $\pm$ 1.28 psi (~103.6 kPa, coefficient of variation (COV) = 9%) with a positive (over-pressure) phase of ~5.41 ms followed by a ~3.95 ms negative (underpressure) phase with peak pressure of -3.96 $\pm$ 0.29 psi (~27.3 kPa, COV = 7%). Impulse (the integration of pressure x time) was measured as 0.03321 $\pm$ 0.00439 psi • sec (COV = 13%), and shock wave velocity near the mouse was 473.00 $\pm$ 10.61 m/s (COV = 2%). All animals survived ABS exposure and exhibited no gross motor impairment upon regaining consciousness nor other signs of significant malaise, besides temporary reductions in activity and ingestive behaviors (See *Any-Maze Cage activity* section).

Concussive brain injury

The CBI procedure was completed in approximately 8 min per mouse with sham-treated mice remaining anesthetized for an equivalent amount of time as their injured counterparts. Skull fractures and cranial or subdural bleeding was never observed with the exception of one mouse that exhibited a skull fracture and was excluded from the remainder of the study and immediately euthanized. Post-operatively no mice sustained scalp inflammation from the incision.

Apnea and righting reflex

Following the induction of anesthesia and injury, the presence/absence and duration (Fig. 2A) of apnea was recorded. Under anesthesia, none of the sham treated mice exhibited apnea (data not shown) and the incidence of observed apnea in the injured groups was 8.3% (4/48 mice) after BTBI and 37% (17/46) after CBI. Fisher's exact test indicated a significant difference in the proportion of mice with apnea after the two injuries (p=0.0011). Due to the lower percentage of mice that exhibited apnea, however, the median duration of apnea was 0 for both groups of injured mice. The median times to initial righting reflex were 287.5 and 172.5 seconds for the CBI and BTBI mice, and 50 and 42 seconds for their respective sham groups (Fig. 2B). Mann-Whitney U tests performed separately for each injury condition compared to its sham group showed that mice that had sustained either CBI or BTBI required a significantly longer amount of time than anesthetized sham controls to right to a prone position following the procedure (CBI: U = 2,020.50, $p < 0.0001$; BTBI: U = 2,593.00, $p < 0.0001$).

Any-Maze Cage activity

CBI and BTBI both reduced wheel running activity (Fig. 3A). When comparing mice with CBI to their sham controls, there was a significant injury by time interaction ($F_{3,63} = 18.95$, $p < 0.0001$). Follow-up one-way ANOVAs for each day showed that there was a significant effect of injury at baseline ($F_{1,29} = 5.74$, $p = 0.0233$) and during the first 24 h following injury ($F_{1,29} = 47.13$, $p < 0.0001$), but not during days 2 and 3 after CBI ($F_{1,29} \geq 3.34$, $p \geq 0.0786$). There was also a significant injury by time interaction effect when comparing mice that had undergone BTBI to their sham controls ($F_{3,88.6} = 8.60$, $p < 0.0001$). The groups did not differ at baseline ($F_{1,29} = 2.71$, $p = 0.1102$), but mice that had sustained blast overpressure spent significantly less time running in the wheel compared to sham controls during the 24 h following the injury ($F_{1,29} = 20.12$, $p < 0.0001$). There were no differences between the groups on the following two days ($F \leq 1.37$, $p \geq 0.2505$).

There was an effect of BTBI on the amount of food consumed after injury ($F_{3,76.6} = 6.60$, $p = 0.0005$) (Fig. 3B), and a significant main effect of injury on the amount of food eaten by mice with CBI ($F_{1,44.9} = 10.50$, $p = 0.0023$). However, the amount of food consumed did not differ between the CBI and sham group as a function of day. The effect of BTBI on food consumption was dependent on the day; mice assigned to injury and sham groups were equal at baseline ($F_{1,27} = 0.00$, $p = 0.9912$), but mice that had sustained blast overpressure consumed significantly less food than sham controls during the 24 h following injury ($F_{1,28} = 23.58$, $p < 0.0001$). There was no effect of BTBI on consumption of food on the second or third days after injury ($F \leq 2.67$, $p \geq 0.1136$).

Water consumption following CBI or BTBI is shown in Fig. 3C. There was a significant day by injury interaction for mice that sustained CBI ($F_{3,82.1} = 22.16$, $p < 0.0001$). Baseline water consumption was equivalent ($F_{1,29} = 0.49$, $p = 0.4911$), but

injured mice drank less water than sham controls in the 24 h following CBI procedures ($F_{1,27} = 46.85$, $p < 0.0001$). Water consumption was equivalent between the groups on the second and third days following injury ($F \leq 0.69$, $p \geq 0.4149$). There was also a significant day by injury interaction effect on the amount of water consumed by mice that sustained injury by BTBI ($F_{3,87.9} = 15.91$, $p < 0.0001$). Injury and sham groups were equivalent during baseline testing ($F_{1,28} = 1.20$, $p = .2834$), but injured mice drank less water during the 24 h following BTBI ($F_{1,29} = 16.81$, $p = 0.0003$). All mice drank the same amounts of water during the second and third days following BTBI or sham procedures ($F \leq 1.85$, $p \geq 0.1842$).

Open field

For mice that were returned to standard group (SG) housing following CBI or sham procedures and tested either 24 or 72 h following injury, there was no effect of injury ($F_{1,55} = 0.02$, $p = 0.8975$) or day by injury interaction effect ($F_{1,55} = 1.94$, $p = 0.1695$) on the distance traveled in the OF arena (Fig. 4A). There was also no effect of injury on distance traveled in the arena in the mice from the AMc housing that were tested 72 h following CBI ($F_{1,30} = 2.08$, $p = 0.1592$). However, there was a significant main effect of BTBI exposure on the total distance traveled in the OF in mice from SG housing tested 24 or 72 h following procedures ($F_{1,60} = 11.56$, $p = 0.0012$; Fig. 4B). Mice that sustained BTBI and were returned to SG housing traveled less distance in the open field arena than their sham counterparts regardless of testing time point. There was no effect of injury on activity levels in mice from the AMc housing tested 72 h following BTBI ($F_{1,29} = 0.05$, $p = 0.8240$; Fig. 4B).

For mice in SG housing that sustained CBI, there was a significant main effect of injury on the distance traveled in the center of the arena (expressed as a percent of the total distance traveled); mice with CBI had greater activity in the center than sham controls ($F_{1,55} = 5.37$, $p = 0.0243$; Fig. 4C). This increase in center activity was also found in the mice from AMc housing tested 72 h following CBI ($F_{1,30} = 5.30$, $p = 0.0285$; Fig. 4C). In the mice from SG housing that were exposed to BTBI (Fig. 4D), there was a significant interaction effect between time and injury on the distance traveled in the center of the arena ($F_{1,60} = 4.66$, $p = 0.0349$); mice that had sustained BTBI had significantly more activity in the center than sham controls did at the 24-h time point ($F_{1,30} = 8.64$, $p = 0.0063$), but not at 72 h ($F_{1,30} = 0.18$, $p = 0.6733$), and mice housed in the AMc showed no group differences after BTBI ($F_{1,29} = 1.46$, $p = 0.2371$).

Y-maze

Spontaneous alternation behavior (SAB) was not affected by CBI in mice from either SG housing (Injury by Day interaction, $F_{1,55} = 1.63$, $p = 0.207$; main effect of Injury, $F_{1,55} = 0.81$, $p = 0.371$) or AMc housing ($F_{1,30} = 1.15$, $p = 0.292$; data not shown). BTBI also did not affect SAB in mice from SG housing (Injury by Day interaction, $F_{1,60} = 0.00$, $p = 0.9644$; main effect of Injury, $F_{1,60} = 0.11$, $p = 0.7455$) or from AMc housing ($F_{1,29} = 1.03$, $p = 0.3181$) (data not shown).

Histological findings

Fig. 5 shows hematoxylin and eosin stained samples from mice kept in SG housing and subjected to CBI and sacrificed at 24 or 72 h PI. CBI resulted in bleeding

that is seen in the left cortex at the site of impact in some but not all mice: at 24 h PI (Fig. 5A), 2 out of 10 mice showed signs of bleeding at the injury site; at 72 h PI, 4 out of 10 mice showed signs of bleeding at the injury site (Fig. 5B). A close examination of the histological specimens indicated no other pathological features were evident after CBI or BTBI. Cortical bleeding was not seen in mice serving as CBI or BTBI sham controls, nor in mice that had sustained BTBI (data not shown).

Fig. 6 (A and B) shows CD11b-positive cells in the optic tract and corpus callosum of mice kept in SG housing and sacrificed either 24 or 72 h following CBI or BTBI. For mice that had sustained CBI, a four-way ANOVA (Time x Injury x Region x Side) on CD11b staining density revealed an interaction effect between side, time and injury ($F_{1,180} = 4.61$, $p = 0.0331$), thus, separate three-way ANOVAs (Injury x Region x Side) were performed for each time point (Fig. 7). In the mice that were sacrificed 24 h following CBI (Fig. 7A), there was a significant injury by region interaction effect ($F_{4,90} = 5.71$, $p = 0.0004$); Bonferroni-corrected planned comparisons showed that CD11b staining density was greater in the optic tract (OPT: $p = 0.0025$), but not other regions measured ($p > 0.179$) in mice that sustained CBI than in sham controls. In addition, there was a main effect of side ($F_{1,90} = 16.35$, $p = 0.0001$), with overall greater staining density on the left (injured) side of the brain than the right side of the brain. There was also a significant injury by region interaction effect at the 72 h time point (Fig. 7B, $F_{4,90} = 6.14$, $p = 0.0002$), with CD11 staining in the optic tract ($p = .0065$) having higher density in injured mice than in sham-treated animals, but other regions had equal staining density between the two groups ($p > 0.2345$). There was also a main effect of side at 72 h, with the left side of the brain having greater CD11 staining density than the right side of the

brain ($F_{1,90} = 8.57$, $p = 0.0043$). In the corpus callosum (Figs. 6B and 7C), a two-way ANOVA (Time x Injury) showed a significant main effect of injury ($F_{1,20} = 5.55$, $p = 0.0287$), with injured mice having greater staining density in this region than sham-treated mice.

In mice that sustained BTBI, a four-way ANOVA (Time x Injury x Region x Side) showed an interaction effect between brain region and injury ($F_{4,180} = 3.40$, $p = 0.0104$). However, Bonferroni-corrected planned contrasts did not find differences between injured and control mice in any of the measured regions (Figs. 7D & E). There were no other interaction effects between the four factors or main effects for BTBI ($F \leq 1.81$, $p \geq 0.1291$). In the corpus callosum (Fig. 7F), there was no effect of injury on CD11b staining density ($F_{1,20} = 1.42$, $p = 0.2466$) or a time by injury interaction effect ($F_{1,20} = 0.41$, $p = 0.5315$), but there was a main effect of time, with greater staining density in all mice sacrificed at 72 h ($F_{1,20} = 4.82$, $p = 0.0401$).

Fig. 8 (A and B) shows orexin-positive cells in the hypothalamus 24 or 72 h following brain injury induced by either CBI or BTBI in mice that were kept in SG housing. There was no effect of either injury type on the number of orexin-positive cells in the hypothalamus in mice that were housed in AMc (CBI: $F_{1,10} = 0.01$, $p = 0.9101$; BTBI: $F_{1,10} = 0.00$, $p = 0.9964$) (data not shown) or in SG housing (CBI: $F_{1,20} < 2.09$, $p > 0.1637$; BTBI: $F_{1,20} < 0.29$, $p > 0.5943$) (Fig. 8C).

DISCUSSION

The effects of the most common forms of mTBI seen in military populations, impact-induced closed-head CBI and exposure to blast overpressure, were assessed in

mice. The Advanced Blast Simulator (ABS) was used to recreate conditions of blast-induced brain injury. Unlike conventional shock tubes, the special geometry of the ABS directly replicates the true wave-dynamics of free-field explosive blast. High blockage ratio from the specimen and its mounting platform and anomalous dynamic pressure from end-wave artefacts cause exaggerated 'blast throw' effects on specimens (128; 190). The large cross-sectional area of the current ABS, however, allows a very low obstruction effect, or blockage ratio, due to the "presented area" of the specimen (5.6%), and the special End-Wave Eliminator device ensures no anomalous waves are introduced due to the reflection of the primary wave at the end of the simulator. Therefore, the specimen configuration within the ABS has been specially designed to provide a model for injuries resulting from free-field blast reflection and diffraction (158; 208). However, the vertical orientation of the mouse holder would result in some degree of enhanced reflected loading and blast-induced acceleration.

The incidence of apnea following exposure to blast overpressure was infrequent. Since there was a consistent delay of ~20 s in removing animals from the ABS before they could be observed directly, some caution is needed in the estimation that only 8.3% of the BTBI mice exhibited apnea. Mice that sustained BTBI, nevertheless, exhibited delayed righting reflex when compared to their sham controls that received an equivalent dose of anesthesia (Fig. 2B), indicating the injurious nature of ABS shock wave exposure. Post-mortem H&E analysis of brain tissue from mice exposed to a single blast overpressure of approximately 15 psi, however, showed no signs of damage, with tissue from injured mice being indistinguishable from their sham controls (images not shown).

The absence of histological findings with H&E staining provided support for the mild nature of this BTBI model.

For CBI, a conventional CCI device was used to mimic the physical impact of closed head TBI. In previous studies, this model had been shown to induce neuropathological and functional changes, including persistent neuroinflammation, particularly in the corpus callosum (209; 222). As with mice that sustained BTBI, mice that experienced CBI displayed a delayed righting reflex, compared to their sham controls (Fig. 2B) and a higher incidence of apnea acutely PI. Post-mortem analysis of H&E stained-tissue from CBI mice revealed some bleeding at the site of impact in two out of ten SG-housed mice sacrificed 24 h PI and four out of ten SG-housed mice sacrificed 72 h PI (Fig. 5). Small focal hemorrhages at the site of impact were also seen in a previous study using the same CCI device to model closed-head repetitive CBI (108).

It was hypothesized that, similar to the collective observations in previous studies (13; 58; 181), CBI and BTBI would result in hypoactivity, a reduction of immunostaining in hypothalamic orexin neurons, increased anxiety, and deficits in working memory. The AMc system enabled examination of baseline activity prior to injury as well as activity during the acute time period following TBI in a non-invasive manner that did not interfere with mouse daily activities. As expected, hypoactivity was observed in mice in both injury groups by reduced levels of home-cage wheel-running, eating, and drinking following either type of injury, but the effects of each injury type were transient with return to baseline levels of activity by 72 h PI. Using a weight drop impact TBI model, Nichols and colleagues reported that mice exhibited transient (days 1 and 2 after injury) decreased activity (130). Rowe and colleagues reported transient (~6 hrs) of inactivity

after fluid percussion injury in mice (163). A reduction in activity level was also seen in the OF for mice that sustained BTBI injury. SG-housed mice traveled less distance overall in the OF when compared to their sham controls at both 24 h and 72 h after BTBI. Tang and colleagues previously reported a transient decrease in food and water intake 24 hrs after weight drop injury in the mouse (194).

All mice that sustained CBI, and SG-housed mice exposed to BTBI at the 24 h time-point exhibited greater activity in the center zone of the OF (Fig. 4C and 4D), suggestive of decreased caution or anxiety in these animals. Activity measures in center versus peripheral zones of OF are often employed as assays of anxiety in rodents (153; 173), with less anxious animals being more willing to explore the open "more dangerous" center area of the arena (153). However, when employing the OF, light-dark box (LDB) and elevated zero maze (EZM) to assess anxiety following TBI, results from the OF contrasted with those from the LDB and EZM, which are more specific tests of anxiety (199). In addition, common anxiolytic agents do not increase center zone activity in C57BL/6J mice (76; 197). OF measures are suggested as a starting point for assessing anxiety and should be followed up with anxiety-specific tests such as the elevated plus (or zero) maze (EPM) or the light-dark box (49; 173). Decreased anxiety-like behaviors in the EPM following single CBI at more chronic time points have been reported (126), but others have reported increased anxiety following CBI or no changes (e.g.(25; 130). Blast studies in mice are few, but Patel and colleagues found greater risk assessment behaviors in the EZM (stretch-attenuated postures, suggestive of heightened anxiety) following BTBI (approximately 31 psi) induced in a shock tube (147). Overall, it has been noted that data from anxiety testing following TBI varies widely between

laboratories, and proper neuropsychiatric symptom modeling remains challenging in pre-clinical TBI modeling (116; 199).

All mice were provided with acetaminophen in their drinking water for 24 h following injury or sham procedures, and there are two concerns to be raised given the reduction in water intake in the injured mice during this time period. First, drinking water is not an ideal delivery method for acute administration of anesthetics or other therapeutics following injury. Second, the sham-treated mice received a higher dose of acetaminophen than the injured animals, and the effects of the drug on the outcome measures in our control animals should be considered. There are few studies on the effects of acetaminophen on mouse behavior; higher doses (100 or 200 mg/kg; intraperotoneal) given to BALB/c mice reduce anxiety (increase number of entries and time spent in the open arms) in the EPM (223) and Vogel conflict test (206), without affecting spontaneous activity in the OF arena (223). In the current study, anxiety-like behaviors of the sham-control mice would have been the most affected as they had a higher dose of analgesic than injured mice; this would serve to increase the activity in the OF center zone of the control mice. Without the (potential) anxiolytic effect of acetaminophen, the observed differences in OF center activity between injured and control mice may have been greater.

Further, there was no change in the number of orexin-positive cells as assessed by immunohistochemistry post-mortem (Fig. 8). The lack of a detectable change in the number of orexin-A-expressing cells may be related to the mild nature of the injuries. The lateral fluid percussion model used by Skopin et al. caused a significant decrease in orexin-A immunostaining, which correlated with chronic decreased wakefulness in rats

(181). However, their injury model was classified as producing moderate injury. In patients, orexin-A in the CSF is decreased after moderate to severe TBI, but not after mTBI (14). Furthermore, in a study examining Parkinson's Disease (PD), which has many symptoms in common with narcolepsy including EDS, researchers found that disease severity positively correlated with loss of orexin cells: patients with more severe PD had less orexin-positive cells than those in earlier clinical stages of PD (195).

In addition to fewer effects from employment of milder TBI treatments, the short survival endpoints of 24 and 72 h could have contributed to the lack of change in orexin-A-expressing cells. A recent study of mild and moderate CCI in mouse showed no significant changes after mTBI at seven days PI; however, at 15 days PI, mTBI mice appeared to have significantly fewer orexin-positive cells than sham controls (196). For moderate TBI, the authors found that injured mice that were sacrificed 15 days PI had a significant decrease in orexin-positive cells when compared to injured mice sacrificed earlier at 7 days PI (196).

Not all studies have reported changes in orexin following TBI. One study that utilized CCI to model TBI in mice employed a deeper depth of impact (2.5mm) than Thomasy and her colleagues (196), and observed no loss of orexin-A-positive cells three days after injury (216). However, there was a decrease in extracellular levels of orexin following TBI in the hypothalamus and the hippocampus (216). Interestingly, two other studies assessing the therapeutic potential of branched chain amino acids after unilateral lateral fluid percussion also noted changes in orexin physiology but not the total number of cells following TBI (59; 105). Lim and colleagues found that injured mice were less active than sham controls at 24 and 33 days PI when locomotor activity was assessed

(105). This decrease in activity correlated with injured mice also spending less time awake during both the light and dark phases and more time in non-rapid eye movement sleep than sham controls during the dark phase as measured by EEG/EMG (59). They also found that orexin neuron activation was reduced in brain-injured mice and not sham controls during the waking state (105). A follow-up study using the same injury model and parameters showed TBI caused a reduction in the density of glutamate immune-labeling within pre-synaptic nerve terminals in the lateral hypothalamus that synapsed onto orexin labeled neurons in an axo-dendritic fashion (59). Based on their findings, the authors concluded that TBI resulted in a reduction of locomotor activity and decreased wakefulness in mice by reducing excitatory inputs to orexin neurons (59). Thus, it is possible that the inability to maintain consolidated wakefulness following TBI may result from disruptions in normal orexin physiology and not just loss of orexin neurons. In line with these mouse studies, Noain and colleagues employed a weight drop TBI model in rats and also did not observe changes in orexin immunostaining (133). Instead, the 28-day study revealed a correlation between increased sleep need and a loss of histamine-positive cells in the tuberomammillary nucleus. The authors did not see any changes in orexin-positive cells nor melanin-concentrating-hormone-positive cells in the hypothalamus (133).

In the current study, the presence of activated microglia was more prominent in the optic tract of mice that sustained CBI and sacrificed at both 24 and 72 h compared to sham controls (Fig. 6A). In addition, CBI mice sacrificed at 24 and 72 h showed a significantly higher density of CD11b-positive cells in the corpus callosum when compared to their sham controls (Fig. 6B). The increased presence of activated microglia

at 72 h PI may reflect the progressive nature of neuroinflammation. However, this increased inflammation at 72 h did not affect home cage activity as wheel running and ingestive behaviors returned to levels comparable to sham control groups by 48 h PI.

Thus, the hypoactivity seen during the first 24 h PI could have resulted from temporary alterations in the aforementioned neuronal networks and neurotransmitter systems involved in the promotion and maintenance of arousal as well as from functional changes in cholinergic, histaminergic, or noradrenergic systems that mediate activity (26; 87). The more persistent effects of TBI on orexin neurons and wakefulness as documented in the clinical literature (13-15) as well as in the two aforementioned rodent studies (181; 196) may result from delayed processes that were not captured in the current study.

Since the current study did not use EEG to assess for changes in wakefulness and sleep, no direct conclusions can be drawn about the effects of BTBI or CBI on mouse sleep-wake physiology. Although activity is a correlate of sleep, non-invasive techniques to indirectly measure central nervous system arousal state and sleep stages may not be sensitive enough to reveal the intricate physiological changes that result in alterations from normal sleep-wake patterns. Measures of daily activities such as wheel running, eating, and drinking, however, are of significant clinical relevance as these behaviors are universal read outs of mammalian behavior and provide insight into functionality after mTBI. Furthermore, vegetative functions as well as behaviors related to energy expenditure are known to be regulated by the orexin system (11; 167). Although orexin is mainly implicated in sleep-wake dysregulation in TBI, the neuropeptide has broad functions that affect behavior through its influence on feeding, motivation, and reward

(11; 167). Thus, wheel running, eating, and drinking behaviors all tie into overall arousal, which is regulated by orexin and known to decrease after TBI in both human and animal studies.

Nevertheless, this study mirrors other recent pre-clinical reports comparing blast, impact, and rotational acceleration effects (187; 191; 203), as well as existing data from clinical studies and other experimental models, that two different types of mTBI could affect, sometimes in subtle ways, the brain differently (178; 191). Pre-clinical reviews have noted dissimilarities in the injuries resulting from different TBI models (30; 179). In general, CBI would be expected to result in a primary focal lesion site localized in the parietal cortex region below the impact site and closely adjacent regions, although more distal changes have been reported, e.g., (22; 108; 112; 204; 215), including what was observed here with CD11b immunoreactivity changes seen in the corpus callosum and optic tracts. Blast-related TBI, however, is known to result in more wide ranging diffuse changes, including injuries to peripheral organs that may contribute to diffuse neuropathological (30; 179).

CBI resulted in a higher incidence of PI apnea and longer righting times on average and in a more persistent decrease in anxiety-like behavior than BTBI. Mice that underwent CBI exhibited neuroinflammation as measured by the density of CD11b staining at both 72 h and 24 h PI. In contrast, BTBI did not result in any measurable level of neuroinflammation. The higher level of neuroinflammation seen after CBI was most likely due to the nature of the localized and direct impact versus the more global shearing forces of BTBI. BTBI, on the other hand, resulted in general hypoactivity in the OF, which was not seen after CBI.

The transient effects of a single mTBI as seen here may speak to the true clinical picture: the presenting symptoms of mTBI in patients are usually mild, can be subtle and transient, and are known to persist in a minority of patients. Importantly, the severity of symptoms that present acutely PI may not reveal the full extent of neurophysiological changes in the brain that eventually lead to worsening clinical outcomes later in life with or without subsequent mTBIs. There is a growing literature on repetitive mTBI, demonstrating that a single milder head injury may not cause apparent deficits, but may leave the brain vulnerable to further injuries (99; 101; 122; 130; 177). In addition, there is evidence that repetitive mTBI can lead to chronic traumatic encephalopathy independent of the presence of concussive symptoms (186; 191). Thus, more work is needed to determine the mechanisms underlying brain injuries by the use of multiple pre-clinical models for the comparison of impact, acceleration, and blast effects, and how these forces interact to cause the behavioral and pathological changes seen in service members exposed to warfare.

Table 1. Final number of mice in each group

	Concussive Brain Injury (CBI)		Advanced Blast Simulator (BTBI)	
	Injured	*Sham*	*Injured*	*Sham*
Standard housing (24 h)*	15	15	16	16
Standard housing (72 h)	15	14	16	16
Any-Maze Cage housing (72 h)	16	16	16	15

* 24 or 72 h refers to time of euthanasia.

Figure 1

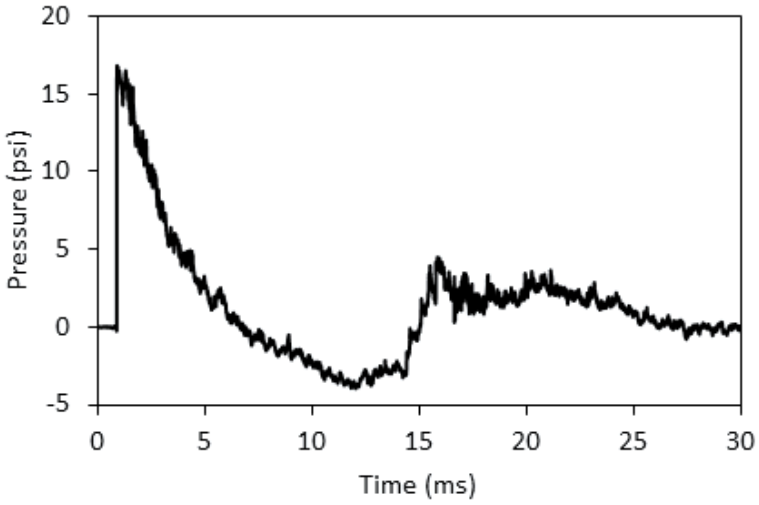

Figure 1. Advanced Blast Simulator time-pressure profile from pencil gauge located adjacent to animal holder. Upon arrival of the shock front, there was a steep change in ambient pressure to ~15 psi, followed by an exponential diminution of pressure. At approximately 5.4 msec after shock wave arrival, the negative phase of the wave was observed for ~4 msec.

Figure 2

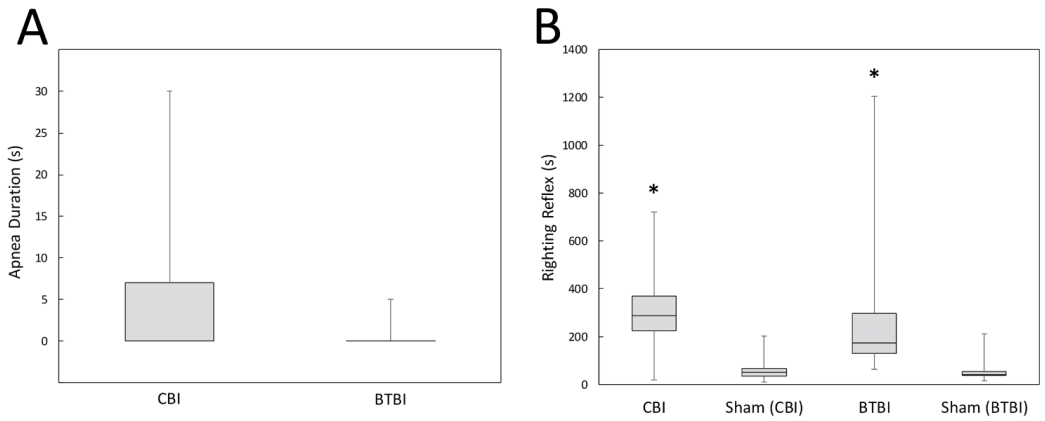

Figure 2. Median apnea and righting reflex times following CBI and BTBI. (A) Box plot of the distribution of the duration of apnea exhibited by injured mice. The median times for apnea for each group was 0 seconds (44 of the 48 BTBI and 29 of the 46 CBI mice exhibited no apnea). (B) The mice exposed to CBI or BTBI exhibited significantly longer righting reflex times compared to sham-treated mice (*p<0.0000000). A direct comparison of the median righting reflex times of the CBI and BTBI mice indicated the CBI-exposed mice exhibited a longer time to recover compared to the BTBI mice (Mann-Whitney U test, U = 2847.50, $p < 0.0001$). The Box plots depict the medians (horizontal line inside boxes), the upper and lower 75 and 25% confidence intervals as the upper and lower horizontal extents of the boxes, and the whiskers show maximum and minimum data ranges.

Figure 3

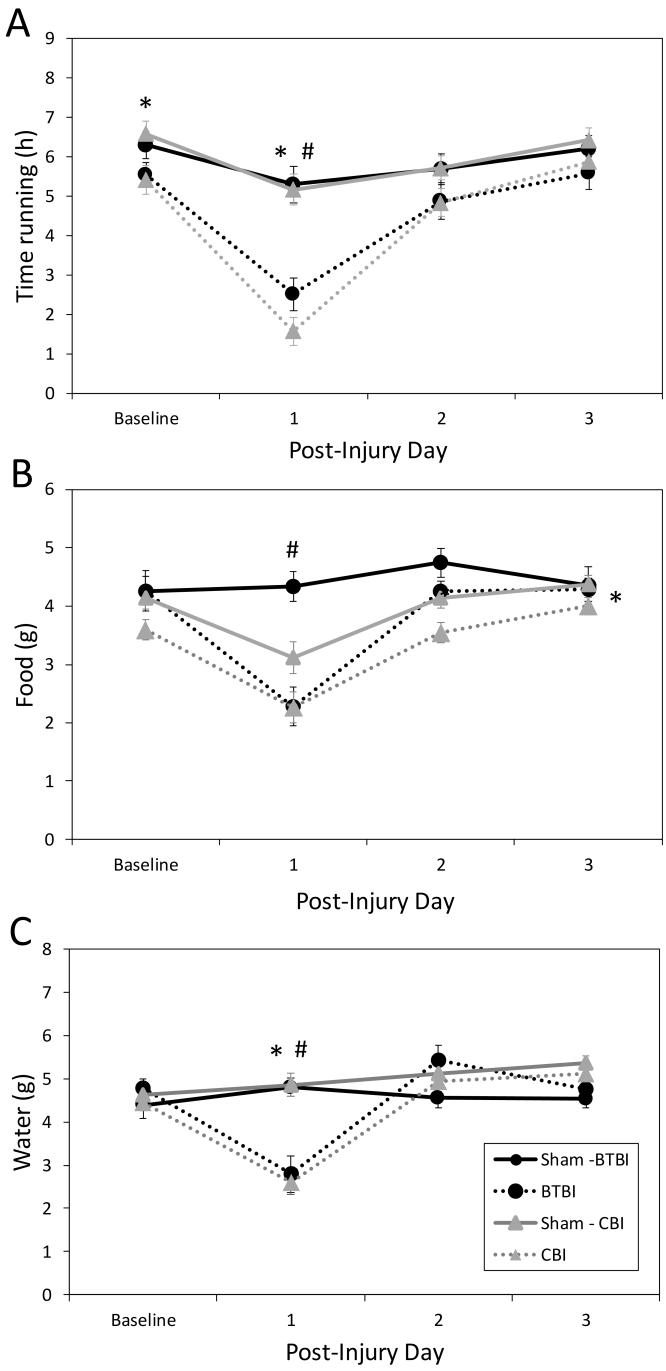

Figure 3. Any-Maze cage activity following CBI, BTBI, or sham treatment. (A) Wheel running following CBI or BTBI. There was a transient decrease in wheel running following both CBI and BTBI. CBI mice spent less time running in the wheel when compared to their sham controls at baseline ($p < 0.0001$) and during the first 24 h after injury ($^*p < 0.0001$). By 48 h post-injury, CBI mice did not differ from their sham controls in the amount of time spent running in the wheel ($p \geq 0.0786$). Mice exposed to BTBI spent significantly less time running in the

wheel than sham controls during the first 24 h after injury (#$p < 0.0001$). By 48 h after injury, there was no difference between BTBI mice and their sham controls ($p \geq 0.2505$). The asterisk (*) represents an effect of CBI on the indicated day; the pound sign (#) represents an effect of BTBI on the indicated day. (B) Food consumption following BTBI or CBI. There was an effect of injury on food consumption in injured animals. There was a significant main effect of CBI on the amount of food consumed (*$p = 0.0023$); however, the amount of food consumed did not differ between mice with CBI and their sham controls as a function of day. In contrast, BTBI mice ate significantly less food than their sham controls during the first 24 h after injury (#$p < 0.0001$). They did not differ from sham controls at baseline ($p = 0.9912$) nor by 48 h post-injury ($p \geq 0.1136$). The asterisk (*) represents a main effect of CBI on food consumption; the pound sign (#) represents an effect of BTBI on the indicated day. (C) Water consumption following BTBI or CBI. CBI mice drank less water than their sham controls during the first 24 h following CBI (*$p < 0.0001$), but not at baseline ($p = 0.4911$) nor by 48 h post-injury ($p \geq 0.4149$). BTBI mice drank less water than their sham counterparts (#$p = 0.0003$) during the first 24 h after injury, but not at baseline ($p = .2834$) nor by 48 h after injury ($p \geq 0.1842$). The asterisk (*) represents an effect of CBI on the indicated day; the pound sign (#) represents an effect of BTBI on the indicated day.

Figure 4

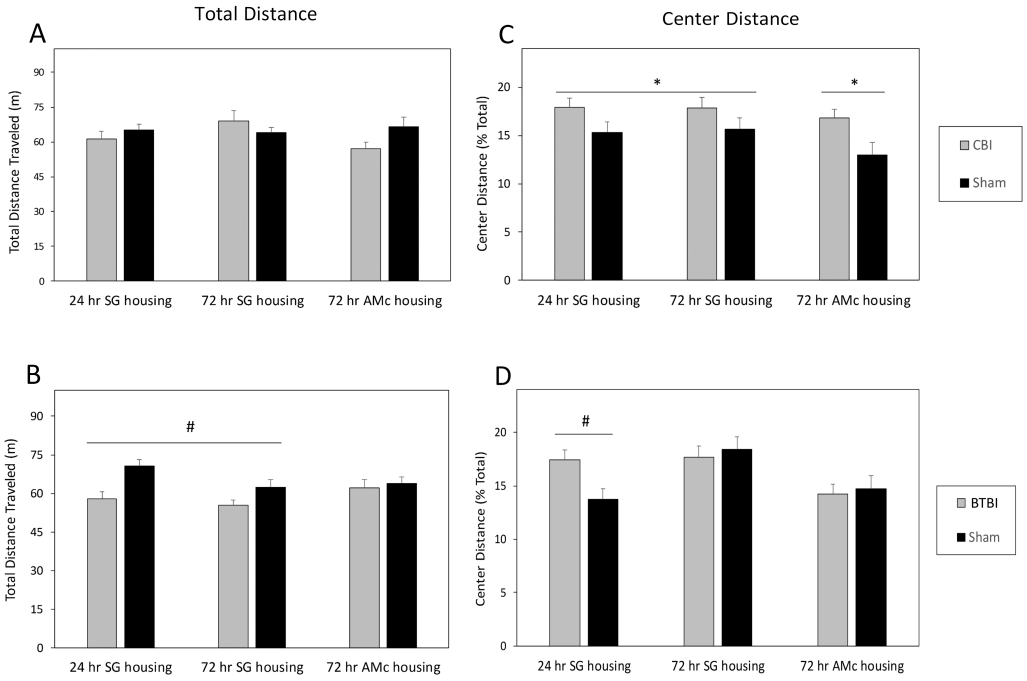

Figure 4. Open field activity following BTBI or CBI. (A) Total distance traveled in open field arena following CBI. When comparing mice that sustained CBI to their sham controls, there was no effect of injury on the total distance traveled in the open field arena for mice that were standard-group housed ($p = 0.8975$), nor was there any day by injury interaction effect ($p = 0.1695$). There was also no effect of injury on the total distance traveled in the open field for mice that were housed in Any-Maze cages ($p = 0.1592$). (B) Total distance traveled in open field arena following BTBI. Injured mice that were housed under standard group conditions traveled significantly less distance in the open field than their sham controls regardless of testing time point (#$p = 0.0012$). Mice housed in Any-maze cages and tested 72 h after BTBI did not differ from their sham controls in the total distance traveled in the open field ($p = 0.8240$). (C) Center distance traveled in the open field following CBI. Injured that were housed in standard groups traveled more in the center of the open field than their sham controls (*$p = 0.0243$). The increase in center activity was also seen in injured mice that were housed in Any-maze cages and tested 72 h after injury (*$p = 0.0285$). (D) Center distance traveled in the open field following BTBI. Mice in SG housing that were exposed to BTBI traveled more in the center of the open field than their sham controls when tested 24 h after injury (#$p = 0.0063$), but did not differ from sham controls when tested 72 h after injury ($p = 0.6733$). No change in the center distance measure was observed in BTBI mice, compared to sham-treated animals, after 72 h of AMc housing. The asterisk (*) represents an effect of CBI on the indicated day; the pound sign (#) represents an effect of BTBI on the indicated day. Table 1 provides a summary of sample sizes for each treatment group.

Figure 5

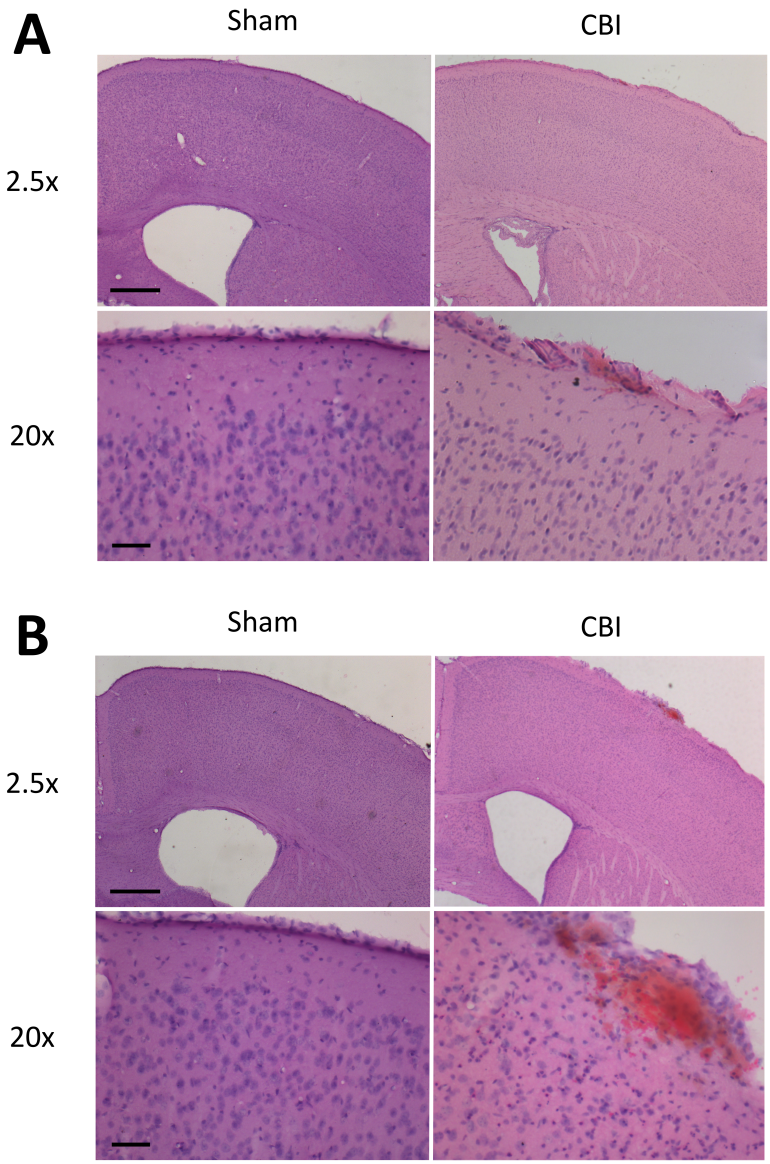

Figure 5. Hematoxylin and eosin staining 24 h and 72 h after CBI. Neuropathology 24 h (A) or 72 h (B) following a single CBI. Sections are shown at 2.5x magnification and 20x magnification. For most of the mice that sustained CBI, there was little evidence of damage to the cerebral cortex. However, as shown in these examples, CBI resulted in bleeding in two out of ten mice sampled at 24 h and four out of ten mice sampled at 72 h after injury. Cortical bleeding was not seen in sham control mice regardless of endpoint. The scale bar for the 2.5x magnification images represents 500 micrometers; scale bar for 20x magnification images represents 50 micrometers.

Figure 6

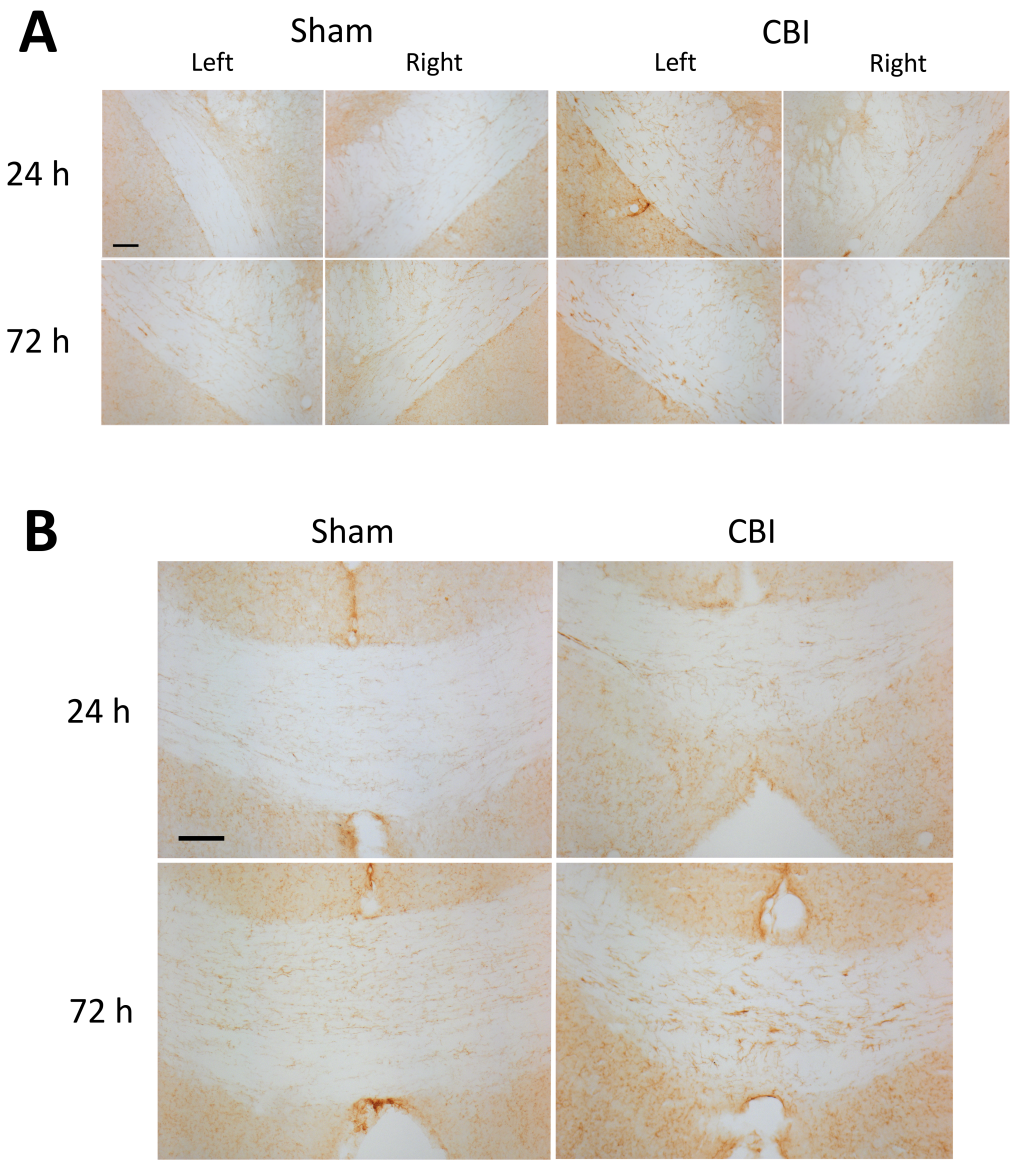

Figure 6. Sections from CBI mice showing CD11b immunostaining in the right and left optic tract (A) and corpus callosum (B) of mice sacrificed at 24 or 72 h after CBI or sham procedures. Images shown at 10x magnification. Both scale bars represent 100 micrometers.

Figure 7

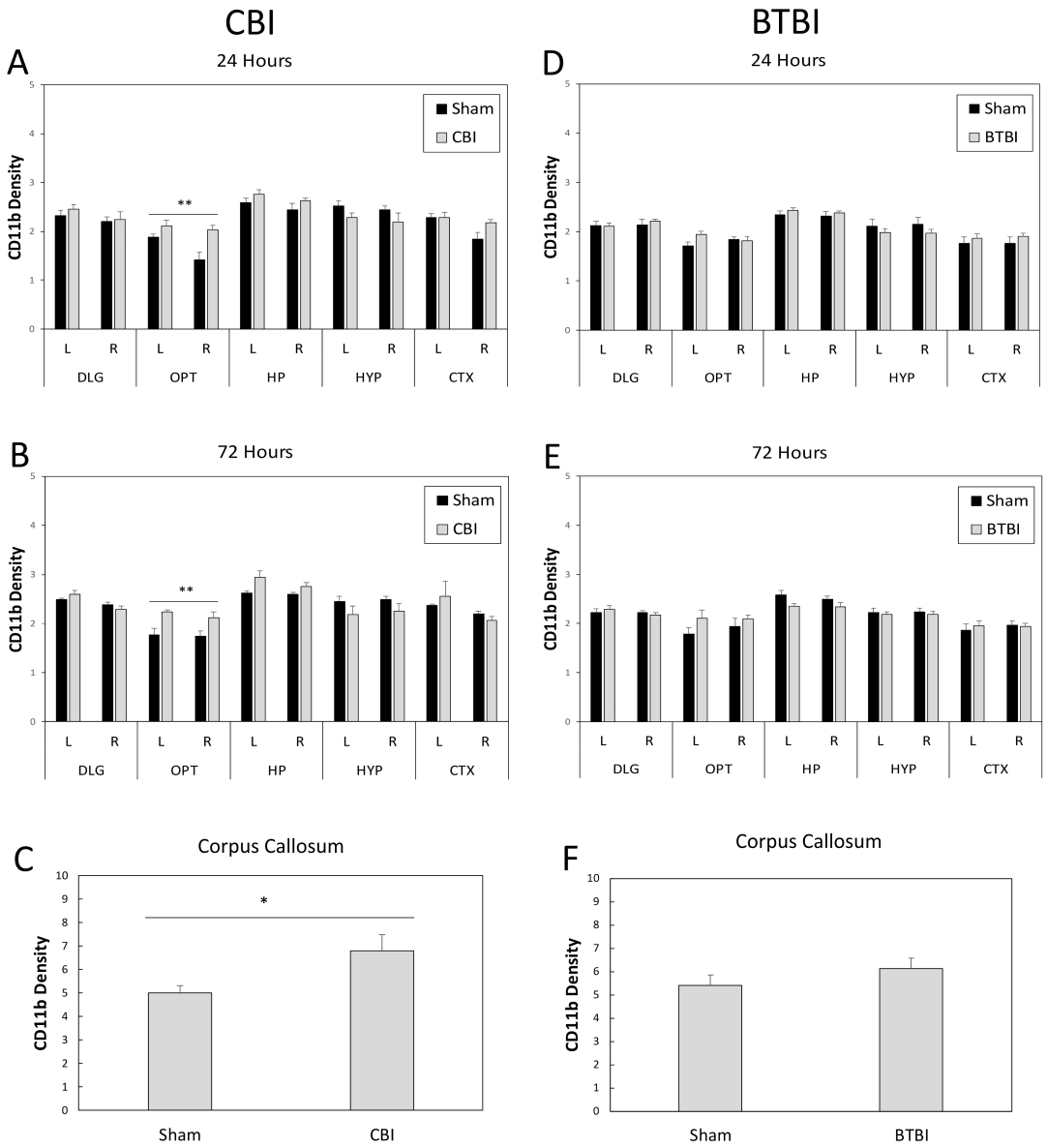

Figure 7. CD11b immunostaining density after CBI or BTBI. Increased microgliosis was seen in the right and left optic tract (OPT: $p = 0.0025$), but not in other brain regions analyzed 24 h after CBI (A). At 72 h post-injury, increased microgliosis was observed in both the OPT bilaterally (p = 0.0065), and corpus callosum (Panel C: $p = 0.0287$). Overall, there was also greater CD11b staining density on the injured (left) side than on the right side for mice sacrificed at 24 h ($p = 0.0001$) and mice sacrificed at 72 h ($p = 0.0043$) after CBI (data not shown). For BTBI mice, there was no difference in CD11b staining density in the brain regions of interest when compared to their sham controls (D, E, F). DLG-R/L = right/left dorsolateral geniculate nucleus; OPT R/L = right/left optic tract; HP-R/L = right/left hippocampus; Hyp-R/L = right/left hypothalamus CTX-R/L = right/left cortex.

Figure 8

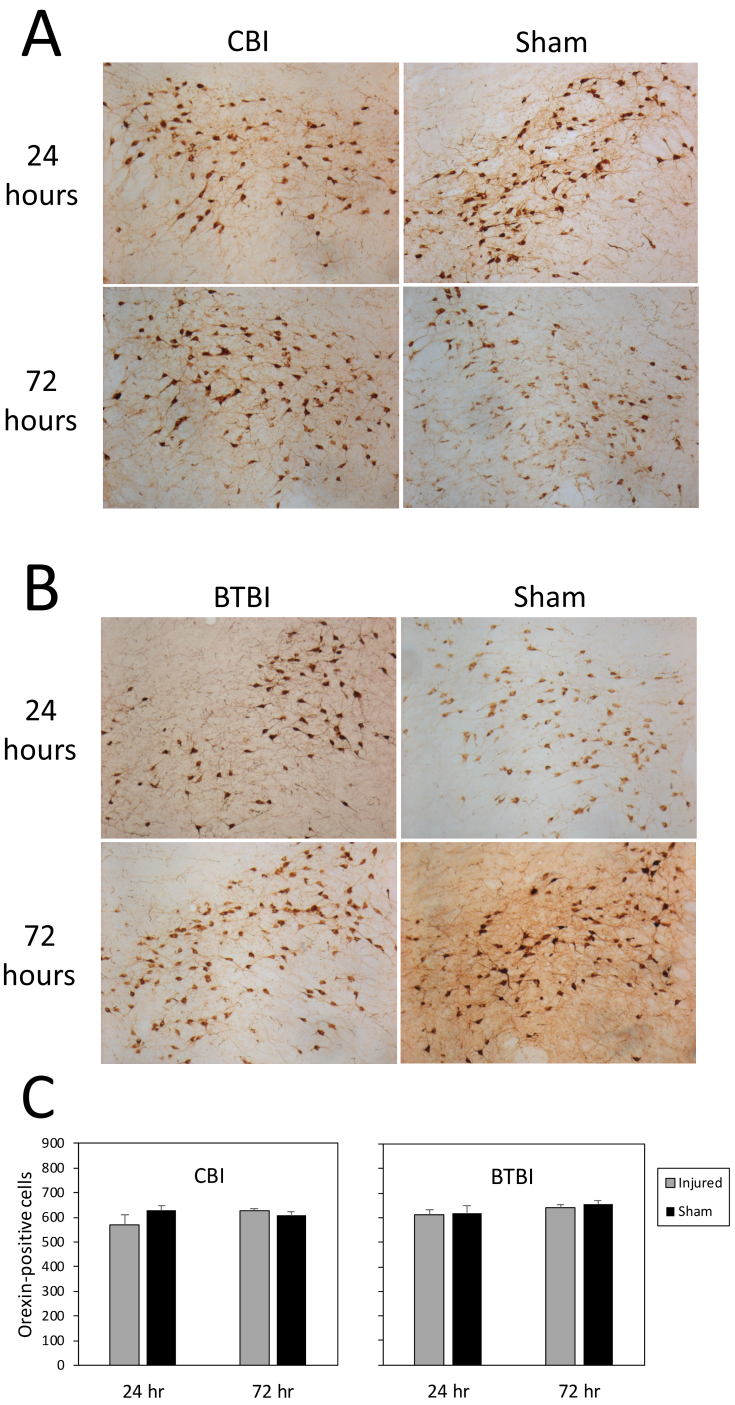

Figure 8. Immunohistochemical staining for the hypothalamic neuropeptide orexin following CBI (A) or BTBI (B) in standard group housed mice sacrificed at 24 or 72 h after injury. Sections shown represent the upper-median sample from each group. (C) Compared to sham group results, there was no effect of either injury type on the number of orexin-positive cells in the hypothalamus.

Figure 9

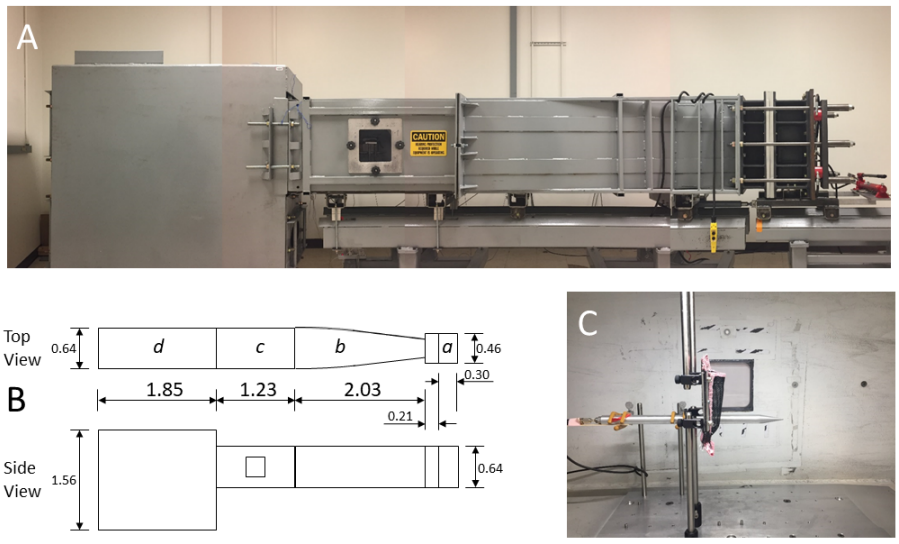

Figure 9. Advanced Blast Simulator Design. (A) Advanced Blast Simulator (ABS). (B) A high pressure shock wave is generated from the driver (*a* in Fig. B), located at the rightmost portion of the ABS, by the rupture of membranes as described in the Results and Methods. Schematic drawings of ABS, viewed from top and side (drawn near to scale). As indicated in the drawing, the ABS consists of high pressure driver (*a*), transition section (*b*), test section (*c*), and end wave eliminator/muffler (*d*). An animal is placed at the center of the test section. Before pressurizing the driver, the membranes are placed at the interface of the driver and transition section (*b*). The length of the transition section is about 2.24 m, and this section widens gradually to make the wave front of the blast wave planar at the center of the test section (seen in top view in B). The height and width of the ABS in the test section (*c*) is 0.64 m throughout and ~1.23 m in length. The distance from the membrane to the center of the test section is 2.86 m. The side access panel in the test section is 0.3 x 0.3 m. Pressure sensors are installed on the sidewall of the test section. (C) Magnified view of the holder located in the test section (not seen in A where view of access panel is obstructed by the hatch door). Behind the holder, the pencil gauge located adjacent to the holder can be seen (horizontal silver rod). The blast waveform is measured by using the pencil gauge and shows the typical characteristics of Friedlander curve, where the pressure recording was triggered by a sensor, located 406 mm upstream, inside of the blast tube.

CHAPTER 3:

Sex differences in behavioral responses following fear conditioning and repeated bilateral frontal region closed head impacts

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ABSTRACT

Military populations are at increased risk for sustaining repetitive concussive brain injury (rCBI), and the frontal lobes are among the most vulnerable sites. These insults can occur during deployments as well as during intensive training exercises. In both scenarios, service members often experience fear and stress. In the current study, female and male C57BL/6J mice received repeated, bilateral frontal concussive brain injury (frCBI) and fear conditioning (FC) to assess how injured mice respond to an adverse condition. All mice were evaluated in the open field (OF) and elevated zero maze (EZM), to assess anxiety-like behavior, and for depressive-like behavior with the tail suspension test. FC resulted in more freezing behavior in all mice that received foot-shocks when evaluated in subsequent context and cue tests, and FC altered mouse behavior in the OF and EZM. Despite their ability to learn during FC training, injured mice froze less than uninjured sham mice. Increased activity in the OF and EZM suggested this difference may be due to impulsive hyperactivity that overrides their natural freezing response to threat. Analysis of CD11b immunostaining density showed increased neuroinflammation in the cortical regions underlying injury sites and also in the optic tract, but not in the amygdala or hippocampus, which are known for their involvement in fear memory. There were notable sex differences, where female mice exhibited more freezing behavior than male mice during FC-related tests, and uninjured female mice had more anxiety-like behavior in the EZM, which was lost in injured female mice. The findings indicate there are some differences between frCBI and other models of rCBI where injury is directed to the parietal lobe, indicating the complexity of neuropsychiatric symptoms arising from injury to different brain regions.

INTRODUCTION

In the United States alone, over 1.7 million TBIs occur annually (62). Approximately, 70 to 90% of treated TBI cases are mild (29). Often milder forms of TBI are underdiagnosed due to the heterogenous nature of presenting symptoms and lack of unified diagnostic criteria (4; 17). Furthermore, those who sustain mTBI may avoid seeking immediate medical attention due to fear of disqualification from athletics or the uniformed services. Unfortunately, participation in contact sports and military missions increase the risk for sustaining multiple mild closed-head concussive brain injuries over ones' lifetime (73; 121; 178).

The most vulnerable brain regions to repetitive concussive impacts are the frontal and temporal lobes (104; 119; 189). Injury to the frontal lobes can lead to deficits that are disruptive to optimal social integration, impulse control, emotional processing, cognitive flexibility, working memory, organizational skills, and planning and prioritizing (40; 119). Furthermore, the prefrontal cortex (PFC) along with the amygdala play important roles in processing positive and negative emotion and governing how internal emotions guide a person's interaction with his or her environment (52). Deficits in emotional regulation have been particularly troublesome in military populations with overlapping symptoms of TBI and post-traumatic stress disorder (PTSD). The increasing suicide rate in certain military populations as well as their vulnerability to developing depression (92; 113; 114) has been suggested to be the consequence of frontal and basal forebrain damage (24). Clinical data demonstrates that service members who sustain multiple TBIs over their lifetime are at higher risk for suicide than those who sustain a single TBI (27).

Thus, repetitive concussive brain injuries pose a major and unique challenge for the military and veteran health systems. Even before deployment, service members may

experience multiple concussive events, resulting from motor vehicle accidents (MVAs), participation in contact sports, and training accidents (121; 178). During combat deployments, service members are exposed to conditions that increase their risk for sustaining TBIs as well as elicit chronic stress and fear that predispose them to developing PTSD, anxiety, and depression.

Some researchers postulate that each successive TBI lowers a person's cognitive reserve (185); further reducing an individual's resilience in the face of emotionally challenging events (27). Similar to the clinical literature (27; 73; 121), rodent studies have demonstrated that a single milder head injury may not cause obvious deficits, but may leave the brain vulnerable to further injuries (99; 101; 122; 130; 177). Previous studies have employed a wide range of injury models, including weight drop (51; 91; 122; 130), closed-head controlled cortical impact (chCCI) on the exposed skull (18; 101; 108; 177), chCCI on a helmet fitted to the mouse's head (149), and chCCI directly on the scalp (202; 209; 222). The number of impacts used in these models ranged from two impacts (one per day for two days) (177) to 42 impacts (six a day for seven days with two hours in between each impact) (149). When comparing a single mTBI to multiple mTBIs, mice receiving more than one mTBI exhibit poorer performance on cognitive tasks than those receiving a single injury (122; 177). Furthermore, mice that sustain repetitive TBI at a rate of one impact per day over five days perform worse than mice that experience wider inter-injury intervals of one week and one month (122).

The majority of closed-head repetitive mTBI (rmTBI) models to date have focused on the effects of repetitive mild concussive brain injury (CBI) administered unilaterally over the parietal lobe where damage to the underlying hippocampus results in

observable changes in learning and memory function (108; 177; 209). In contrast, most mouse models of TBI to the frontal cortices to date have utilized invasive procedures such as CCI on the exposed brain via craniotomy (36; 39; 102; 137; 182; 205), aspiration of brain matter (118; 176), lateral fluid percussion (140; 141), and cryoinjury (97).

Therefore, it is necessary to investigate frontal repetitive closed-head mild CBI (frCBI) to better understand the onset of symptoms associated with frontal lobe injury and how these symptoms affect one's ability to navigate adverse life events in order to develop more effective treatments that will enable patients to maintain good quality of life. In this study, male and female C57BL/6J mice were subjected to bilateral frCBI. After four consecutive injuries or sham procedures (once per day), all mice were assessed for anxiety-like behaviors in the open field (OF) and elevated zero maze (EZM), for depressive-like behavior with the tail suspension test (TST), and response to fear conditioning. Neuroinflammation was assessed with CD11b immunohistochemistry. We hypothesize that repetitive injuries to the frontal lobe will result in deficits in emotional processing that contributes to impaired fear learning based on previous studies (108; 202). We expect to find a marked increase in activated microglia as a marker of neuroinflammation bilaterally in the frontal brain regions, amygdala, and hippocampi.

METHODS

Animal and housing

Male and female C57BL/6J mice (Cat. No. 0664), 8 weeks old, from Jackson Laboratories (Bar Harbor, ME) were housed under standard-group conditions (three to five mice per cage) and allowed to acclimate to housing facilities for three to five days

prior to frCBI or sham procedures. The housing room was on a standard 12-h light-dark cycle. Food and water were available *ad libitum*. The facilities are humidity- and temperature-controlled, and accredited by the Association for the Advancement and Accreditation of Laboratory Animal Care. All procedures were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences (Bethesda, MD).

Eight experimental groups (n = 18/group) were included in the current study (Table 2). Mice of each sex were randomly assigned to receive four consecutive CBIs or exposed to four sham procedures. These mice were then subdivided to undergo fear conditioning (FC) during which foot-shocks were administered or to experience control conditions during which the mice were placed in the FC apparatus but foot-shocks were withheld. All mice were euthanized for tissue collection on Day 25 upon completion of all behavioral testing.

Frontal Repeat Concussive Brain Injuries (frCBI)

Male and female mice were randomly assigned to undergo bilateral frCBI or sham procedures for a total of two impacts per side over four consecutive days with an inter-injury interval of 24 hours. All mice were anesthetized with a gas mixture of isoflurane (Forane, Baxter Healthcare Corporation, Deerfield, IL) and 100% oxygen (induction: 3% isoflurane; maintenance: 1.5-2% isoflurane). Fur overlying the scalp was shaved with an electric razor and further removed by the application of Nair depilatory cream (Church & Dwight, Princeton, NJ). The Leica Impact One controlled cortical impact (CCI) device (Leica Microsystems, Buffalo Grove, IL) was used to strike the hairless scalp overlying

the frontal lobes to simulate non-invasive closed-head CBI. Specifically, each mouse was positioned in a stereotactic device with atraumatic ear bars, an incisor bar, and nose cone through which the anesthesia was delivered. The location of bregma was determined by tactile sensation and its location marked on the scalp with a permanent marker. The isoflurane was discontinued and a 3-mm diameter steel tip was used to strike the scalp at 5 m/s for 100 ms at the following approximate coordinates relative to bregma: AP 2.0 mm, ML 1.5 mm, and a depth of 1.5 mm (65). Any period of apnea following injury was recorded. Mice in sham groups underwent the same conditions and were maintained under anesthesia for the same duration as their injured counterparts each day for four days; however, no impacts were delivered. Immediately following each CBI or sham procedure, mice were placed in the supine position for assessment of the righting reflex. They were then returned to their home cages upon full pronation and given drinking water supplemented with acetaminophen (1 mg/ml; ~200 mg/kg b.w. for 24 h).

Open Field (OF)

On post-injury Days 4, 15, and 22 mice were tested in an open field (OF) environment for 20 min sessions as previously described by Tucker and colleagues (200). The OF apparatus (Stoelting, Wood Dale, IL) was 40 cm x 40 cm and had opaque walls that were 34 cm high. Illumination was maintained at 175 lux. Mice were placed in the center of the OF, and an overhead camera tracked movements of the mice. Any-Maze software provided measures, including the total distance traveled and the distance traveled in the center of the arena (20 cm x 20 cm) as defined by the Any-Maze software.

Delayed Fear Conditioning (FC)

FC was performed in a similar fashion as previously described (108; 202). On Day 7 after the final frCBI or sham procedure, mice were trained to associate neutral stimuli (the conditioned stimuli: the context and the cue/tone) with an aversive stimulus (the unconditioned stimulus: foot shock) over the span of six minutes. At the start of the training trial, mice were placed individually into the FC context in Plexiglas chambers that measured 17 cm x 17 cm with 4 lux lighting (Ugo Basile, Varise, Italy). The FC chambers had salient black and white checkerboard or striped backdrops that served as visual cues as well as an odor cue (mint, vanilla, or citrus). Mice were allowed to habituate to the context for two minutes prior to the onset of the 30 second tone (3 KHz, 80 dB), which co-terminated with a 2 second, 0.5 mA foot shock. After an interval of one minute, the tone and foot shock pairing were delivered again. Mouse activity was analyzed for an additional minute before termination of testing and mice were returned to their home cages. Similar to the groups that received foot shock, control groups were exposed to the tone and contextual cues in the FC chambers, but did not receive any foot shocks.

Following training, mice were tested for memory of the FC context on Day 8 after the final frCBI or sham procedure. Context-dependent memory was evaluated over a 5-minute test session during which mice were individually returned to the same FC chamber in which training occurred. The same visual and odor cues were used. Mice behavior was continuously monitored with cameras as described below. Then on Day 9 following the final frCBI or sham procedure, mice underwent memory testing for the FC cue (tone). For this 7-minute cue test, the context was significantly altered, including lighting level, visual, tactile, and olfactory cues. Mice were allowed to acclimate for three

minutes prior to delivery of the same tone from the training session for three minutes. Behavior was monitored for an additional minute before termination of the cue test. Upon completion of each FC context test or cue test, mice were returned to their home cages. Contextual and cued fear were tested again on Days 14 and 21 after the final frCBI or sham procedure, using the same protocol described above. For each subsequent cue test, the contextual stimuli were altered significantly so that the context for each cue test was different. Cameras in each chamber were used to record mouse movements during all training and testing sessions. Any-Maze software was employed to calculate freezing behavior based on the following parameters: minimum freeze duration– 250 ms; freezing on threshold– 30; freezing off threshold– 40 (no units).

Elevated Zero Maze (EZM)

On Day 24 following the final frCBI or sham procedure, all mice were tested for five minutes in the EZM (Stoelting) as previously described (199). Briefly, the EZM was an elevated circular platform 49 cm above the ground partitioned into four equal quadrants: two open quadrants with 1 cm high edges separated by two closed quadrants with 16 cm high walls that are dark and opaque. Additional lighting was provided by fluorescent lamps. Light levels were approximately 1600 lux in the open quadrants and 200 lux in the closed quadrants. Mice were individually placed into the EZM at a randomly chosen boundary between a closed quadrant and an open quadrant so that they were facing the closed quadrant, and allowed to freely explore the EZM. A ceiling camera was used to track the movement of each mouse. Any-Maze software calculated

the amount of time each mouse spent in the open versus closed quadrants as well as the total distance they traveled during each testing session.

Tail Suspension Test (TST)

On Day 25 after the final frCBI or sham procedure, mice underwent one final behavioral test: the TST for depressive-like behaviors. The TST was six minutes long and was performed as previously described (28; 201). Mice were suspended by their tails from laboratory shelves using laboratory tape that was approximately 25 cm long and 1.27 cm wide and placed approximately 1 cm from the tips of their tails. Cylindrical polycarbonate tubing (4 cm length, 1.3 cm inside diameter, and 1.6 cm outside; McMaster-Carr, Santa Fe Springs, CA; #8585K41) was used to cover each mouse's tail to prevent tail-climbing. Partitions between each mouse prevented it from seeing other mice that were being tested. Padding was placed on the surface below the shelves to prevent injuries in case of a fall. A standard video camera was used to record each test session. These videos were later imported into Any-Maze software for scoring by an observer, who was blinded to all experimental conditions of the animals, using a key press to calculate the total time each mouse spent completely immobile. Mice that spent more time immobile were interpreted as exhibiting a lack of motivation and therefore more depressive-like behaviors.

Histology and Immunohistochemistry

Upon completion of behavioral testing, mice were anesthetized and transcardially perfused with cold phosphate buffer solution, followed by 4% paraformaldehyde in

phosphate buffer. The brains were extracted and further fixed in paraformaldehyde for 24 h. The brains were then placed in 20% sucrose for 72 h before freezing. Coronal sections, 30 μm thick, were produced using a microtome.

Brain sections from six mice from each experimental group were selected for CD11b immunostaining. The sections were washed in Tris-buffered saline with 0.05% Triton (TBST) before incubation at room temperature (RT) for 30 min with 0.3% hydrogen peroxide, to block endogenous peroxidase. The sections were washed again in TBST before incubation in blocking buffer (rabbit serum, TBST with 0.20% Triton, and 10% bovine serum albumin (BSA)) for 1 h at RT. The sections were stained with CD11b (1:500; rat anti-CD11b, ThermoScientific, Cat. No. MA1-80091) overnight at 4°C. The following day, the sections were washed in TBST and incubated in a goat anti-rat secondary antibody [1:300; Biotin-SP (long spacer) AffiniPure goat anti-rat IgG (H+L), Jackson ImmunoResearch Laboratories, Inc. Cat. No. 112-065-003] in modified blocking buffer (TBST with 0.05% Triton, rabbit serum, and BSA) for 1 h at room temperature (RT). The sections were then washed with TBST before incubation in ABC solution from the Vectastain ABC HRP Kit (Vector Laboratories, Inc., Burlingame, CA; Cat. No. PK-4000) for 45 min at RT. A final 2 min wash with TBST preceded development with DAB from the DAB Peroxidase (HRP) Substrate Kit (with nickel), 3,3'-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA; Cat. No. SK-4100). The free-floating sections were mounted onto glass slides and cover-slipped for analysis.

Images of five brain regions were captured at 10x magnification, including the corpus callosum, dorsal hippocampi, the amygdala, cerebral cortices (motor cortex regions, M1 and M2, *per* Franklin & Paxinos) (65), and optic tracts. Densitometry was

performed using a Zeiss Axioskop microscope with an attached AxioCam MR.5 camera and ImageJ software as described by Collins and colleagues (45). The brain regions of interest were traced via freehand selection and the mean grey density was determined using the measurement feature. Average density values were calculated using 3-4 sections from each animal by subtracting the background from the mean grey density. The background of each image was calculated by measuring a selected area that lacked immunostaining. All measurements were taken by a microscopist blinded to the treatment conditions of mice from which histological samples were obtained.

For H&E staining, coronal samples between 2.96 mm rostral to bregma and 3.64 mm caudal to bregma based on the mouse brain atlas (65) were selected from six mice from each of the treatment groups for analysis of gross hemorrhage and cellular morphology. Sections were mounted onto glass slides and dried prior to staining. The sections were rinsed in distilled water for 1 min three times, and then submerged in 100% ethanol for 2 min. The sections were stained with hematoxylin (Sigma-Aldrich; Cat. No. GHS132-1L) for 3 min and immediately rinsed under running tap water for 5 min. They were then quickly submerged in acid ethanol (0.125 mL of 37% hydrochloric in 50 mL of 70% ethanol solution) 12 times. After, the sections were rinsed under running tap water for 3 min followed by a 2-min rinse in distilled water. The sections were stained with a 1:2 eosin solution (50% eosin solution, Sigma-Aldrich; Cat. No. HT110316 and 50% deionized water) for 30 seconds, and then placed in running tap water for 3 min. The sections were placed in a 95% ethanol solution for 2 min twice and then in 100% ethanol for 2 min twice before being moved to xylene for 2 min twice. Glass cover slips were

applied for microscopy, and the samples were assessed for the presence of bleeding or cellular injury at 2.5x magnification and 20x magnification

Statistical Analysis

Statistical analyses were performed with SPSS (version 20; IBM SPSS Statistics, Armonk, NY) and SigmaPlot 12.3 (Systat, Software, Inc., San Jose, CA). A mixed model analysis of variance (ANOVA) was performed to assess Sex x Day alterations in apnea and righting reflex. Body weight data during the TBI procedures was assessed using a mixed model ANOVA for Injury x Sex x Day, and the body weight data on post-injury Day 25 at the completion of the study was evaluated by a three-factor ANOVA (Injury x Sex x FC Treatment). Mauchly's test indicated there was a significant degree of sphericity ($W = 0.586$, $p = 0.000$). Therefore, Greenhouse-Geisser corrections for the degrees of freedom were used.

OF data for total and center distance traveled were analyzed with four-way ANOVAs with injury, sex, and shock during FC training as fixed factors and day as a repeated measure. EZM and TST data were analyzed separately using three-way ANOVAs treating injury, sex, and shock during FC training as fixed factors.

Data from FC training (Day 7), context testing (Days 8, 14, and 21), cue testing (Days 9, 14, and 21) were analyzed separately. Data from FC training were transformed to square root values to minimize violations of homogeneity of variance, and then analyzed using a four-way ANOVA with injury, sex, and shock during FC training as fixed factors and time (30s segments) as a repeated measure. Follow-up three-way ANOVAs with sex, time (30s segments), and shock as fixed factors were performed

separately for injured mice and uninjured mice. Data from context testing were also transformed to square root values, and a four-way ANOVA was performed with injury, sex, and shock as fixed factors and day as a repeated measure. This was followed by separate three-way ANOVAs with injury, sex, and shock as fixed factors for each testing day. For cue testing data, baseline data from first three minutes of test sessions was analyzed separately from data collected during tone presentation, which took place during minutes four through six of testing. Four-way ANOVAs were used to analyze both the baseline and tone data with injury, sex, and shock as fixed factors and day as a repeated measure. In addition, follow-up one-way ANOVAs were performed for each day on data collected during tone presentation with shock as a fixed factor.

For CD11b staining density data, three-way ANOVAs with injury, sex, and shock treatment as fixed factors were performed separately for each brain region of interest (AMY, HP, OT, VPM, M1, and M2) using SigmaPlot. Because data obtained from the OT failed the equal variance test, a second ANOVA was performed after reciprocal data transformation was done to meet the assumption for normality.

Main effects and effects of interaction were considered significant when the *p*-value was less than 0.05. Data summarized in figures generally represent the mean +/- standard error of the mean unless otherwise specified.

RESULTS

Apnea and Righting Reflex

Following frCBI, the duration of apnea was evaluated in injured mice only (sham-treated mice exhibited no apnea during anesthesia). Analysis of apnea duration in male and

female mice after frCBI (Fig. 10A) indicated there was a significant decrease in apnea duration across days (Greenhouse-Geiser $F_{1.881,99.701} = 16.974$, $p = 0.001$), but no differences between the sexes ($F_{1,53} = 0.503$, $p = 0.481$). Pairwise comparisons indicated the apnea duration was significantly longer on Day 1 than what was observed on Days 2-4, and that the duration of apnea on Day 2 was also longer than the duration on Day 3 (all results at least $p = 0.027$). Righting reflex measures indicated differences between the groups from injury ($F_{1.0,140.0} = 315.250$, $p = 0.000$), and a Days x Injury difference ($F_{1.990,278.535} = 54.083$, $p = 0.000$), but no sex difference ($F_{1, 140} = 0.728$, $p = 0.395$). Pairwise comparisons indicated the mean duration of the righting reflex for the sham animals did not differ over days, but the duration of the righting reflex in injured mice was shorter after later injuries (Day 1 > Day 2> Days 3 and 4) (Fig. 10B). Body weight was also assessed after each injury or sham treatment. A mixed model analysis of variance for the factors, Injury x Sex x Day was used to evaluate changes in body weight. There was no overall significant difference in body weight due to Injury ($F_{1, 140} = 1.783$, $p = 0.184$), and no Injury x Sex interaction effect $F_{1, 340} = 0.162$, $p = 0.688$), but there was a significant effect related to Sex ($F_{1, 140} = 607.339$, $p = 0.000$). There were significant within-subjects effects with differences for Day ($F_{2.195, 307.297} = 55.788$, $p = 0.000$), Day x Injury ($F_{2.195, 307.297} = 21.284$, $p = 0.000$), and Day x Sex ($F_{2.195, 307.297} = 10.488$, $p = 0.000$), while the Day x Injury x Sex factor was not significant ($F_{2.195, 307.297} = 1.417$, $p = 0.243$). Post hoc tests indicated that compared to mean body weights on Days 1 and 2, there were significant reductions in body weight in the frCBI animals on Days 3 and 4 for the males over days, but not for females, and that there were significant reductions in body weight from Day 1 in the frCBI-treated animals, but not in the sham groups.

Compared to body weight on Day 1, there were significant reductions in body weight in the males over Days 2-4, but not for females. On the day of euthanasia (PID 25), there was a significant difference in the body weights of the male and female mice ($F_{1,136}$ = 189.41, $p < 0.001$), but no differences as a result of frCBI or FC (data not shown).

Behavioral Studies

Open Field (OF)

Mice were tested in the OF on Days 4, 15, and 22 following the final frCBI or sham procedure. A four-way ANOVA (Injury x Sex x Shock x Day) revealed a main effect of injury on the total distance traveled ($F_{1,136} = 9.53$, $p = 0.0024$); mice that experienced frCBI traveled more distance in the OF overall than uninjured sham mice (Fig. 11A). There was also a main effect of day ($F_{2,272} = 230.54$, $p < 0.0001$); both injured mice and sham mice showed a significant decrease in overall distance travelled on Days 15 and 22 compared to Day 4. When comparing female mice to male mice, there was a Sex by Day interaction effect ($F_{2,272} = 10.20$, $p < 0.0001$). On Day 4 post-injury (Fig. 11B), female mice in general traveled more distance in the OF than male mice ($p = 0.0069$). When comparing mice that underwent FC to their non-shocked controls, there was a day by shock interaction effect ($F_{2,272} = 5.12$, $p = 0.0066$). On Day 22 following the final frCBI or sham procedure (Fig. 11C), mice that experienced foot shocks during FC training traveled less distance overall in the OF than mice that did not receive foot shocks ($p = 0.0252$). There were no effects of injury, day, shock, or sex when mice were assessed for distance traveled in the center of the OF.

Fear Conditioning Training

Fig. 12 summarizes the freezing time of injured and sham-treated mice during FC training on Day 7 following the final frCBI or sham treatment as a function of shock experience. A four-way Injury x Sex x Shock x Time (minute) ANOVA was performed and there was a three-way interaction effect for Injury x Time x Shock ($F_{9,1224} = 1.94$, $p = 0.0425$). For interpretation, separate three-way Sex x Shock x Time ANOVAs were performed for the injured and sham-treated groups. For the frCBI mice (Fig. 12A), there was a significant interaction effect for the Time x Shock factors ($F_{9,612} = 26.37$, $p < 0.0001$). An analysis of each epoch during fear conditioning indicated significant differences between the injured shocked and injured non-shocked groups regardless of sex starting at the presentation of the second tone and throughout the remainder of the training session (epochs 180-210 s, 210-240 s, 240-270 s, and 270-300 s; $p < 0.001$). frCBI mice exposed to shock froze more than frCBI mice in non-shocked groups. For mice that underwent sham procedures for frCBI (Fig. 12B), there was a similar significant interaction effect for Time x Shock ($F_{9,612} = 15.61$, $p < 0.0001$), where the groups differed in terms of duration of freezing for 30s after the termination of the first tone with non-shock mice freezing more than mice that were shocked (150-180 s , $p = 0.0220$). In contrast, shocked mice froze more than non-shocked mice during the second tone and during its pairing with foot-shock, as well during the last 30s of the training session (epochs 180-210 s, $p = 0.005$; 210-240 s, $p < 0.0001$; and 270-300 s, $p < 0.001$).

Fear Conditioning Context Tests

Fig. 13 shows the percentage of time that mice displayed freezing behavior during testing for memory of the FC context on Days 8, 14, and 21 after the final frCBI or sham procedure (Days 1, 7, and 14 after FC training). A four-way ANOVA (Injury x Sex x Shock x Day) indicated a significant main effect for Injury ($F_{1,136} = 8.09$, $p = 0.0051$), and significant interaction effects for Day x Shock ($F_{1,172} = 46.75$, $p < 0.0001$) and Day x Sex ($F_{1,136} = 4.29$, $p = 0.0147$). Three-way ANOVAs were performed separately for each day. On Days 8 (Fig. 13A) and 21 (Fig. 13C), there was a main effect of Injury ($F_{1,136} = 4.35$, $p = 0.0389$ and $F_{1,136} = 13.08$, $p < 0.0004$, respectively) where the injured mice group exhibited less freezing behavior than the sham injured group, but on Day 14 (Fig. 13B), the groups were not different ($F_{1,136} = 1.84$, $p = 0.2301$). Shock also had a significant effect upon behavior, where mice that received foot-shock during FC training froze for longer durations during context testing compared to non-shocked mice regardless of whether they received frCBI (Day 8: $F_{1,136} = 174.31$, $p < 0.0001$; Day 14: $F_{1,136} = 30.86$, $p < 0.0001$; Day 21: $F_{1,136} = 28.02$, $p < 0.0001$). In addition, on Days 14 and 21, male mice exhibited less overall freezing compared to female mice ($F_{1,136} = 9.99$, $p = 0.0019$ and $F_{1,136} = 4.63$, $p = 0.0333$); however, they did not differ from female mice on Day 8.

Fear Conditioning Cue Tests

Fig. 14 summarizes freezing response profiles of mice during the cue test, which was carried out on Days 9, 14, and 21 following the final frCBI or sham treatments (2, 7, and 14 days after FC training). Analyses were performed separately for freezing

behavior during the baseline period (Fig. 15A) and the presentation of the tone (Fig. 15B).

For the baseline portion (first three minutes) of the FC cue tests, a four-way ANOVA (Injury x Sex x Shock x Day) revealed a main effect of Injury, ($F_{1,135} = 8.71$, $p = 0.0039$) where frCBI groups exhibited less freezing than non-injured sham groups (Fig. 15A) when the data was collapsed across days, sex, and shock treatment. There was a significant interaction effect for Sex x Day ($F_{2,290} = 3.32$, $p = 0.0375$), however, males and females did not differ on any given test day. There was also a Day x Shock interaction effect ($F_{2,290} = 6.66$, $p = 0.0015$); mice that experienced foot-shocks during training froze significantly more than non-shocked mice on Days 9 ($p < 0.0001$) and 14 ($p = 0.0042$). They did not, however, differ from non-shocked controls on Day 21 ($p = 1.0$).

A four-way ANOVA (Injury x Sex x Shock x Day) was also performed for the tone portion of the cue test (minutes four through six of the test), revealing a main effect of Injury ($F_{1,135} = 4.46$, $p = 0.0365$; Fig. 15B). Mice that received frCBI froze significantly less than non-injured control mice. A main effect of sex was also seen ($F_{1,135} = 8.23$, $p = 0.0048$) where female mice froze for longer durations than males. Furthermore, there was a Day x Shock Interaction ($F_{2,268} = 30.48$, $p < 0.0001$); mice that were exposed to shock treatment during FC training froze more than mice that underwent the sham FC protocol ($p < 0.0001$). Follow-up one-way ANOVAs for each day revealed that mice that experienced foot-shocks during FC training froze significantly more than non-shock mice during all three cue trials ($p < 0.0001$).

Elevated Zero Maze (EZM)

Fig. 16A summarizes the amount of time each group spent in the open quadrants of the EZM on Day 24 after the final frCBI or sham procedure (Day 17 after FC training). A three-way ANOVA with Injury, Sex, and Shock as fixed factors showed an Injury x Sex x Shock interaction ($F_{1,136} = 5.15$, $p = 0.0248$). Follow-up two-way ANOVAs performed separately for male mice and female mice showed no effects of injury, shock, or Injury x Shock interaction for male mice. In contrast, there was an Injury x Shock interaction ($F_{1,68} = 6.03$, $p = 0.0167$) for female mice. When comparing uninjured female mice that received foot-shocks to their FC controls, shocked mice spent significantly less time in the open quadrant than non-shocked mice ($p = 0.0092$). For total distance traveled (Fig. 16B), a three-way ANOVA with Injury, Sex, and Shock as fixed factors revealed a main effect of injury ($F_{1,136} = 8.758$, $p = 0.004$) and a main effect of shock ($F_{1,136} = 8.610$, $p = 0.004$). Injured mice traveled more distance in the EZM than uninjured sham mice, and shocked mice traveled less distance overall than mice that did not receive foot-shocks during FC training.

Tail Suspension Test (TST)

TST was performed on Day 25 following the final frCBI or sham procedure (18 days after FC training). A three-way ANOVA (Injury x Sex x Shock) revealed no significant differences among groups (data not shown).

Histological Findings

Hematoxylin & Eosin

Fig. 17 shows hematoxylin and eosin stained brain sections from male and female mice from both frCBI and uninjured sham groups. frCBI resulted in cortical bleeding bilaterally at the site of impact in 1 out of 12 male mice and unilaterally at the site of impact in 4 out of 12 for a total of 5 out of 12 male mice. Brain sections from uninjured sham male mice did not display any bleeding. On closer inspection, one sample from the frCBI male group showed changes in cellular morphology despite the absence of cortical bleeding. As for brain sections from frCBI female mice, 4 out 12 samples showed unilateral cortical bleeding at the site of impact and 4 out of 12 samples showed bilateral cortical bleeding at the site of impact for a total of 8 out of 12 female mice. There were no differences in proportion of instances of bleeding due to sex. Brain sections from uninjured sham female mice did not have any signs of cortical bleeding.

CD11b

Three-way ANOVAs (Sex x Injury x Shock) on CD11b staining density were performed separately for each of the following brain regions: primary motor cortex (M1), secondary motor cortex (M2), amygdala (AMY), hippocampus (HP), optic tract (OT), and ventral posteromedial nucleus of the thalamus (VPM). For M1 (Fig. 18), there was a main effect of injury ($F_{1,40} = 6.56$ $p = 0.014$); there was significantly more CD11b staining density in brain sections from both male and female frCBI mice compared to uninjured sham mice. No effect of sex or shock was detected. Similarly, for M2, there was a main effect of injury ($F_{1,40} = 11.216$, $p = 0.002$); brain sections from frCBI mice regardless of sex showed more CD11b staining density than uninjured sham mice. There was no effect of sex or shock on CD11b staining density for M2. CD11b density was also

greater after frCBI in the OT ($F_{1,40} = 100.677$ $p < 0.001$). For the remaining brain regions of interest (AMY, HP, and VPM), there were no significant differences among groups in CD11b staining density.

DISCUSSION

This study assessed the effects of fear conditioning on female and male mice that previously sustained bilateral concussive injuries to the frontal lobes (two CBIs per side with an inter-injury interval of 24 h). As expected, injured animals exhibited apnea, which was not seen in uninjured mice. The duration of apnea after the initial injury on Day 1 was significantly longer than after all subsequent injuries (Fig. 10A). A trend in a shortening of apnea duration after frCBI was also seen between Days 2 and 3. Apneic events following TBI have been previously reported (72; 192) and at least one report found an overall decrease in apnea duration after repeated closed head impact injuries (22). Sex effects on apnea following repeated closed head impacts have been previously reported by Tucker and colleagues: female mice had longer durations of apnea than male mice on the second day of injury (202). As expected, injured mice exhibited delayed righting reflexes when compared to uninjured mice, but no sex differences were seen. The righting reflex time in injured animals significantly decreased with repeated injuries, which has been documented in other studies of rmTBI in mice (22; 130; 202; 222). Only one study showed an increase in the righting reflex time between the first injury and second injury (51). The mechanism underlying the shorter recovery of the righting reflex after repeated injury remains poorly understood. This finding is unexpected given that mice sustaining repeated injury demonstrate poorer neurocognitive outcomes than mice

that sustain a single injury (99; 122; 177). One author postulated that the decrease in righting reflex time with successive injuries may be an adaptive mechanism (22). This physiological response and adaption to repeated insults may promote survival.

Examination of brain tissue indicated there was cortical bleeding at the sites of injury in 8 out of 12 female mice and 5 out of 12 male mice that underwent frCBI, but this may reflect minor variation. The female mice were overall smaller, but reportedly there are no substantial differences in the frontal bone width when comparing male and female C57bl/6J mice (93). The differences in injured female mice does not appear to alter their physiological response to injury when compared to injured male mice. Injured female mice did not differ from injured male mice in their duration of apnea in this study. Furthermore, there were no significant differences seen between female and male mice when assessing righting reflex time.

The mice were assessed for anxiety-like behaviors (OF and EZM) and depressive-like behavior (TST) following frCBI and fear conditioning. Mice that experienced foot-shocks froze significantly more than non-shocked mice during FC training (Day 7) and during testing for memory of the FC context (Days 8, 14, and 21) and auditory cue (Days 9, 14, and 21). The increased freezing time was seen in all mice that received foot-shocks, regardless of injury status on testing days subsequent to FC, indicating the effectiveness of FC in inducing sustained changes in behavior and that injury appears to have not altered mouse responses to this external stressor.

During FC training, no difference in freezing behavior was observed between injured mice and uninjured sham controls, indicating that the frCBI model did not blunt the capacity of mice to learn the association between tone and foot shock. In contrast,

mice in the frCBI group froze less than uninjured sham control mice during contextual fear testing one day after FC training and two weeks after FC training, but not one week after FC training. This difference in freezing behavior is consistent with findings from other studies examining FC after rCBI and suggests frCBI, similar to unilateral rCBI over the parietal lobe, leads to a decreased response to threat context (108; 202). Analogous to the FC context test, frCBI mice froze significantly less than uninjured sham mice during both the baseline and tone portions of FC cue testing. However, this finding has been more variable in the literature. One study found that decreased freezing seen during cued fear memory testing was sex-dependent and that only injured male mice froze less in response to the FC cue when compared to uninjured mice (202). Other studies found no differences between injured and uninjured sham mice during cued tone testing (99; 108). One possible explanation may be that the association between the tone and foot-shocks during cued fear learning is more easily made and more resilient to extinction than the association made during the pairing of the context with foot-shocks (150).

More importantly, whether the decrease in freezing behavior seen in this current study is due to deficits in fear memory, increased impulsivity, hyperactivity, or a combination of all three is unknown. Previous studies have implicated deficits in memory as the cause of decreased freezing response to subsequent exposure to the FC context (108; 202). The anatomical basis of contextual fear learning and cued fear learning during FC has been well-studied in rodent models and it has been shown that both the hippocampus (HP) and amygdala (AMY) are involved in contextual fear learning; whereas, only an intact amygdala is required for cued fear learning (150). In a study assessing FC after rCBI in male mice, the authors noted changes in the

electrophysiological properties of the hippocampus in addition to decreased freezing behavior in injured mice as compared to uninjured sham mice when tested in the FC context. Although their findings correlated changes in response to the FC context with alterations in hippocampal function, no other behavioral tests were employed to further explore whether deficits in memory alone were behind the observed differences between experimental groups (108). In another study utilizing similar injury parameters to Logue et. al (108) the authors found that in addition to the decreased freezing behavior, rCBI mice also showed increased activity in the OF (202). Similar to mice that received rCBI over the parietal lobe, mice that received frCBI traveled more distance in the OF across all testing days (Days 4, 15, and 22) than uninjured sham mice (Fig. 11A). Despite this hyperactivity, injured mice still displayed an ability to learn and habituate to their surroundings as evident by significant decreases in distance traveled on Days 15 and 22, mirroring the behavior of uninjured mice. Therefore, it is possible that frCBI may result in hyperactivity that interferes with the freezing response to learned fear.

Hyperactivity may also explain the results in the EZM. Injured mice traveled an overall greater distance in the EZM than uninjured sham mice (Fig. 16B). Furthermore, when comparing uninjured female sham mice that were shocked during FC training to non-shocked uninjured female mice, the shocked mice spent less time in the open quadrants than their non-shocked counterparts. This behavior has been interpreted as increased anxiety and/or caution to potential threat, and an expected response after FC. This expected change in behavior was not seen in the frCBI female mice group that experienced foot-shocks. Rather than spending less time in the open quadrants, they displayed similar exploratory behavior when compared to injured female mice that did

not experience foot-shocks. The lack of change in the behavior of injured female mice who underwent FC may be the result of hyperactivity that perhaps leads to motor impulsivity overriding the normal response to a threatening environment. In the OF, another test for anxiety-like behavior, however, there were no significant differences in center distance traveled among injured mice and uninjured sham mice regardless of sex and experience during FC.

Similar findings were demonstrated in male mice—after sustaining five consecutive TBIs with an inter-injury interval of 24 hours that were sustained in a CHIMERA model, where injured mice spent significantly more time in the open arms of the elevated plus maze (EPM) than sham controls (134).The same authors further noted that both injured and sham mice traveled similar distances overall in the EPM, and therefore, were more likely exhibiting disinhibition and risk-taking behavior and not just simply motor hyperactivity (134). Another study modeling frontal CCI in male rats have demonstrated that brain-injured rats show more impulsivity that non-injured controls (212).

Evidence of inflammation in the cortex was observed in the brains of mice that underwent frCBI. Compared to uninjured mice, there were significant increases in CD11b staining density in both the M1 and M2 regions of the cortex in injured mice. After unilateral rCBI over the parietal cortex, injured mice showed increased astrogliosis in perilesional cortex when compared to uninjured sham control mice (202). Contrary to our hypothesis, however, there were no significant differences in CD11b staining density in either the HP or the AMY when injured mice were compared to their uninjured controls. It may be that the lack of changes in these brain regions is related to the distal

location of the AMY and HP relative to the impact site (2.0 mm anterior to the bregma suture). In contrast, with a parietal region impact there was increased astrogliosis in the HP on the injured side when compared to the non-injured side in rCBI mice; impact site 2.5 mm posterior to the bregma suture directly over the dorsal HP (although there were no differences when comparing the HP of injured mice to uninjured sham mice) (202). An increase in the level of the cytokine interleukin-12 (IL-12) was seen in the cortical samples obtained from rats that sustained bilateral frontal CCI (212). Furthermore, the authors of this study saw a correlation between impulsivity and IL-12 levels, suggesting that alterations in rodent behavior following frontal CCI may be mediated by neuroinflammation (212).

Whereas previous studies commonly utilize only male mice for their studies, we assessed the effects of bilateral frontal mTBI in female and male mice. It was hypothesized that sex differences would manifest in response to injury and exposure to post-injury stress based on clinical findings of higher rates of certain affective disorders (96), anxiety disorders (96; 106), and PTSD (106) in women. In line with the clinical literature, we saw some notable sex differences. Female mice froze significantly more than male mice in the FC context one week and two weeks after FC training as well as during the tone portion of cue testing two days, one week, and two weeks after FC training. The findings from the FC context and cue tests, then, found that female mice overall have a more robust response to threat than male mice. Likewise, there was a difference in the EZM where uninjured female mice spent less time in the open quadrants of the EZM after they experienced foot-shocks; this Injury x Shock interaction was not observed in male mice.

Interestingly, the frontal injuries did not alter measures of anxiety in female mice in the EZM. A previous study with injury directed over the parietal regions found female mice exhibited less overall anxiety than male mice in the EZM, where female mice that received the parietal rCBI spent more time in the open quadrants when compared to both uninjured female mice and male mice on Day 22 after the final CBI (202). When assessing mice that underwent parietal rCBI in the EZM one year after the last injury, however, the authors found that injured male mice spent more time in the open quadrants than uninjured male mice, and that injured female mice did not differ from uninjured female mice (201). The differences in the present findings in the EZM could be a manifestation of the difference in injury location. In the OF and TST, our frCBI mice behaved similarly to other studies performed in our laboratory utilizing single and repetitive TBI models (199; 200; 202). Female mice traveled overall more distance than male mice. There were no differences among mice groups when assessed for depressive-like behavior in the TST, which was unexpected.

In summary, bilateral frCBI resulted in behavioral differences not seen in unilateral rCBI studies where impacts are directed over the parietal cortex. In particular, the data from the EZM revealed differences between female and male mice, suggesting uninjured female mice display a normal response to anxiogenic conditions after experiencing an adverse event, and that this behavior changes after brain injury. Behavior in the OF supports that frCBI results in motor hyperactivity in both injured female and injured male mice, providing an explanation for decreased freezing behavior during testing in the FC context and with the FC cue as well as increased activity in the EZM.

More importantly, injured mice in this study demonstrated an ability to learn and respond to threat, suggesting frCBI results in impulsive motor hyperactivity that overrides fear instinct and that these changes in behavior may be mediated by neuroinflammation. Thus, when modeling mTBI, it may be important to take into consideration the location of injury when evaluating for specific behavioral outcomes. Furthermore, although not addressed in this study, a better understanding of how social context affects behavioral testing is needed when assessing anxiety- and depressive-like behaviors. One study, for example, has noted differences in exploratory behavior in female mice tested in an anxiogenic environment when the rodents were housed individually versus when housed in groups (144). A supportive social network is important in recovery from psychiatric illness in humans. Consideration of how social context influences complex neuropsychiatric processes such as anxiety, fear, and depression is essential in modeling TBI in an effort to develop better treatments.

Table 2. Final number of mice in each group

Sex	Male		Female	
Injury	Sham	frCBI	Sham	frCBI
Fear Conditioning	18	18	18	18
No Fear Conditioning	18	18	18	18

Figure 10

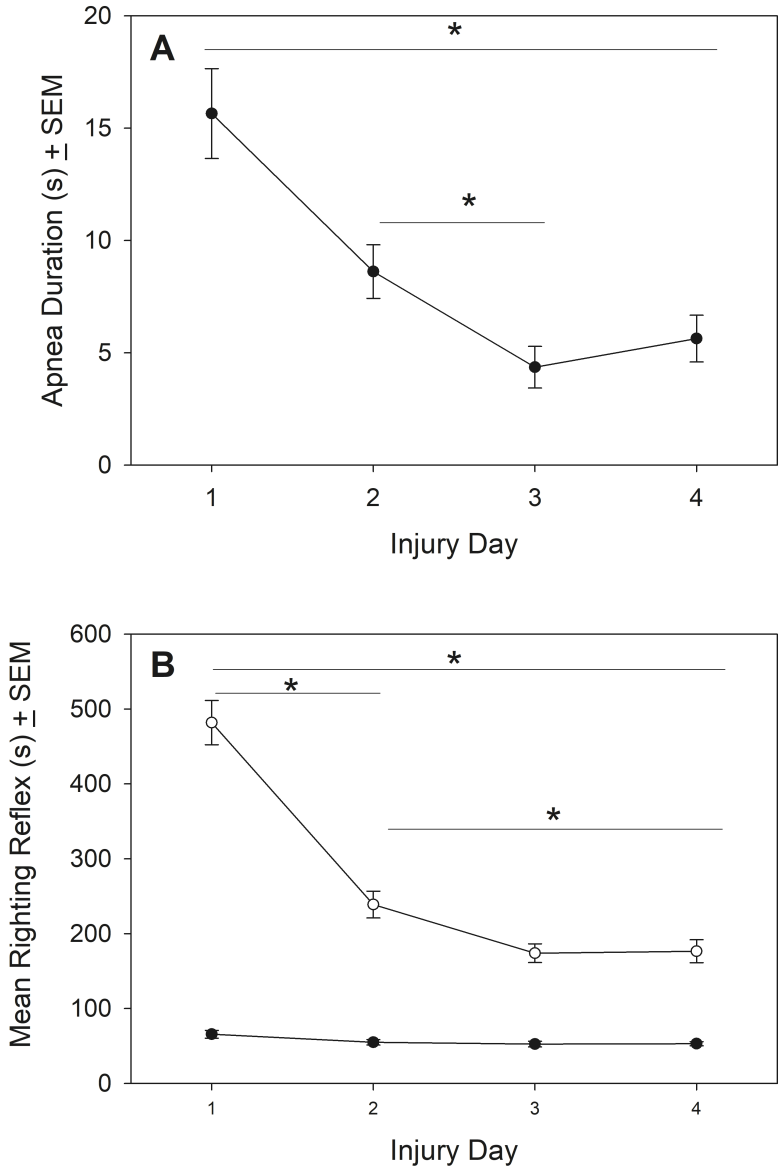

Figure 10. Duration of apnea in injured mice (A) and time to recovery of the righting reflex (B) following each frontal concussive brain injury (frCBI), shown as means with standard errors of the means. The mean duration of apnea and the righting reflex time significantly decreased after the first injury and then again after the second injury. The asterisk (*) represents the Injury x Day interaction for apnea (Day 1 > Days 2 > Days 3 and 4) and righting reflex (Day 1 > Day 2 > Days 3 and 4). Sham-treated mice did not exhibit apnea, and did not exhibit differences in righting reflex as a function of Day (dark circles in B).

Figure 11

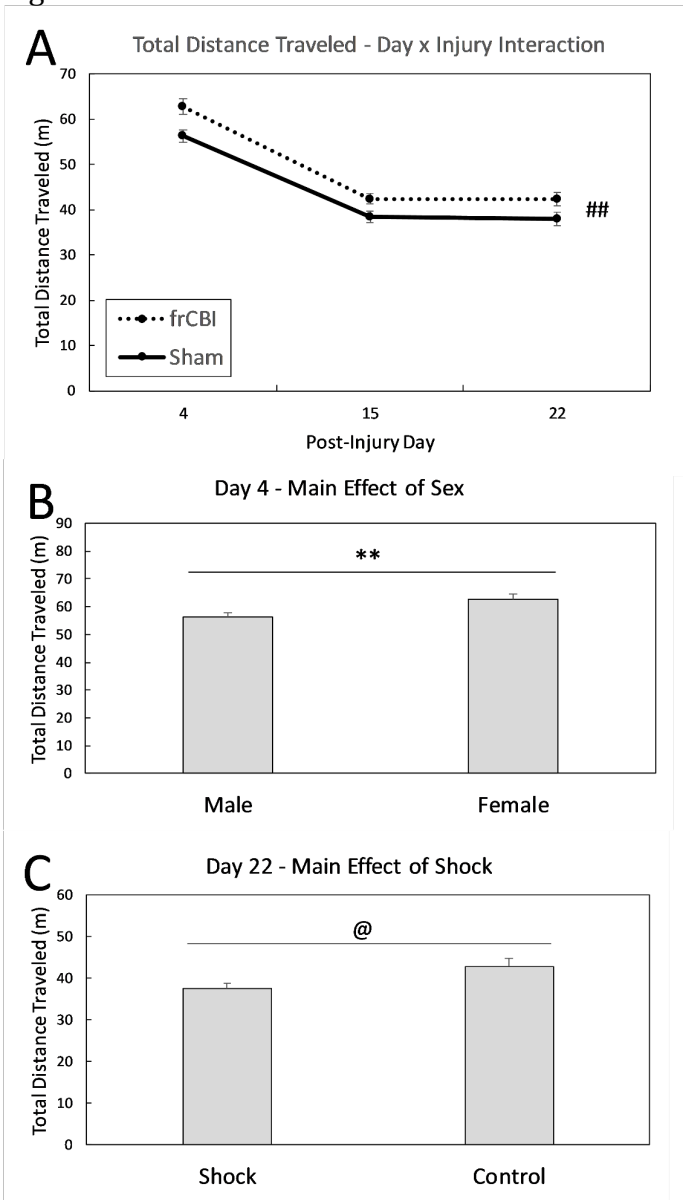

Figure 11. Activity in the open field (OF) following frCBI and fear conditioning. Total distance traveled in the open field on Days 4, 15, and 22 after the final frCBI or sham procedure collapsed across sex (A). There was a main effect of Injury on the total distance traveled (#, $p = 0.0024$) with injured mice traveling more distance overall than sham mice regardless of sex. There was also a main effect of Day ($p < 0.0001$) with all mice travelling less overall distance in the OF on Days 15 and 22 when compared to Day 4. There was a Sex x Day interaction effect (B; $p < 0.0001$). On day 4, female mice travelled significantly more distance than male mice regardless of experimental group (**, $p = < 0.0069$). Furthermore, there was a Day x Shock interaction effect (C; 0.0066). On day 22, mice that experienced foot shocks during fear condition training on day 7 traveled less distance overall in the OF than mice that did not receive foot

shocks (@; p = 0.0252). The pound (#) sign represents a main effect of Injury. The double asterisks (**) represents a Sex x Day interaction effect on Day 4. The at (@) sign represents a Day x Shock interaction effect on Day 22.

Figure 12

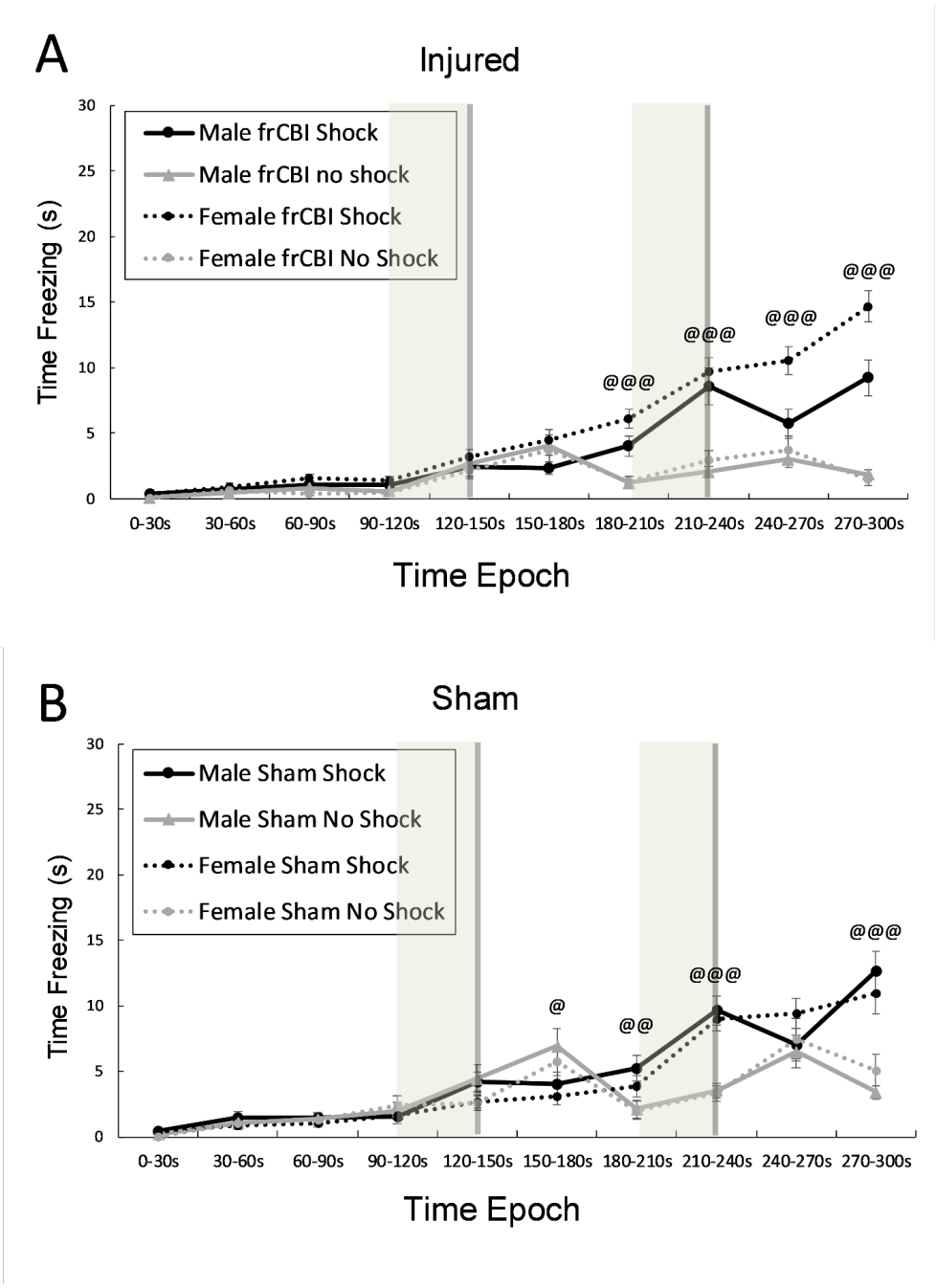

Figure 12. Freezing behavior during fear conditioning training on Day 7 after the final injury or sham procedure when a neutral stimulus (30-second, 3 KHz, 80dB tone) was paired with an aversive stimulus (two-second, 0.5 mA foot-shocks). The light gray regions represent the presence of the tone whereas the dark gray lines represent foot-shock. For injured mice (A), foot-shock during FC training resulted in significantly more freezing than non-shock injured mice during the

presentation of the second tone, the second shock, and for the last minute of the training session (@@@, epochs 180-210s, 210-240s, 240-270s, and 270-300s; $p < 0.001$). For uninjured mice (B), more freezing was seen in non-shocked mice than shocked mice during the 30 seconds after termination of the first tone and shock pairing (@, 150-180s, $p = 0.0220$). During the second tone, the second shock, and the last 30 seconds, shocked mice froze more than non-shocked mice (@@, 180-210s, $p = 0.005$; @@@, 210-240s, $p < 0.0001$; and @@@, 270-300s, $p < 0.001$).

Figure 13

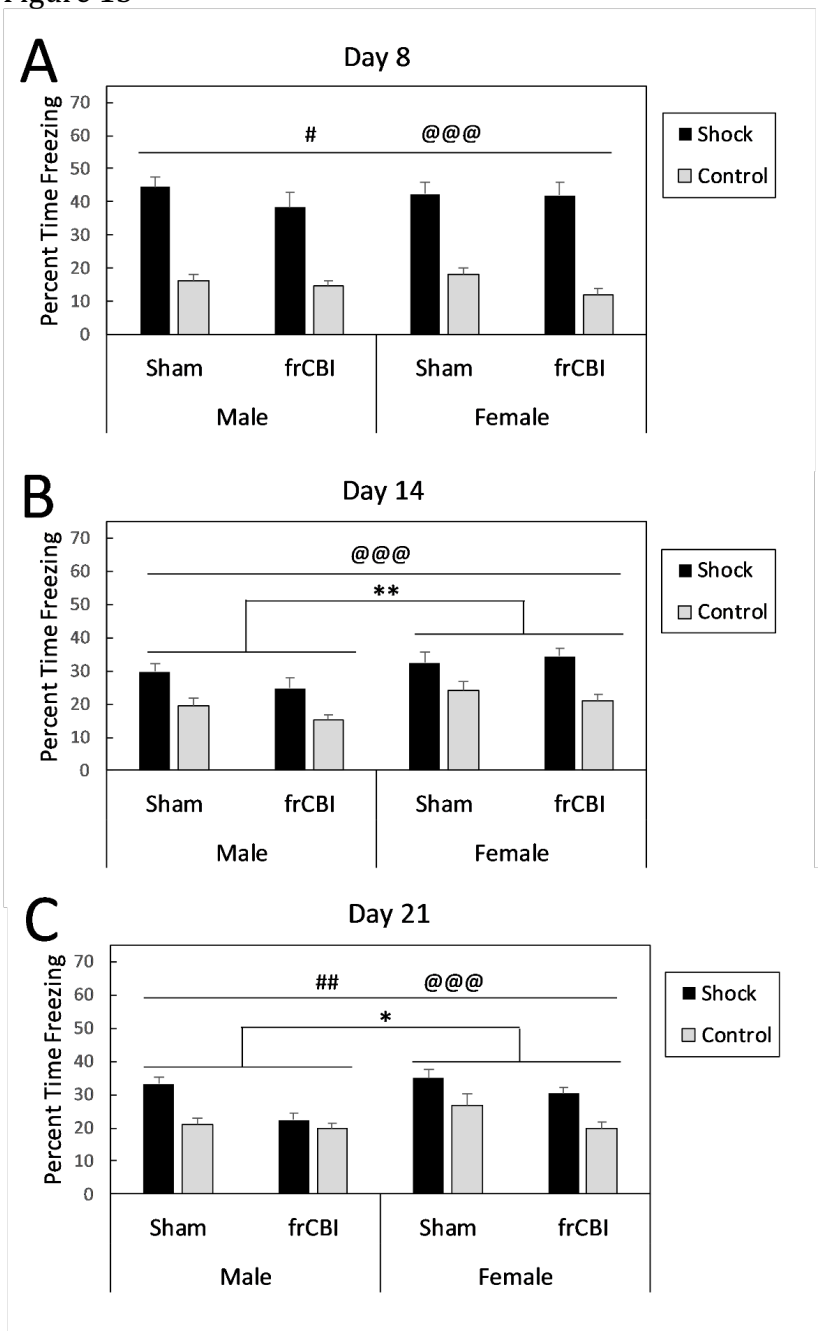

Figure 13. Freezing behavior in injured and uninjured mice during testing for contextual memory on Days 8 (A), 14 (B), and 21 (C) after the final injury or sham procedure. On Day 8, there was a main effect of Injury with injured mice exhibiting less freezing behavior than uninjured sham mice (#, $p = 0.0389$), and main effect of Shock with shocked mice freezing significantly more than non-shock mice (@@@, $p < 0.0001$). On Day 14, there was main effect of Shock where shocked mice froze significantly more than non-shock mice (@@@, $p < 0.0001$), and a main effect of Sex where female mice significantly froze more than male mice in the fear conditioning context (**, $p = 0.0019$). On Day 21,

there was a main effect of Injury with injured mice freezing less than uninjured sham mice (##, $p < 0.0004$) as well as main effect of Shock with shocked mice freezing more than non-shock mice (@@@, $p < 0.0001$). There was also a main effect of Sex on Day 21 with female mice freezing more than male mice (*, $p = 0.0333$). The pound sign (#) represents the main effect of injury. The asterisk (*) represents main effect of sex. The at symbols (@@@) represents main effect of shock.

Figure 14

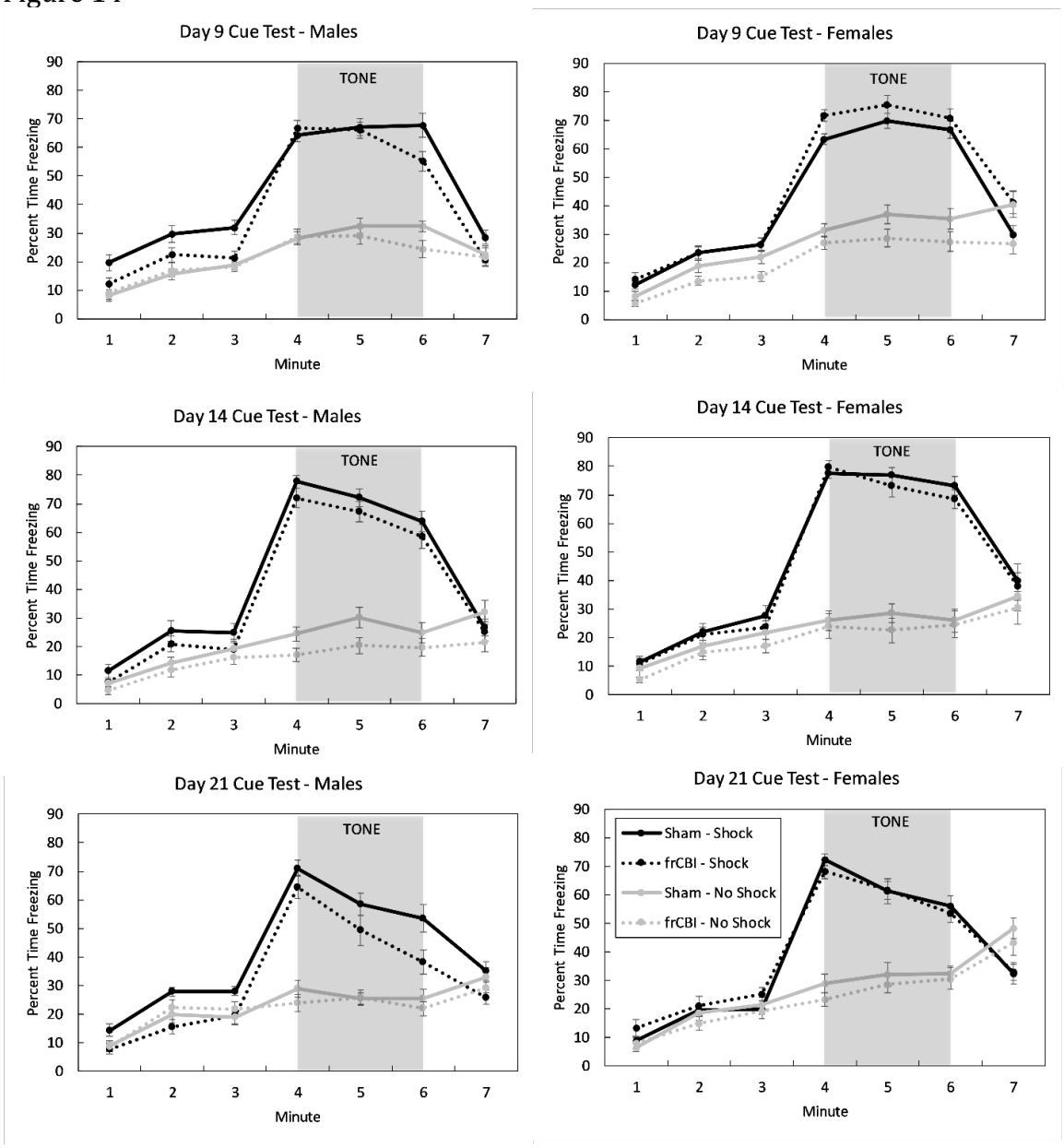

Figure 14. Summary of freezing behavior over time (min) in injured and uninjured mice during testing for memory of the fear conditioning cue on Days 9, 14, and 21.

Figure 15

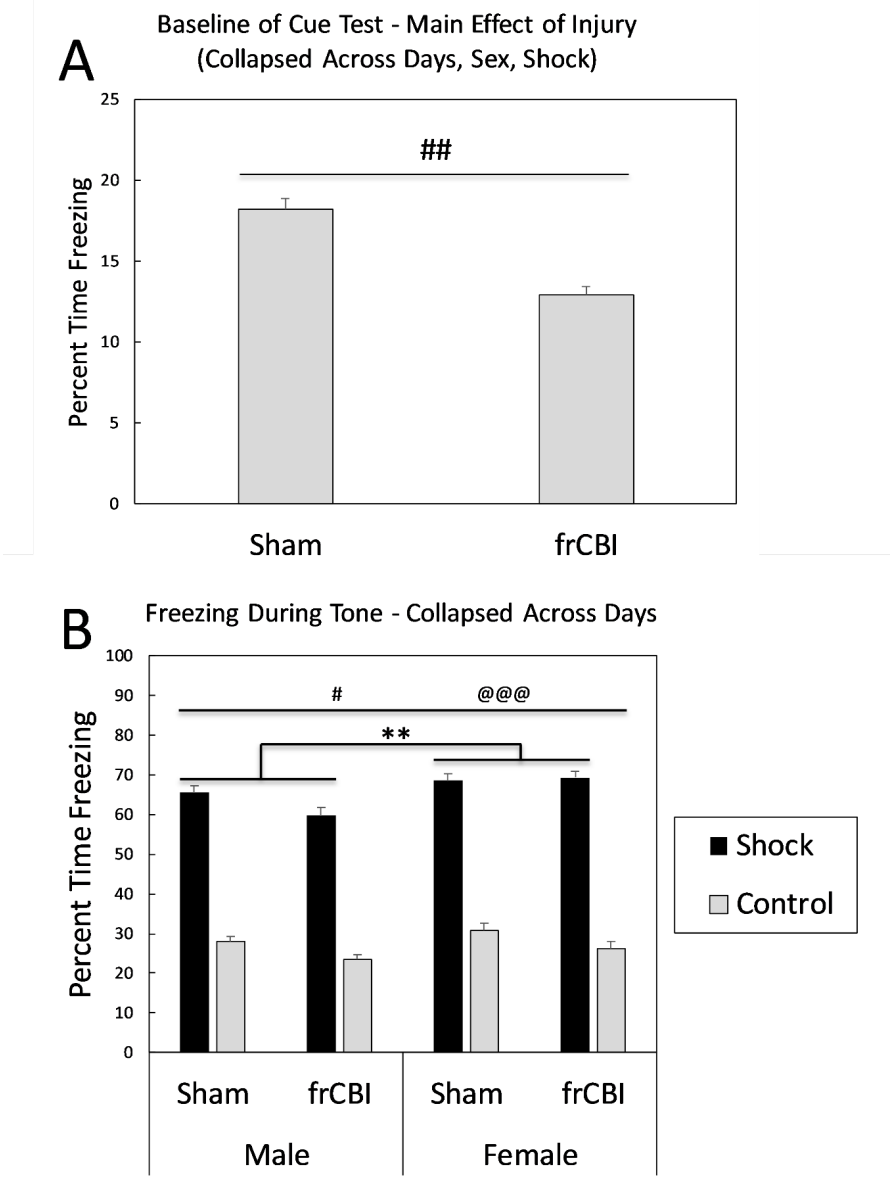

Figure 15. The baseline portion (A) and the tone portion (B) of the fear conditioning cue testing were analyzed separately. When collapsed across Days, Sex, and Shock status, injured mice showed significantly less freezing than uninjured mice during the baseline portion of cue testing (##, $p = 0.039$). During the tone portion of cue testing, there was a main effect of Injury with injured mice freezing significantly less than uninjured sham mice (#, $p = 0.0365$) and a main effect of sex with female mice freezing for longer durations than male mice (**, $p = 0.0048$) across all days. Furthermore, shocked mice froze more than non-shocked mice during all cue testing trials (@@@, $p < 0.0001$). The pound sign (#) represents a main effect of injury. The asterisks (**) represents a main effect of sex. The at symbols (@@@) represents a Day x Shock interaction effect.

Figure 16

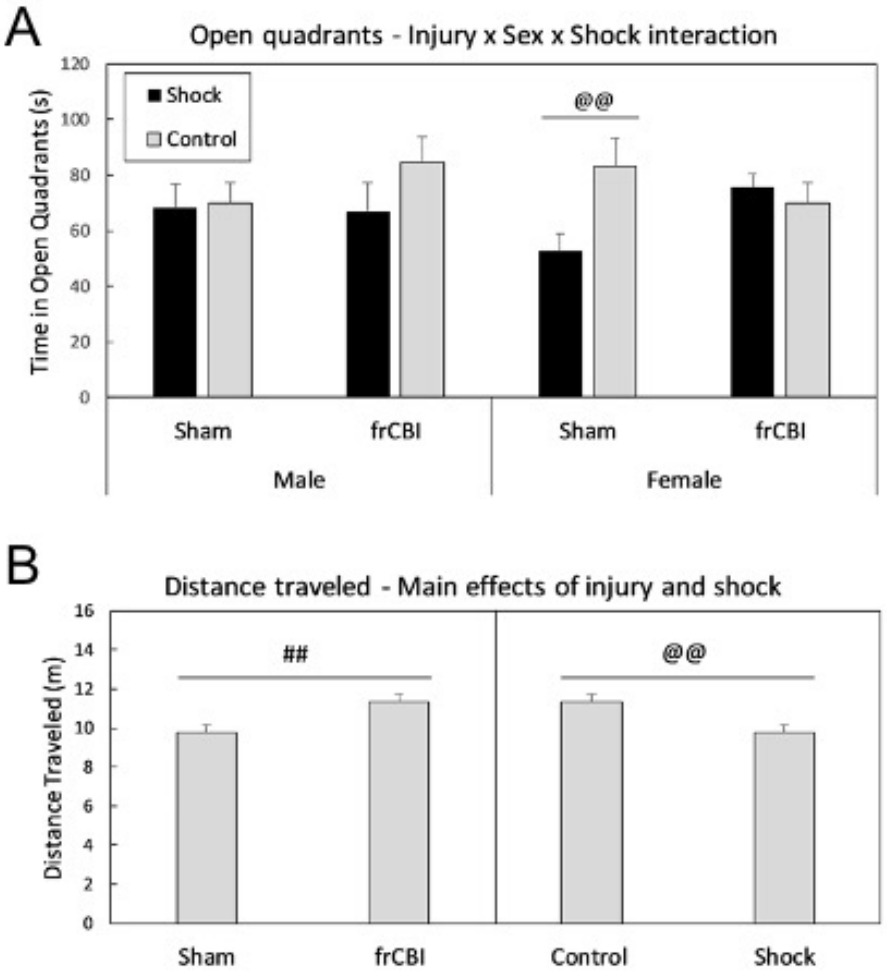

Figure 16. Anxiety-like behavior in injured and uninjured mice after fear conditioning. When time spent in the open quadrants of the elevated zero maze (EZM) was analyzed (A), there was an Injury x Shock interaction effect for female mice ($p = 0.0167$). Uninjured female mice that received foot-shocks during fear conditioning training spent significantly less time in the open quadrants (anxiogenic regions) than non-shocked mice (@@, $p = 0.0092$). When analyzing the total distance traveled in the EZM, there was a main effect of shock (@@, $p = 0.004$) and main effect of injury (##, $p = 0.004$). Injured mice traveled more distance than sham mice. Shocked mice traveled less distance overall than mice that did not receive foot-shocks during fear conditioning training.

Figure 17

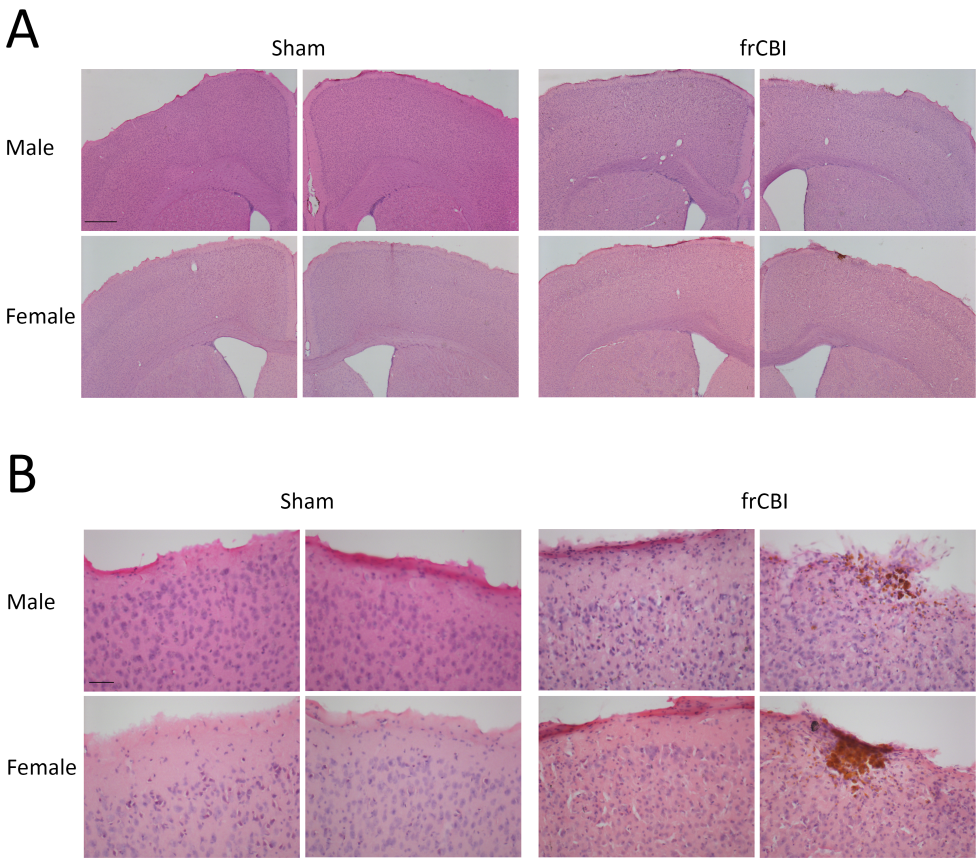

Figure 17. Hematoxylin and eosin staining 25 days following the final frontal repetitive concussive brain injury (frCBI) or sham procedure. Sections are shown bilaterally at 2.5x magnification (A) and 20x magnification (B) for both female and male mice. frCBI resulted in cortical bleeding bilaterally at the sites underlying impact in 1 out of 12 male mice and unilaterally at the site underlying impact in 4 out of 12 for a total of 5 out of 12 male mice. As for brain sections from frCBI female mice, 4 out 12 samples showed unilateral cortical bleeding at the site underlying impact and 4 out of 12 samples showed bilateral cortical bleeding at the sites underlying impact for a total of 8 out of 12 female mice. Cortical bleeding was not seen in any sham-treated mice. The scale bar for the 2.5x magnification images represents 500 μm; scale bar for 20x magnification images represents 50 μm.

Figure 18

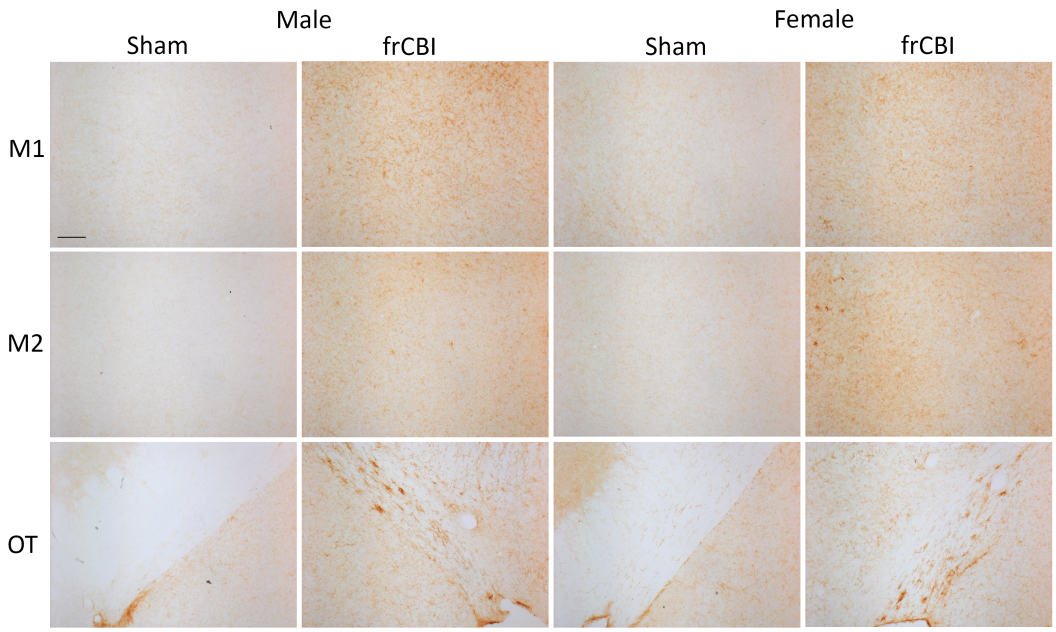

Figure 18. Sections showing CD11b immunostaining in cortical brain regions M1, M2, and the optic tract (OT) of mice 25 days after four frontal repetitive concussive brain injuries or sham procedure. Images shown at 10x magnification. The scale bar represents 100 μm.

CHAPTER 4: Discussion

TRAUMATIC BRAIN INJURY (TBI) IN MILITARY POPULATIONS

TBI continues to pose a huge challenge for military and veteran health systems as service members are at increased risk for sustaining different types of TBI while on deployment as well as multiple TBIs over their lifetimes (80; 107; 178). Furthermore, service members sustaining TBI during combat operations are exposed to life-threatening situations that elicit high levels of fear and stress. In an attempt to better capture the diversity of TBI in military service members, single and repetitive injury models were utilized in wildtype mice to assess functional and behavioral outcomes after a single blast-induced TBI (BTBI), a single concussive brain injury (CBI), and repetitive CBI to frontal brain regions.

PHYSIOLOGICAL CHANGES IMMEDIATELY FOLLOWING INJURY

The occurrence of apnea after injury was previously reported (72; 99; 152; 192; 209; 222). However, not all studies provided details when reporting this physiological parameter. Apnea was only seen in injured mice: 8.3% of BTBI mice and 37% of CBI mice (213). Because there was a consistent delay of 20 seconds in removing BTBI mice from the Advanced Blast Simulator for observation, apnea measurements in these mice were less accurate. For frontal repetitive CBI (frCBI) mice, most mice showed apnea after at least one of the four injuries with only 2 mice out of 72 mice (2.8%) in the injury groups showing no apnea across all four injuries. When trending the duration of apnea across days, there was a significant decrease in the average apnea duration after the

second injury relative to the first injury and after the third injury relative to the second injury regardless of sex (Fig. 10A). Uninjured sham-treated mice in this study also did not show any apnea, which was consistent with uninjured mice that served as controls for the BTBI and CBI models (213). In previous studies of repetitive injuries, a decrease in apnea duration with subsequent injuries was reported (22; 125; 222).

There are some data to suggest that injured mice that did not spontaneously resume breathing after 30 seconds on their own and require resuscitation, exhibit poorer cognitive performance than injured mice that did resume breathing on their own (72). The authors also observed more pathological changes in mice with prolonged apnea (72). Taken together, the results of their study suggested apnea may be a valuable surrogate marker in predicting outcome after a single injury (72). However, with repetitive injuries, the utility of apnea as a surrogate marker for predicting neuropsychiatric outcome remained unclear.

Similar to apnea, the time it took mice to regain the righting reflex served as another physiological measure that was observed immediately after injury or sham procedures. Suppression of the righting reflex has been used as an indirect measure of loss of consciousness since mice have an innate tendency to turn from a supine position to prone position when awake. All injured mice displayed significant delayed righting when compared to their respective sham-treated controls, which were exposed to equivalent amounts of anesthesia. Interestingly, the time to righting decreased with subsequent injuries as seen in frCBI mice (Chapter 3, Fig. 10B). This finding was described in previous studies (22; 130; 201; 222), and may be indicative of adaptation to injury that was unrelated to behavioral outcome since mice sustaining repeated injury

were shown to demonstrate poorer neurocognitive outcomes than mice that sustained a single injury (99; 122; 177; 222).

TRANSIENT DECREASE IN HOME CAGE ACTIVITES AFTER A SINGLE BTBI OR CBI

In general, hypoactivity was noted in both BTBI mice and CBI mice (213). BTBI mice showed decreased activity in wheel-running, eating, and drinking 24 hours after injury (Chapter 2, Fig. 3). This decrease in daily activities was not significant at 48 hours after injury with activity levels returning to baseline by 72 hours post-injury. CBI mice also showed a transient decrease in wheel-running and drinking, but not food intake 24 hours after injury. With regards to feeding behavior, CBI mice showed an overall decrease that was not dependent on day (Chapter 2, Fig. 3).

The transient hypoactivity seen in BTBI mice and CBI mice was contrary to the hypothesis that mild TBI (mTBI) would result in sustained behavioral changes indicative of excessive daytime sleepiness. However, the transient alterations in activity level as measured by time spent immobile during the 12-hour dark cycles (when mice are typically active) and 12-hour light cycles (when mice typically sleep) on post-injury days 2 to 9 were seen in another study utilizing weight drop to model mTBI (130). The authors noted mice that received three injuries spent more time immobile during the dark phase of post-injury days 2 and 3, and thus exhibited hypoactivity during a mouse's typical waking hours (130). In contrast, mice that received a single injury spent more time immobile during the light phase of post-injury days 2, 3, 4, and 6 when compared sham-treated mice and repeated mTBI mice, indicating more hypoactivity during a mouse's resting hours than normal (130).

In another study, modeling mild and moderate midline fluid percussion injury, the authors noted an increase in mean percent sleep time (indirectly measured via motion and respiratory sensors) during the first six hours after injury when injured mice were compared to sham-treated mice (163). This increase in percent sleep was not seen after six hours post-injury, and interestingly independent of injury severity (163). Based on these results, the author then assessed the effects of a second midline fluid percussion injury within the six-hour window during which injured mice showed increased sleep (162). They found that mice receiving a second injury before recovery of normal sleep behavior performed poorer on motor tasks as well as showed increased anxiety-like behavior when compared to mice receiving a second injury after recovery of normal sleep behavior (162). Their findings implicated increased sleep behavior as a potential marker of increased vulnerability to repeat injury (162).

Although sleep was not assessed in BTBI and CBI mice, hypoactivity within the first 24 hours that fully resolved by 72 hours similarly indicated a window of increased need to rest and increased vulnerability to repeat injury. This was consistent with a weight drop study in which a repeat injury interval of 24 hours resulted in worse outcomes than wider inter-injury intervals of seven days or more (122). Another study showed that mice sustaining five CBIs with an inter-injury level of 48 hours did not differ in motor and memory tasks when compared to mice that sustained injuries with an inter-injury interval of 24 hours (22). Further evaluating the need for increased rest and sleep after injury is crucial in determining return-to-duty as well as return-to-play guidelines. Current guidelines from the Department of Defense recommend a minimum recovery period of 24 hours for first-time mTBI (1); however, extending this mandatory recovery

period may be crucial for the avoidance of further injury during a window of increased vulnerability for development of lasting motor, cognitive, and psychiatric symptoms.

Although loss of neurons containing orexin-A have been widely implicated in excessive daytime sleepiness following TBI (13; 181; 196), immunostaining for orexin-A-positive cells after BTBI and CBI did not show any significant change in cell numbers (Chapter 2, Fig. 8). Midline fluid percussion also did not result in loss of orexin-A-positive cells despite injured mice exhibiting an increase in percent sleep (162). Other studies investigating the link between alterations in sleep and orexin-A-positive neurons after TBI also reported no change in the number of orexin-A-positive cells (59; 105; 216). These studies did, however, detect changes in orexin-A physiology as well as decreased wakefulness or decreased activity (59; 105; 216). Thus, post-mortem cell counts may not have reflected the full impact of injury on neuronal function.

The relationship between hypoactivity, increased sleep drive, and sleep quality after TBI is still unclear. Some pre-clinical studies demonstrated an increase in or fragmentation of sleep in rodents following TBI (105; 162). Another pre-clinical study, however, did not demonstrate any alterations in sleep quality in rodents post-injury, but rather only fragmentation during the wake period (196). Because sleep is essential to recovery from TBI (90), further assessment of how mTBI affects sleep and its relationship to symptoms, such as excessive daytime sleepiness, is essential to developing more effective treatments. One study found that chronic insomnia and short sleep duration in mTBI patients increases their likelihood of poor functional outcomes (90). Furthermore, poor sleep quality has been linked to increased anxiety, aggressiveness, and impulsivity (53). Other clinical studies have also noted similar effects of sleep

deprivation and sleep insufficiency on anxiety and mood (54; 123; 131; 148; 165; 193; 219).

BEHAVIORAL OUTCOMES FOLLOWING BTBI, CBI, AND FRCBI

Anxiety and depression are commonly reported in patients who have sustained TBI (77; 78; 89; 172; 183; 185). Despite these clinical reports, mice that sustained BTBI or CBI demonstrated behaviors that were suggestive of decreased anxiety in the open field (OF) when tested for anxiety-like behaviors at 24 and 72 hours post-injury (Chapter 2, Fig. 4C & 4D). An increase in distance traveled in the anxiogenic region (center) of the OF was not seen in mice that sustained frCBI, which were tested 4, 15, and 22 days after the final CBI. Sham female mice from this study, however, did spend less time in the anxiogenic regions of the elevated zero maze (EZM) when exposed to foot-shocks during fear conditioning, and this increase in anxiety-like behavior was lost with frCBI (Chapter 3, Fig. 16A). Depressive-like behaviors were assessed only in frCBI mice, which did not differ from uninjured sham mice during the tail suspension test (TST).

Similar findings were observed in mice that sustained repetitive midline fluid percussion (162) or repetitive CBI (202). When tested in the OF, mice did not show increased anxiety-like behavior compared to injured sham mice (162). Injured mice also did not show depressive-like behavior when tested with the forced swim test (162) nor the TST (202). In contrast, injured mice exhibited behavior that was interpreted as decreased anxiety compared to uninjured sham mice when tested in the EZM (202). This behavioral alteration was also noted in a study of repetitive Closed-Head Impact Model

of Engineered Rotational Acceleration (CHIMERA), where injured mice spent more time in anxiogenic regions of the elevated plus maze (134).

When tested in the EZM one year post-injury, injured male mice, but not female mice, spent more time in the open and anxiogenic regions of the EZM than uninjured sham mice (201). Also, unexpectedly, both female and male injured mice in this study exhibited more mobility during the TST than uninjured sham mice (201). Traditionally, more time spent immobile during the TST and forced swim test is interpreted as lack of motivation and depressive-like behavior (116; 188). Tucker and colleagues also noted increased distance traveled in the OF in injured male mice when compared to uninjured sham controls (202). Thus, they interpreted their results from the EZM and TST as emotional dysregulation resulting in hyperactivity (201).

In the present work (Chapter 3), mice that underwent frCBI also demonstrated greater distances traveled in the OF and EZM when compared to uninjured control mice (Fig. 11A & Fig. 16B). Furthermore, testing for memory of the context and cue after fear conditioning resulted in frCBI mice freezing less than uninjured sham mice (Fig. 13 & Fig. 15). Similar observations were reported after fear conditioning in mice that were subjected to repetitive CBI over the parietal lobe (108; 202). In these studies, the authors implicated memory deficits as the source of decreased freezing behavior during fear conditioning testing (108; 202). However, frCBI did not differ from uninjured sham mice during fear conditioning training where mice exposed to the aversive stimulus still demonstrated more freezing behavior than non-shocked mice. Furthermore, like uninjured sham mice, frCBI showed a similar habituation pattern to the OF upon repeated exposures. Taken together, the data suggested that frCBI mice were capable of learning

and that their memory was not compromised, but rather they were exhibiting hyperactivity leading to impulsive behaviors, which would be consistent with damage to the frontal lobes (61; 119). Hyperactivity and impulsivity made interpretation of data from other behavioral assays for anxiety-like and depressive-like behaviors difficult as many of these assays relied on measures related to movement.

NEUROINFLAMMATION AFTER TBI

There is evidence from clinical and pre-clinical studies to suggest that impulsivity may be mediated by neuroinflammation (48; 212). Specifically cortical inflammation may alter or impair different aspects of neurotransmission (6; 81; 161; 210), and as a result may lead to comprised neurotransmitter systems (monoamines, dopamine, and serotonin) that regulate impulsivity (19; 110; 217). Consistent with this hypothesis, frCBI mice exhibited a marked neuroinflammatory response in cortical regions underlying the sites of injury bilaterally (Chapter 3, Fig. 18). There was also evidence of bleeding in some, but not all frCBI mice (Chapter 3, Fig. 17). Cortical bleeding was also seen in some, but not all CBI mice that received a single injury; however, these mice did not demonstrate cortical neuroinflammation as measured by CD11b immunostaining density for activated microglia (213).

IMPULSIVITY IN CLINICAL STUDIES AFTER TBI

Impulsivity has a significant impact on daily function (85; 160) and can be problematic following TBI. Impulsive behavior can lead to poor decision-making and risk-taking behaviors that lead to increased morbidity and mortality (23; 27; 85; 135;

146). It is a complex behavior that has a multifaceted clinical presentation (143). Clinically TBI can result in motor impulsivity described as response disinhibition (127; 143; 160). TBI can also result in choice impulsivity, which is defined by decision-making that results in immediate perceived rewards despite the risk of long-term negative consequences (55; 120; 143; 174; 218). Risk-taking is another component of impulsivity that has been documented in TBI patients (47; 115; 129; 169; 220).

FUTURE DIRECTIONS

Because of the detrimental effects of hyperactivity and impulsivity, more studies are required to further assess this behavioral dimension after TBI in rodent models; particularly in the setting of how it affects fear learning and decision-making. These factors can affect behavioral outcomes and complicate the assessment of anxiety-like and depressive-like behaviors post-injury. The heterogenous nature of TBI and the wide range of clinical symptoms that span across different categories of neurologic and psychiatric diagnoses require a symptoms-based approach to treatment. Thus, this approach may also be useful in interpreting findings when modeling mTBI, since subtle behavioral differences have been observed following different types of mTBI (213) and location of direct impact in CBI models may influence outcomes (Chapter 3).

Also important is the inclusion of female rodents in pre-clinical studies. As seen in frCBI mice, some sex differences were noted when mice were tested in the fear conditioning context as well as for memory of the fear conditioning cue. Female mice froze more than male mice in general when presented with stimuli associated with foot-shocks (Chapter 3, Fig. 13 & Fig. 15). Female mice from the frCBI study also

demonstrated increased anxiety-like behavior in EZM after receiving foot-shocks during fear conditioning training that was not seen after injury, whereas, no differences in behavior were noted for male mice (Chapter 3, Fig. 16A). Sex differences were also observed in other studies of repetitive injury (201; 202; 209), but were not always consistent. Most studies to date have focused on behavioral and histological outcomes following TBI in male mice (99; 108; 134; 162) despite documentation of increasing reports of TBI in women (46) as well as differences in clinical outcomes between men and women after TBI (96; 106). Capturing these differences—even if subtle—are important in developing targeted treatments. It may also be prudent to take into account how the social needs of the species may affect behavioral outcomes, especially when assessing anxiety-like and depressive-like behaviors after injury (144). Strong support networks are essential in the recovery of patients from any chronic disease, including TBI.

As populations grow and industrialization continues to evolve in developing nations, there will be an increase in traffic and traffic-related accidents. The race for economic development and profit can sideline safety, resulting in increased work-related TBIs. Furthermore, the competition for limited natural resources will continue to drive tension between countries and potentially lead to future wars, further exposing populations to explosive blast and the risk of TBI. Thus, continued efforts to better elucidate mechanisms of injury, as well as mechanisms by which symptoms arise, are essential and require a multidisciplinary approach in defining pathology and providing individualized and targeted treatment options.

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