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CEREBRAL BARRIER BREAKDOWNS FOLLOWING HIGH VELOCITY IMPACTS
AND ROTATIONAL ACCELERATION: NEUROPATHOLOGICAL AND
BEHAVIORAL ASSESSMENT

by

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ABSTRACT

Cerebral barrier breakdowns following high velocity impacts and rotational acceleration:
neuropathological and behavioral assessment

Eileen Hongxia Hinton McNamara, Doctor of Philosophy, 2022

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Rotational acceleration and high velocity impacts are the predominant causes of traumatic brain injury (TBI). In this project, the effects of shock wave exposure and mechanical impact-rotational acceleration were investigated in male and female mice using two pre-clinical TBI models. In the first study, functional outcomes, neuronal activation, and blood-brain barrier (BBB) permeability were evaluated in mice after ventral exposure to primary (shock wave) and tertiary (impact/acceleration) effects in an Advanced Blast Simulator. Behavioral testing indicated no differences on anxiety- or depressive-like tests after blast TBI, but mice demonstrated greater activity levels on the elevated zero maze test, where female mice travelled the farthest. On the tail suspension test, injured mice displayed reduced immobility compared to uninjured mice; possibly an indication of an agitated behavior state in this task. There were sex differences in c-Fos expression in the paraventricular thalamic nucleus and greater c-Fos activation in the amygdala of female mice that may correlate with reported hyperactivity in females post-

injury. Four hours after shock wave exposure, BBB permeability was evident on the superficial brain parenchyma of injured animals, but not sham mice. This study demonstrated behavioral and neuronal activation correlates in female rodents after blast injury, which could be relevant to post-traumatic stress disorder in women.

TBI research has primarily focused on effects related to the brain, but reports of traumatic meningeal enhancement in TBI patients may have clinical significance. The second study focused on the consequences of impact-rotational acceleration on the meninges and visual system after one or four injuries produced by the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) device. In male mice, meningeal dynamic contrast enhancement was elevated soon after single or repetitive impacts. Enhancement returned to below baseline one week later for single injured male mice, but remained elevated in male and female mice following multiple impacts. Meningeal T2 pre-contrast analysis showed significant increases only after single CHIMERA impact in injured mice. Since vision is impaired after CHIMERA, visual pathway regions were also analyzed through imaging and GFAP histology. Initial enhancement in the optic tract (OPT), lateral geniculate nucleus, and superior colliculus (SC) was observed after injury, with decreased signal one week later, but astrogliosis in the OPT and SC was still evident.

These studies explored functional, neuroimaging, and histological outcomes that demonstrated: 1. The importance of including males and females in pre-clinical studies of TBI, 2. Shock wave exposure with primary and tertiary effects results in disruption of the BBB, neuronal activation in the amygdala, and an agitated-like behavioral response, and 3. Impact-rotational injuries disrupt the integrity of the cerebral meninges, perhaps

leading to important sequelae that are relevant to clinical diagnosis and treatments. Taken together, the studies demonstrated multifaceted TBI effects that will enable further evaluation of mechanisms relevant to injuries sustained by military personnel and civilians populations.

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CHAPTER 1: Introduction

TRAUMATIC BRAIN INJURY (TBI)

Traumatic brain injury (TBI) is a challenging global source of morbidity that affects the lives of the individual and their families, and it places financial and medical resource stress on society. Without a current FDA-approved treatment for TBI, even the more common milder forms are a growing long-term economic and medical concern (115). James and colleagues recently reported there were approximately 27 million new cases of TBI each year, and estimated the world prevalence as 55.5 million cases, particularly in younger and older populations (122). TBI-related emergency department visits, hospitalizations, and deaths indicate the most common causes of head injuries include falls and motor vehicle accidents, and a variety of other insults from acceleration and deceleration forces from violence, spousal and child abuse, and sports, as well as from firearms and industrial accidents (72; 122). TBI is particularly relevant for military populations during training and from conflicts in Iraq and Afghanistan (253). Military personnel and athletes are often exposed to repetitive closed-head injuries, which are a risk factor for chronic disease, such as chronic traumatic encephalopathy (166). In addition to repetitive impact risks, there may be a potential sex difference in head trauma with higher rates of concussions reported in female collegiate athletes (46). Further investigation of these susceptibilities is needed to develop safer sports and training guidelines and a greater understanding of the underlying biological mechanisms.

Pre-clinical modeling of TBI includes approaches that result in greater or lesser degrees of focal and diffuse effects (82; 143). To study primarily focal effects, open-head

models that require a craniectomy are used to direct a sudden energetic mechanical impact to the surface of the dura mater. This can be implemented by a metallic cylinder of the controlled cortical impact model (CCI) (146) or by a rapid high pressure fluid pulse as is employed in fluid percussion models (FPI) (60). Both approaches have been used for decades in pre-clinical TBI studies and are considered reproducible and a valuable source for understanding the biological and behavioral effects related to local cellular damage and axonal trauma. Closed-head models have been utilized less but include the weight drop model that causes an impact and acceleration of the head resulting in diffuse injury along the neuraxis (161). Models that employ closed-head acceleration/deceleration forces are seen as having clinical relevance, but if the impact causes skull or cerebral deformation, or penetration, the model does not mimic the most common human injury processes. Clinical TBI cases are more often associated with lesser degrees of focal injury and no conspicuous cerebral pathology. In clinical cases, skull durability protects brain tissue from focal contusion and the impact causes injury as a result of the rapid inertial changes in head velocity. Designs, such as the CHIMERA, closely replicate such conditions (183). Some designs, such as the CHIMERA with the assistance of a polylactic acid (PLA) interface (16) and rotation-only injury applications with no impact, enable diffuse pathology without a single point of impact. Diffuse TBI can also arise from blast wave exposure. Specifically, in addition to primary shock wave effects from a blast (discussed below) the blast wind that follows a supersonic shock wave can cause impacts of objects with the victim (secondary blast effects) as well as propel the victim into immovable objects (tertiary blast effects) that both result in impact-

acceleration TBI. The two animal models employed for this project included closed-head designs that closely mimic clinical biomechanisms of rotational acceleration injury.

Blast TBI (bTBI)

Blast TBI characteristics

Blast exposure is a leading cause of TBI in military personnel and blast TBI (bTBI) is considered the invisible wound in combat zones. Blast TBI was a frequent cause of casualties in recent Mideast conflicts when Warfighters encountered improvised explosive devices. Shock wave exposures also can occur in training with explosives (e.g., breacher training) and artillery practice. Milder injury is the most common diagnosis with about an 80% prevalence compared to moderate and severe TBI cases (72). Depending upon the circumstances, injury can result from the four main components of bTBI, including the *primary* injury effect caused by the violent shock wave front that travels through the brain and body in the microsecond time frame from the almost instantaneous initial expansion of the atmosphere at supersonic velocity; *secondary* “blast wind” effects that propel fragments and debris from the explosion; *tertiary* effects from bodily displacement caused by acceleration and deceleration of the body and head against the ground and solid immovable structures; and lastly *quaternary* miscellaneous factors, such as burns (34). The causes of primary blast-related neurological symptoms resulting from single and repeated exposures to shock waves are not fully understood, but casualties from blast are often injuries resulting from the combination that includes primary shock wave effect in conjunction with impact injuries that result from rapid displacement and impact deceleration (53; 106; 189; 216).

Since free-field explosions are a challenge due to safety considerations, local setting, animal placement, and alterations in climate conditions, laboratory studies have attempted to replicate shock wave conditions under better controlled conditions. Translation of studies from the laboratory to real-world casualty effects, however, is a challenge. Animal size, the peak pressure, and shock wave duration affect the level of injury caused by primary blast injury (24; 198), and studies indicate bTBI displays unique characteristics that distinguish it from other forms of TBI and these have been a challenge to replicate in the laboratory. Distinct human pathological characteristics of astroglial scarring in post-mortem brains of bTBI victims that included astrogliosis near penetrating cerebral vessels have been described, indicating that bTBI uniquely affects the human brain (245). In addition to brain damage, peripheral organs such as the lungs and vasculature are particularly vulnerable following blast exposure (78; 139). These observable disruptions in vasculature support the idea that the blood-brain barrier (BBB) becomes disturbed during bTBI. In the present study, different approaches with the ABS and CHIMERA models were taken to assess these primary and tertiary injury effects.

Advanced Blast Simulator (ABS)

The increasing incidence and unique pathology of bTBI exposure requires the establishment of pre-clinical models of blast to better understand the injury effects for clinical translation. However, the research field has been hindered by the diversity in shock tube designs that creates a lack of standardization in relating published findings. Many of the devices for modeling bTBI cause combined complex injuries that cannot be attributed solely to shock wave effects, but to tube design (2; 110; 112; 151; 256; 283). For example, blockage within the shock tube due to small tube dimensions, exit-jet

effects from placing the specimen outside of the tube, and tertiary injury from lack of animal restraint are common challenges in device design (184). The pre-clinical modeling of bTBI, then, varies greatly and most designs continue to struggle in isolating the primary shock wave alone without additional injury to the head from secondary and tertiary effects. Most reported pre-clinical damage from blast in non-optimally designed shock tubes arises from dynamic pressure artifacts, i.e., blast wind. Unlike the shock wave overpressure, which is believed to not be significantly affected by the target's dimensions, the blast wind can cause significant concussive effects related to the size (mass) and surface area of the animal, with greater effects in smaller animals related to violent head and/or body movement (24; 241). Specifically, dynamic impulse effects on a small animal have a significant impact on shock wave-related acceleration/deceleration alone, resulting in more severe brain injury (221).

Body orientation to a shock wave is a critical variable for injury severity. A vertical, standing orientation, leaving the ventral thoracoabdominal region exposed, for example, presents a greater surface area for shock wave impact and enables a greater degree of thoracic injury (44; 85; 139). Finite element modeling of prone, vertical, or head-only ABS exposure revealed that the body orientation affected the peak pressure and that prone positioning caused the least amount of pressure, stress, and strain damage, whereas vertical positioning increased damage that enabled the most body movement and possibly skull deformation during blast (265). Blast-induced skull flexure and changes in flow dynamics due to head geometry variability could lead to differences in intracranial pressure and possible cavitation (22; 79; 238). In a study comparing prone versus side orientations within the ABS, greater damage is also displayed with thoracic polytrauma

associated with side positioning (101; 109). Even with less biomechanical force, changes in brain function, BBB impairment, and microvascular disturbances have been reported in animals with prone placement as well (78; 241). The position of the body during shock wave exposure, then, can alter the degree and nature of damage inflicted and should be considered in experimental design and interpretation.

The Advanced Blast Simulator (ABS) model is one of the few shock tube designs that minimizes the common confounding factors seen in other studies (184; 241). The ABS, originally developed by Ritzel and colleagues, is considered a state-of-the-art design for simulation of shock waves in the laboratory setting (221; 241). The shock waves generated by the ABS allow for high reproducibility and are designed to emulate free-field explosive blasts (241). The ABS produces a Friedlander-like curve that arrives as a planar wave front with the characteristic positive peak pressure spike, followed by a negative phase with a slight secondary shock wave. The dimensions of the ABS, relative to specimens, reduces potential blockage of the tube. Likewise, an end-wave eliminator located at the rear reduces wave reflection. Previous work to characterize our ABS device found that an average shock wave travels at about 480 m/s, also mimicking actual blast conditions. Secondary and quaternary injuries are limited because fragments from the membrane are minimal (abating secondary blast effects) and travel at slower speeds, and compressed air driven shock tubes eliminate the exposure to burns or toxic inhalations caused by explosives (abating quaternary blast effects) (220).

CHIMERA TBI

Biomechanical rotational acceleration

Impact-acceleration injuries are a common event associated with military training exercises and deployment. Injuries arise from high impacts of the head and body with stationary objects, such as falls during training, sports and exercise, and vehicular accidents, as well as during deployment. Inertial forces are thought to be a significant component of the injury process. Injury severity is associated with increases in the magnitude and duration of acceleration, with rotational acceleration thought to be the predominant component (83; 94; 107; 143; 179; 193; 226). Pre-clinical models and human cadaver studies have shown that the magnitude, duration, and direction of rotational acceleration are key contributors to TBI (6; 82; 94; 107). Rotational acceleration is more closely associated with concussion and diffuse axonal injury, whereas linear acceleration is linked with skull fracture (48; 83; 136; 160; 161; 170). To mimic impact-acceleration effects, the CHIMERA device was employed. The CHIMERA induces rapid head acceleration under controlled conditions that is interpreted as primarily affecting diffuse pathology related to the white matter integrity and inflammation (168; 183).

Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA)

The application of imaging in CHIMERA models may provide relevance to clinical cases and allow for repeated assessment of animals over time. Three out of the four CHIMERA neuroimaging papers utilized diffusion MRI (dMRI), while the fourth implemented T2-weighted MRI (91; 140; 186; 239). Investigators used *ex vivo* dMRI to show variability in fractional anisotropy (FA) values for white matter regions and tracts (131; 248). FA values are typically reduced in damaged white matter tracts, but differences in biological mechanisms of injury and variations in scanning parameters

could contribute to the mixture of dMRI outcomes (114). Since CHIMERA mainly induces white matter injury, dMRI was preferred by investigators because it detects CHIMERA's subtle white matter damage (91; 140). Primary regions of interest (ROIs) in all four papers included white matter tracts, such as the corpus callosum (91; 186; 239), optic tracts (91; 140), and anterior commissure (239). The brachium of the superior colliculus (91) and hippocampus (239) were also identified as affected areas (91; 239). Subtle changes were observed in the DTI data, but no structural damage or alterations in anatomical volumes or signal were observed in T2-weighted MRI, (91; 186). Collectively the DTI results demonstrated changes primarily in white matter tracts following CHIMERA. In particular, the optic tract displayed reduced values in most of the DTI metrics, but still did not show changes for the T2-weighted images (91). The optic tract was also a key focus for Komlosh and colleagues, who discovered asymmetrical values in the left and right optic tracts, which can be common in diffuse axonal injury (140). The optic tract was identified by the computer-learning forest analysis program as a primary ROI for injury classification in both DTI-based and histology-based analyses. White matter tracts (particularly the optic tract) appear to be a key area of investigation for CHIMERA and are ideal to study with dMRI because of its high coherence and simple geometry (91; 140). For further review of CHIMERA literature see McNamara et al., 2020 (168).

Following our CHIMERA review paper (168), eleven additional research papers using the CHIMERA device have been published: Tucker et al., 2021; Feng et al., 2021; Button et al., 2021; Chen et al., 2021; Desai et al., 2021; Reyes et al., 2021; two publications by To et al., 2021; Sun et al., 2022; Hutchinson et al., 2022; and Vonder

Haar et al., 2022 (**Table 1**). Briefly, male and female mice were examined by Tucker and colleagues (4x 0.7 J CHIMERA) and Desai and coworkers (3x 0.55 J CHIMERA) for behavioral changes after injury. Investigators described hippocampal impairments in Morris Water Maze and changes in trace fear conditioning with more deficits observed in male mice (56; 260). The rat CHIMERA used by Feng and colleagues (6x 2 J CHIMERA, 3 per day), however, did not observe differences in trace fear conditioning, but instead found reduced cued fear memory with delayed fear conditioning and novel object recognition impairment, which they attribute to reduced mitochondrial function in the medial prefrontal cortex (73). Vonder Haar and colleagues (3–8x, 2.4–3.2 J CHIMERA) also used the rat CHIMERA to test different piston surfaces with either a rubber or metal tip. Behavioral tests for five-choice serial reaction time and delay discounting task displayed attentional deficits after multiple impacts. Differences between the two tasks showed motor impulsivity increase in the five-choice task, but choice impulsivity decrease in delay discounting. Changes in microglia and astrocytic expression appeared in related cortical regions, striatum, and nucleus accumbens (90). Button and coworkers (1x 2.5 J with PLA interface) described a new mouse plasma GFAP assay with male and female wildtype and APP/PS1 mice with increased GFAP 6 hours post injury (30). Sun and colleagues (1x 0.9 J CHIMERA) reported immediate formation of axonal varicosities in male and female mice after impact in the corpus callosum, external capsule, and cortex layer VI, but did not observe sex differences. Other CHIMERA energy levels (0.5 J and 0.7 J) were investigated and impact strength affected axonal varicosity size and abundance, but not the overall patterning (250). Damage in the optic tract was consistent with previous CHIMERA literature with

increased silver stain, GFAP, and Iba-1 stains and reduced visual evoked potential N1 amplitude (38; 56; 168; 260). Chen and colleagues (3x 0.55 J CHIMERA) also described visual deficits lasting up to 3.5 months post-injury in male and female mice (38). Desai and colleagues found long-term therapeutic effects of a polyunsaturated fatty acid diet on injury-induced visual and memory impairments. The diet attenuated gliosis and axonal damage due to CHIMERA (56). The same group also analyzed *in vivo* and *ex vivo* DTI and reported no statistical improvements in white matter damage, which may differ from their reported pathological evidence of attenuated axonal damage in injured diet-treated animals because different time points were examined. Imaging data by Reyes and coworkers were consistent with previous literature showing reduced FA in the OPT and brachium of the superior colliculus (218). Like the neuroimaging studies described above, To and colleagues (1x 5.1 m/sec or 5.36 m/sec CHIMERA) also used *ex vivo* DTI in male mice, which displayed increased FA after lower impact, but decreased FA after higher impact mainly in the grey matter. Surprisingly, the optic tract was not studied (255). In a separate study by the same group (1x 5.2-5.4 m/s CHIMERA) *in vivo* T2-weighted images, DTI, fMRI, and behavior were assessed. Inconsistent with previous literature, structural changes were observed with increased ventricle and tissue volume two days post-injury, but decreased tissue volume one week after injury (168; 255). Overall, imaging and behavioral changes did not temporally match. Specifically, increased FA was primarily observed in the grey matter one and two weeks post-injury. The authors suggest different recovery phases with behavioral deficits occurring more quickly and imaging changes appearing later (255). A novel study (range of energy levels, 1-4x CHIMERA with custom helmet interface) examining male ferret MRI with a

battery of MRI sequencing parameters at different time points from 3 hours after CHIMERA to 19 weeks post-injury discovered meningeal injury on T2 FLAIR 3 hours after severe injury. Disruption to cerebral blood flow and the BBB were also reported. In addition to the cerebral meninges, contrast enhancement was observed in the thalamus and brainstem. Increases in T2 and decreased FA values for DTI occurred in the brainstem as well (113).

The Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA, **Figure 1**) was selected for the second study because of the reliable, non-surgical, and free head rotation design (168; 183). The device's ability to induce cerebral distortion was supported by a live imaging study of ferrets during CHIMERA, which indicated that brain movement lagged behind the trajectory of the skull (277). A human live imaging study with similar, but non-injurious neck rotations or extensions, also showed changes in strain following different biomechanical rotations with overall higher strain values after rotational movements (137). Biomechanical sex differences have been described with greater peak acceleration and head-neck displacement in females compared to males and decreased head stability in females (254).

BTBI AND LIMBIC SYSTEM

Behavior

It is well documented that bTBI can affect limbic areas and that TBI is a risk for behavioral disorders, most commonly anxiety and depression often related to PTSD. Clinical studies and reports associated with post-mortem findings of chronic bTBI cases revealed histories of enduring neuropsychiatric symptoms (154; 224; 234; 245). Although no gross pathological changes after mild bTBI are reported in rodent models, transient

alterations in home cage activity or increased anxiety with PTSD-like behavior are observed (66; 121; 231; 269). Functional outcomes related to the hippocampus and amygdala following blast in rodents have been assessed for elicitation of contextual fear in the conditioned fear test (66; 97; 187; 236). One study noted early effects at three days and one week after exposure, however, the deficits were diminished eight weeks after single or repetitive bTBI (187). Studies of bTBI and fear conditioning suggest that there could be a delayed onset of PTSD (21; 66; 141; 142). Some behaviors, such as home cage activity might be altered immediately after bTBI, whereas limbic related behaviors, such as heightened cued memory in fear conditioning may appear months after blast exposure (142; 269). Longer time points for behavioral tasks could enable delayed or biphasic behavioral changes to appear after blast (8; 205; 248).

Sex differences

Clinical data show apparent sex differences in the development of psychiatric disorders after mild TBI (18). For bTBI, few clinical studies have provided data, but PTSD is seen in association with other symptoms after TBI, including depression (18; 154), and anxiety, with higher reports of depression in females (120). Much of the bTBI literature focuses only on male animal models (2; 78; 110; 139; 155; 256; 283). However, rodent studies that included both sexes, but not TBI, have reported sex differences. One group described increased risk behavior in male rats in response to negative outcomes compared to female rats (119). In general, female rodents exhibit more activity compared to males during behavioral tasks. The latter observation has been noted as a potential inherent difference that can make data interpretation challenging when studies focus on a single sex (258; 259). One hypothesis for sex differences in anxiety-like behavior is the

higher ratio of corticotropin-releasing factor (CRF)1 to CRF2 neurons in females compared to males. Female mice displayed an increased number of CRF neurons in the amygdala, whereas males demonstrated decreased CRF amygdala activation after bTBI (231).

Amygdala and paraventricular nucleus of the thalamus (PVT)

The basolateral amygdala (BLA) has been studied extensively with regards to its key role in fear and anxiety-like behaviors. Following bTBI, previous studies have reported there are changes to neuronal cytostructure and gene expression in the basolateral nucleus of the amygdala (BLA), and behavioral tasks find enhanced contextual fear response is accompanied by increased activity in the amygdala after TBI (21; 214). Pre-clinical and clinical imaging data indicate more amygdala activation and long-term anxiety post-bTBI (121; 163). These changes in the amygdala are important observations since fear conditioning may have relevance to PTSD, including hyperarousal (66; 204). Region-specific imaging differences in brain metabolism particularly in the amygdala, BBB permeability, and deficits in white matter have also been seen following blast exposure (121; 229). One theory for increased anxiety and hyperactivity after TBI could be the impairment of GABA in the amygdala following injury through loss of GABAergic interneurons and decreased GABA signaling (21).

The paraventricular nucleus of the thalamus (PVT) is another important area mediating limbic responses, including stress, arousal, and motivated behaviors by its projections to the central and basolateral amygdala and prelimbic and infralimbic cortices (11; 135; 227). Some PVT pathways include the PVT–central amygdala–hypothalamus and brainstem system related to autonomic fear and a suggested prelimbic–PVT–lateral

central amygdala pathway implicated in fear learning and fear memory retrieval (61; 135; 199; 203). The PVT and central amygdala facilitate the maintenance of fear memory and freezing behavior. Increased c-Fos activation in these regions was reported in a single prolonged stress mouse model of PTSD (11; 199; 203). When the PVT-nucleus accumbens projections were optogenetically silenced, decreased avoidance behavior occurred during the active avoidance task, but silencing PVT-central amygdala projections increased avoidance, suggesting that the central amygdala may play more of a role in passive instead of active avoidance. In fear memory, the PVT is thought to assist with connecting fear associations to behavioral outcomes rather than acting as a mediator of fear classical conditioning (152). A marker of neuronal activation, c-Fos, becomes elevated in the PVT after stressors, such as the forced swim test and elevated plus maze (15). The PVT's connection to depressive-like behavior is less understood, but one study described reduced tail suspension test immobility with PVT inhibition (15; 128). As a stress memory hub, the PVT is an important area of limbic response study.

c-Fos

One approach to observing limbic brain region responses to activation is the induction of immediate early gene (IEG) expression. Levels of IEGs such as Fos are generally undetectable in the brain under healthy conditions and can serve as a useful marker of brain-region specific neuronal depolarization following a range of extracellular stimuli, including behavioral experiences and injury. Neuronal activation by extracellular stimulation initiates calcium influx that leads to the rapid phosphorylation of the transcription factor, cyclic AMP response element-binding (CREB) protein. A variety of kinases phosphorylate CREB, leading to CREB dimerization and translocation to the cell

nucleus, where the dimer binds to promotor sequences of the *fos* gene, initiating *fos* transcription (77; 169). Since CREB is constitutively present in cells, *fos* transcription does not require protein synthesis for its activation and translation. Subsequent and often coordinated with the induction of *jun* transcription and Jun production, c-Fos protein dimerizes with a variety of Jun proteins to form the dimer AP-1 that regulates subsequent transcriptional events in response to a wide range of tissue adaptive events, including TBI cell injury, immune responses, cellular proliferation and apoptosis (276).

Following mild TBI, several IEGs, including Fos and Jun, are upregulated with the highest expression between 2-4 hours post-injury, and changes in c-Fos have also been reported as early as one hour after blast exposure (195). C-Fos and *fos* mRNA levels display increases in the hippocampus and amygdala after CCI and mild FPI TBI (4; 84; 207; 211; 282). Studies of human contusional brain injury indicate that higher levels of c-Fos in cortical tissue are associated with poorer patient outcomes (276). Only one study has examined IEG response after bTBI and a restraint stressor, which found sex differences in c-Fos response in the paraventricular nucleus of the hypothalamus (232). Additional studies have detected c-Fos activation in limbic and visual regions after blast (64; 217). Learning and memory, particularly fear memory, have also been identified as being regulated by c-Fos (127).

MENINGES PHYSIOLOGY

Anatomy

The three layers of the meninges (**Figure 2**) offer a physical barrier and neurochemical isolation for the brain. The outermost layer, the dura, is arrayed with a mesh-like microstructure that contains a high content of collagen, vasculature, trigeminal

nerve innervation, and lymph vessels. The arteries, veins, and fenestrated vasculature all assist in the transport of neuroimmune cells and signals (42; 130; 172). The thick dura mater (also called the pachymeninges; pachy meaning thick from the Greek pachys) is directly attached to the skull. It is composed of two epithelial layers: an outer, endosteal layer that is adherent to the inner surface of the calvarium (skull cap) and an inner meningeal layer that in certain anatomical regions separates to form septa that surround the venous sinuses, which drain cerebral blood. The dura, arachnoid, and pia layers have different functioning fibroblasts during embryonic development that contribute to meningeal formation (42; 58). The arachnoid layer consists of web-like trabeculae structures made of collagen between the dural meninges and the pia. In gyrencephalic species the arachnoid layer bridges over sulci and its inner surface forms the outer layer to contain the cerebrospinal fluid (CSF) where the subarachnoid space allows cell transport through CSF flow (42; 213). The thin pia mater closely follows the surface of the cerebrum, including dipping down into the sulci. The pial layer covers the surface of the brain and arteries in the subarachnoid space, but provides inconsistent protection for veins (162; 274). The arachnoid and pia are collectively known as the leptomeninges (leptós, Greek meaning thin or slight).

Like the brain, the skull and underlying meninges are heterogeneous and exhibit variability in thickness depending on the cranial location (285). Studies of murine meninges report inter-subject and tissue preparation variability, adding to the difficulty in measuring meningeal thickness. Humans are estimated to have a dural thickness of about 1 mm, whereas pigs are estimated to have a range between 0.35 mm-1.11 mm. The arachnoid thickness is thought to be 200 μm in humans and $27.06 \pm 5.57 \mu\text{m}$ in pigs

(156; 162; 272). In rats, dural thickness is recorded as 80 μm , but there is also much variability in the reporting of rat dura mater thickness estimates, and there is debate whether smaller mammals have subarachnoid space and arachnoid trabeculae at all (70; 156; 272), which were not observed in mice in some studies (167; 191). Human arachnoid trabeculae are heterogeneously distributed and may affect brain deformation after TBI (20).

Barrier properties

Across species (invertebrates to mammals), the meninges are an important protective tissue with stiff physical properties, compared to the central nervous system tissue that they encase (130; 156). The cranial meninges have been examined as an essential contributor for the regulation of healthy brain function, particularly in their role in meningitis, as a site of headache pain, and the neuroimmune responses to infection and injury. Despite the meninges' potential protective properties during TBI, much is still unknown about meningeal responses after injury (70). Some studies show that the meninges help to protect the brain against indentation-type deformations and may reduce strains from other forms of TBI, but possibly only at low energy levels (272). As with astrocytic glial scars after injury, fibrotic scar formation from perivascular fibroblasts influence post-injury inflammatory responses (55). Electron microscope investigation of mouse parietal dura suggest that most blood vessels are located on the dorsal but not the ventral meninges (172) and lymphatic vessels have a basal distribution pattern and are observed in the dura mater alongside channels in the exterior of the skull bone (5; 99; 162). Small channels in the skull have been cited as a possible passage route for cells to

gain access to the meninges (99), but meningeal vasculature also presents opportunities for molecular exchange.

While the dura mater has fenestrated vessels that easily allow molecules to enter the meninges, the pial layer contains blood vessels with tight junctions that enable only smaller molecules (~3 kDa in size) to pass (55; 162; 228). The tight junctions in pial vasculature create a direct barrier to the underlying brain parenchyma, but differ from BBB vasculature in the absence of astrocytic endfeet. The primary physical meningeal barrier lies in the arachnoid layer, which has an arachnoid barrier with tight junctions that selectively monitors the passage of molecules (55; 162; 180). Tight junctions in the arachnoid barrier are conserved across species and provide critical control to the CSF since most molecules in the CSF are able to reach the brain (55; 117). Macromolecules and immune cells preferentially drain through meningeal lymphatic vessels (5).

Neuroimmune response

As noted previously, the meninges provides critical functions beyond acting as a simple physical barrier. For example, studies in the last decade have particularly focused on their role in active immunological surveillance. The meninges contains a gamut of innate resident meningeal cells, including dural macrophages, mast cells, dendritic cells, T cells, B cells, and neutrophils (**Figure 3**), with many of these cell populations distributed near dural sinuses. Peripheral immune cells also pass through the meninges and enter the CNS (55; 125; 228; 233). Recent investigation has discovered critical roles of meningeal lymphatics, where cellular transportation occurs through the basal lymphatic vessels, which are larger than the dorsal vessel, and enable CSF drainage, but also T cell infiltration (5). The meninges are an important basin for T cells (47) that are

involved in learning and memory, such as CD4 T cells that produce IL-4 which are necessary for cognitive function regulation. Cognitive tasks lead to meningeal T cell accumulation, whereas decreases in meningeal T cell-derived IL-4 lead to proinflammatory cytokines and behavioral impairments (54). After TBI, more inflammation occurs if lymphatics are impaired (23). Meningeal macrophages express Class MHC II, which present antigens to CD4 T cells. Additional functions have been identified with other cell populations. There are different subsets of macrophages, such as homeostatic and disease state cells (125), with elongated morphologies that reside along dural blood vessels (130). A study of leptomeningeal macrophages demonstrated that the primary antigen presenting cells for T cells during disease states are from infiltrating dendritic and monocyte-derived cells rather than endogenous macrophages. Meningeal macrophages, however, possibly assist in the recruitment of immune cell infiltration and quickly create macrophages subsets during inflammation (125). Likewise, mast cells in the meninges act as early regulators of barrier integrity. Mast cells and chemoattractants can assist in neutrophil and T cell recruitment. Neutrophils arrive within hours and non-inflammatory macrophages arrive days following injury (42; 233; 274). Finally, microglia are recruited to injury sites by meningeal macrophage death and form a stable honeycomb structure (a few extended processes) between astrocytes in the glial limitans. After astrocytic death, microglia respond with jellyfish morphologies (only one extended process) on the glial limitans to act as a physical barrier to prevent reactive oxygen species from entering the brain (225). McGavern and colleagues demonstrated meningeal repair occurred one week following meningeal compression injury. Initially after injury, peripheral macrophages invaded and inflammatory, classical myelomonocytic cells

scavenged dead cells for removal. Afterwards, non-classical monocytes promoted meningeal angiogenesis. Seven days post-injury, dead cells were removed and new vasculature was created with fibrinogen and platelet formation (233). Meningeal immune cells and responses may play a role after TBI and could lead to secondary damage to the underlying brain parenchyma or assist in injury recovery, but further study is needed.

BLOOD-BRAIN BARRIER (BBB) PHYSIOLOGY

Anatomy

The blood-brain barrier (BBB, **Figure 4**) was first described by Paul Ehrlich in 1885 and is one of three major interfaces of the brain: the BBB, blood-CSF barrier, and blood-meningeal barrier (162). The BBB consists of a neurovascular unit with endothelial cells, basement membrane, pericytes, astrocytes, and microglia. Endothelial cells in the BBB are unique and differ from other endothelial cells throughout the body. BBB endothelial cells surround cerebral vessels, are not fenestrated, consist of tight junctions, and selectively allow for molecular transport. The main proteins that comprise tight junctions are claudins, occludins, and junction adhesion molecules as well as accessory zonula-occludens in the cytoplasm. Molecules are able to pass via passive diffusion, facilitated diffusion, or active transport (65; 126; 162). The glial limitans is a thin layer composed of astrocytic end feet directly below the meningeal pia layer and, although not part of the meninges, the glial limitans is an outer layer of the BBB (228; 263).

Barrier properties

The BBB provides physical separation between the central nervous system and peripheral blood to prevent harmful substances from entering the brain. It is an essential element in brain homeostasis and is one of the first sites to be altered following TBI (78;

110). Under healthy conditions, the BBB maintains homeostasis, controls levels of neurotransmitters, provides a barrier to large plasma molecules, and protects against neurotoxins. Like insults to the meninges, BBB breakdown presents concern for secondary inflammation and damage. Numerous secondary cascade events, including neuroinflammation, involve BBB dysfunction (78; 110; 256). Neurons are always within 20 μm of a capillary, displaying the vital role of vasculature in the central nervous system (62; 190). However, not all brain regions possess BBB protection. The choroid plexus and circumventricular organs, such as the subfornical organ, do not have tight junction barriers (162). The barrier created by the BBB is also dynamic with control over the influx and efflux exchange of small molecules. When the BBB is damaged, the neurochemical environment is disrupted and toxic molecules are able to enter the brain. Acute cerebral vascular impairment and BBB protein dysregulation have been reported after single and multiple ABS exposure, respectively (101; 102; 222). In a bTBI model with non-human primates, astrocytic end-feet hypertrophy and elevated levels of S100 β and aquaporin-4 indicated BBB breakdown. In addition, co-labeled apoptotic markers showed cell death in oligodendrocytes and astrocytes (151). Cerebral blood flow impairment may result after blast-induced BBB damage and could contribute to longer-term deficits. Biphasic BBB breakdown has been reported after CCI with initial BBB openings from 5 minutes to 24 hours and then a second delayed extravasation 3 days after injury (13; 17). A similar BBB biphasic window was observed after bTBI from 0.25 to 75 hours post-blast. Interestingly, the biphasic pattern was only seen after two repeated bTBI, but not after a single bTBI (149). Consistent with this, a different study with single blast exposure only reported BBB changes shortly after injury, but no biphasic effect

(129). BBB permeability, however, also presents therapeutic delivery opportunity for drugs that are typically unable to cross the BBB (1; 92; 190).

MAGNETIC RESONANCE IMAGING (MRI) AND MILD TBI

Noninvasive, *in vivo* imaging is an important tool for clinical diagnosis of TBI. MRI, however, is primarily reserved for cases of moderate or severe TBI (100; 200). The majority of cases are mild TBI, which are clinically defined as not having detectable computerized tomography imaging changes. The lack of sensitivity of traditional imaging methods may contribute to the underdiagnosis of mild TBI (100; 131). Diffusion MRI is favorable for studying mild TBI because of its sensitivity to detecting microstructural changes in water diffusion within tissue (114; 131). As described previously, a common pre-clinical approach for dMRI is utilizing *ex vivo* brain tissue for imaging, which is unrestrained by time limitations and can allow for higher resolution images compared to *in vivo* scanning (131). Diffusion MRI has utility for detecting changes in axonal geometry, which can manifest as axonal varicosities and retraction bulbs causing a decrease in diffusivity (27; 28; 114; 153). Pre-clinical experiments that incorporate imaging often cross-correlate observed changes in imaging with pathological data. Many advances have been made to improve neuroimaging techniques for TBI, but imaging data alone cannot provide information about the mechanism of injury (91; 114; 140).

TRAUMATIC MENINGEAL ENHANCEMENT (TME)

General TME

Contrast enhancement by the administration of agents, such as gadolinium permit greater visualization of the meninges. Distinct enhancement patterns resulting from post-surgery, meningitis, or cancer are associated with inflammation, and contrast agent

accumulation appears as a thick and continuous image profile, indicating physiological vascular and interstitial damage (132; 246; 251). Following mild TBI in humans, traumatic meningeal enhancement (TME, **Figure 5**) has been observed even when other trauma-related imaging changes are not detected (33; 51; 225; 233). TME is not observed after stroke (40) and is distinct from MRI patterns after BBB breakdown because the appearance of TME is limited to the meninges, whereas BBB enhancement occurs in the BBB and immediately spreads to underlying brain parenchyma (263). Early papers involving case studies during medical procedures have described TME, but only recently has more extensive investigation begun into this phenomenon (246). TME presents a more sensitive diagnostic tool for patients with mild TBI.

TME and the meninges may also be an important therapeutic target for the prevention of deleterious secondary damage. TME leads to systemic inflammatory response as indicated by 76 inflammation-related genes that are expressed differently in the peripheral blood of TME versus non-TME patients (148) and could cause an immune response with secondary damage to the brain parenchyma through oxidative stress (225). TME likely indicates the impairments of blood-CSF and BBB with contrast agent extravasation from the blood to CSF and delayed permeability into the subarachnoid space (263). A strong correlation was established between meningeal enhancement and subdural hematoma and loss of consciousness (132). Detection of TME may assist in the prevention of secondary damage especially in cases of mild TBI (148; 225; 263).

TME after mild TBI

The long term implications of TME or the reaction of the meninges after injury are not well understood, but the meninges may be a key component in the TBI recovery

process or therapeutic target for the prevention of subsequent parenchymal damage (42; 225; 263). TME possibly indicates clinically significant injury that includes brain lesions (40; 132). In a study of mild TBI, BBB disruption did not appear on T2-weighted FLAIR MRI, but meningeal enhancement was observed (40). Possible BBB damage is reported in athletes with mild TBI (188), however, and delayed extravasation of gadolinium from TME into the subarachnoid space has been reported and may indicate meningeal and BBB breakdowns (263). The location of TME in patients varies with some enhancement appearing diffuse while others are focal. Common TME sites are the falx cerebri (33; 40; 51; 132; 148; 225; 263), frontal convexity (40; 132; 148; 225; 263), temporal convexity (33; 225; 263), and cerebellum (33; 263). Microhemorrhages due to TBI also vary relative to TME with some adjacent and some contralateral to the site of injury. Traumatic microbleeds have been observed near sites of TME and delayed subarachnoid space contrast extravasation, but do not completely overlap on scans. Microbleeds are persistent on neuroimaging, suggesting that microbleeds indicate injury severity and could be a marker of chronic TBI damage (263). TME duration in humans is uncertain, where enhancement quickly becomes visible after injury, but there is a wide range of reported TME persistence from as short as one day (132), to weeks (233; 263), to multiple year-long enhancement (68; 132; 263). One study observed about 83% of TME cases resolved by about 19 days post-injury, but 17% of TME cases persisted during repetitive imaging around 87 days after injury (233). Human cases of TME are only reported in about half of mild TBI patients, so the detection of meningeal enhancement can be difficult (40; 225). Both the clinical ramifications and underlying mechanisms of TME need investigation.

Mechanisms of TME

The mechanisms for how fluids, such as neuroimaging contrast agents, are able to accumulate in the meninges are still debated. The dura and pia have fenestrated vessels, but the arachnoid vessels have tight junctions. Since the meninges do not have a BBB, the arachnoid and pia to some extent are the only layers that present selectivity through tight junctions, but after injury the barrier is impaired (162). The arachnoid barrier, a physical barrier that the arachnoid vasculature provides through tight junctions, may be structurally impaired after injury allowing the contrast agent to infiltrate (180; 274). Both the arachnoid tear theory and vascular impairment in the arachnoid barrier have been suggested mechanisms of fluid extravasation and accumulation after injury (132; 181; 263). Stress to arachnoid trabeculae and villi, bridging veins, and nearby meningeal lymphatics, as well as the shearing of dural border cells, may also create a pocket of potential space post-injury between the dura and arachnoid layers allowing for contrast enhancement to appear (95; 148; 225; 263; 272). This theory of TME (**Figure 6**), due to newly created subdural space, has been cited by several investigators (51; 148; 263). Groups also described the presence of contrast agent in free fluid that causes T1 relaxation times to be equivalent to that of CSF (33; 225). TME, however, is not observed in all TBI cases and there is reported variability in dural mechanics and thickness, but the physical design of the subdural space may allow for movement during injury to diffuse damage (29; 93; 148; 180; 225; 272). It is also unclear whether contrast agent is able to perfuse underlying brain parenchyma as previously shown by some groups (157), but not others (263). Delayed extravasation of contrast agent into the subarachnoid space was observed in some mild TBI patients who displayed immediate T2-FLAIR meningeal

enhancement (263). A mini review of TME location, duration, and possible mechanism after mild TBI is provided in **Table 2**.

MANUSCRIPTS INTRODUCTION

Taken together, there are a range of experimental questions that could be addressed related to cerebral barrier breakdowns following high velocity impacts and rotational-related TBI. In this thesis, I have focused on two pre-clinical models: 1. ABS shock wave exposure that includes primary overpressure and tertiary acceleration/deceleration injuries and 2. The CHIMERA design with surgery-free, rapid impact rotational-acceleration. For the ABS study, I hypothesized that shock wave exposure will activate limbic regions, particularly the amygdala, resulting in anxiety- and depressive-like behaviors and that this response would be especially pronounced in injured females since women are more likely to develop neuropsychological disorders like depression after TBI. In the CHIMERA study, I hypothesized that dynamic contrast-enhanced MRI will display contrast enhancement in the meninges immediately after injury, but will return to baseline levels by one week post-injury. This would be consistent with previous studies displaying meningeal revascularization one week after compression injury (233). White matter tracts were hypothesized to display enhancement and inflammation immediately after CHIMERA and increased astrogliosis due to pro-inflammatory reactions in injured mice characterized in CHIMERA literature. This project focused on behavioral and neuropathological outcomes from shock wave and impact-acceleration exposure.

Table 1: Review of CHIMERA literature following McNamara et al., 2020.

Table 1. Review of CHIMERA Literature Following McNamara et al., 2020*						
Publication	Animals	Injury parameters	Morbidity	Behavior	Electrophysiology/Imaging	Pathology
Button et al., 2021	Male and female C57BL/6 or APOE3 3.5 to 5 months	1 x 2.5 J with PLA interface 1 mg/kg Meloxicam 5%/2-4% Isoflurane	Not reported			Blood plasma showed elevated GFAP 6 hours post-injury
Chen et al., 2021	Male and female C57BL/6 and GPR110 knockouts 2-4 months	1x or 3x 0.55 J 24 hrs apart 5% Isoflurane	Not reported			A8 or synaptamide attenuated TNF mRNA and OPT deficits in wildtype mice
Desai et al., 2021	C57BL6/NCr 3 months	3x 0.55 J 24 hrs apart Isoflurane	Not reported	MWM performance was not as impaired as deficient diet group		High n-3 polyunsaturated fatty acid diet attenuated gliosis in OPT, CC, HP and OPT deficits
Feng et al., 2021	Male Sprague-Dawley rats	6x 2 J, 3 impacts/day spaced 1 min apart Dexmedetomidine Impact near bregma	No mortality	Reduced cued fear memory with delayed fear conditioning, no trace fear conditioning changes, novel object recognition impaired	<i>In vivo</i> single unit recordings increased mEPSC amplitude, decreased mIPSC frequency in mPFC; FCCP infusion showed impaired mitochondrial function	Increased GFAP in mPFC and dentate gyrus, increased Iba-1 in dentate gyrus
Hutchinson et al., 2022	Male Ferret 6-26 months	Variety 1-5x with 15-75 psi spaced 5 mins apart Custom helmet 5% Isoflurane	Not reported		T2 hyperintensity and DTI decreased FA in brain stem, contrast enhancement in brain stem, thalamus T2 FLAIR meningeal enhancement	
Reyes et al., 2020	Male C57BL/6 3-4 months	3x 0.55 J 24 hrs apart 5% Isoflurane in O ₂ impact near bregma	No mortality		<i>In vivo</i> and <i>ex vivo</i> DTI displayed reduced FA in OPT and brachium of superior colliculus, but adequate omega-3 polyunsaturated fatty acid diet did not attenuate white matter damage	
To et al., 2021 roadmap	Male mice 3-4 months	1x 5.2-5.4 m/s 2-3% Isoflurane in 60% air 40% O ₂	Not reported	Behavioral deficits up to one week post-injury and recovered by two weeks	<i>In vivo</i> DTI, NODDI, and fMRI changes one week post-injury and continued two weeks	
To et al., 2021 ultra-high-field	Male C57BL/6 7-7.5 months	High concussion (5.36 m/s) and low concussion (<5.1 m/s)	Not reported		<i>Ex vivo</i> : Low concussion group showed increased FA and decreased ODI in grey	Increased GFAP in HP, CC, and fimbria, increased Iba-1 in

			Range 4.7 to 5.7 m/s 2-3% Isoflurane in 60% air 40% O ₂			matter, but little white matter damage <i>Ex vivo</i> : High concussion group had increased mean diffusivity, decreased ODI and neurite density index in grey and white matter; decreased FA in white matter	dentate gyrus and CC, decreased Iba-1 in caudate putamen, and increased silver stain in retrosplenial cortex dentate gyrus, CC, internal capsule, fimbria
Tucker et al., 2021	Male and female C57BL/6J 8 weeks	4x 0.7 J 24 hrs apart 3% Isoflurane in 100% O ₂ , 2.5% anesthesia impact dorsal cortical region	7.5% mortality	MWM and trace fear conditioning showed hippocampal- dependent deficits with greater impairment in male mice			Silver stain and GFAP in white matter and brainstem including the CC, fimbria, and cerebral peduncle; no GABAergic parvalbumin changes
Sun et al., 2022	Male and female C57BL/6J and Thy1-YFP-H 8-12 weeks	1x 0.9 J 1 mg/kg Meloxicam 4% Isoflurane in O ₂ Midpoint between bregma and lambda	3% mortality				Axonal varicosities in external capsule, CC, and cortex layer VI within 2 mins after impact
Vonder Haar et al., 2022	Male Long Evans rats 5-6.25 months	4-8x 3.2 J rubber tip 3-6x 2.45 J metal tip 5x 2.4 J 5 mg/kg ketoprofen 5% Isoflurane	No mortality with rubber tip 3% mortality with metal tip 12% total mortality	5CSRT and DDT attentional deficits 5CSRT motor impulsivity increase, DDT choice impulsivity decrease			6x and 8x fewer reactive microglia in mPFC, increased in OFC and NAc, decreased GFAP+ area in dSTR 3x and 4x increased reactive microglia in OFC and NAc, increased GFAP in mPFC, OFC, dSTR, and NAc, decreased GFAP+ area in NAc

* Abbreviations: 5CSRT (five-choice serial reaction time); A8 (dimethylsynaptamide); CC (corpus callosum); DDT (delay discounting task); dSTR (dorsal stratum); DTI (diffusion tensor imaging); FA (fractional anisotropy); FLAIR (fluid-attenuated inverted recovery); GPR110 (G-protein receptor 110); HP (hippocampus); mEPSC (miniature excitatory postsynaptic current); mIPSC (miniature inhibitory postsynaptic current); mPFC (medial prefrontal cortex); MWM (Morris Water Maze); NAc (nucleus accumbens); NODDI (neurite orientation density dispersion imaging); ODI (orientation dispersion index); OFC (orbitofrontal cortex); OPT (optic tract)

Table 2: Mini review of traumatic meningeal enhancement from mild TBI.

Table 2. Mini Review of Traumatic Meningeal Enhancement from Mild TBI*					
Publication	Number of participants	Time after injury (within)	Location	Duration	Stated mechanism
Castro et al., 2016	17 all TMI	96 hours	13 regions: temporal, occipital lobe, falx, cerebral tentorium	Not stated	Gd in space with free fluid
Chiara Ricciardi et al., 2017	22 TBI, 10 TME+	48 hours	4 diffuse, 3 focal frontal convexity, 3 falx	Not stated	Not stated
Davis et al., 2020	25 total	48 hours	Falx and right convexity	Not stated	Gd in subdural space
Hutchinson et al., 2022	11 Ferrets, 2 severe impact	3 hours, 1 week, 4 weeks	Dorsal midline meninges near olfactory bulb and brainstem	T1W-MDEFT 3 hours, little 1 week, none at 4 weeks T2 FLAIR only 3 hours	Not stated
Kim, et al., 2014	54 total, 32 TME+	14 days	12 diffuse, 8 frontal lobe some ipsilateral some contrecoup, 12 falx	3 with repeated scans, 2 still TME 1 week later and 1 resolved 2 years	Not stated
Livingston, et al., 2017	40 total, 17 TMI	48 hours	Frontal lobe, falx	Not stated	Dura or subdural space
Rizk et al, 2020	30 total, 16 TME	48 hours	Not stated	63% resolved 1 year later	Not stated
Roth et al., 2014	142 total; 69 had TME+	48 hours	Focal frontal, temporal lobes, falx	Not stated	Gd in space with free fluid
Russo et al., 2018	209 total, 104 TME+	48 hours	Not stated	Resolved in 83% patients average by day 18.9 from injury, 17% still TME on day 87	Not stated
Turtzo et al., 2020	Study 1: 36 total, 12 TME+ Study 2: 32 total, 23 TME+, 18 ECSAS	6 hours then 3 hours after first scan	TME: 30 regions, falx, vertex, frontal, temporal, and parietal occipital lobes, cerebellar tentorium, and superior sagittal sinus ECSAS: falx, convexity, cerebellum	50% still TME 1 week later	Gd in subdural space, bridging veins, arachnoid trabecula and villi, and meningeal lymphatics shear stress then delayed to subarachnoid space

*Abbreviations: ECSAS (extravasation of contrast into the subarachnoid space), falx (falx cerebri), FLAIR (fluid-attenuated inverted recovery); TME+ (traumatic meningeal enhancement positive), TMI (traumatic meningeal injury); T1W-MDEFT (T1-weighted modified driven equilibrium Fourier transform)

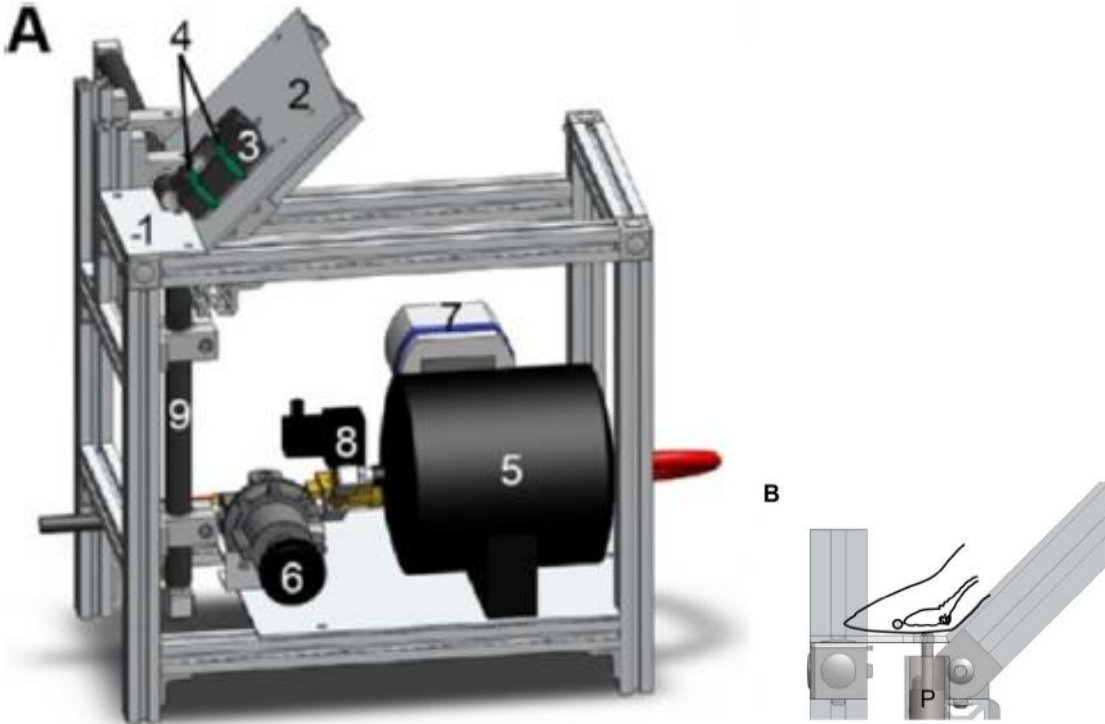


Figure 1: The mouse CHIMERA device.

A) The mouse CHIMERA device used for single and repetitive CHIMERA experiments. In the figure an animated mouse is placed in a supine position on the animal bed where **B)** the piston is driven upward to impact the dorsal head near bregma. Figure reprinted with permission (183).

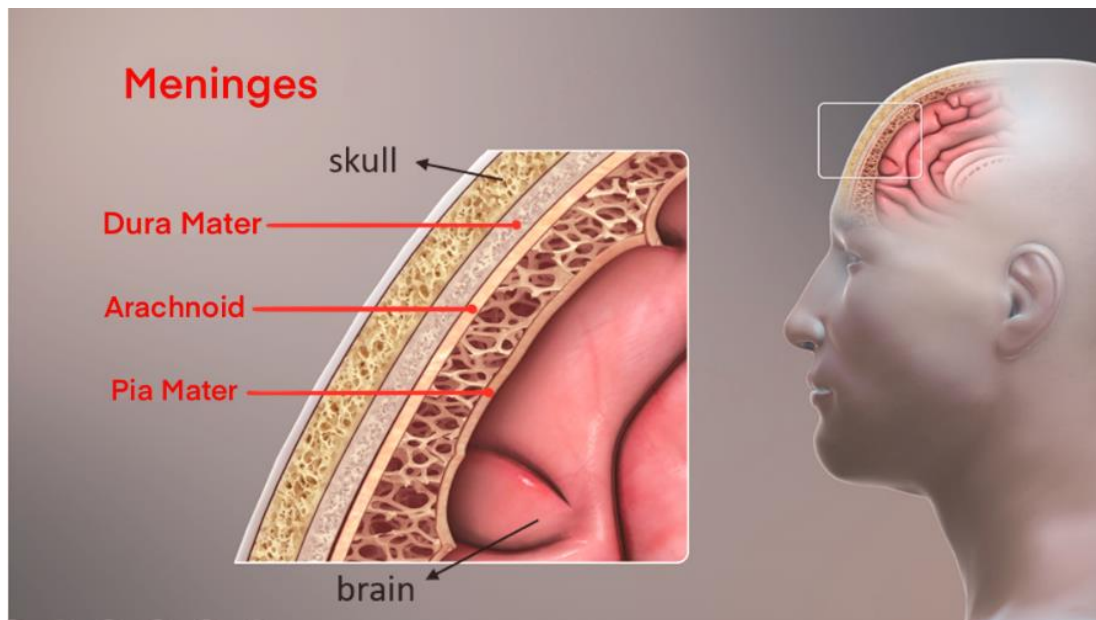


Figure 2: Meningeal layers.

The meninges consist of three layers of tissue between the skull and brain. The dura mater is the thick outer most layer, the arachnoid layer primarily contains cerebrospinal fluid in the subarachnoid space, and the thin pia mater attaches to the brain parenchyma. Figure reprinted and adapted with permission (43).

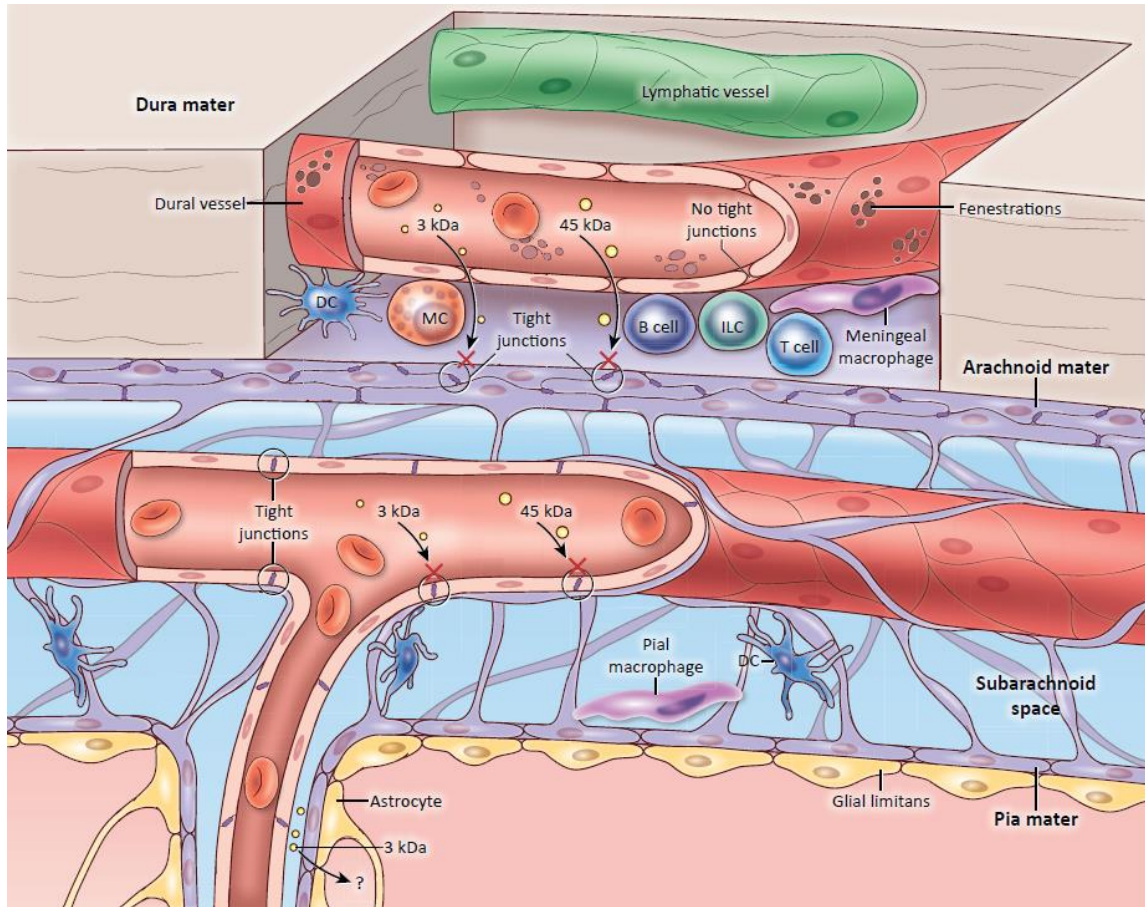


Figure 3: Meningeal anatomy and immune cells.

Detailed schematic of healthy meningeal anatomy and relevant neuroimmune cells, such as meningeal macrophages. Vessels in the dura are fenestrated and allow different sized molecules to pass through, but vessels and dura-arachnoid border tissue (arachnoid barrier) in the subarachnoid space have tight junctions, which provide a selective physical barrier. Figure reprinted with permission (228).

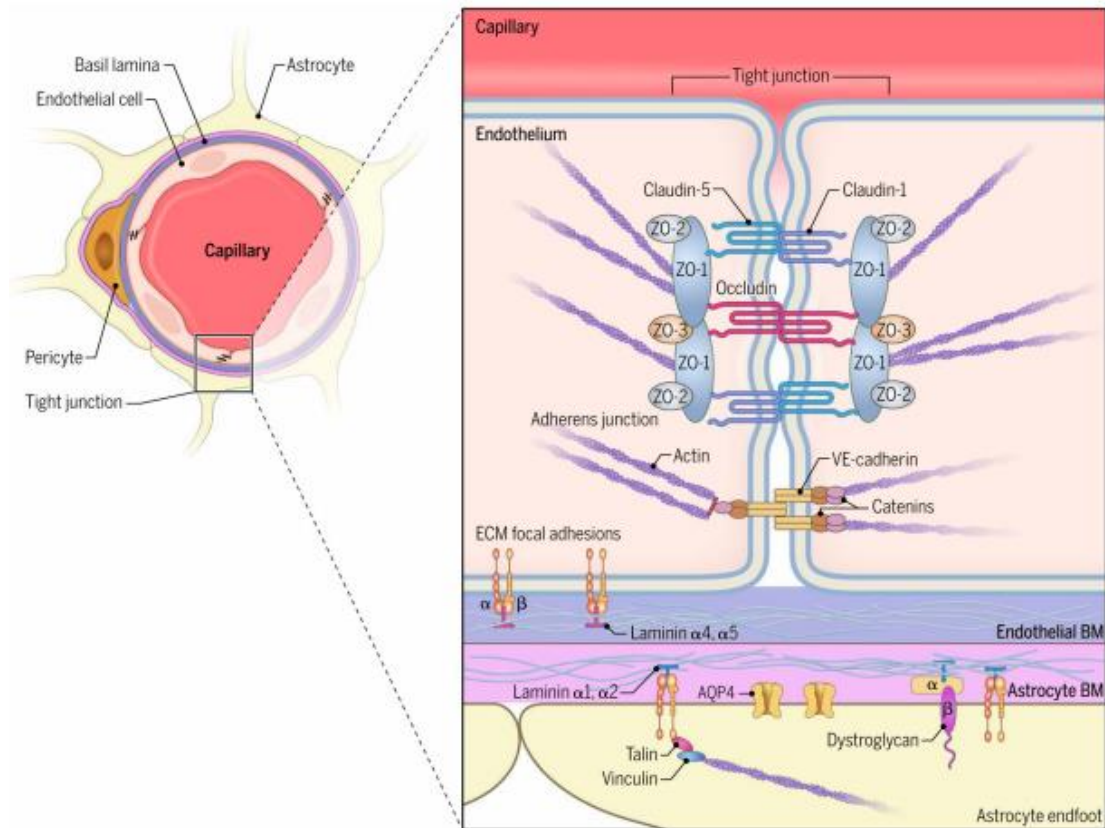


Figure 4: Transverse schematic of the blood-brain barrier.

The astrocytic endfeet compose the outer layer of the BBB followed by layers of laminin outlining the endothelial cells with tight junctions that lead to the blood vessel. The main proteins in the tight junction that assist with transportation between the tight junction gap are claudin, occludin, and zonula occludens. Figure reprinted with permission from AAAS (162).

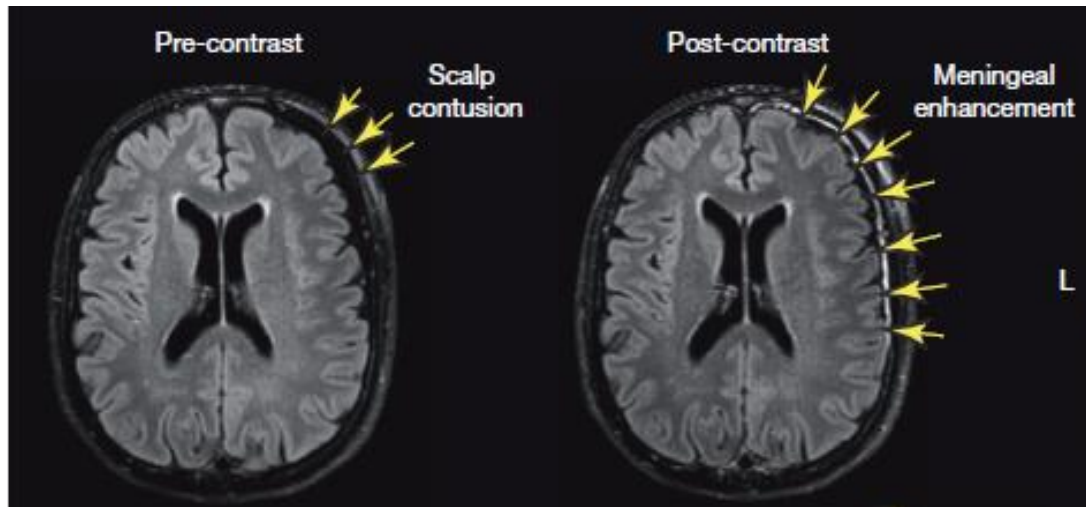


Figure 5: Human TME viewed on T2 FLAIR MRI.

TME observed through FLAIR T2-weighted post-contrast imaging of a patient 19 hours after falling down. The pre-contrast T2-weighted image shows scalp contusion. TME is evident after gadolinium administration, on the post-contrast image. Figure reprinted with permission (225).

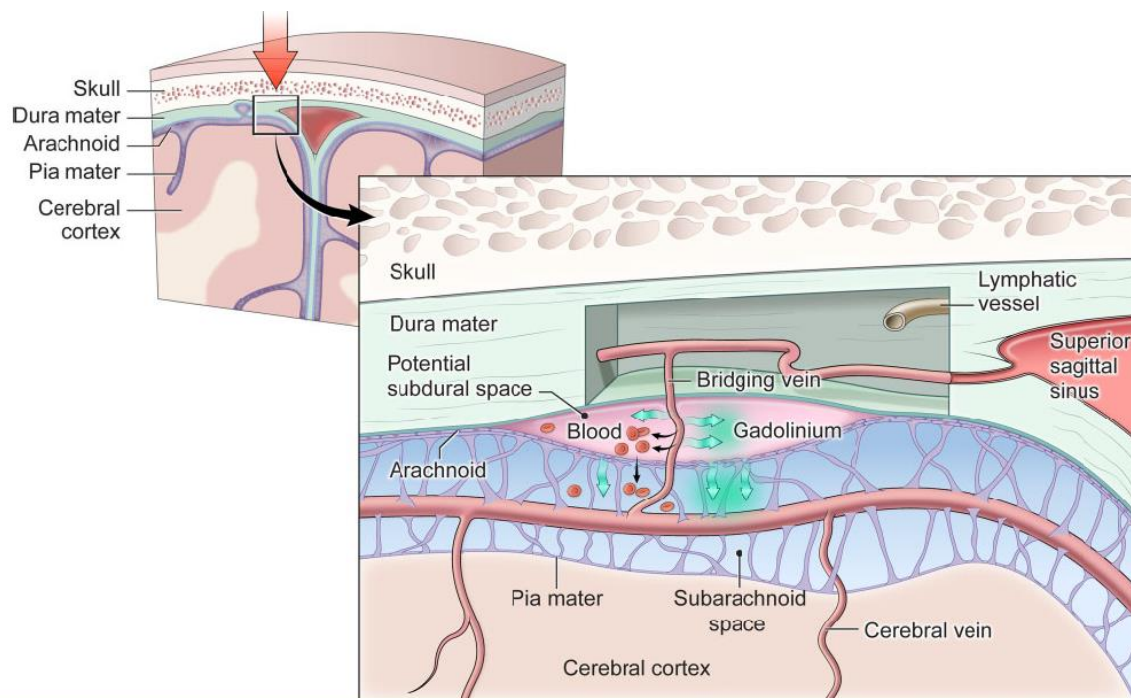


Figure 6: A potential TME mechanism after mild TBI.

Damage to bridging veins in the dura and meningeal lymphatic vessels, as well as stress to arachnoid trabecula and villi may help in the creation of a subdural space for gadolinium contrast agent to leak from the blood and accumulate. Figure reprinted with permission (263).

CHAPTER 2: Limbic responses following shock wave exposure in male and female mice

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ABSTRACT

Blast traumatic brain injury (bTBI) presents a serious threat to military personnel and often results in psychiatric conditions related to limbic system dysfunction. In this study, the functional outcomes for anxiety- and depressive-like behaviors and neuronal activation were evaluated in male and female mice after exposure to an Advanced Blast Simulator (ABS) shock wave. Mice were placed in a ventrally exposed orientation inside of the ABS test section and received primary and tertiary shock wave insults of approximately 15 psi peak pressure. Evans blue staining indicated cases of blood-brain barrier breach in the superficial cerebral cortex four, but not 24 hours after blast, but the severity was variable. Behavioral testing with the elevated plus maze (EPM) or elevated zero maze (EZM), sucrose preference test (SPT), and tail suspension test (TST) or forced swim test (FST) were conducted 8 days–3.5 weeks after shock wave exposure. There was a sex difference, but no injury effect, for distance travelled in the EZM where female mice travelled significantly farther than males. The SPT and FST did not indicate group differences; however, injured mice were less immobile than sham mice during the TST; possibly indicating more agitated behavior. In a separate cohort of animals, the

expression of the immediate early gene, c-Fos, was detected four hours after undergoing bTBI or sham procedures. No differences in c-Fos expression were found in the cerebral cortex, but female mice in general displayed enhanced c-Fos activation in the paraventricular nucleus of the thalamus (PVT) compared to male mice. In the amygdala, more c-Fos-positive cells were observed in injured animals compared to sham mice. The observed sex differences in the PVT and c-Fos activation in the amygdala may correlate with the reported hyperactivity of females post-injury. This study demonstrates, albeit with mild effects, behavioral and neuronal activation correlates in female rodents after blast injury that could be relevant to the incidence of increased post-traumatic stress disorder in women.

Keywords: anxiety, blast traumatic brain injury, blood-brain barrier, c-Fos, depression, limbic, post-traumatic stress disorder, righting reflex

1. INTRODUCTION

Blast exposure is the leading cause of traumatic brain injury (TBI) in military personnel and a serious threat to civilian populations in proximity to regional conflicts and civil unrest. Blast TBI (bTBI) is considered the “invisible wound” in modern day combat zones, such as Iraq and Afghanistan, and milder injury with ~80% prevalence is the most common form. Blast exposure has been associated with a variety of psychiatric conditions, including post-traumatic stress disorder (PTSD), depression, and anxiety (271). Post-mortem examination of chronic bTBI cases also found histories of enduring neuropsychiatric symptoms (154; 224; 234; 245).

Pre-clinical reports have shown CNS limbic system areas are particularly vulnerable to bTBI, and are often associated with neurobehavioral changes related to anxiety, depression, and PTSD (21; 66; 142). Alterations in the basolateral amygdala (BLA) have been reported following bTBI with changes to neuronal cytostructure, gene expression, and neuroimmune responses with associated anxiety-like behavior (21; 214; 236). Likewise, blast injury in rats altered a marker associated with PTSD, stathmin 1, in the amygdala, but not in the hippocampus (66). However, ultrastructural rat hippocampal changes have been reported after blast (35). The paraventricular nucleus of the thalamus (PVT) is another important area for emotion-based responses, especially fear. The PVT is primarily involved in stress, arousal, and motivated behaviors with projections to the amygdala and limbic cortex (11; 135; 227), and it was recently found to mediate PVT-central amygdala freezing responses (152). The PVT's connection to depressive-like behavior is less understood, but reduced tail suspension test immobility with PVT inhibition has been reported (15; 128). Other cortical changes after bTBI include a decrease in Thy-1 stained cortical neuronal afferents, possibly from the medial prefrontal cortex, which terminate in the BLA (97). These limbic regions are also involved in fear conditioning; a common pre-clinical model for evaluating PTSD. Blast exposure increased responses in the acoustic startle reflex and anxiety-related behaviors in the elevated plus and zero mazes (10; 279). Pre-clinical and clinical imaging data following blast exposure correspondingly indicate greater amygdala activation and long-term anxiety post-bTBI, including region-specific imaging differences in brain metabolism in the amygdala and blood-brain barrier (121; 163; 229).

Acute immediate early gene (IEG) responses in the cerebrum are observed after a broad range of stimuli including cell insults, immune activation, apoptosis, neuronal depolarization, and learning and memory experiences (36; 50; 77; 212). Alterations in c-Fos expression may play a role in encoding transient stimuli to long-term genetic changes. A marker of neuronal activation, c-Fos expression becomes elevated in the PVT after stressors such as the forced swim test and elevated plus maze (15; 50; 77). Changes in c-Fos have been reported as early as 1–3 hours after blast exposure in the hippocampus and amygdala (64; 195; 217; 237), and elevated levels can persist (232; 237). As described earlier, the PVT and central amygdala are involved in fear conditioning, and increased c-Fos activation in these regions was reported in a single prolonged stress mouse model of PTSD (11; 199; 203). Only one study has examined IEG response seven days after a restraint stressor and bTBI, and found sex differences in c-Fos response in the paraventricular nucleus of the hypothalamus (232). Sex differences in functional outcome are also observed with more activity displayed by females compared to males after TBI (258; 259). One group described increased risk behavior in males in response to negative outcomes, lack of reward during a gambling task with the choice to select a more secure option, compared to female rats (119).

The objective of this exploratory study was to determine how a shock wave exposure alters physiological and behavioral outcomes. To assess the effects of primary and tertiary shock wave injury, the Advanced Blast Simulator (ABS) was utilized as a reliable state-of-the-art model of bTBI (241). The elevated plus and zero maze (EPM, EZM), sucrose preference test (SPT), tail suspension test (TST), and forced swim test (FST) were performed to assess anxiety- and depressive-like behaviors after ABS, while

blood-brain barrier (BBB) and c-Fos neuronal activation were studied for acute pathological changes.

2. MATERIALS AND METHODS

2.1 Animals

Eight-week old male and cycling female C57BL/6J mice (00664) were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in an AAALAC-accredited animal facility for at least three days of acclimation before the experiments were started. All procedures were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee. Until the SPT was conducted, all animals were group-housed (five per cage), had access to food (Harlan Teklad Global Diets 2018, 18% protein) and water *ad libitum*, and were maintained on a standard 12 h: 12 h light-dark cycle. Animals that underwent the SPT were divided into separate cages with standard enrichment (cotton nestlets and huts) for the duration of the study, but singly housed mice were still able to view neighboring cages. All experimental procedures were performed by female investigators.

2.2 Advanced Blast Simulator (ABS)

Male and female mice were randomly assigned to injured or sham conditions. Injured groups were exposed to a single blast overpressure ~ 15 psi peak pressure using the USUHS Advanced Blast Simulator (ABS) as previously described (269). Briefly, the ABS contains a driver and driven chamber with pressure transducers placed within the inner wall to monitor incident pressure and shock wave velocity. A pencil gauge probe immediately adjacent to the mouse holder measured incident pressure (Quartz, free-field, ICP blast pressure pencil probe, 50 psi, 104.2 mV/psi, 137B23A, PCB Piezotronics). A

membrane consisting 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: 14.5×10 grids/in², wire diameter: 0.635 mm) separated the driver and the driven chambers. Sham-treated mice were anesthetized and placed near the blast chamber, but were not exposed to the blast wave. All mice were first placed in an isoflurane induction chamber (3% isoflurane in 100% oxygen for 4–6 min) and once anesthetized, head and body wraps (a modified tongue depressor and Vet Wrap) were used to minimize movement before the mouse was placed inside the simulator. A hatch in the driven chamber allowed the ABS mouse to be secured in a mesh holder (same material as the membrane, 14 cm $\times$ 15 cm with a cross-sectional areal occlusion of ~5.6% for the shock wave) supported by metal posts (12.7 mm diameter, about 2.9 m distal to the driver membrane) in a vertical orientation, exposing the ventral surface of the mouse to the oncoming blast wave. Once the hatch to the driven chamber was tightly sealed with the mouse inside the ABS, compressed air was allowed to accumulate in the driver end, until the pressure (150–160 psi) was great enough to rupture the membrane and the shock wave travelled down the ABS to where the animal was placed (about 2.9 m away from the membrane). After the blast wave was delivered, the mouse was removed from the mesh pocket and assessed for the occurrence of apnea. Sham-treated mice were placed in a clean cage in a supine position outside of the ABS, and the latency to recover the righting reflex was recorded for all mice. Once both ABS and sham animals regained consciousness, they 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). Animals were weighed both immediately before and one day after ABS exposure.

2.3 Behavioral Testing

Animals were randomly assigned to different behavioral task cohorts. The five cohorts consisted of $n = 18\text{--}20$ mice evenly distributed between males and females as well as injured and sham conditions (**Figure 1**). Following ABS as described above, each cohort underwent one of two testing paradigms: Elevated Plus Maze (EPM, 8 days post-injury) then Sucrose Preference Test (SPT, 2 weeks post-injury) and Tail Suspension Test (TST, 3.5 weeks post-injury), or Elevated Zero Maze (EZM, 8 days post-injury) then Sucrose Preference Test (SPT, 2 weeks post-injury) and Forced Swim Test (FST, 3.5 weeks post-injury).

2.4 Elevated Plus Maze (EPM)

The EPM (Stoelting Wood Dale, IL, USA) was conducted eight days following ABS to assess anxiety-like behavior (202). The EPM is a cross-shaped platform with equal 35 cm length and 5 cm width arms raised 50 cm above the floor. Two opposite side arms are “open,” with 1 cm high edges and the remaining arms are “closed” with opaque, dark 16 cm high walls (**Figure 1**). Animals were allowed to acclimate to the room for 30 minutes before testing began. Overhead fluorescent lights illuminated the maze during testing with 1600 lux illuminance for the open arms and 200 lux for the closed arms. To start the test, individual mice were placed at the 5 cm center square region and allowed to explore the maze for 5 minutes. A ceiling camera and Any-Maze software (Stoelting) tracked animal movement throughout the test and were used to calculate the time spent in the open and closed arms, distance travelled, and number of entrances to the open arms during the session.

2.5 Elevated Zero Maze (EZM)

The Stoelting EZM (244) as previously described by Tucker et al. (258) was performed eight days post-injury to examine anxiety-like behavior. Briefly, the EZM is a 60 cm diameter ring platform raised 50 cm above the floor. The ring is divided into four equally sized areas with two opposite side “open” quadrants and two remaining “closed” quadrants (**Figure 1**). The open quadrants have 1 cm high edges and are exposed to 1600 lux light from overhead fluorescent lamps whereas the closed quadrants have 16 cm high dark, opaque walls and have a brightness of 200 lux. All quadrants have 5 cm width lanes. Animals spent 30 minutes acclimating to the room before starting the test. At the beginning of the test, mice were placed individually at an arbitrary boundary between an open and closed quadrant facing the closed quadrant. Animals were allowed to freely explore the maze for five minutes and a ceiling camera tracked the animal movement throughout the test. Any-Maze software was used to calculate the time spent in the open and closed quadrants, distance travelled, and the number of entrances to the open quadrants during the test.

2.6 Sucrose Preference Test (SPT)

The SPT was administered two weeks following injury as a measure of anhedonia. Due to laboratory spatial and time constraints, housed mice cages were randomly selected so that ~57% of the mice were tested. Cages of mice not selected for testing remained group housed. Individually housed mice were evaluated over the course of five days. Mice were offered two 20 mL bottles of 1% sucrose diluted with water placed about 7.5 cm apart at equal heights in their home cages for the first two days of testing to acclimate them to the sweet taste and to the new bottles. The amount of sucrose consumed was measured by weighing each bottle daily. On the third day, one bottle was

replaced with filtered tap water, so that each mouse was given a choice of drinking solutions of sucrose or tap water. On the fourth day, the positions of the bottles were switched to control for potential side bias. SPT bottles were again weighed and the sucrose preference ratio was calculated for the final 48 hours of testing with the following

$$\text{equation: } \textit{sucrose preference ratio} = \frac{\textit{consumed sucrose}}{\textit{consumed water} + \textit{consumed sucrose}} \times 100$$

2.7 Tail Suspension Test (TST)

The TST to measure depressive-like behavior was performed 3.5 weeks after ABS. As previously described by Can et al. (31), mice were suspended from their tails from laboratory benches with tape (12 mm wide, 24 cm long). The tape was adhered about 1 cm from the tip of the tail and a 4 cm length hollow polycarbonate tube (1.3 cm inner diameter, McMaster-Carr, Santa Fe Springs, CA #8585K41) was placed around the base of the tail to prevent tail-climbing during the test. Padding was placed below the mice in case of falls and mice were monitored throughout the six minute test. A standard video camera was used to record the sessions and videos were uploaded into Any-Maze. The time spent immobile (defined as animals with minimal movement) during the last five minutes to account for initial test acclimation of the session was later scored using Any-Maze with manual key presses by an investigator blinded to injury condition where a computer key was held down for the duration of animal immobility.

2.8 Forced Swim Test (FST)

The Porsolt FST (208) to study learned helplessness was conducted 3.5 weeks post-injury. As described by Tucker et al. (258), FST chambers (Stoelting) were clear 42 cm in height and 19 cm diameter Plexiglas cylinders. The chambers were filled to a depth

of about 25 cm with water at 25°C. Mice were placed into the cylinders for six minutes and allowed to swim or float. Mice were closely monitored from a separate room. Once the test concluded, mice were gently dried with paper towels and placed in a clean cage under a heat lamp to dry. FST cylinders were rinsed and replaced with fresh water for each animal. A standard video camera was used to record sessions and videos were imported into Any-Maze with key presses to measure immobility (defined as animals floating on the water surface with minimal movements). The first minute of the test was not scored to account for initial acclimation to the FST, so an investigator blinded to injury condition only scored the last five minutes of the test.

2.9 Immunohistochemistry

A separate cohort of animals, which did not undergo behavioral testing, were exposed to a single ABS shock wave (about 15 psi) and examined for the presence of Evans blue as a marker of blood-brain barrier disruption. An animal restrainer was used to intravenously administer Evans blue (2% diluted in buffer, 0.1 mL per animal) via the tail vein immediately prior to ABS exposure. The mice were euthanized 4 hours ($n = 25$, $n = 6-7$ per injury and sex condition) or 24 hours ($n = 15$, with Evans blue injection after ABS) post-injury and tissue was collected for c-Fos immunohistochemistry from only the 4 hour group. Briefly, mice were anesthetized with a mixture of ketamine and xylazine and then transcardially perfused with cold phosphate buffer solution (0.1 M) and 4% paraformaldehyde in phosphate buffer. Brains were dissected and further fixed in paraformaldehyde for an additional 24 hours. They were then transferred to 20% sucrose solution in phosphate buffer for 72 hours before freezing the tissue and storage (-80°C) until sectioning. A Leica microtome was used to cut 30 μ m thick coronal sections. The

tissue was initially washed in tris-buffered saline with 0.05% triton (TBS-T). Sections were then processed with 0.3% hydrogen peroxide for 30 minutes and afterwards washed with TBS-T again before blocking buffer (TBS-T with 0.20% triton, goat serum, and 10% bovine serum albumin; BSA) incubation for 1 hour at room temperature. C-Fos (1:1000; Millipore Sigma Cat: ABE457, Lot: 3585299) antibody was applied to the sections before storage at 4°C overnight. The next day, sections were washed with TBS-T and secondary antibody goat-anti-rabbit IgG (1:1000 Jackson ImmunoResearch Cat: 111-065-003, Lot: 117316) was applied in blocking buffer (TBS-T with 0.05% triton, goat serum, and 10% BSA) for 1 hour at room temperature. Sections were again washed with TBS-T before incubation in ABC solution (Vectastain ABC HRP Kit, PK-4000, Vector Laboratories) for 45 minutes at room temperature. The tissue was washed a final time with TBS-T prior to DAB development with the DAB Substrate Kit, Peroxidase (HRP), with Nickel, 3,3'-diaminobenzidine (SK-4100, Vector Laboratories) for 1 minute. The free-floating sections were mounted onto glass slides and left to dry overnight. Lastly, sections were dehydrated in ethanol gradients (75–100%), cleared in xylene, and cover slipped with Permount mounting media the next day for analysis. Positive, activated neural tissue from restrained ABS-exposed mice, and negative, tissue processed without primary antibody, controls were included in all immunohistochemistry procedures.

Three regions of interest (ROIs), bilateral cerebral cortex, bilateral amygdala, and the paraventricular nucleus of the thalamus (PVT), were analyzed for c-Fos staining. Six mice per group were selected for Carl Zeiss El-Einsatz model #451485 light microscope imaging. Images for the PVT were taken at 100x magnification, the cerebral cortex at 50x, and the amygdala was captured at 25x total magnification. To quantify c-Fos in each

ROI, the threshold and particle analysis functions on Image J software (NIH) were used for cell counts on black/white c-Fos images. The particle count was employed as an estimate for the number of c-Fos positive cells. The values were averaged for three or more sections per animal. Cresyl violet (Chroma-Gesellschaft Schmid & Co 1 A 396) and H&E (hematoxylin Sigma-Aldrich Cat: GHS132-1L, Lot: SLCH6216 and eosin Sigma-Aldrich Cat: HT110332-1L, Lot: SLCJ2544) staining were performed to indicate anatomical consistency for the ROIs and for microbleed analysis, respectively.

2.10 Statistics

Graphpad Prism version 8.42 (GraphPad Software, San Diego, CA) and SPSS version 27.0.1.0 (IBM, Armonk, NY) were used for statistical analysis and figure generation. Body weights were measured on the day of injury and 24 hours later. Since there was a noticeable difference in baseline (before injury) body weights, a two-way Injury x Sex analysis of variance (ANOVA) was performed for the mice as a percentage of body weight loss after sham or injury treatment. Mann Whitney U-tests were performed to analyze righting time data using within sex comparisons and between sex comparisons for male ABS and female ABS groups. Photographs of the brains from the cohort of mice that were euthanized after Evans blue infusion and ABS exposure were visually ranked for degree of staining. The rankings for Evans blue staining intensity were evaluated with respect to righting reflex to determine if there was an association between staining and the duration of the righting reflex. The SPSS program (Version 28.0.1.0) to determine Kendall's tau (τ) was employed and a bootstrapped 95% confidence interval for the correlation coefficient, based upon 1000 bootstrapped samples, was performed.

Two-way ANOVAs (injury x sex) were performed for all behavioral tasks (EPM, EZM, SPT, FST, and TST). Behavior graphs depict the scores for individual animals and the embedded horizontal bar shows the arithmetic means for each group. Histology for c-Fos was analyzed using two-factor (injury x sex) ANOVAs. $p < .05$ was considered significant.

3. RESULTS

3.1 Shock Wave Characteristics, and Mouse Body Weight, Righting Times, Apnea, Morbidity

The ABS produced a characteristic Friedlander-like curve with a consistent peak pressure across the study (mean 15.56 psi, coefficient of variation = 4.5%) (**Figure 2**). The shock wave velocity was approximately 469.73 m/s and the positive and negative phases were 5.62 and 8.40 msec, respectively, with an average impulse (pressure x time) of .0358 psi*sec.

Figure 3 summarizes the body weights of individual female and male mice before and after sham or ABS exposure. Since the baseline (pre-injury) body weights for the mice in the ABS and sham groups were not equivalent, an Injury x Sex ANOVA was computed using the percentage change in body weights after ABS injury or sham treatment compared to pre-injury body weights. The average percent change in body weight was -2.23% and 0.574% for the female and male mice, respectively, on the day after sham treatment, while the females lost -8.22% and the males -5.79% of their preinjury body weight after ABS exposure. The ANOVA indicated there was a significant overall main effect between the females and males in percentage of body weight loss before to after sham or ABS treatment (-2.619% change, $F_{1,76} = 24.214$, $p < .001$) and a difference in the percentage of body weight loss after ABS injury compared

to sham treatment (-7.002% , $F_{1,76} = 134.584$, $p < .001$), but no interaction effect to suggest the injuries had a different effect on female and male mice ($F_{1,76} = 0.123$, $p = .727$).

Behavior and Evans blue-treated cohorts were combined for righting reflex analysis. Mann Whitney U analyses showed injured animals required a longer time to regain consciousness compared to sham mice. Female shock wave exposed mice (462.5 sec median) had increased righting times compared to female sham mice (46.0 sec median; $U = 3$, $p < .0001$) and male shock wave-exposed mice (316.0 sec median) displayed longer righting reflexes than male sham animals (43.0 sec median; $U = 1.5$, $p < .0001$). A third Mann Whitney test showed injured male and female mice were significantly different, with male animals exhibiting overall shorter reflex durations than female mice ($U = 370.5$, $p = .0026$, **Figure 3**). Five cases of apnea, ranging from about five to thirty seconds, were observed in two male and three female injured animals. One case with about five seconds of apnea corresponded to the longest female ABS righting time. Six animals died following ABS. Five female mice died immediately after injury and one male mouse died four days after ABS exposure.

3.2 Anxiety-like behavior

For the EPM and EZM, injured mice overall spent less time in the open regions, but the two-way ANOVAs were not statistically significant (**Figure 4A, 4B**). No statistical differences were detected for either EPM or EZM test in terms of the amount of time spent in or the number of entries into the open areas (**Figure 4C, 4D**). No difference in the total distance travelled during EPM testing was evident, but for the EZM there was a main effect of sex in which female animals travelled a greater distance compared to male mice ($F_{1,45} = 6.999$, $p = .0112$, **Figure 4E, 4F**).

3.3 Depressive-like behavior

The two-way ANOVA for the SPT data did not detect statistical differences between the sham and injured or male and female mice for sucrose intake. However, the two-way ANOVA for the TST data showed a main effect of injury ($F_{1,45} = 6.763$, $p = .0125$) with shock wave exposed animals spending less time immobile than sham-treated mice. No sex differences were reported for the TST. The FST two-way ANOVA indicated no group difference for immobility (**Figure 5**).

3.4 Behavioral testing sequence effects

Post hoc analyses to determine whether or not prior behavioral tests affected later testing performance was assessed. Specifically, we evaluated whether or not the EZM or EPM may have affected performance on the SPT, and if subgroups of mice that were or were not evaluated on the SPT may have affected performance on the subsequent TST and FST. The performance ratio measures in mice that previously received testing on the EPM and EZM were compared using a three-factor (Injury x Sex x EPM/EZM Testing). ANOVA indicated there was no significant main effect of EPM vs. EZM on the subsequent SPT ($F_{1,49} = 1.131$, $p = 0.293$). Likewise, a three-factor ANOVA (Injury x Sex x SPT Testing/No SPT Testing) indicated there was no difference on either the TST or the FST as a function of mice having been tested on the SPT (and single housing) compared to mice that had not been used on this test (and remained in group housing). The main effect for Injury on the TST was significant, as expected ($F_{1,41} = 8.206$, $p = 0.007$), indicating the time immobile on the TST was less in injured mice compared to the sham animals. For the FST, there were no significant differences between groups on any factors.

3.5 Histology

Evans blue staining

Evans blue staining was evaluated in a separate cohort of sham and injured animals four hours post-ABS. No staining appeared on the brain parenchyma of sham animals (**Figure 6**). Of note was the observed variability in Evans Blue deposition in the cerebral cortex following injury, and there was a trend (albeit with a small sample size) for the appearance of a more intense uptake in some females. There appeared to be one case of subdural hemorrhage isolated in the left hemisphere of an injured female (top photograph for the Female ABS mice in Figure 6) and general staining near the superior sagittal sinus in several animals. A smaller cohort of mice were analyzed for the presence of Evans blue 24 hours after shock wave exposure, but no staining was evident in either the injured or sham animals (**Figure 6**). Kendall's tau was computed to determine whether or not there was an association between the intensity of Evans blue staining four hours after ABS injury and the duration of the righting reflex. There was a significant association, where longer righting reflex righting duration was associated with more intense Evans blue staining ($\tau = .580$, 95% confidence interval = .029-.924, $p = .030$). A summary of the measures is presented in **Figure 7**.

H&E and Cresyl Violet

Qualitative analysis of H&E did not reveal any microbleeds in any brain regions in the samples from ABS or sham animals (data not shown). Adjacent sections immunolabeled for c-Fos were stained with Cresyl Violet to confirm consistent anatomical location of the amygdala (data not shown).

c-Fos

Data are presented collapsed across anatomical sides. No significant differences were observed in the cerebral cortex (**Figures 8A, 8D**). There was a main effect of sex for staining in the PVT ($F_{1,14.497} = 15.781, p = .001$) with females exhibiting more c-Fos staining compared to male mice (**Figure 8B, 8E**). There was a main effect of injury in the amygdala ($F_{1,44.567} = 16.036, p = .001$) with ABS-injured mice expressing more c-Fos positive cells compared to sham mice (**Figures 8C, 8F**).

4. DISCUSSION

4.1 Physiological changes after bTBI

The current study found that male and female mice experienced longer righting reflex times after shock wave exposure compared to sham animals, and female ABS-exposed mice expressed longer periods of unconsciousness than male injured mice. The sex differences observed for righting times may be correlated with variations in body weight. As expected, males were larger than females, which may affect injury-related biomechanics during the shock wave exposure, causing the smaller female mice to move more during the blast event. The decrease in body weight following shock wave, as well as the increase in righting reflex for animals in the injured condition, indicate that the shock wave produced physiological effects. The loss in body weight suggested that, acutely, injured animals were physically unable or uninterested in eating following ABS. The observed weight loss and longer righting reflexes after ABS are consistent with a recent ABS study in the laboratory with identical conditions (187), as well as other reports (242). Previous experiments demonstrated only transient changes in water and food consumption post-blast with vertical orientation exposure, or no differences in male and female body weight after ABS when animals were placed in a prone position (232;

269). The sex differences in weight and righting time immediately following bTBI suggest that female mice sustained greater damage initially compared to male mice.

4.2 Effects of bTBI on the blood-brain barrier

Behavioral and neuropathological changes have been reported in blast studies in which the animal is placed in a vertical orientation (139; 187; 269) and in a prone position (8; 12; 231; 241). Body orientation may be particularly relevant to cases of tertiary blast effects. The BBB, for example, is an essential element in brain homeostasis and is one of the first sites to be altered following bTBI, and is a structure particularly vulnerable to impact compression (78; 110). Acute cerebral vascular impairment and BBB protein dysregulation have been reported after single and multiple ABS exposure, respectively (101; 102; 223), and our data support these findings with acute BBB permeability to Evans blue four hours following ABS. Finite element modeling and shock tube experiments demonstrate that the prone orientation produces a lesser degree of damage compared to other body orientations (101; 109; 265). In this study the vertical orientation was employed and Evans blue staining provided evidence of BBB disruption, but it was evident only on the dorsal surface of the brain. This suggests that the fixed vertical animal positioning enabled tertiary, acceleration artifacts from the shock wave-associated blast wind that pushed the mouse backwards, compressing the top of the head into the mesh holder. Interestingly, Unnikrishnan and colleagues used almost identical peak pressure (100 kPa or 14.5 psi) modeling of vertical orientation inside of the ABS, and reported higher pressure on the ventral brain than the dorsal side (265).

Assessment of the degree of Evans blue staining on the dorsal surface of the neuraxis, four hours after ABS injury, was associated with the duration of the righting

reflex, suggesting longer righting reflex duration was reflected in greater severity of BBB compromise (**Figure 7**). However, although Evans blue staining was present in some injured animals four hours after bTBI, H&E staining did not indicate the presence of microbleeds and no c-Fos changes were observed in the cerebral cortex, suggesting that the extent of the damage was limited to albumin extravasation to the superficial surface, but not deeper cortical layers (275). The damage, however, might be transient since only subtle changes in behavior were observed at later time points (281). Another study demonstrated the extravasation of dyes smaller than 70 kDa with barrier integrity restoration one day post-blast (111), which is consistent with the observed absence of Evans blue 24 hours following shock wave exposure in the current study, indicating that the BBB may be repaired in rodents one day after bTBI. Evans blue has been used historically, but caution must be used with extravasation interpretation and its quantification continues to present challenges (240). Taken together, many potential artifacts are present in pre-clinical bTBI designs and additional complications with Evans blue quantification, including observed vulnerability in stain intensity, hinder definitive conclusions from being made regarding BBB disruption in this study.

4.3 Acute changes in c-Fos activation in the amygdala and PVT following bTBI

In addition to BBB disruption, there were increases in c-Fos activation in the amygdala (injured males and females) and the overall PVT (females only) following bTBI. The c-Fos activation reported in this study supports previous investigations of behavioral changes after blast injury through limbic activation. In previous studies, increases in c-Fos appeared in the central and basolateral amygdala and subsequent increases occurred in anxiety-like behavior and fear circuitry (195; 227). Reports of

changes in cytostructure after bTBI also may shed light on possible mechanisms of hyperactivity in the amygdala with increased dendritic branching and spine density after injury (214; 215). The sex differences in c-Fos seen in the PVT may correspond to the higher hypothalamic-pituitary-adrenal axis activation in females and maladaptive behaviors in response to stress (119; 227). The acute changes in neuronal activation observed primarily in the amygdala after ABS and overall c-Fos sex differences in the PVT were consistent with some of the behavioral alterations seen at later time points.

4.4 Effects of blast exposure on depressive- and anxiety-like behaviors

The mild and transient nature of the bTBI as assessed histologically was associated with only subtle changes in behavior. Previous bTBI studies described increased anxiety-like behavior in the EZM and EPM in injured rats and mice (10; 66; 231; 279). However, the present study did not find significant differences for time in the open region, nor for the number of entrances into the open zones for EPM and EZM. Some of the differences in reported anxiety-like behavior could be due to the type of shock tube used. One group reporting anxiety-like behaviors following bTBI used a compact, focal blast tube with animal head-only exposure (10), whereas another group used a full body shock tube, but with multiple blast injuries (66). A different design of repetitive, automated blast exposures was implemented in an air blast chamber with anxiety-like behavior performed five minutes after injuries (279). Russell and colleagues employed an injury paradigm that closely resembled the current study, but animals were placed in a prone instead of vertical orientation (231). These differences in animal body orientation and blast tube design alter injury biomechanics and therefore functional outcomes (184; 265).

While there was no change in the total distance travelled in the EPM, females travelled farther than males in the EZM. Hyperactivity has been observed in females during open field tests following controlled cortical impact (258; 259). Likewise, a recent study with repetitive concussive injuries directed at the frontal region likewise observed increased distance travelled in the open field test, and that female mice travelled farther in an acute test four days after injury, but not at later time points (268). The higher levels of activity, particularly in females as observed in the EPM and in open field in other studies, could be related to activational effects of estrogen (175; 192), but further investigation is needed to understand the mechanism and duration of this increased activity in female mice after blast.

Behavioral tasks measuring depressive-like behaviors showed varying trends. No sex differences were found, but the injured group had reduced immobility in the TST compared to sham-treated controls, an unexpected finding. Reduced immobility after blast exposure may indicate injury-induced agitation or a panic-like reaction and has been reported after other concussive TBI models (7; 262). Interestingly, a study of CRF neurons after bTBI and restraint-induced stress found sex differences with females showing increased amygdala CRF2 gene expression and hippocampal decreased expression, whereas males displayed the opposite (231). Increased neuronal activation in the amygdala of injured females was consistent with the current study. Hyperarousal is an element of PTSD and as discussed previously, blast is able to induce PTSD-like traits where greater activity level during the TST in injured animals may correspond to the hyperactivity observed in female mice in the EZM. (66; 204). Activity level, however, has been criticized as an inaccurate measurement of anxiety (147; 260). The TST and

FST are arguably models of acute stress, which could be interpreted as changes in anxiety rather than depression (7; 147; 185; 258). Further study is needed to define the relationship between bTBI and subsequent hyperactivity.

4.5 STUDY LIMITATIONS AND FUTURE PROSPECTS

Results from behavioral tests can depend on the animal strain and sex (164). Overall, female rodents exhibit more activity compared to males during behavioral tasks and this inherent difference can make data interpretation challenging (258; 259). Females may also have subtle neuroprotective effects associated with estrogen and progesterone, but estrous cycle stage does not appear to have a predominant effect on behavioral outcome after TBI (270). Likewise, housing conditions with respect to sex differences in behavior should also be considered. Animals in the current study were singly housed for the SPT and remained individually housed for subsequent testing with the TST and FST. In this study, single housing did not affect depressive-like behavior. Increased corticosterone and anxiety-like behavior have been reported in female rodents that are singly housed, whereas individually housed males display less anxiety-like behavior (26; 197). A potential confound of this study's histological data included additional animal restraint during intravenous Evans blue administration, but injured and sham-treated mice underwent the same injection procedure. Previous literature demonstrated that blast alone without a stressor could increase contextual fear conditioning (66; 204; 205). TST and FST alone can cause changes in c-Fos activation (104; 280), so separate cohorts of animals were included for behavioral testing and pathology in this study, and no behavioral stress confounds occurred for c-Fos staining (49; 104; 280). Since c-Fos activation has been reported in other areas after bTBI, such as the paraventricular nucleus

of the hypothalamus and hippocampus, additional brain regions should be investigated (64; 195; 232) and given reports of different PVT cellular divisions, specific PVT circuitry should be identified (80; 135). Future directions could also include investigation of multiple blast exposures and bTBI with chronic stress as more clinically relevant designs for military populations (141; 196). The evaluation of later time points after blast for behavioral tasks may indicate delayed or biphasic behavioral changes, such as slowed onset or immediate deficits followed by a recovery and then prolonged alterations (8; 205; 248). This study further highlights the importance of investigating potential sex differences, and indicates overall variance in limbic activation and functional outcome between male and female rodents following bTBI exposure.

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AUTHOR CONTRIBUTIONS

EHM, LBT, JTM contributed to the experiment design and development, PAV assisted with ABS training, EHM, AHF, YK performed ABS procedures, EHM and LBT conducted behavioral testing, AHF assisted with tail injection training, EHM performed tail injections, histology was performed by EHM and JL, EHM and JTM performed statistical analyses and wrote the manuscript, EHM, LBT, JTM edited the manuscript, EHM and JTM finalized the manuscript. The opinions, interpretations, conclusions and

recommendations are those of the authors and are not necessarily endorsed by the U.S. Army, Department of Defense, the U.S. Government or the Uniformed Services University of the Health Sciences. The use of trade names does not constitute an official endorsement or approval of the use of reagents or commercial hardware or software. This document may not be cited for purposes of advertisement.

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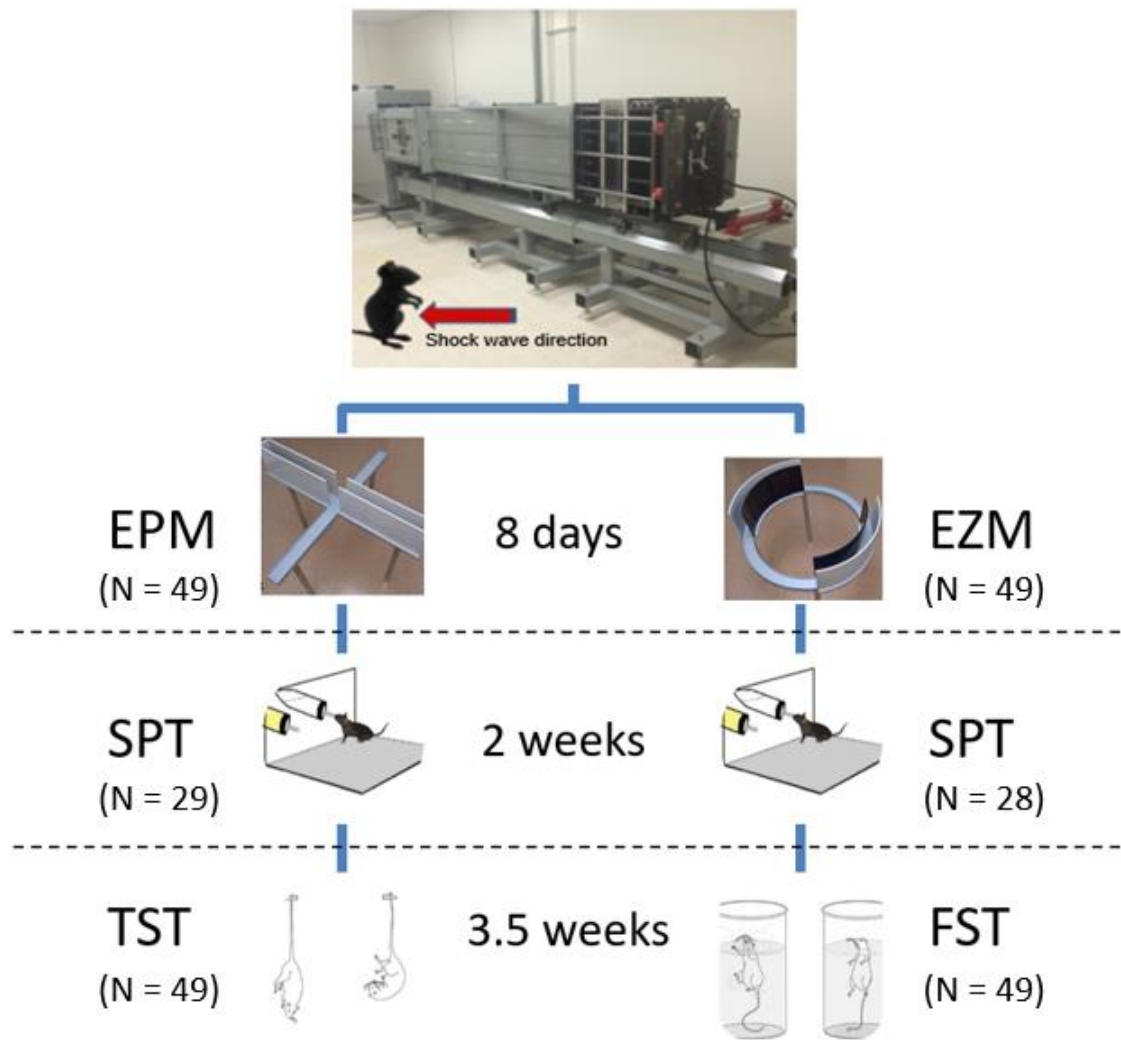


Figure 7: ABS and behavioral testing paradigms.

A single shock wave (~15 psi) was delivered with the mouse in an upright position with the ventral surface of the mouse exposed to the oncoming shock wave (see 269 for a detailed description of the ABS). Eight days post-injury, animals underwent either EPM or EZM testing followed by the SPT two weeks post-injury or sham treatment, and lastly either TST or FST 3.5 weeks following ABS. Images used with permission from (3; 25; 261).

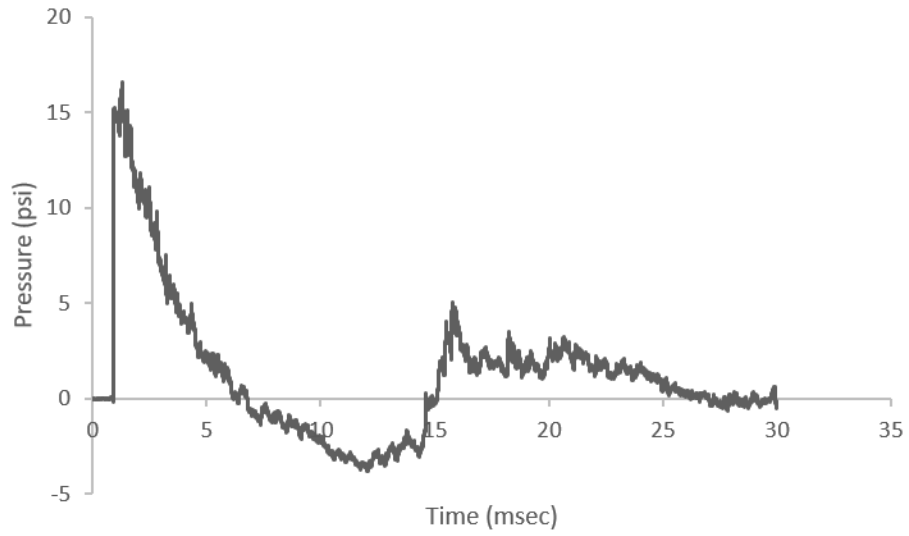


Figure 8: Representative Friedlander-like ABS waveform.

The ABS device produced a waveform with an average of 15.56 psi peak incident pressure and 469.74 m/s shock wave velocity. The shock wave positive phase duration was 5.62 msec and negative phase duration was 8.40 msec.

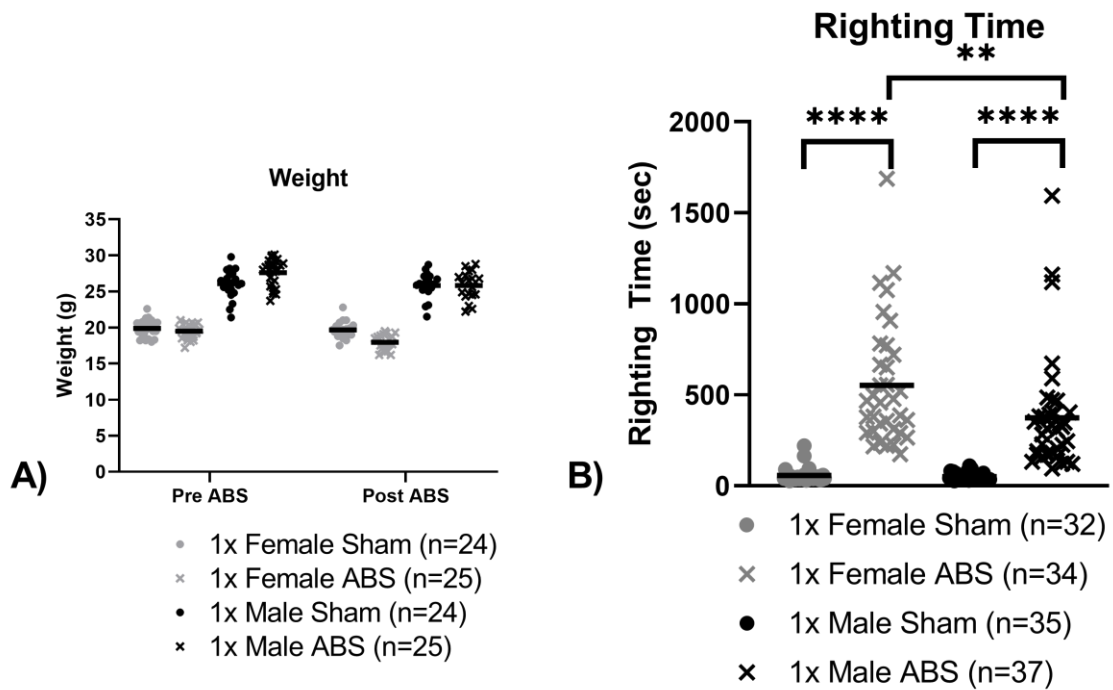
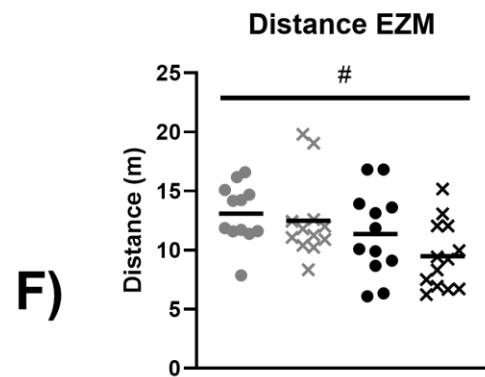
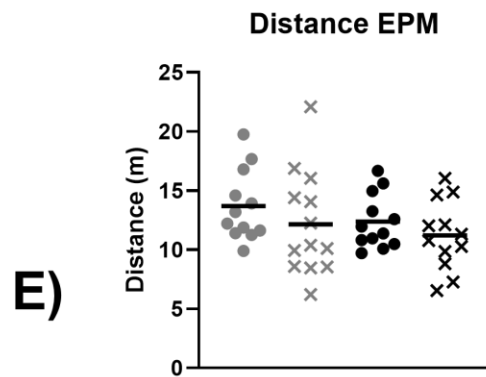
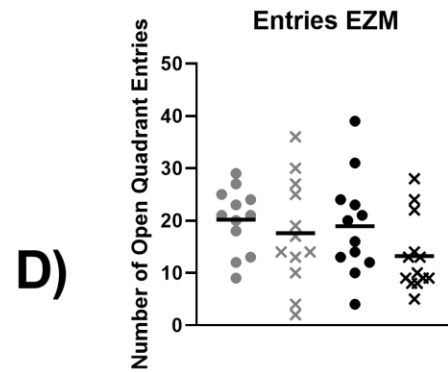
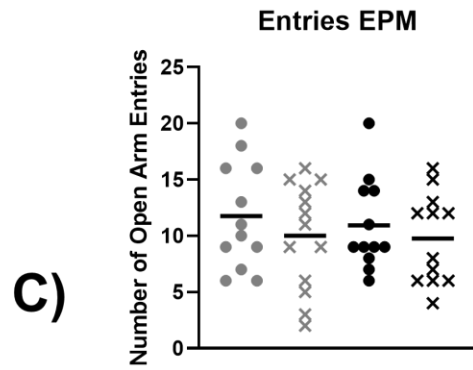
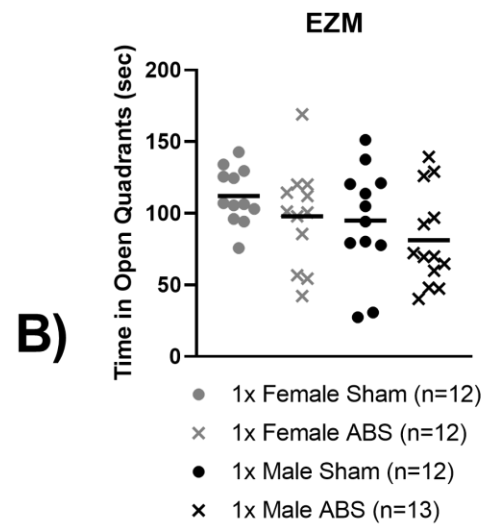
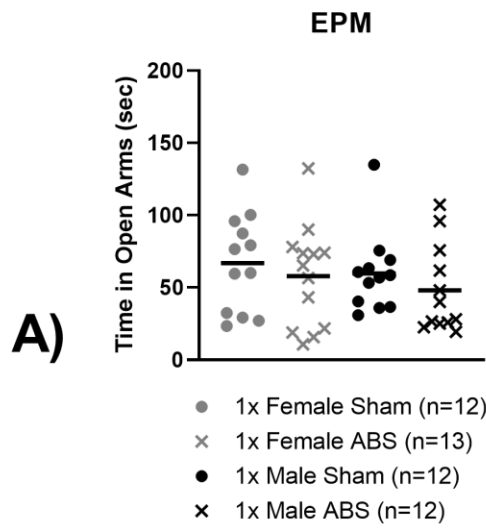


Figure 9: Weight and righting time changes after bTBI.

A) Animal weight before and one day after ABS exposure for the behavior cohort of mice. Due to differences in baseline body weights, the percentage change in body weight for each mouse was computed for pre- and post-injury sham or ABS treatment (data not shown; see text). ANOVA indicated there was a significant greater difference in females compared to males overall and a significantly greater percentage of body weight loss in ABS animals, but no sex differences due to injury. **B)** Righting times of all cohorts, behavior and Evans blue, animals immediately after a single blast. The Mann Whitney U test ($U = 3$, $p < .0001$) demonstrated that the duration of the righting reflex was longer for shock wave exposed females compared to female sham mice. Male injured mice also demonstrated a longer righting reflex compared to sham counterparts ($U = 1.5$, $p < .0001$). A comparison of righting reflex duration in injured mice indicated the responses after shock wave exposure in males and females were significantly different ($U = 370.5$, $p = .0026$) with longer righting times for female mice. Bars indicate means. * indicates $p \leq .05$, ** indicates $p \leq .01$, **** indicates $p \leq .0001$. No significant correlations (data not shown) were found between righting reflex and loss of body weight ($\tau = .000$, $p = 1.000$) or percentage loss of body weight ($\tau = .032$, $p = .846$) for females, or righting reflex and loss of body weight ($\tau = -.167$, $p = .312$) or percentage loss of body weight ($\tau = -.105$, $p = .516$) for males.

Figure 10: No changes in anxiety-like behaviors on the EPM or EZM after injury, but a sex difference was displayed for EZM distance travelled.

The results from the **A)** EPM and **B)** EZM testing show no difference in terms of time spent in the open areas for injury or sex. The number of open region entries also did not significantly differ for **C)** EPM or **D)** EZM. ABS group and sham animals for each behavioral task. **E)** The total distance travelled was not statistically different between injured (ABS) or sham mice for EPM. **F)** Results from the EZM testing indicated there was a main effect of sex where females travelled significantly farther than male mice ($p = .0112$). Bars indicate means. # indicates main effect of sex $p \leq .05$.



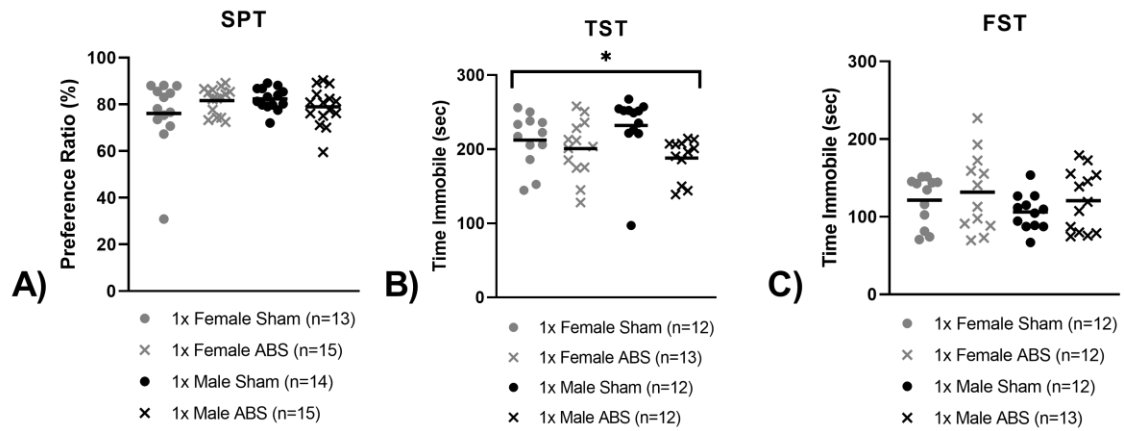


Figure 11: Decreased time immobile on the TST following bTBI.

A) SPT did not show any significant difference in sucrose consumption as an effect of shock wave injury or sham treatment. **B)** TST displayed a significant effect of injury with ABS mice spending less time immobile compared to sham mice. **C)** The time immobile on the FST was not significant. Bars indicate means. * indicates $p \leq .05$ for main effect for the difference between uninjured and ABS exposed mice.

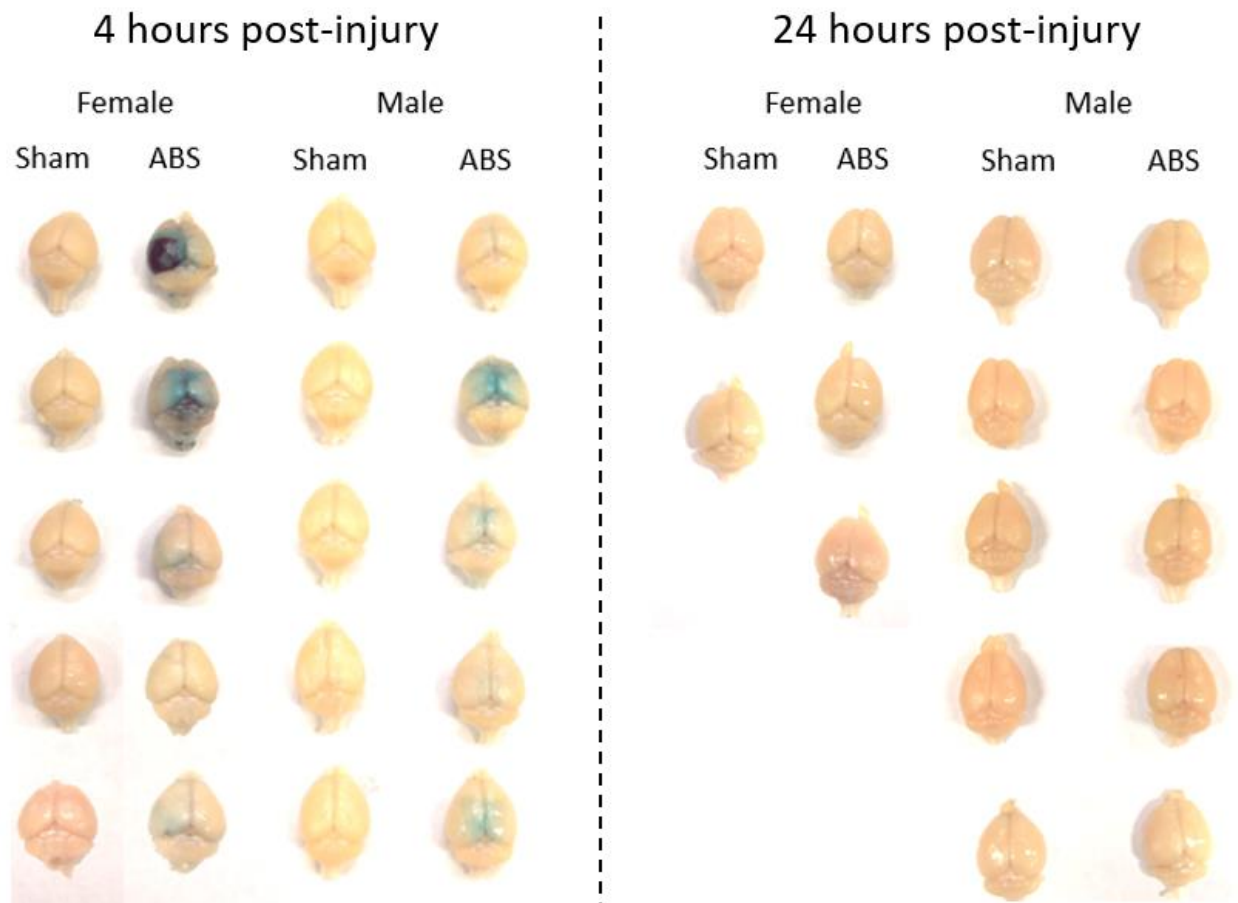


Figure 12: Evans blue was evident 4 but not 24 hours after shock wave exposure. Evans blue staining was present on the brain parenchyma of ABS-exposed male and female mice four hours after injury, but was not apparent on the brains of sham animals. One day after shock wave exposure, Evans blue was not observed on the brain parenchyma of either ABS-injured male and female or sham animals.

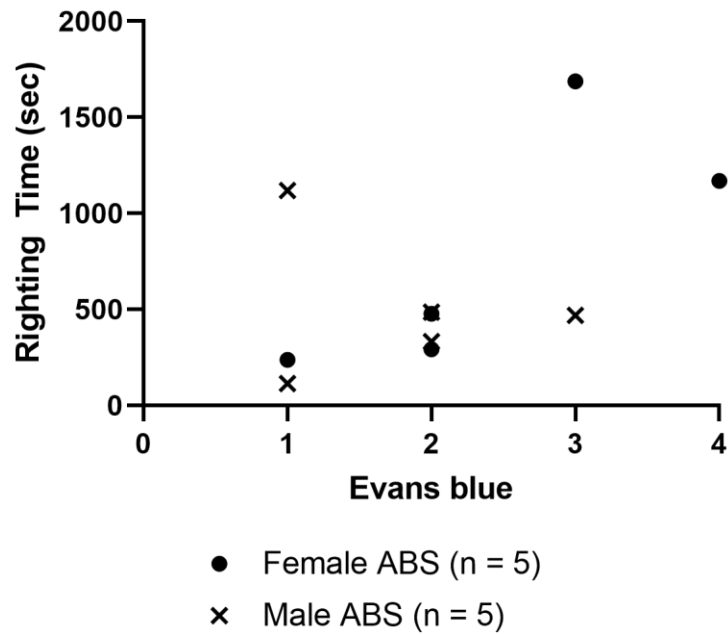


Figure 13: Correlation of righting reflex time and Evans blue staining 4 hours post-injury. The scatterplot displays the righting time of injured male and female mice 4 hours after shock wave exposure with respect to the amount of Evans blue staining intensity ranked 1 (least) – 4 (most), and Kendall tau analysis indicated a significant correlation between stain and the duration of the righting reflex ($\tau = .508, p < .030$). No correlations (data not shown) were detected between Evans blue ranking and peak ABS pressure ($\tau = -.025, p < .925$), Evans blue and body weight ($\tau = .090, p < .719$), or righting time and peak pressure ($\tau = .244, p < .325$).

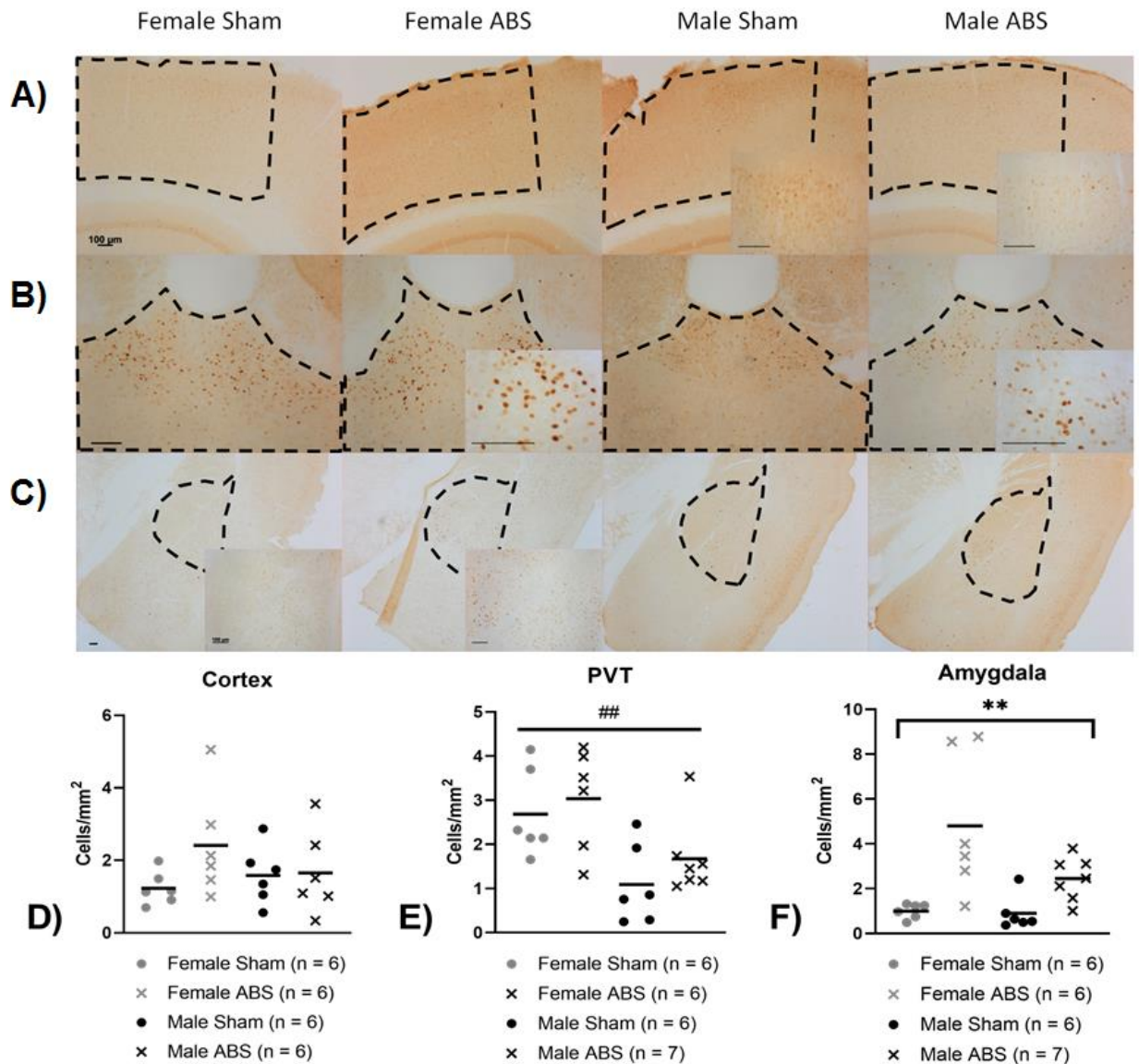


Figure 14: C-Fos differences by sex and injury in the PVT and amygdala, respectively.

Representative images of the **A)** cerebral cortex, **B)** PVT, and **C)** amygdala (basolateral and central nucleus) with regions of interested outlined in dashed lines and magnified inserts of areas within the measured regions. All scale bars indicate 100 μ m. **D)** The cerebral cortex did not show any significant difference between the groups for injury or sex. **E)** The PVT displayed an overall effect of sex in which females had more c-Fos positive cells compared to males. **F)** There was a main effect of injury in the amygdala, where injured mice had greater c-Fos cell density than sham mice. Bars indicate means. ## indicates main effect of sex $p \leq .001$, ** indicates main effect of injury $p \leq .001$.

CHAPTER 3: Meningeal and visual pathway MRI analysis after single and repetitive Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA)-induced disruption in male and female mice

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ABSTRACT

The consequences of forceful rotational acceleration on the central nervous system are not fully understood. While traumatic brain injury research primarily has focused on effects related to the brain parenchyma, reports of traumatic meningeal enhancement in TBI patients may possess clinical significance. The objective of this study was to evaluate the meninges and brain for changes in dynamic contrast enhancement (DCE) magnetic resonance imaging following CHIMERA-induced cerebral insult. Adult male and female mice received one (1x; n=19 CHIMERA, n=19 Sham) or four (4x one/day; n=18 CHIMERA, n=12 Sham) injuries. Each animal underwent three MRI scans: one week before injury, immediately after the final injury, and one week post-injury. Compared to baseline readings and measures in sham animals, meningeal DCE in males was increased after single impact and repetitive injury. In female mice, DCE was elevated relative to their baseline level after a single impact. One week after CHIMERA, the meningeal enhancement returned to below baseline for single injured male mice, but compared to uninjured mice remained elevated in both sexes in the

multiple impact groups. Pre DCE meningeal T2-weighted relaxation time was increased only after 1x CHIMERA in injured mice. Since vision is impaired after CHIMERA, visual pathway regions were analyzed through imaging and GFAP histology. Initial DCE in the lateral geniculate nucleus (LGN) and superior colliculus (SC) and T2 increases in the optic tract (OPT) and LGN were observed after injury with decreases in DCE and T2 one week later. Astrogliosis was apparent in the OPT and SC with increased GFAP staining seven days post-injury. To our knowledge, this is the first study to examine meningeal integrity after CHIMERA in both male and female rodents. DCE-MRI may serve as a useful approach for pre-clinical models of meningeal injury that will enable further evaluation of the underlying mechanisms.

Keywords: CHIMERA (closed-head impact model of engineered rotational acceleration), MRI (magnetic resonance imaging), Meninges, Traumatic brain injury, Traumatic meningeal enhancement, Visual pathway

INTRODUCTION

Traumatic brain injury (TBI), including the more common milder forms, are a growing long-term economic and medical burden (115). A recent study found there were approximately 27 million new cases of TBI each year and estimated the world prevalence to be around 55.5 million cases (122). The most common causes include falls, motor vehicle accidents, and similar impact injuries involving acceleration and deceleration forces (72; 122). TBI is particularly relevant for military populations and is considered the invisible wound and signature injury from the conflicts in Iraq and Afghanistan (253).

Repetitive closed-head injuries are also a risk factor for chronic functional impairment and degenerative brain diseases, including chronic traumatic encephalopathy (166). There is a potential for sex differences related to higher rates of concussions reported in female collegiate athletes and biomechanical factors, such as reduced head stability (46; 254). In many species, from invertebrates to mammals, the cranial meninges are a significant structure and act as a protective tissue with stiff physical properties compared to the central nervous system tissue that it encases (130; 156). While there has been a focus on brain effects, the consequences of impact rotational acceleration on the meninges have received little attention.

Contrast enhancement in MRI by the administration of agents such as gadolinium permit better visualization of the meninges. Human cases of traumatic meningeal enhancement (TME) are only reported in about half of mild TBI patients who receive a MRI (40; 225). However, TME has been observed, even when other trauma-related imaging changes are not detected (33; 51; 225; 233). Possible blood-brain barrier damage has also been reported in athletes (188) and in patients that manifest TME. Little is known about the long-term implications of meningeal changes after injury, but the meninges may be an important site of insult after concussion, a key component in the TBI recovery process, and a therapeutic target for the prevention of subsequent parenchymal damage (42; 225; 263). While the TME mechanism for contrast agent extravasation and accumulation in the meninges is unknown, the clinical ramifications and underlying mechanisms of TME both require investigation.

The focus of this study was to determine the effects of head impact-acceleration upon meningeal integrity as a possible pre-clinical model of TME. In addition, visual

impairments have been recognized as a frequent sequelae to head impact injuries (86). Male and female rodents were included in this study since males are the most common victims of TBI, but more cases of concussion have been reported in female collegiate athletes and biomechanical sex differences have been described in humans (45; 254). The Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) was selected for this study because of the reliable, non-surgical, and free head rotation design (168; 183). To identify meningeal changes from contrast agent perfusion after single (1x) or repetitive (4x, separated 24 hours apart) CHIMERA, we acquired dynamic contrast enhancement (DCE) MR images at Baseline, Day 1 immediately after impact, and one week post-injury. Relaxation times of T2-weighted images were also assessed for alterations in inflammation. Since it is well documented that the visual pathway becomes impaired after CHIMERA, the optic tract (OPT), lateral geniculate nucleus (LGN), and superior colliculus (SC) were included for analysis (38; 39; 56; 57; 89; 218; 260). We hypothesized that murine meninges and brain regions will show increased DCE and T2 immediately after CHIMERA injury and will be sustained after repetitive impacts, indicating meningeal impairment, increased blood-brain barrier permeability, and inflammation. Demonstrating a pre-clinical model of TME is critical for further investigation of this novel marker. Although it has received some attention, clinical studies indicate a significant portion of impact acceleration injuries result in contrast enhancement as a reflection of meningeal impairment. These changes may underlie structural and functional complaints, including edema of the meninges related to headache, and secondary effects from inflammation and disruption of the meningeal barrier.

METHODS

Animals

A total of 68 seven-to-eight-week old male and female C57BL/6J mice (00664) were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in an AAALAC-accredited animal facility for at least three days of acclimation before the experiments were started. All procedures were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee. Animals were group-housed (4–5/cage), had access to food and water *ad libitum*, and were maintained on a standard 12 h: 12 h light-dark cycle.

Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA)

Animals were randomly assigned to the injured or sham conditions (Table 1). Groups were further divided into either single (1x, n = 19) or repetitive (4x, 1/day, n = 18) CHIMERA-related TBI with appropriate sham counterparts (1x n = 19, 4x n = 12). All mice were anesthetized with isoflurane (3% Forane, Baxter Healthcare Corporation, Deerfield, IL, with 100% O₂) inside an induction chamber for approximately two minutes. Respiration was monitored, and when deeply unconscious the mice were transferred to the CHIMERA animal bed and placed in a supine position at a 30° angle with the animal head laid flat horizontally on the head plate. Instrument crosshair markings were used for head alignment for impact of the region surrounding bregma. Velcro straps secured the body, but also allowed free rotation of the head and upper torso. During placement, a nose cone on the animal bed delivered isoflurane (2% isoflurane with 100% O₂) for about three minutes before being removed just prior to impact. For the injured groups, the CHIMERA device piston was driven upward to impact the closed

mouse head dorsally (~0.6 J, 4.9 m/s velocity). After impact, the mouse was removed from the holder and positioned in a supine orientation in a clean cage to assess the time to return of the righting reflex, a surrogate measurement of return to consciousness. Once the animals turned upright, they were placed back into their home cage. Acetaminophen diluted in water (3% concentration) was administered after injury to all animals. Sham animals were handled identically, including anesthesia treatment of equal duration, placement on the CHIMERA platform, and acetaminophen administration, but were not exposed to the impact.

Magnetic Resonance Imaging (MRI) acquisition

In vivo images were collected with a Bruker 7T MR scanner (Bruker, Billerica, MA) at three time points. First, a baseline MRI (Baseline) was conducted one week before CHIMERA injuries or sham treatment. The second MRI (Day 1) was initiated within 15 minutes after the last CHIMERA injury or sham treatment, and the third scan (Day 7) took place one week post-injury (**Figure 1**). A transmit birdcage coil (Bruker, Billerica, MA) in combination with an actively decoupled 4 channel mouse head receive array coil (Bruker, Billerica, MA) was used. Pre-contrast and post-contrast T2-weighted images were acquired for each scan. All measurements, T2 and DCE, were acquired during each of the three scans per animal: T2W/PDW/T2 map 2D RARE TR = 4000 ms, echo time TE = 10, 30, 50, 70, 90, 110 ms, ETL = 2, NA = 2, FOV 13.8x13.8 mm², matrix 92x92, NS = 25, fat suppression, BW 36 kHz, time 6:08 min. DCE 3D FLASH TR/TE = 13/2 ms, flip angle 13°, NA = 1, NR = 11, FOV 13.8x13.8x50 (13.8x13.8x 25 initial cohorts) mm³, matrix 92x92x50(25), no fat suppression, BW 85 kHz, time 11 min. Prior to each scan, animals were weighed and placed in an isoflurane anesthesia (4%

isoflurane in medical air) induction chamber. A syringe and catheter containing the contrast agent ProHance (Bracco Diagnostics Inc, Milan Italy; 50% Gd in saline) was prepared and the needle tip was directed to the intraperitoneal cavity, but not injected. The mouse was then transferred to the MRI bed, placed in a prone stereotaxic holder for head alignment, and positioned on a circulating water warming pad while maintained under isoflurane through a nose cone. Each MRI session was approximately 55 minutes in duration and animals were monitored by temperature (maintained 34–37°C) and respiration (maintained 30–70 breaths/min) with appropriate adjustments made to the isoflurane anesthesia (maintained between 1.5–2.0% isoflurane in medical air) while inside the scanner. At the start of the DCE acquisition, ProHance was intraperitoneally injected (0.5 mL 1:1 ProHance:Saline per mouse) with an 11:00 min DCE acquisition time where the injection commenced beginning 2 minutes into the sequence. Experimenters were blinded to the animal conditions for MRI acquisition and analysis. Following the third scan, animals were euthanized and tissue was collected for histology.

To accommodate time constraints on the treatment sequences, the mice were utilized in cohorts that were divided by single or repetitive injuries and balanced for treatment condition. There were a total of five cohorts. Cohorts 1–2 included 4x CHIMERA and sham counterparts, while Cohorts 3–5 consisted of 1x CHIMERA and sham treatment (Table 1). One male 1x CHIMERA mouse died immediately following impact and the baseline data from this animal was not included in the analysis. One female 4x CHIMERA mouse displayed hind leg paralysis following the fourth impact. The scan (Day 1) indicated intracerebral hemorrhaging and this mouse was euthanized resulting in no Day 7 scan. Finally, one male 1x Sham mouse died just before the Day 7

scan, possibly due to anesthesia exposure, and the Day 7 data from this animal was not available.

Data processing

MRI data were processed using Matlab R2020a (The MathWorks Inc, Natick, MA, USA). T2-relaxation time maps were computed from the multi-echo T2-weighted images. For DCE, the Area Under the Curve (AUC) was calculated to provide a more robust metric for image enhancement (116). Details of the image processing are provided in the Supplemental Material.

MRI data were analyzed for changes in enhancement using a region-of-interest (ROI) approach; which were selected based on previous CHIMERA literature (168). ROIs were hand drawn on the pre-contrast 30 ms TE T2-weighted image and on the average of the first three DCE image frames from each scan session using VivoQuant Software version 3.0 (inviCRO LLC, Boston, MA) and ITK-SNAP version 3.8 (Yushkevich, P.A. and Gerig, G., NIH) software (**Supplemental Figure 1**). A bilateral ROI was drawn in the muscle to normalize the data. Animal and voxel exclusion criteria were applied to omit scans with poor contrast uptake and to remove potential errors in ROI drawings (see MRI data processing in the Supplemental Material). As discussed in the Supplemental Material, only T2 pre-contrast data was analyzed because T2 pre-contrast is a clinically relevant measurement and is comparable to T2 post-contrast in this study.

Immunohistochemistry

Immediately following Day 7 MRI scans, mice were anesthetized with a mixture of ketamine and xylazine and then transcardially perfused with cold phosphate buffer

solution (0.10 M) and 4% paraformaldehyde in phosphate buffer. Brains were dissected and further fixed in paraformaldehyde for an additional 24 hours. They were then transferred to 20% sucrose solution in phosphate buffer for 72 hours before freezing the tissue and stored at -80°C until sectioning. A Leica microtome was used to cut 30 µm thick coronal sections. The tissue was stained at least one week after sectioning and were washed in tris-buffered saline with 0.05% triton (TBS-T). Sections were then processed with 0.3% hydrogen peroxide for 30 minutes and afterwards washed with TBS-T again before blocking buffer (TBS-T with 0.20% triton, 10% goat serum (Vector Laboratories, S-100), and 0.02% bovine serum albumin (BSA, Sigma-Aldrich, A7906) incubation for 1 hour at room temperature. Glial fibrillary acidic protein (GFAP) antibody (1:500; Thermofisher Cat: Ab-6 ASTRO06, Lot: 1376P1810J) was applied to the sections before storage at 4°C overnight. The next day, sections were washed with TBS-T and secondary antibody goat-anti-mouse IgG (1:500 Jackson ImmunoResearch Cat: 115-065-003, Lot: 92050) was applied diluted in blocking buffer for 1 hour at room temperature. Sections were again washed with TBS-T before incubation in ABC solution from Vectastain ABC HRP Kit (PK-4000, Vector Laboratories) for 45 minutes at room temperature. The tissue was washed a final time with TBS-T prior to DAB development with DAB Peroxidase (HRP) Substrate Kit (with nickel), 3,3'-diaminobenzidine (SK-4100, Vector Laboratories) for 3 minutes. The free-floating sections were mounted onto glass slides and left to dry overnight. Lastly, sections were dehydrated in ethanol gradients (75–100%), cleared in xylene twice, and cover slipped with Permount mounting media (Fisher Chemical).

Visual pathway ROIs: optic tract (OPT), lateral geniculate nucleus (LGN), and superior colliculus (SC) were investigated for GFAP staining. Twelve mice (six female and six male mice) per group where successful MRI scans were obtained on all three scans, were randomly selected for Carl Zeiss El-Einsatz model #451485 light microscope imaging. Images for the OPT and LGN ROIs were taken at 10x magnification, whereas the SC were captured at 5x magnification. To quantify GFAP in each ROI, percent area stained was measured for black/white GFAP images with the threshold function on Image J software (NIH). GFAP values were averaged for three or more sections per animal. Additional regions were investigated, corpus callosum, anterior commissure, and hippocampal fimbria (Supplemental Table 3), but no differences in GFAP were observed.

Statistics

Graphpad Prism version 8.42 (GraphPad Software, San Diego, CA) was used for figure generation. Data are reported for means or medians, as appropriate, and standard error of the mean (SEM) and coefficient of variation (COV). Statistical analyses were performed using SPSS (IBM, Armonk, NY) and SigmaPlot (Systat Software Inc, San Jose, CA). The body weight data for the 1x CHIMERA and 1x Sham groups were analyzed as a two factor (Sex x Day) analysis of variance (ANOVA) and the 4x CHIMERA and 4x Sham groups were assessed with a three factor mixed model ANOVA (Injury x Sex x Day) with Days as a repeated measure. Righting reflex data did not meet the assumptions of normality and was analyzed using separate Kruskal-Wallis ANOVAs for 1x CHIMERA data and separate analyses each day for the 4x CHIMERA experiment. For MRI meningeal analysis, 1x and 4x CHIMERA data were analyzed separately with a three-factor linear mixed model analysis (Injury $\times$ Sex $\times$ Day), with Days treated as a

within subjects variable. *Post hoc* pairwise comparisons were made using the Bonferroni correction method. Brain regions were analyzed with a four-factor linear mixed model (Brain Region $\times$ Injury $\times$ Sex $\times$ Day) for the brainstem, cerebellum, cerebral cortex, corpus callosum, dorsal hippocampus, lateral geniculate nucleus, and superior colliculus, and multiple comparisons were evaluated with the Bonferroni correction (Supplemental Material). The optic tract was analyzed separately for 1x and 4x AUC because optic tract mean AUC values greatly differed compared to other brain regions. Since different levels of resolution were performed for 1x and 4x cohorts, effect sizes were computed using the *Hedge's g* formula (equation in Supplemental Material). The Mann Whitney U-test was used to analyze GFAP histology. GFAP data were also collapsed across sex. $p \leq .05$ was considered significant.

RESULTS

CHIMERA velocity and animal body weight were consistent throughout the experiments

The mean maximum energy and the velocity impact for the CHIMERA were consistent across cohort trials and over the days that CHIMERA was performed. The average energy level and piston exit velocity for the cohorts after a single CHIMERA impact ($n = 19$) were 0.60 J (± 0.005 J; COV = 0.15%) and 4.90 m/s (± 0.02 m/s; COV = 1.8%). The repetitive CHIMERA experiment ($n = 18$) had average energies and velocities of 0.59 J (± 0.005 J; COV = 0.14%) and 4.87 m/s (± 0.02 m/s; COV = 1.7%), respectively, across the days that CHIMERA was performed. Animal body weight across single and repetitive CHIMERA experiments differed between males ($n = 35$) with an average of 24.82 g (± 0.75 g, SEM) and females ($n = 33$) with an average of 19.21 g (± 0.43 g, SEM) as expected. Neither single nor repetitive treatments affected body weights

by injury condition; sex differences were the only effect on animal body weight (Supplemental Figure 2).

Righting reflex duration increased after CHIMERA and sex differences appeared in the 4x CHIMERA groups

Only two brief cases of apnea, about 2 s, immediately after a single impact were observed in one female and one male. Righting reflex was recorded after animals received anesthesia and sham treatment or anesthesia and CHIMERA (Figure 2A). Overall, males and females that sustained single and repetitive CHIMERA insults had longer righting reflex times compared to sham animals, suggesting an increase in the return to consciousness from the impacts. The median time for the sham mice to exhibit a righting reflex was 18 s, while the response in the 1x CHIMERA-injured animals was significantly longer ($H_3 = 27.042$, $p < .001$) with a median time of 96 s. There were no sex differences so the data were combined in Figure 2A.

Ten instances of apnea divided evenly between males and females were observed across the 72 impact trials in the 4x CHIMERA group. Apnea mainly occurred following the first impact, but two cases appeared immediately after the third and fourth injuries. Apnea lasted no more than 10 s in duration. 4x CHIMERA animals also sustained longer righting times compared to sham mice, on each day they received a CHIMERA injury (Figure 2B). A Kruskal-Wallis ANOVA was performed on the data for each day, and indicated the median duration of the righting reflex was significantly longer in both male and female 4x CHIMERA mice on Day 1 (medians: 90 and 84 s, respectively, $H_3 = 23.08$, $p < .001$) than the duration in male and female sham animals (17 and 23 s, respectively). On later Days 2–4, the female 4x CHIMERA animals continued to have longer righting reflex durations compared to female sham animals, while the male 4x

CHIMERA animals exhibited longer duration reflex responses, but were not different from levels measured in the 4x Sham male mice.

The meninges displayed contrast enhancement at Day 1 following 1x and 4x CHIMERA with no apparent changes in the cortex

Analysis of AUC changes in the meninges from a single CHIMERA insult indicated there was a significant Injury $\times$ Sex $\times$ Day interaction effect ($F_{2, 30.599} = 6.188, p = .006$). Male 1x CHIMERA injured mice consistently exhibited greater changes in meninges AUC over time. For the female mice, there was a significant increase in the AUC on Day 1 compared to Baseline ($p = .024$) in injured animals, and a similar increase was observed in the male animals ($p = .014$). Injured males also demonstrated a greater mean normalized AUC on Day 1 compared to Day 7 ($p < .001$). Comparison of between group effects for the males on Day 1 also indicated the 1x CHIMERA animals exhibited a greater mean AUC than the sham males ($p = .002$), but the females at this time were not different due to injury ($p = .453$). By Day 7, however, the male 1x CHIMERA mice showed a significant AUC decrease compared to sham males ($p = .022$; **Figure 3A**).

Comparison of AUC measures in the meninges after repetitive CHIMERA impacts found a significant 2-way Injury $\times$ Day interaction ($F_{2, 25.681} = 5.613, p = .010$). No sex differences were detected, so the data presents findings collapsed across sex (**Figure 3B**). Pairwise comparisons indicated greater enhancement on Day 1 in the mice that sustained CHIMERA injury compared to sham animals ($p = .005$). This enhancement difference continued one week post-injury, albeit with levels lower than were seen on Day 1, but the difference persisted ($p < .001$), and AUC levels for the 4x CHIMERA mice meninges continued to be greater compared to sham mice on Day 7 ($p = .035$). AUC

values on Day 1 after CHIMERA injury were also significantly greater than Baseline ($p < .001$) and higher than Day 7 ($p < .001$).

Since the meninges is a thin structure, there is a chance alterations in DCE are associated with inclusion of underlying cerebral cortex that is captured as a portion of the meninges ROIs. An Injury x Sex x Day analysis indicated the AUC measures of the cerebral cortex directly inferior to the meninges ROIs did not display any significant differences as a function of the injury, sex, or across days. (**Figure 4A**). Likewise, no significant changes were observed after 4x CHIMERA in the cortex over time (**Figure 4B**).

Analysis of the 1x CHIMERA T2-relaxation time data in the meninges (**Figure 3C**) found significant 2-way interactions of Injury $\times$ Day ($F_{2, 33.699} = 5.453, p = .009$) and Sex $\times$ Day ($F_{2, 33.699} = 3.808, p = .032$). However, *post hoc* assessments for differences related to Sex $\times$ Day were not different. The Injury $\times$ Day difference indicated the average T2 value was greater for the 1x CHIMERA group than the sham group on Day 1 ($p = .010$). The injured and sham groups did not differ at Baseline or on Day 7 ($p = 0.112$). On Day 1, T2 assessment in injured mice were also significantly greater than at Baseline ($p = .004$) and Day 7 ($p = .007$). The 3-way ANOVA of T2 values for the 4x CHIMERA and sham group indicated no significant meningeal changes (**Figure 3D**).

Initial enhancement was observed in regions of the visual pathway after CHIMERA followed by decreases one week post-injury

No AUC changes were detected in the OPT. The four-factor ANOVA for 1x CHIMERA AUC levels in the brain ROIs demonstrated a Brain Region x Injury x Sex x Day interaction ($F_{18,822.967} = 1.841, p = .018$). The LGN displayed an effect of injury on Day 1 with an initial AUC increase after injury in males compared to sham mice ($p =$

.023), but then levels of AUC were decreased on Day 7 ($p = .002$). Within-subjects comparisons revealed injured males (**Figure 5A**) also had reduced AUC values on Day 7 than measures at Baseline ($p = .007$) and Day 1 ($p < .001$). The SC showed a similar pattern. Compared to sham animals, AUC in 1x CHIMERA injured males was increased on Day 1 ($p = .024$) followed by a decrease one week post-injury ($p = .008$). Bonferroni analyses also indicated there was an AUC decrease on Day 7 for CHIMERA males compared to Baseline ($p = .002$) and Day 1 ($p = .003$; **Figure 5B**). Interestingly, injured female mice demonstrated a decrease in mean AUC values compared to sham animals on Day 1 ($p = .021$). No significant differences in AUC measures were observed after 4x CHIMERA injuries.

No significant changes in T2-relaxation were seen after 1x CHIMERA injury. A four-factor ANOVA of the T2 relaxation measures for the 4x CHIMERA data indicated there was a significant Brain Region x Injury x Day interaction ($F_{14,592.324} = 3.208, p < .001$) and Injury x Sex x Day interaction ($F_{2,594.253} = 3.432, p = .033$). The latter interaction effect suggested there was an overall decrease in injured male and female mice across all brain regions on Day 7 compared to sham animals. On Day 1, the OPT exhibited an initial increase in T2-relaxation in the 4x CHIMERA mice compared to sham animals ($p < .001$), and this level in injured mice was greater on Day 1 compared to Baseline ($p < .001$) and Day 7 ($p < .001$). A pattern of decrease in T2 relaxation time was observed for injured animals compared to sham on Day 7 for the OPT ($p < .001$) and LGN ($p = .036$), whereas the sham mice had higher Day 7 within-subject levels compared to Baseline ($p = .001$) and Day 1 ($p = .001$; **Figure 5C**). *Post hoc* analyses for

the LGN displayed higher Day 1 compared to Day 7 values for injured animals ($p = .012$; **Figure 5D**). No T2-relaxation changes were detected in the SC.

GFAP increases in the visual pathway

Representative images of GFAP staining are shown in **Figure 6A**. The OPT exhibited significant changes in astrogliosis following injury. Percent area stained analysis for GFAP in the OPT displayed a significant increase in astrogliosis of injured animals after 1x CHIMERA ($n = 12$; median value 28.10; $U = 28$, $p = .0100$) compared to sham (median value 22.04; **Figure 6B**). Likewise, mice subjected to repetitive impacts had increased GFAP in the OPT ($n = 12$; median value 24.55; $U = 34$, $p = .0284$) compared to 4x sham mice (median value 20.27; **Figure 6C**). GFAP ROIs for the LGN and SC did not display significant differences between 1x CHIMERA and sham conditions (**Figure 6D & F**). However, repetitive injury was associated with changes in the SC. Animals that received 4x CHIMERA impacts (median value = 18.53), compared to sham mice (median value = 11.56) had increased GFAP staining in the SC region ($U = 11$, $p = .0001$; **Figure 6G**). Similar to findings after a single injury, no significant changes were observed in the LGN after 4x CHIMERA (**Figure 6E**).

With potential relevance to the meninges, the glial limitans were visually examined at 40x for qualitative astrocytic changes, but no observable differences between groups were found (data not shown).

DISCUSSION

1x CHIMERA and 4x CHIMERA induced a loss of consciousness and sex differences occurred during repetitive impacts

The rationale for the impact sequence was selected to match previous experiments (168). The repetitive 4x CHIMERA design selected chiefly corresponds with other studies in terms of using repeated impacts 24 hours apart (168), repetitive injuries with similar energy parameters known to result in no or few fatalities (37; 186), and an energy level (0.6 J) below the threshold of skull fracture, but that still induced changes (183).

CHIMERA injured animals exhibited greater durations of loss of consciousness after a single impact compared to the sham mice, but no sex differences were observed. The 4x CHIMERA group also experienced longer periods of unconsciousness following each impact they received, compared to uninjured matched animals, and a sex difference was observed. Possible reasons for the observed sex difference may be related to CHIMERA biomechanics and body weight. Males were heavier than females, which may pose different injury mechanics or decreased rotational acceleration movement after impact. In a recent repetitive CHIMERA study that used a higher impact level (0.7 J), longer righting reflexes were seen for both injured males and females compared to sham counterparts (260). This study may also have relevance for observed body effects with repeated injuries. We did not observe a reduction in body weight in males, in contrast to this previous repetitive TBI study that used the CHIMERA device at 0.7 J, and found a change in males (260).

Significant meningeal enhancement occurred after 1x CHIMERA and 4x CHIMERA

Meningeal enhancement after injury was observed after both the 1x and 4x CHIMERA injury. However, while single and repetitive CHIMERA exposed groups demonstrated increased AUC values for the meningeal ROI compared to sham counterparts on Day 1, the single CHIMERA mice appeared to return to below AUC

Baseline levels one week post-injury. This suggests that barrier impairment was resolved a week following single impact, whereas the repetitive CHIMERA mice continued to exhibit elevated enhancement on Day 7, indicating a persistent effect of injury for animals that underwent multiple impacts. Since the slice thickness for the DCE imaging was different for the 4x (1000 microns) compared to the 1x (500 microns) cohorts, direct comparisons of the relative change after impact(s) cannot be considered. However, changes relative to the respective sham group AUC levels on Day 1 suggested similar large effect sizes for 1x CHIMERA male mice and their respective sham group (Hedge's $g = 1.64$) and for the 4x CHIMERA mice compared to their sham counterparts (Hedge's $g = 1.10$). A similar effect of repeated injury has been seen in a compression injury model in the meninges. After a single injury, meningeal reconstitution was demonstrated with observed revascularization following a week after injury. When a second compression injury was introduced within one day of the initial injury, however, meningeal revascularization was impaired (233).

Resolution of TME suggests meningeal repair and decreased inflammation (233). Clinical studies indicate the duration of meningeal enhancement in humans can vary (68; 132; 263), but most TME cases resolve one week to one month post-injury (233). Neuroimaging in the current study after a single injury is consistent with meningeal recovery, but longer time points post-injury should be included in future studies to better understand the time course of the observed meningeal enhancement, the relationship to injury severity, as well as possible profile differences in responses in male and female animals. In clinical populations, sex differences with respect to TME are also presently unknown. However, the current results with persistent AUC enhancement in injured male

mice seven days after 1x CHIMERA suggest male meninges may be more sensitive to an impact (138; 173). The potential sex differences observed in the current pre-clinical data differs from most clinical data suggest that female patients have higher concussion risk and poorer outcomes than males. Female student athletes appear to be particularly susceptible to posttraumatic migraines as well as migraines in general, which could be directly related to meningeal damage. There may also be overall sex differences in the type of symptoms reported after TBI (138; 173). Additional reports, however, argue that DTI showed more damage and longer recovery in male patients and that possible baseline sex differences in imaging might be present with hypointense susceptibility weighted images in males compared to females (71; 98). Further investigation of TBI-related sex differences is needed, as clinical neuroimaging reports consist of small sample sizes and results remain unclear in relation to injury severity.

Elevated T2 relaxation times in the meninges after impact in the 1x CHIMERA injured animals

T2-weighted images can inform about robust structural changes. Increased signal related to water content may be indicative of edema and inflammation, whereas hypointensity is associated with hemorrhage (98; 145). The 1x CHIMERA injured animals displayed higher T2 values compared to sham mice on Day 1, suggesting edema and inflammation in the meninges after impact, which has been reported to rapidly occur in the minutes and hours after cerebral insult (14). By Day 7, however, T2 relaxation times were not significantly different from baseline, suggesting meningeal inflammation had subsided within one week post-injury. The lack of T2 change in the meninges after 4x CHIMERA on Day 1 was surprising, but possibly a result of the longer passage of time (3 days) from initial injury or from the neuroprotective effects of repeated isoflurane

exposure during the MRI scans (approximately one hour) on the days preceding injuries on Days 2–4 (247). The 4x CHIMERA animals received four consecutive days of isoflurane, so the increased repetitive, amount of neuroprotective exposure may have had anti-inflammatory outcomes. Human TME is primarily assessed using T2-FLAIR sequences and only one study has reported some concurrent brain parenchymal T2*-weighted imaging differences in mild TBI patients, but no meningeal T2 relaxation time changes, as used in the current study, have been previously discussed (40).

No MR cortical changes were observed

Subsequent to the observed meningeal damage, brain ROIs were also analyzed for indicators of injury and astrogliosis (see Supplementary Material). Unlike previously described open-head models of TBI, the cortex in this closed-head study did not show MRI contrast agent enhancement (145; 284). Imaging studies with controlled cortical impact in rats at similar time points showed T2 and DCE enhancement in the cortex. Corresponding cortical GFAP levels were also increased in one study, but only male rats were examined (284). Other closed-head designs, such as lateral impact and weight-drop models, displayed greater damage than the CHIMERA and reported BBB damage with contrast agent extravasation into the brain parenchyma. These TBI models appear to mimic severe injury and advanced pathology, whereas the CHIMERA may capture subtle changes (252; 266).

The lack of change in the cortex could be due to the nature of CHIMERA-induced injuries, where here, alterations to the brain parenchymal surface were not apparent *in vivo*. The absence of observable cortical damage *in vivo* is consistent with repetitive CHIMERA studies, which also did not show gross pathology in the cortical tissue near

the site of impact (260). Likewise, no parenchymal gadolinium extravasation was detected in a clinical study of TME (263). The parameters used in this experiment may induce a milder degree of injury relative to MRI visible contusions reported in the clinical scenario.

Finally, coronal measures of DCE in the meninges were obtained at two mm caudal to bregma. In addition to assessing the response in this region, the cortex ROI in this study played an additional role as a control for the meningeal ROI. Because of partial volume effects, it was not possible to draw the meningeal ROI at the level of bregma, directly underneath the CHIMERA site of impact. The cortex ROI, however, expanded from bregma to two mm caudal to bregma, and did not show any changes suggesting that the differences observed in the meningeal ROI in corresponding posterior bregma sections reflected changes to the meninges. Edema has been observed through T2 imaging after a pre-clinical model of controlled cortical impact TBI in rats (145; 284). Consistent with previous CHIMERA imaging studies, however, no T2 changes were reported in the cortex (91; 186). The absence of T2 enhancement in the cortex, as well as an absence of histological damage, implies that the CHIMERA injury was mild in nature and did not cause significant contusion to the brain parenchymal surface.

MR alterations in the visual pathway

Differences in MR measures were observed as a function of single and multiple insults. The observed pattern of 1x CHIMERA AUC and 4x CHIMERA T2-relaxation decreases on Day 7 in these brain regions are consistent with reported susceptibility-weighted imaging hypointensities after concussion (98). Previous neuroimaging studies following CHIMERA focused on *ex vivo* DTI, with only one *in vivo* DTI study, and these

mainly reported decreases in fractional anisotropy and diffusivity increased in white matter tracts post-injury (91; 140; 218; 239), but no reports of T2 changes were noted (91; 186). The decreased T2-relaxation observed by Day 7 may indicate a robust anti-inflammatory response or potential hemorrhage one week after injury (145). In the OPT, Day 7 sham displayed greater T2 intensity than Baseline sham where no change overtime was expected. Although the observed MRI differences in the reported brain regions reached significance, the changes were subtle and warrant further investigation to better elucidate the underlying mechanisms. In parallel with observed differences in the visual system, functional impairment is a frequently reported consequence of CHIMERA injury. The immediate Day 1 MRI changes in areas related to the visual pathway are consistent with previous CHIMERA studies that reported functional impairments on visual tasks, such as the visual cliff test and Morris Water Maze, as well as decreased visual evoked potentials after single and multiple impacts (38; 56; 57). Visual deficits, however, were observed at later time points than the current study's reported Day 7 recovery (38; 57).

GFAP changes in the visual pathway

Increased GFAP staining has been reported at both acute and longer-term time points after CHIMERA. Although other CHIMERA studies described elevated GFAP changes up to one week post-injury in several regions, such as the OPT, corpus callosum, hippocampus, cortex, and SC (37; 91; 144; 260), the current study only observed GFAP changes in the OPT and SC (Supplemental Table 3). The LGN is a primary termination for OPT axons and an absence of GFAP staining changes was not expected. This may imply that the astrogliosis in the SC is not the results of optic nerve trauma, but a consequence of the anatomical position of this structure. CHIMERA injury to the

parenchyma may affect superficial locations such as OPT and SC that are more prone to injury from displacement. A weight drop study reported increased GFAP in the OPT, LGN, and SC seven days after injury, but also noted that these regions may have different time lines of inflammation presentation (69).

A *post hoc* assessment was performed to determine if GFAP staining was associated with AUC or T2 MRI results in corresponding regions. Imaging and histology in this study were not well correlated, with Pearson correlations of .01-.51. Strong imaging and pathology cross-correlates were reported, however, by a different study. Post-mortem tissue from TBI patients was imaged using *ex vivo* multidimensional MRI and displayed a strong positive correlation in the corpus callosum with higher multidimensional intensity and increased diffuse axonal injury severity indicated by amyloid precursor protein histology (19). Although GFAP in the current study could potentially appear as T2 enhancement because of the inflammation associated with astrogliosis, perhaps the imaging constraints described earlier or the degree of inflammation and lack of edema by Day 7 were factors lending to a difference for what was reported in this clinical report.

Limitations and Future Directions

Across the study, the CHIMERA device was found to deliver consistent impacts. There may be a degree of imprecision for the impact location across animals and injuries since the positioning of the scalp above the piston relies on investigator judgement. Nevertheless, the 5 mm piston diameter relative to the dimensions of the mouse scalp suggest head placement on the device is repeatable by an experienced operator. The CHIMERA device is an ideal pre-clinical model. It is a non-surgical procedure allowing a

brief exposure to anesthesia at the time of injury, which was essential to the avoidance of meningeal enhancement due to lengthy operative procedures. However, the MRI scanning required prolonged isoflurane use. Although no direct link between isoflurane's effects on contrast agent is evident and the effect of repetitive scans is unclear, anesthetics can alter MRI results by changing animal body temperature and metabolism. Many variables can affect imaging and hypothermia due to anesthetics is a primary concern (103). A recent study showed that isoflurane exposure during MRI reduced histological blood-brain barrier damage compared to animals that did not undergo prolonged sedation (59). The MRI differences observed in some sham animals could be related to these biological variable responses to isoflurane since individual differences in anesthetic stabilization were observed despite standardized imaging procedures/setup. Future studies could include the use of alternative sedation agents as a substitute for isoflurane during imaging.

MRI analysis was restricted by image resolution. No initial whole brain analyses was conducted because of the slice thickness, so that voxel-based analysis was not optimal. Acquisition resolution resulted in deeper and more medial ROIs being more susceptible to partial volume effects during MRI analysis. A surface coil was utilized throughout the current study, therefore signal-to-noise was decreased further from the coil. Like the brain, the skull and underlying meninges are heterogeneous with variability in thickness depending on the location and species measured. Studies of murine meninges report inter-subject and tissue preparation variability, adding to the complexity in measuring meningeal thickness (70; 156; 191; 272). While composition is fairly consistent across species, dural thickness has been reported to be about 50 μm in rats

compared to approximately 564 μm in humans, but variability in tissue preservation methods can greatly alter this measurement (133; 272). There may also be differences among rodent meningeal structure with well characterized subarachnoid space reported in rats, whereas mice may not possess such space or arachnoid trabeculae (191; 235). This variability in meningeal thickness and its size relative to the MRI resolution means that the meninges ROI contains some other structures; however, the cortex ROI provided a control to differentiate between the meninges and underlying brain parenchyma. A rodent meningeal model is ideal in terms of logistics, but future TME study should include larger animal models that more closely resemble human meningeal properties (48). The current study employed an intraperitoneal route for contrast agent injection. The intraperitoneal route has been employed previously in rodent blast studies (229), and has been seen as the better alternative when the study requires multiple injection events (206; 209). While intravenous administration is more clinically relevant, tail vein access is challenging and repetitive local access is an effective alternative with lessened local vessel damage.

While the resolution of MRI was at the limits for exclusive capture of voxels that were circumscribed to the meningeal layer, the results suggest that a pre-clinical model of TME can be achieved using the CHIMERA device, which can allow for further study to determine the mechanism of action. To understand the mechanisms relevant to TME and potential protective meningeal properties, *ex vivo* analysis of the meninges could provide detailed structural data to show anatomical changes in the meninges after injury (93; 132; 148; 172; 180; 181; 225; 263; 272). Additional approaches, such as meningeal two-photon microscopy has been successfully employed and could assist in studying the

pathway for neuroimmune exchange in the meninges that contribute to the observed meningeal enhancement (130; 225; 233; 257). Lastly, determining comparable differences between single and repetitive injury and potential behavioral deficits related to meningeal damage is critical to defining functional impairments. TME in humans may provide a sensitive imaging biomarker for mild TBI and could present a therapeutic target to prevent additional inflammatory damage to the parenchyma.

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AUTHOR CONTRIBUTIONS:

EHM, BD, JTM contributed to study design and development, A. Knutsen and EHM worked on MRI data quantification and analyses, A. Korotcov and AB performed MRI acquisition, EHM, JL, CK, AAG, HS assisted with MRI animal monitoring, AHF provided CHIMERA device training, EHM performed CHIMERA procedures, EHM and JL collected tissue, EHM, CK, AAG contributed to histology, EHM and CK quantified histology, A. Knutsen and A. Korotcov wrote MRI methods sections of the manuscript, EHM and JTM did statistical analyses and wrote the manuscript, EHM, A. Knutsen, A. Korotcov, BD, JTM edited the manuscript, EHM and JTM finalized the manuscript with authors' inputs.

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Cohort	Injury	Sham	Resolution for T2W (μm^3)	DCE Resolution (μm^3)
1	4x n = 12	4x n = 3	150 x 150 x 1000	150 x 150 x 1000
2	4x n = 6	4x n = 9	150 x 150 x 1000	150 x 150 x 1000
3	1x n = 8	1x n = 4	150 x 150 x 1000	150 x 150 x 500
4	1x n = 6	1x n = 8	150 x 150 x 1000	150 x 150 x 500
5	1x n = 5	1x n = 7	150 x 150 x 1000	150 x 150 x 500

Table 3: MRI cohort sample sizes.

Pre-contrast and post-contrast T2-weighted images were acquired during each MRI. ProHance gadolinium-based contrast agent was injected during every scan and DCE values were acquired with a slightly higher resolution for the single CHIMERA experiment versus the repetitive CHIMERA group.

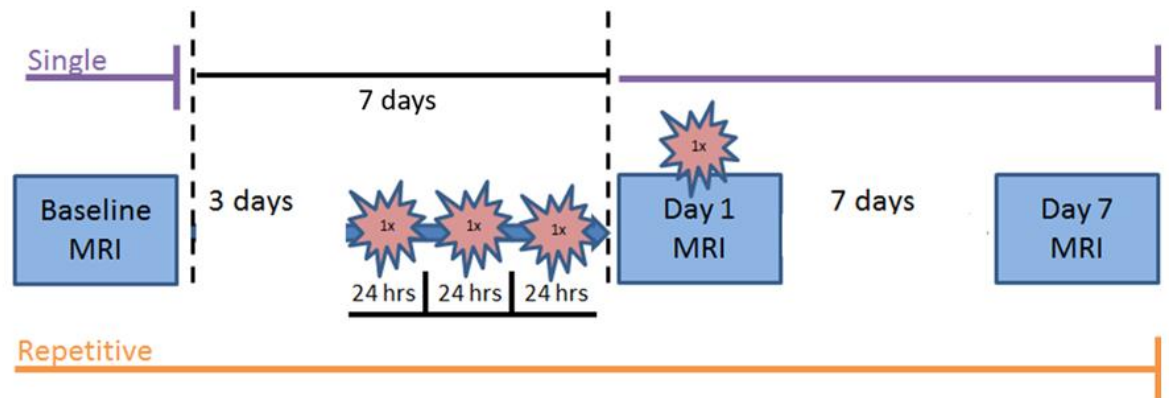


Figure 15: Schematic of the experimental procedure timeline for single and repetitive CHIMERA experiments.

Timeline for single (purple, top) and repetitive (orange, bottom) experiments. For all groups three MRI scans were performed at Baseline (one week before the final CHIMERA), on Day 1 (immediately after the single or last CHIMERA), and Day 7 (one week following the final injury). The single CHIMERA animals only received one impact immediately before Day 1 scanning, while repetitive CHIMERA mice underwent three impacts separated 24 hours apart on the three days before the fourth and final impact that was performed immediately before Day 1 scanning. Total scan time was approximately 55 minutes for each MRI. ROIs used for MRI analyses are displayed in Supplemental Figure 1 and histology ROIs are outlined in Figure 6A.

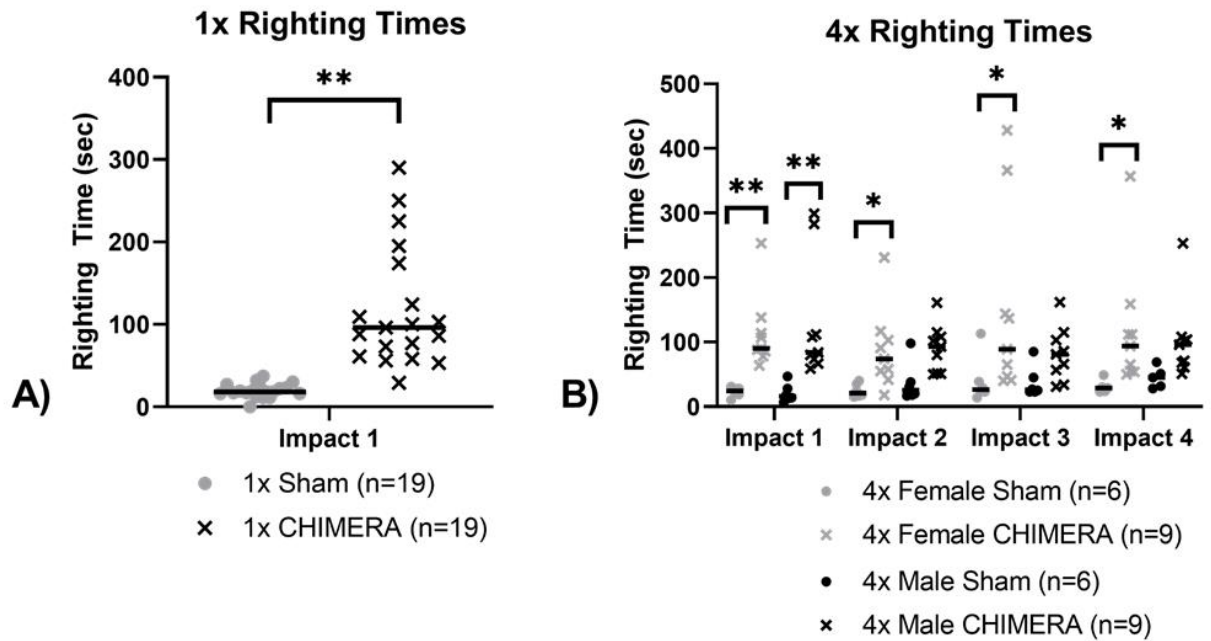


Figure 16: Righting times increased after single and repetitive CHIMERA.

A) Righting times after 1x CHIMERA. Injured groups (n = 19) for single impact displayed longer righting times and loss of return to consciousness compared to sham counterparts (n = 19, $H_3 = 27.042$, $p < .001$). B) On Day 1 for 4x CHIMERA, both male CHIMERA (n = 9) and female CHIMERA (n = 9) injured mice sustained increased righting reflexes compared to sham ($H_3 = 23.08$, $p < .001$). A difference in sex was observed on Days 2-4, where the female 4x CHIMERA injured animals continued to have longer righting reflex durations compared to female 4x Sham (n = 6). On later days, the male 4x CHIMERA animals were not different from levels measured in the 4x Sham male mice (n = 6). Bars indicate medians. * indicates $p \leq .05$, ** indicates $p \leq .001$.

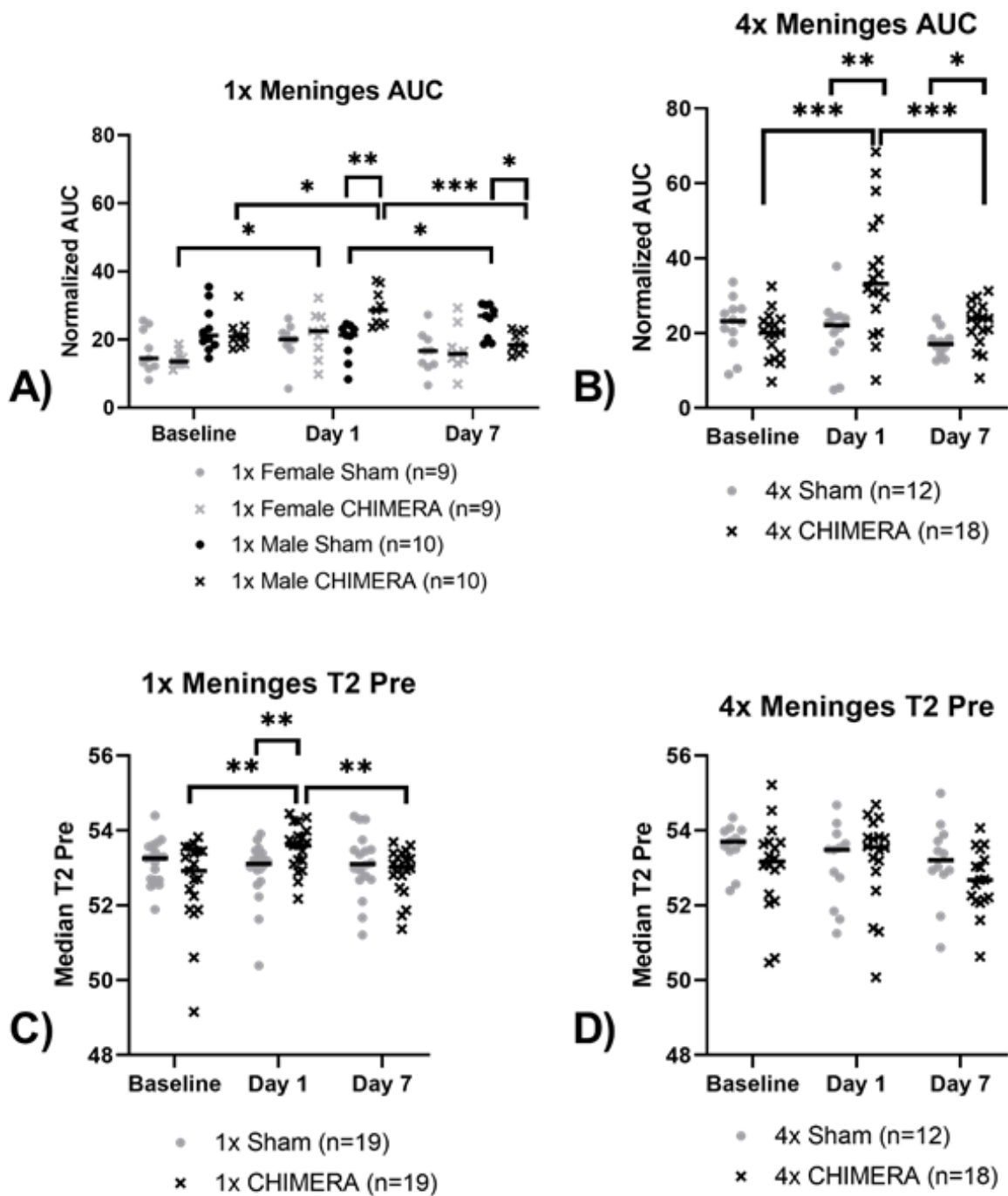


Figure 17: Meningeal MRI enhancement observed after injury in 1x CHIMERA and 4x CHIMERA conditions.

A) Analysis of the 1x CHIMERA meningeal ROIs showed an interaction of Injury x Sex x Day ($p = .006$). The male CHIMERA mice ($n = 10$) AUC measures were significantly greater compared to sham mice ($n = 10$) on Day 1 ($p = .002$). CHIMERA animals had elevated AUC values on Day 1 compared to

their Baseline for males ($p = .014$) and females ($p = .024$), on Day 7 injured males demonstrated decreased AUC compared to sham ($p = .022$), and within-subject comparison for 1x CHIMERA males displayed a decrease between Day 1 and Day 7 AUC levels ($p < .001$). **B)** The 4x CHIMERA experiment revealed an interaction between Injury x Day ($p = .010$). On Day 1 after the fourth impact, there was an effect of injury with greater enhancement in the CHIMERA mice ($n = 18$) compared to sham animals ($n = 12$, $p = .005$). The CHIMERA group enhancement was also sustained one week post-injury ($p = .035$). Day 1 CHIMERA were significantly greater than Baseline CHIMERA ($p < .001$) and higher than Day 7 CHIMERA ($p < .001$). **C)** T2-relaxation analysis revealed Injury x Day ($p = .009$) interaction after 1x CHIMERA. On Day 1 T2 values increased in the CHIMERA group ($n = 19$) compared to sham mice ($n = 19$, $p = .010$). CHIMERA on Day 1 was also greater than CHIMERA at Baseline ($p = .004$) or on Day 7 ($p = .007$). **D)** Following 4x CHIMERA, no significant changes were observed. Bars indicate medians. No sex differences were found after single or repetitive CHIMERA, so male and female results were combined. * indicates $p \leq .05$, ** indicates $p \leq .01$, *** indicates $p \leq .001$.

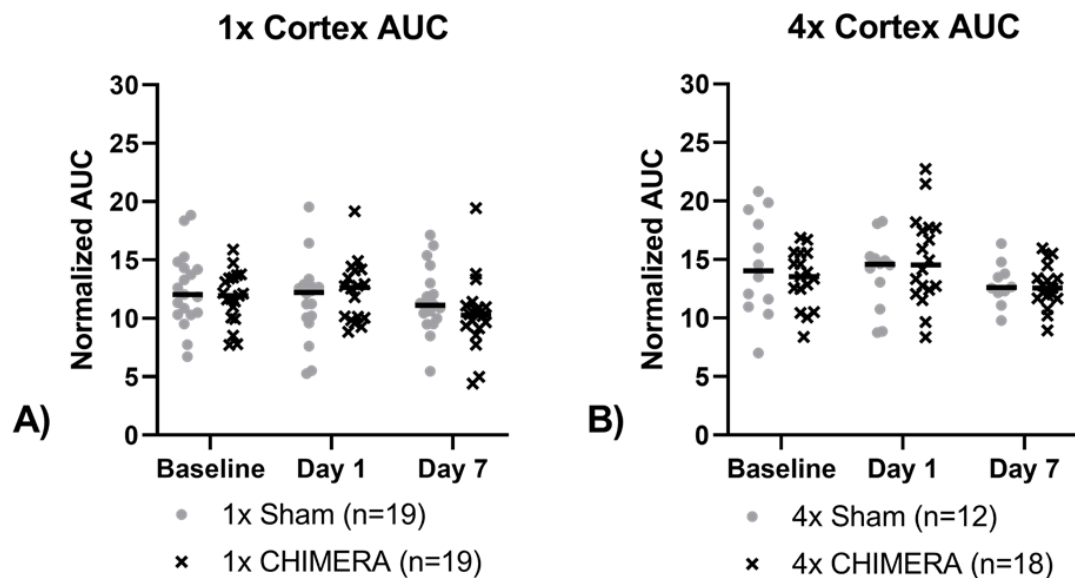


Figure 18: No significant cortical differences in DCE.

As measured by AUC, no cortical differences were observed after **A)** single or **B)** repetitive CHIMERA. No sex differences were observed in the cortex after single or repetitive CHIMERA, so male and female results were combined.

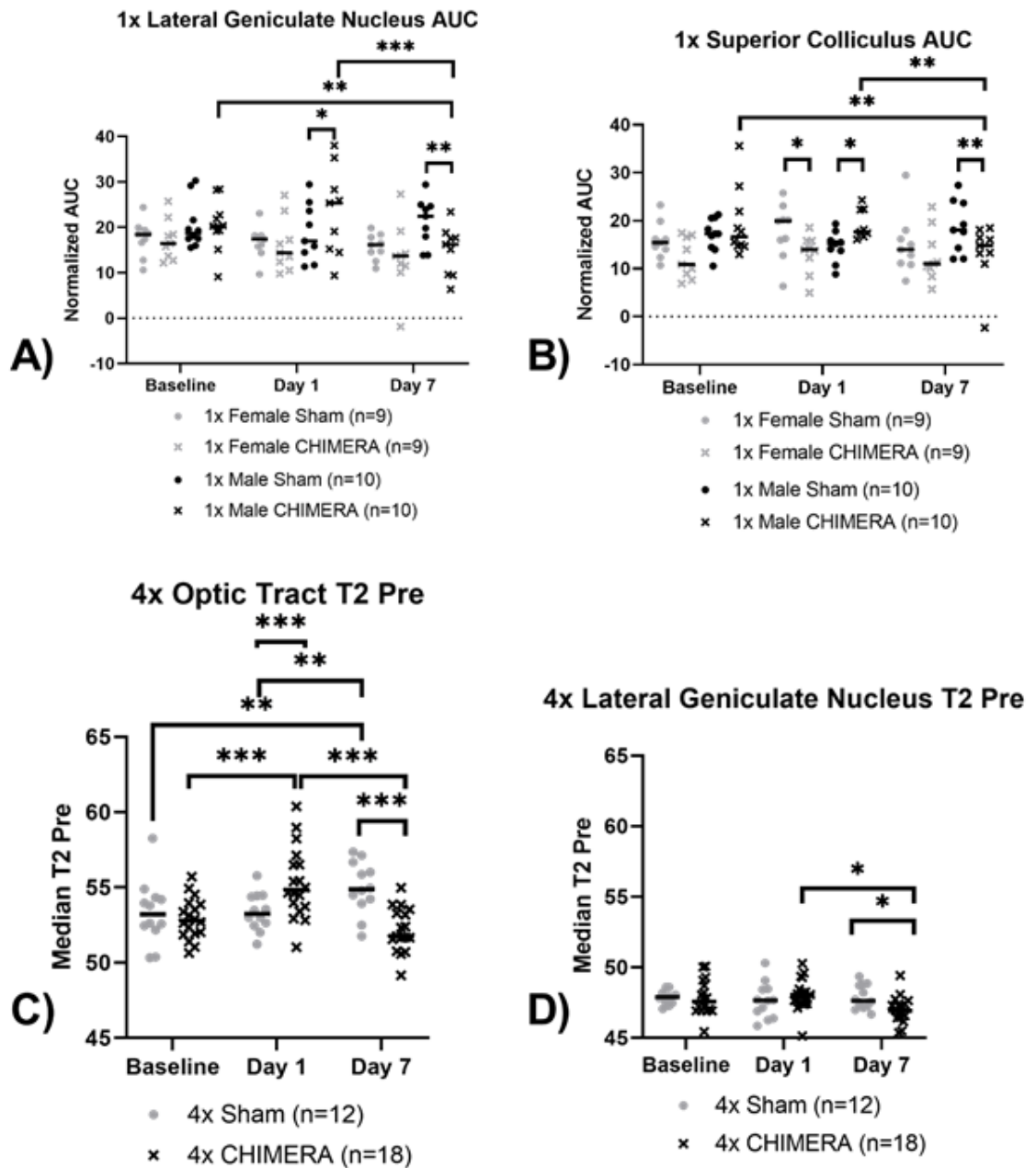


Figure 19: ROIs for visual areas showed different changes after CHIMERA.

A) The LGN displayed an effect of injury on Day 1 with an initial increase in AUC measures after injury in males compared to sham males ($p = .023$), but then there was a decrease in AUC on Day 7 ($p = .002$). Within subjects comparison revealed injured males had higher Baseline ($p = .007$) and Day 1 ($p < .001$) levels compared to Day 7. **B)** The SC showed a similar pattern with an increase in AUC values on Day 1 in 1x CHIMERA males compared to sham ($p = .024$) followed by a decrease one week post-injury ($p = .008$). Injured female mice demonstrated a decrease in AUC measures in the SC on Day 1 compared

to sham females ($p = .021$). Bonferroni analyses also indicated there was decrease in AUC on Day 7 for the CHIMERA males compared to measures at Baseline ($p = .002$) and Day 1 ($p = .003$). **C)** A pattern of decreased T2 values was observed for injured animals compared to sham on Day 7 for the OPT ($p < .001$) and LGN ($p = .036$). The OPT showed an acute increase in T2 relaxation time on Day 1 for the 4x CHIMERA mice compared to sham mice ($p < .001$). The 4x CHIMERA animals had higher Day 1 within group levels compared to Baseline ($p < .001$) and Day 7 ($p < .001$), whereas the Sham had higher Day 7 within-subject levels compared to Baseline ($p = .001$) and Day 1 ($p = .001$). **D)** *Post hoc* analyses for the LGN displayed higher Day 1 compared to Day 7 values for injured animals ($p = .012$). Bars indicate medians. * indicates $p \leq .05$, ** indicates $p \leq .01$, *** indicates $p \leq .001$.

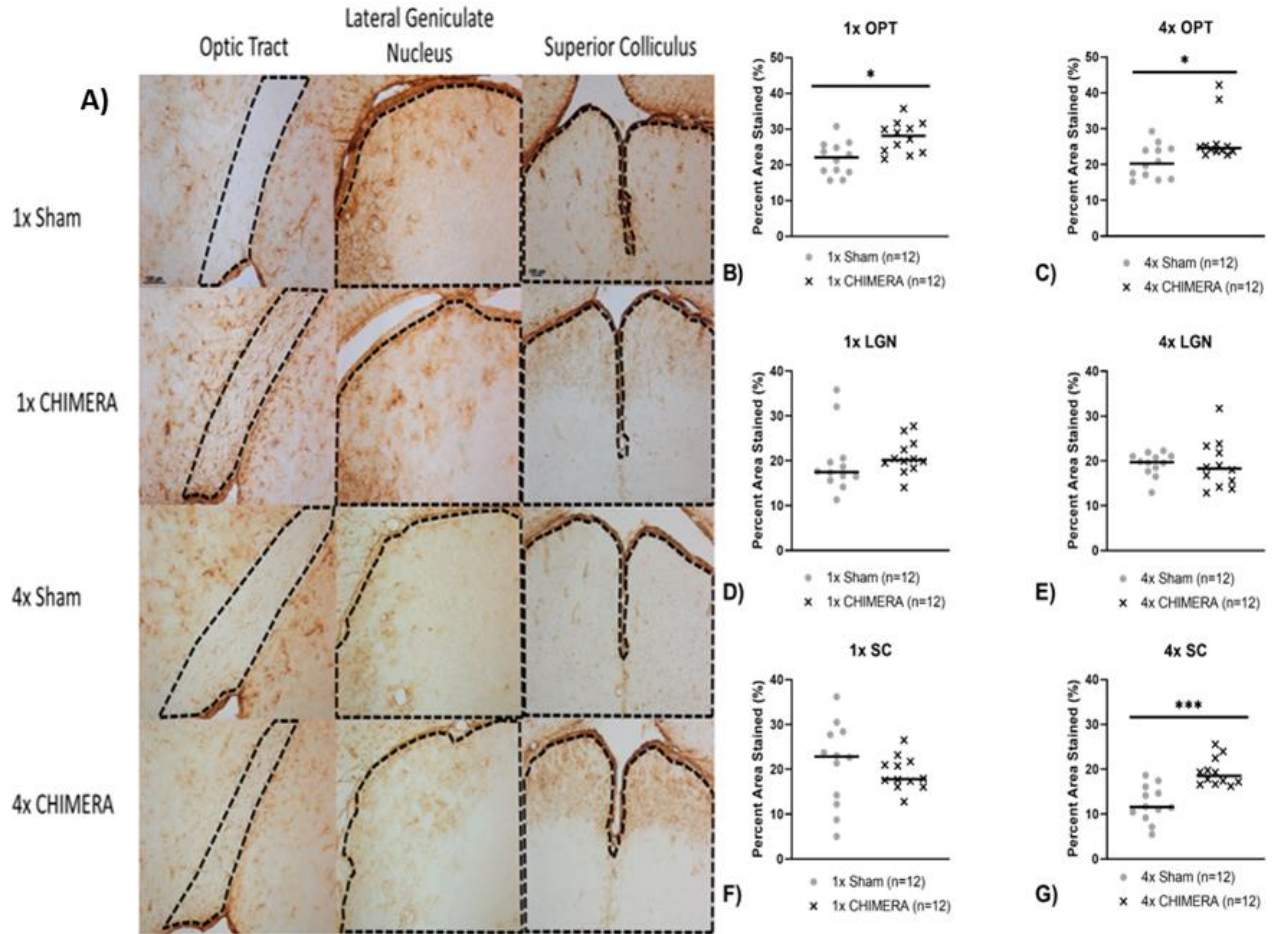


Figure 20: GFAP analyses of the visual pathway.

A) Representative GFAP images from the OPT, LGN, and SC (regions are circled with a dashed line). B) Percent area stained analysis for GFAP in the OPT displayed a significant increase in astrogliosis after 1x CHIMERA. Following 1x CHIMERA, there was an increase in GFAP in injured animals ($n = 12$; median value 28.10; $U = 28$, $p = .0100$) compared to sham animals ($n = 12$; median value 22.04). C) After 4x CHIMERA OPT astrogliosis was also observed in injured mice ($n = 12$; median value 24.55; $U = 34$, $p = .0284$) compared to 4x Sham mice (median value 20.27). D and E) No significant changes in the LGN after 1x CHIMERA (sham median = 17.50, 1x CHIMERA median = 20.15) or 4x CHIMERA (sham median = 19.71, 4x CHIMERA median = 18.29). F) GFAP in the SC after 1x CHIMERA (median = 17.83) was not significantly different than sham (median = 22.87). G) GFAP analysis in the SC showed a significant difference ($U = 11$, $p = .0001$) between 4x CHIMERA ($n = 12$; median value = 18.53) and sham ($n = 12$; median value = 11.56) with elevated GFAP staining in injured animals one week after the fourth impact. * indicates $p \leq .05$, ** indicates $p \leq .01$ *** indicates $p \leq .001$.

SUPPLEMENTAL MATERIAL

MRI data processing

All registration steps were performed using the Advanced Normalization Tools software (9). All Bruker 2dseq files were converted to the NIFTI file format. An N4-bias field correction was applied to the first frame of the DCE data and the computed bias field was applied to the other DCE image frames (264). To correct for potential motion, frames 2 through 10 (Cohorts 1–2) or 20 (Cohorts 3–5) were rigidly registered to frame 1 using the Mattes mutual information algorithm as the cost function. The transformation was restricted to be within the acquisition plane (i.e., no through-plane motion was allowed). The first three image frames were averaged together and used to draw the ROIs.

To correct for small motions within the scan session, the T2-weighted post-contrast images were rigidly registered to the T2-weighted pre-contrast image using the mattes cost function. The transformation matrix was restricted to be within the image plane. The computed transformation matrices were applied to the post-contrast T2-relaxation map using cubic b-spline interpolation. An N4-bias field correction was applied to the 30 ms TE of the pre-contrast T2-weighted image set (264). The computed bias field was applied to all other TEs and TRs for the pre- and post-contrast T2-weighted images (all Cohorts). T2-relaxation maps were computed using a nonlinear fit in Matlab to solve for S_0 and T_2 in the following function:

$$S_i(x_j) = S_0(x_j)e^{-\frac{TE_i}{T_2(x_j)}}$$

where S_i is signal intensity of the i^{th} TE of the j^{th} voxel, S_0 is the signal amplitude at a TE of 0 ms, and T_2 is the estimated relaxation parameter in ms.

Scans with low DCE values, three standard deviations below the mean baseline values, in the muscle ROI were identified as unsuccessful intraperitoneal gadolinium injections and were excluded. For unsuccessful Baseline or Day 7 injections, the scan was repeated on the following day. Ten animals from the 1x CHIMERA or 1x Sham groups received an additional scan or second gadolinium dose because of failed initial injections. In total 8/204 scans (1x Male Sham Day 1, 1x Male Sham Day 7, 1x Male CHIMERA Day 1, 1x Male CHIMERA Day 7, 4x Female CHIMERA Baseline, 4x Female CHIMERA Day 7, and two 4x Male Sham Day 7) were omitted due to death (2 scans), bad injections (5 scans), or ghost artifact (1 scan) discovered during image processing. To ensure outlier voxels did not bias the results, a Gaussian mixed model was applied to identify the number of voxel distributions present within each ROI of the Baseline scans; voxels outside ± 3 standard deviations from the mean of the estimated fit were excluded. The median value was then computed within each ROI for all metrics.

For DCE, the Area Under the Curve (AUC) was calculated to provide a more robust metric for image enhancement (116). The DCE percent enhancement (DCEPE) was calculated for each image frame using the formula for percent signal change from Baseline.

$$DCEPE_i(\mathbf{x}_j) = \frac{DCE_i(\mathbf{x}_j) - DCE_{pre}(\mathbf{x}_j)}{DCE_{pre}(\mathbf{x}_j)} * 100$$

Where DCE_i is the DCE signal in the j^{th} voxel of the i^{th} image frame and DCE_{pre} (pre-contrast) is the signal in frame 2 of 10 (Cohorts 1–2) or 3 of 20 (Cohorts 3–5) of the j^{th} voxel. The AUC value was computed by applying the *cumtrapz* function in Matlab to the average DCEPE series in each ROI (frames 2–10 for Cohorts 1–2, and frames 3–20 for Cohorts 3–5). To account for variable amounts of contrast agent uptake across scans

and animals, the AUC values in each ROI were normalized by the AUC value in the muscle ROI.

Statistics (continued from Methods)

T2 pre-contrast (T2 pre) is a clinically relevant metric for edema, impairment of the blood-brain barrier, and inflammation. However, when T2 post-contrast data was compared to T2 pre-contrast by T2 (i.e., (post-pre)/pre), changes in T2 post did not greatly differ from T2 pre values, so only T2 pre is reported.

The acquired scan data after 1x and 4x CHIMERA treatments were also analyzed for the brain with a four-factor linear mixed model (Brain Region x Injury x Sex x Day) with pertinent comparisons reported with Bonferroni correction. Preliminary inspection indicated the values for the mean AUC for the optic tract greatly differed compared to other brain regions. This structure was analyzed separately from the other brain ROIs.

Finally, as an estimate of the impact of CHIMERA on imaging values across the cohorts of animals that received one or repetitive injuries, Hedge's *g* statistic (96) was computed to estimate effect size using the following:

$$g = \frac{M_1 - M_2}{SD_{pooled}}, \text{ where } SD_{pooled} = \sqrt{\frac{(n_1 - 1)SD_1^2 + (n_2 - 1)SD_2^2}{n_1 + n_2 - 2}}, \text{ and } g \cong d \left[1 - \frac{3}{4(n_1 + n_2) - 9} \right].$$

Where *M* refers to the expected means obtained from the analyses of variance, *n*₁ is the sample size for sample₁ and *s*₁ is the standard deviation for mean₁. *n*₂ and *s*₂ are the sample size and standard deviation for sample₂, respectively. *g* is the unbiased version of Cohen's *d* (67).

Additional brain region analyses

Evaluation of brain regions after 1x CHIMERA with a four-factor (Brain Region x Injury x Sex x Day) interaction ($p = .018$) suggested the corpus callosum ($p = .012$), hippocampus ($p = .008$), lateral geniculate nucleus (LGN, Day 1 $p = .023$, Day 7 $p = .002$), and superior colliculus (Day 1 $p = .024$, Day 7 $p = .008$) exhibited DCE changes in male mice. Only the superior colliculus displayed a significant decrease in AUC in injured females on Day 7 ($p = .021$). Three-factor interactions for T2 brain analyses appeared with Brain Region x Injury x Day ($p < .001$) and Injury x Sex x Day ($p = .021$). However, several regions had lower T2 pre values one week after 4x CHIMERA including the optic tract (OPT, Day 1 $p < .001$, Day 7 $p < .001$), LGN ($p = .036$), and brainstem ($p = .016$). GFAP quantification showed astrogliosis changes only in the OPT for 1x CHIMERA ($p = .0100$) and 4x CHIMERA ($p = .0284$) and superior colliculus ($p = .0001$) after 4x CHIMERA.

Table 4: Summary of AUC results for 1x CHIMERA and 4x CHIMERA, *p*-values from ANOVAs.

AUC 4x CHIMERA Summary of <i>p</i> -values from ANOVAs*							
Region	Injury	Sex	Day	Brain Region	Injury x Day (Day)	Brain Region x Sex	Injury x Sex x Day
Meninges	ns	$p < .001$	$p = .013$	n/a	ns	n/a	n/a
							$p = .006$ male 1x CHIMERA > 1x Sham on D1; $p = .002$ male 1x CHIMERA < 1x Sham on D1; $p = .022$
Brain Regions	ns	$p = .005$	$p < .001$	$p < .001$	$p = .005$	$p = .035$	$p = .018$ Corpus Callosum: male 1x CHIMERA < 1x Sham on Day 7; $p = .012$ Hippocampus: male 1x CHIMERA < 1x Sham on Day 7; $p = .008$ Lateral Geniculate Nucleus: male 1x CHIMERA > 1x Sham on Day 1; $p = .023$ Male 1x CHIMERA < 1x Sham on Day 7; $p = .002$

											Superior Colliculus: female 1x CHIMERA < 1x Sham on Day 1; p = .021 Male 1x CHIMERA > 1x Sham on Day 1; p = .024 Male 1x CHIMERA < 1x Sham on Day 7; p = .008
Optic Tract	ns	ns	ns	n/a	ns	n/a	ns	n/a			n/a
AUC 4x CHIMERA Summary of p-values from ANOVAs*											
Meninges	p = .014	ns	p = .009	n/a	p = .010 4x CHIMERA > 4x Sham on Day 1; p = .005 4x CHIMERA > 4x Sham on Day 7; p = .035	n/a	ns	n/a	ns		n/a
Brain Regions	ns	p = .041	p < .001	p < .001	ns	p = .049 cerebellum	ns	ns	ns		ns
Optic Tract	ns	ns	ns	n/a	ns	n/a	ns	n/a	ns		n/a

*Abbreviations: n/a, not applicable; ns, not significant.

Table 5: Summary of T2 relaxation values for 1x CHIMERA and 4x CHIMERA, p -values from ANOVAs.

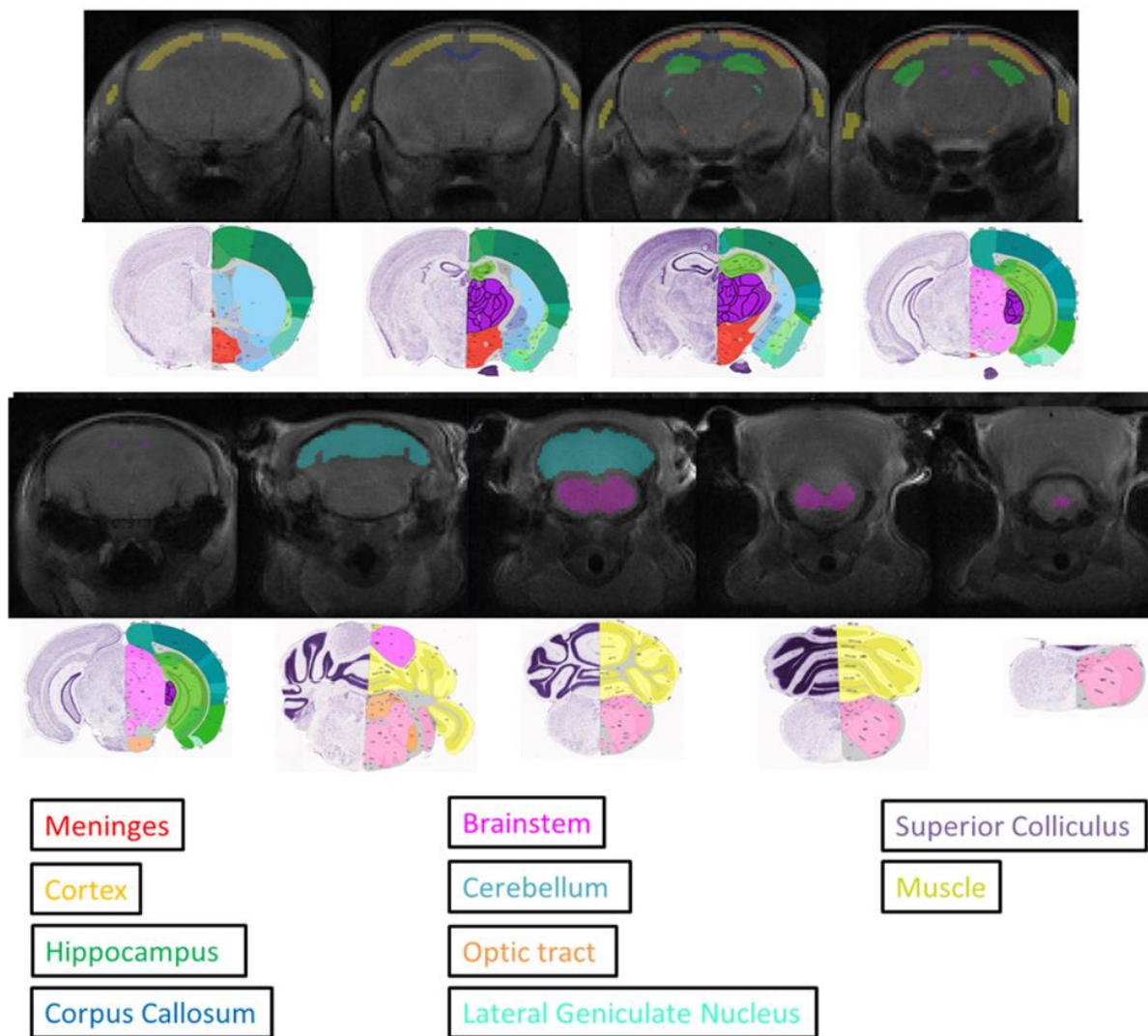
T2 1x CHIMERA Summary of <i>p</i> -values from ANOVAs*								
Region	Injury	Sex	Day	Brain Regions	Injury x Day (Day)	Sex x Day	Brain Region x Injury x Day	Injury x Sex x Day
Meninges	ns	ns	ns	n/a	p = .009 1x CHIMERA > 1x Sham on Day 1; p = .010	p = .032	n/a	ns
Brain Regions	ns	ns	ns	p < .001	ns	ns	ns	ns
T2 4x CHIMERA Summary of <i>p</i> -values from ANOVAs*								
Meninges	ns	ns	ns	n/a	ns	ns	n/a	n/a
Brain Regions	p = .037	p = .001	p = .019	p < .001	p < .001	p = .001	p < .001 Optic Tract: 4x CHIMERA > 4x Sham on Day 1; p < .001 4x CHIMERA < 4x Sham on Day 7; p < .001 Lateral Geniculate Nucleus: 4x CHIMERA < 4x Sham on Day 7; p = .036 Brainstem: 4x CHIMERA < 4x Sham on Day 7; p = .016	p = .021

*Abbreviations: n/a, not applicable; ns, not significant.

Table 6: Summary of all CHIMERA data for significant regions.

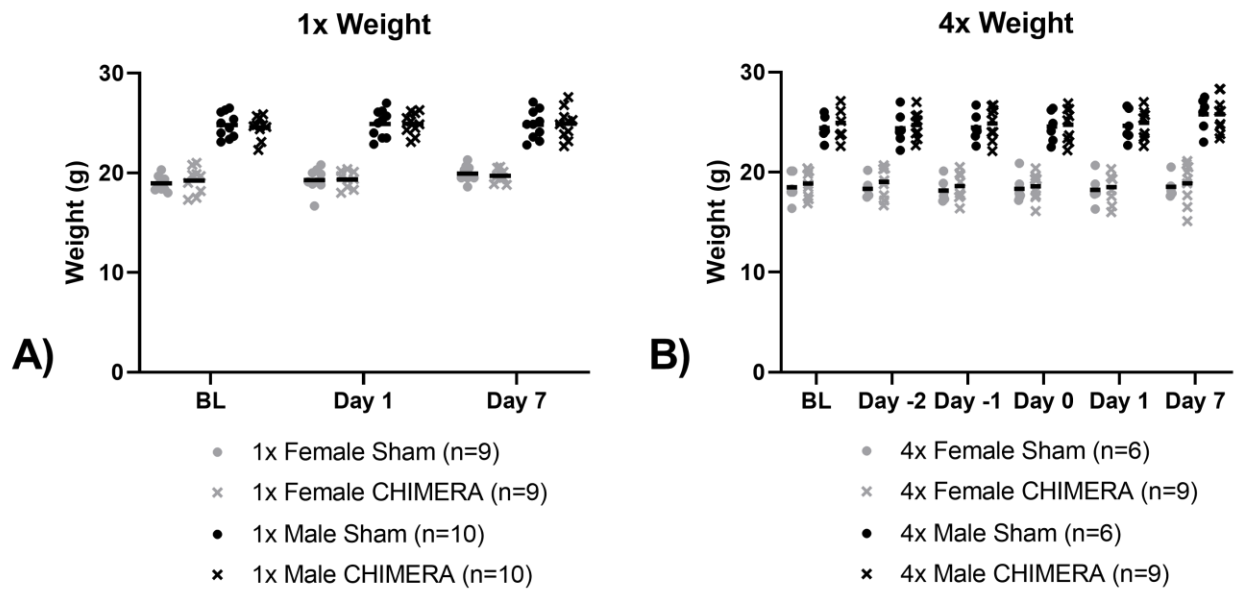
Summary of all data for significant regions*			
Region	AUC	T2	GFAP
Meninges	1x male ↑ on Day 1 then ↓ on Day 7 4x ↑ on Day 1 and Day 7	1x ↑ on Day 1	NA
Corpus Callosum	1x CC male ↓ on Day 7	ns	ns
Hippocampus	1x male ↓ on Day 7	ns	NA
Optic Tract	ns	4x ↑ on Day 1 then ↓ on Day 7	1x ↑ on Day 7 4x ↑ on Day 7
Lateral Geniculate Nucleus	1x male ↑ on Day 1 then ↓ on Day 7	4x ↓ on Day 7	ns
Superior Colliculus	1x male ↑ on Day 1 then ↓ on Day 7 1x female ↓ on Day 1	ns	4x ↑ on Day 7
Brainstem	ns	4x ↓ on Day 7	NA

*Summary of all data for significant regions. Injury level severity, trend direction with respect to CHIMERA group, and day of interaction is provided for regions where significant changes were observed.



Supplemental Figure 21: Hand-drawn depiction of the ten ROIs.

ROIs (meninges=red, hippocampus=green, corpus callosum=dark blue, cerebellum=teal, brainstem=pink, optic tract=orange, lateral geniculate nucleus=dark green, superior colliculus=purple, muscle=yellow, cortex=gold) across several slices with estimated corresponding Allen Mouse Brain Atlas levels below for anatomical orientation.



Supplemental Figure 22: Animal body weight over time after CHIMERA.
 Body weights across days for A) single and B) repetitive CHIMERA cohorts.
 Males had higher body weights compared to females, but there was no
 significant difference between injured and sham groups. The bars indicate
 means.

CHAPTER 4: Discussion

EARLY RECOGNITION OF PRE-CLINICAL TBI MODELING NEEDS AND CHALLENGES

The diagnosis and treatment of TBI has a long tradition in civilian and military trauma medicine, including early reports of battlefield-related bTBI (32; 88; 134). With emerging recognition of the utility of experimental approaches, pre-clinical modeling of TBI slowly had its genesis (210). An early focus was on concussion, and Denny-Brown and Russell were some of the original investigators who demonstrated that concussion resulting from a weight drop in cats required a free-moving head to transfer the impact-acceleration throughout the neuraxis (52). Seminal follow-up and more detailed work by early researchers, such as Holbourn, Ommaya, Gennarelli, Margulies and Thibault, and Hardy, laid the foundations for the study of TBI, particularly as a consequence of early cognizance of the rising frequency of vehicular accidents with the expansion of highways in the U.S. (83; 94; 107; 159; 194). These early pre-clinical experiments focused upon making the models relevant to human cases by drawing attention to issues of scaling from humans to smaller animal models. Holbourn emphasized rotational acceleration as a key factor of injury, and adapted one of the main scaling theories of the time, mass scaling, to TBI research. Further studies in this tradition were undertaken by Ommaya (63). From this work it was predicted that the level of rotational acceleration required to inflict equal injury on different sized brains is inversely proportional to two-thirds power of the brain mass ratio (107; 194). Ommaya explored dimensional scaling between humans and monkeys, identifying the peak rotational acceleration to induce concussion and the effect of duration (193; 194). Gennarelli and colleagues also brought attention to the importance of magnitude and duration of rotational acceleration as factors to consider (83). A study

by Stemper and colleagues observed that higher rotational acceleration magnitude resulted in longer loss of consciousness compared to lower magnitudes, but did not identify a similar pattern with increased acceleration duration. Acceleration duration, however, appeared to affect DTI results more than the acceleration pulse magnitude (249). Margulies and Thibault, also extending earlier work, studied head angular acceleration scaling and created rotational injury criterion for human diffuse axonal injury (159; 160). The mass and inertia ratios, however, are argued by some to not accurately predict brain strain because of the lack of accountability for species brain variability. For application to larger animals an alternative was proposed by Wu and colleagues for the study of nonhuman primate models that is a frequency-based scaling method for comparison to clinical trauma (278). The Head Injury Criterion was created by Versace to provide a scaling ratio between stiffness and length of the head and is now commonly used for vehicle safety testing, but primarily models linear acceleration (267; 273). Issues related to impact-acceleration scaling have not been resolved. Scaling is a multifaceted challenge that should carefully consider insult parameters such as mass, inertia, brain geometry, and material properties of different tissues (249). Even with proper scaling, however, a major assumption is still made that successful scaling will cause similar outcomes between species (198).

Neuropathological studies of bTBI have a similar history to that of the events from impact-acceleration injuries; broader interest did not emerge until the second half of the last century. Medical cases of bTBI were recorded during the First (178) and Second (41) World Wars but did not receive much attention until the more recent Mideast conflicts (134). However, according to Phillips and colleagues (219), blast was primarily

a military focus and the first known animal studies were recorded by Franchino Rusca in Switzerland in 1914 (230), and in reports on David Hooker's studies in 1918-1919 from the U.S. (108). From both clinical and animal observation, an early realization was that victims would occasionally have no exterior signs of injury despite the effects of shock waves on the air-filled lungs and other internal soft tissue. Further focus on TBI effects again were not fully appreciated until the most recent conflicts, but there was a long tradition in military psychiatry for associating blast effects with psychological trauma including shell shock (an early term) now identified as PTSD (124).

THE UTILITY OF CHIMERA TO STUDY ROTATIONAL ACCELERATION EFFECTS IN PRE-CLINICAL TBI

As noted, the CHIMERA device is a recently introduced model of impact-acceleration effects. This model is relevant to physical displacements from impacts to the victim as well as to the tertiary effects from blasts. The CHIMERA model has several positive features for pre-clinical studies. It is a closed-head procedure that obviates intervening factors related to craniectomies that are employed in other classic pre-clinical models such as FPI and CCI. Likewise, there are quantifiable readouts of the impact conditions and effects on the head and torso. Finally, the device is reproducible, as evident by the low variation in impact energies that were observed in the current study.

For pre-clinical application of CHIMERA, there is a need to balance injury intensity within a range that results in tissue damage from acceleration, but minimizes the possibility of skull fracture as an added compression injury. In addition, the kind of injury reported in patients must be replicated in smaller laboratory animals so that the model reproduces biomechanical parameters that scale with human physiology. By using the formula, $KE = 0.5 \times M_e \times \Delta V^2$, where M_e is the effective mass and is approximated by

the mouse head mass (3.4 g) and ΔV as a change in head velocity, Namjoshi and others determined a mouse-to-man scale factor (183). Based on this equal stress/equal velocity model, the scaling factor λ was derived as $\lambda = (\text{mass of human brain}/\text{mass of mouse brain})^{1/3} = 13.8$ (183), and a mathematical study estimated the small brain mass of rodents would require an angular acceleration of 350 krad/s^2 to produce mild TBI (74).

Namjoshi and colleagues determined the comparability with human injury that included a comparison for scaling of a few rotational models by reporting change in velocity, angular velocity, linear and angular acceleration, and Head Injury Criterion (183). With employment at 0.5 J, the CHIMERA device was seen as similar to professional football impacts and the change in angular velocity as comparable to Olympic boxing (182; 183). An initial study has also explored the CHIMERA's effects on brain motion. Live imaging of implanted fiducials in a ferret brain during CHIMERA with a PLA interface at energies of 17-56 J, showed ferret brain motion lagged behind the skull (277). The sudden changes in velocity and non-centroidal impact (rotation without linear motion) reportedly lead to brain acceleration and deceleration with possible skull deformation. Fuller characterization is needed to understand the utility of CHIMERA as a pre-clinical model. Additional dose-response studies for species, such as the experiment conducted by Namjoshi and colleagues in mice (182), should be undertaken so that the CHIMERA can be scaled properly for differing animal models.

ADDITIONAL FACTORS AFFECTING ROTATIONAL ACCELERATION MODELING

Other species differences must be recognized. For example, species variability in skull and brain composition can alter the biomechanics. Rodents have more flexible bone structure than humans, which allows for reduced rates of velocity change and milder

injury (48; 107; 118; 182; 193). One solution is the use of a plate or helmet to dissipate the focal nature of impact force from the piston. Standardization for the plate or helmet designs are needed (16; 30; 165; 239). Finally, more CHIMERA studies are needed with larger, gyrencephalic models, such as species historically included in rotational studies (75; 83; 171). Differences in strain have been reported between lissencephalic and gyrencephalic brains. A finite element model comparing a human brain with or without sulci showed that the sulci mediate some of the force thereby reducing the stress in the brain during acceleration (105). However, a present challenge is that the CHIMERA's design is presently restricted to rodent and ferret models (183). Finally, there have been studies that report the neuropathological effects of CHIMERA TBI, but additional characterization of the model is needed, including modeling and descriptions of specific regional injuries (168). For example, the weight drop model mimicking impact/rotation injury in rats finds that head rotation leads to brain strain particularly in the corpus callosum, whereas the brainstem is affected by multiple factors (285). This is consistent with non-injurious head movements studied in human participants where neck rotation created more strain in the corpus callosum, while neck extension resulted in brainstem strain (137).

BIOMECHANICAL EFFECTS FROM SHOCK WAVES

Several factors are hypothesized as mediators of blast TBI. These include thoracic mechanisms, skull flexure, and stress wave transmission (22; 44; 176; 177). Ritzel and coworkers described acceleration and deceleration forces during primary shock wave exposure in their biomechanical modeling study. Spheres of differing sizes and masses were examined inside of a shock tube to assist with bTBI scaling. Both acceleration and

deceleration occurred during the positive phase, while only deceleration was present during the negative phase of the bTBI waveform. When bTBI dynamics were tested using a human skull, however, it was estimated that the head does not undergo significant acceleration/deceleration with short-duration blast, but injury instead results from diffracting skull flexure and tissue cavitation (79; 221). Linear and rotational acceleration, however, was postulated to occur during shock reflection and diffraction. The authors listed shock wave loading during the diffraction phase, blast wind, Basset force (the rate of change of momentum of the boundary layer), and mass effect as considerations for scaling (221). There are differences in scaling between blast and rotational injuries where bTBI typically leads to low momentum and low strain, but a high strain rate in body displacement (198). Bowen and colleagues helped to pioneer bTBI scaling with differing species and described animal mass-dependent changes in lung scaling (24). Taken together, these studies demonstrate some overlap in bTBI and impact-rotational injury with the presence of acceleration/deceleration forces and the damaging effect of unrestrained head movement.

COMPARISONS BETWEEN bTBI AND IMPACT-ROTATIONAL ACCELERATION MODELS

Several studies have sought to make comparisons between bTBI and impact-rotational acceleration injuries. Stemper and colleagues used rats to examine two designs with the Medical College of Wisconsin rotational injury model and bTBI that included head restraint to reduce secondary and tertiary bTBI injury (248). Rotational injury led to longer righting reflex times after injury compared to bTBI, but the severity levels of rotational and bTBI injuries were chosen to have similar righting recovery times to enable comparison. Increased activity was observed one week after rotational injury, but

bTBI-exposed animals showed initial decreased activity post-bTBI. Since many bTBI studies struggle with isolating primary shock wave injury, a hallmark study by Goldstein and colleagues demonstrated the effects of bTBI with and without head restraint. Mice placed inside of the shock tube in a prone orientation with free head movement led to a “bobblehead effect” and hippocampal-dependent learning impairments one month post-injury. When mice were exposed to the shock wave with head immobilization, however, subsequent hippocampal deficits were prevented. Blast TBI with unrestrained head movement led to inertia injuries and shear strain (85). A later study by Tagge and coworkers incorporated the same bTBI design with head restraint (85) for comparison with a lateral impact-rotation TBI model. Awake mice with no anesthesia underwent lateral head impact with matching head kinematics as recorded in the bTBI model. Similar outcomes as Stemper and colleagues’ comparison were observed where impact-rotational acceleration resulted in injury and neurobehavioral impairments soon after injury, but despite similar head kinematics, bTBI only demonstrated long-term neuropathology. Pressure-sensitive film of each TBI model displayed different energy loads. Impact injury showed localized, high pressure loading at the site of impact, whereas diffuse, lower pressure loading was seen with bTBI. Follow-up computational simulations provided evidence that the greater immediate damage from impact rotation injury was not because of intracranial pressure, but instead could be due to increased intraparenchymal shear stress. The shear stress exhibited after impact injury was higher and longer in duration over a large area with seven times greater peak shear stress compared to bTBI. DTI studies suggest that blast with head restraint and rotational injury lead to changes at grey and white matter interfaces (28; 249). Although impact rotation

injury and bTBI can lead to similar long-term neuropathology, the authors conclude that there are different underlying mechanisms with varying energy loads and shear stress in the brain. Cortical function disruption and the neuronal excitation theory of concussion rather than structural damage were noted as the main causes of impairment (252). In contrast to Goldstein and colleagues' discovery and suppression of tertiary head movement during bTBI, Ritzel and coworkers described acceleration and deceleration forces during primary shock wave exposure in their biomechanical modeling study as described in the previous section.

While a direct comparison between studies in this project, ABS and CHIMERA, was not performed as in the experiments described above, the presence of tertiary effects from impact acceleration was evident during ABS experiments. Although head restraint was implemented in the ABS study, the force from the blast overpressure appeared to push the animal into the mesh pouch that restrained the mouse inside of the ABS chamber. The head restraint used during ABS experiments was a mixture of rigid tongue depressor and flexible vet wrap, which could have enabled some movement. The blast shock wave may have propelled the dorsal mouse head to hit the tongue depressor and push the whole animal deeper into the mesh pouch holder. This interpretation of injury effects is supported by the Evans blue extravasation observed on the dorsal brain parenchyma on some injured animals. More investigation would be needed to determine whether the energy loads or head kinematics from ABS and CHIMERA were comparable, but in the current project, longer righting reflex times were generally observed for ABS injured animals (median 462.5 sec female and 370.5 sec male mice) versus single CHIMERA injured mice (median 96 sec), which differs from the righting

reflex results seen by Stemper and colleagues (248). The differences in righting times between models could be due to animal orientation to the blast wave and varying blast tube and rotational acceleration devices implemented. Although the ABS has an end-wave eliminator to prevent shock wave reflection, artifacts could still occur and may contribute to acceleration/deceleration effects during ABS or during the primary shock wave itself as displayed by spherical models (221). More investigation is needed to determine whether a similar pattern of short-term behavioral deficits from rotational acceleration and longer-term impairments from blast shock wave exposure are evident (248; 252).

FUTURE DIRECTIONS

Relative to the findings in the two studies performed, there are exciting prospects for further investigation. These are outlined as commentary related to *in vivo* imaging, further investigations of TBI effects from impact rotational and blast injuries, and interrelating behavior with acute changes in IEG responses.

Alternative imaging approaches

TME is detected in humans using T2-weighted fluid-attenuated inversion recovery (FLAIR), which suppresses CSF and allows for better visualization of enhancement due to damage. FLAIR can also work with T1 contrast enhancement (132). T1-weighted images form the basis for DCE and are ideal for enhancement related to hypervascular events or contrast agent extravasation (246). DCE FLAIR was reported to show high accuracy in detecting traumatic meningeal injury in patients with over 90% sensitivity (33), but a different study found TME was easier to identify on T2 FLAIR rather than T1-weighted images with only a 62% overlap (51). Future investigation

should include a pre-clinical model of TME using FLAIR T2-weighted image analysis to provide more clinically relevant data. However, FLAIR in rodents might be challenging given the limited signal-to-noise ratio. Another approach could include multidimensional MRI, which was recently demonstrated to have a high cross-correlation between imaging and subsequent pathology (19). Implementing an alternative imaging sequencing may reduce the partial volume effects that distorted bregma level measurements for the meninx ROI in the current study. In addition to changes in MRI sequence parameters, the route of contrast agent administration should be further explored. Clinically, contrast agents for imaging are delivered via intravenous (i.v.) injection, but the pre-clinical design and repeated measures for within-subject comparison of the current study made i.v. administration difficult. To achieve i.v. delivery in rodents, the tail vein is often accessed during *in vivo* imaging, but repetitive tail vein injections have been shown to cause vessel damage (209). Intraperitoneal (i.p.) gadolinium administration was chosen as an alternative for i.v. contrast agent injection and has been utilized by other investigators (113; 229) and reported to have equivalent cerebral blood flow results (174; 206). The absorption of gadolinium is described as reaching the peak steady state about 15-45 minutes after i.p. administration (174; 206; 209), but clear contrast distinction is observable within 7.5 minutes after injection (174). A steady state of uptake in the brain was seen about 5-7 minutes after i.p. injection in the current project, but given the previous literature future directions should include a longer DCE acquisition time to ensure peak absorption of gadolinium is reached. Intraperitoneal contrast agent delivery provides a safe and reliable supply of gadolinium during scanning, but has been noted to accumulate in the CSF and can diffuse into periventricular structures (206).

Future study for *ex vivo* meningeal and BBB analyses

Further investigation should include corresponding *ex vivo* quantification of barrier impairment. In the initial experiments related to my thesis, several approaches to BBB quantification were explored. Preliminary immunoglobulin G (IgG) data were collected to accompany the reported Evans blue BBB results and displayed labeling increases and microvasculature damage in the brains of bTBI mice, supporting BBB disruption. Over time, however, IgG staining did not prove to be consistent. Another strategy employed was the detection of BBB proteins, such as claudin-5 and occludin, but these markers also appeared inconsistent. Smaller molecular markers, dextrans, compared to Evans blue were applied to determine whether an extended time line of BBB disruption was indicated, but imaging of the dextrans was challenging. Although several histological approaches were employed, additional techniques for BBB quantification could include electron microscopy and aquaporin-4 labeling.

Meningeal *ex vivo* histological methods were pursued to provide TME correlation, but meningeal preparation proved to be difficult. A meningeal whole mount has been described by other investigators (150; 233), but replication with various technical approaches including fresh or fixed tissue, different buffer solutions, varying time points, a multitude of antibodies, and tissue clearing with the PEGASOS protocol (123) were unsuccessful. Next, flow cytometry was investigated to quantify different proinflammatory cells in the meninges after injury and has been utilized by others (158), but meningeal tissue dissociation was a significant hurdle. The mouse meninges fragility was a challenge in removing the meninges from the skull cap of the mouse and may have distorted the morphological integrity of the tissue, suggesting that *in vivo* imaging is a more promising approach in future studies. Future study could also include 2-photon

examination of the meninges, which has been successfully employed (225; 233).

Additionally, inclusion of spinal cord meningeal analysis has been described (158) and could be particularly relevant for the unknown effect of CHIMERA on possible spinal cord injury.

A cross-model comparison like the studies described previously (248; 252) to compare ABS-induced bTBI and CHIMERA could be beneficial. Preliminary data with CHIMERA and Evans blue staining showed little change at two to four hours after a single impact at 0.7 J, but some dye extravasation was observed in the optic tract, trigeminal nerve, and brainstem in a small sample of injured mice (unpublished observations). Likewise, further investigation of DCE-MRI after bTBI is needed. One study conducted DCE analyses one week and one month after shock wave exposure with mice in an open field bTBI design. A brain barrier permeability index was computed based on the number of enhanced voxels and was found to be significantly increased only at one month after bTBI, suggesting a delayed effect of blast (229). Finally, behavioral testing following CHIMERA is warranted for comparison with previous literature (168) and to explore possible functional outcomes related to TME. Longer timelines for behavioral testing could enable biphasic responses to be observed. And lastly, repetitive injury for bTBI and direct comparison of single and repetitive CHIMERA should be explored and could be more clinically relevant with multiple injury exposures.

Further prospects for relating behavior and c-Fos correlations

In the ABS study, direct correlation between the observed c-Fos and behavioral changes could not be deduced. The quantification of c-Fos only allows for short-term examination of neuronal activation and c-Fos is not solely responsible for fear memory.

For closer investigation of c-Fos' role after injury, c-Fos knockout animals should be studied. Complete c-Fos knockouts lead to developmental impairments, so conditional knockouts could be employed (201). A Cre-loxP mouse knockout was developed by Fleischmann and colleagues to determine c-Fos' role in hippocampal long-term potentiation. Investigators showed that c-Fos knockout mice had impaired long-term potentiation and that the c-Fos pathway was also induced by the NMDA receptor (76). Another group used a different knockout approach by inserting c-Fos antisense oligonucleotides to block c-Fos function in order to study fear memory. Antisense rats displayed learning and fear memory impairments one week, but not two days after inhibitory avoidance training, demonstrating c-Fos' role in gene expression for fear-related memory persistence (127). Future directions could include the study of other transcription factors, such as *Erg1* and *arc* or Δ FosB, an alternative, slower responding variant of the Fos pathway; all of which have been implicated in PTSD (77). Pairing electrophysiology and behavior, such as chemogenetics, or in vivo calcium imaging and retrograde tracers, could enable more direct comparison between observed hyperactive behavior and IEG activation (81; 169). In situ hybridization and qPCR for c-Fos and c-Fos Western blot could further quantify IEG levels. Given the many pathways in which the PVT is involved and a recent report of different cellular divisions within the PVT, future study should include the identification of specific PVT circuitry related to bTBI to determine the mechanisms of action after ABS (80; 135). No sex differences in the PVT were observed in studies of fear memory (152; 203), but the current study found sex differences in the number of c-Fos⁺ neurons in the PVT. A different study determined sex differences in the number of activated CRF neurons in the paraventricular nucleus of

the hypothalamus, so further investigation of CRF neurons in the PVT may be warranted (231). The prefrontal cortex should be further investigated for changes in c-Fos because of the prelimbic cortex's role in fear (61) and only the cerebral cortex posterior to the prefrontal cortex was analyzed in the current project. Lastly, housing and handling conditions should be carefully considered when analyzing behavioral tasks. Mouse handling methods, such as the tail handling technique induce stress and anxiety in mice (87) and sometimes result in increased time spent immobile during the forced swim test in male mice (243). Although the tail handling method was used throughout all experiments in the current project, during the tail suspension test less time spent immobile was observed in shock wave-exposed mice.

SUMMARY

In this project, two pre-clinical models were employed to explore the neuropathological and functional effects associated with common sources of TBI that are sustained by our military personnel. The CHIMERA study was the first in the field to describe a similar pattern of traumatic meningeal enhancement in a pre-clinical mouse model. This is a significant advancement because the meninges have been understudied in the TBI field, but could present a potential biomarker for mild TBI diagnosis, and a therapeutic target for meningeal vascular or arachnoid barrier repair, as well as assist in injury recovery. Further investigation into the underlying mechanisms of this phenomenon is now possible with a relevant pre-clinical design.

The ABS study provided additional pre-clinical evidence of sex differences after TBI. Women report higher rates of neuropsychiatric symptoms, particularly anxiety and depression related to TBI, but this has been difficult to replicate in animal models, partly

because of the lack of female animals and sex difference analyses in most studies. This study showed behavioral and neuronal activation sex differences that could be related to post-traumatic stress disorder after shock wave exposure.

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