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Blast-induced brain injury in rats leads to transient vestibulomotor deficits and persistent orofacial pain

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Abstract

Blast-induced traumatic brain injury (blast-TBI) is associated with vestibulomotor dysfunction, persistent post-traumatic headaches, and post-traumatic stress disorder (PTSD), requiring extensive treatments and reducing quality-of-life. Treatment and prevention of these devastating outcomes requires an understanding of their underlying pathophysiology through studies that take advantage of animal models. Here we report that cranium directed blast-TBI in rats results in signs of pain that last at least 8 weeks after injury. These occur without significantly elevated behavioural markers of anxiety-like conditions, and are not associated with glial up-regulation in sensory thalamic nuclei. These injuries also produce transient vestibulomotor abnormalities that resolve within three weeks of injury. Thus, blast-TBI in rats recapitulates aspects of the human condition.

Introduction

High-order explosives, such as landmines or improvised explosive devices (IEDs), create blast waves upon detonation. In an explosion, solid or liquid fuel is rapidly converted to gas, which forms a wave of high pressure. As the blast wave passes through the body, it induces primary blast injuries [1,2]. Primary blast injury to the brain induces neurotrauma, even in the absence of head movement resulting in coup/contrecoup or rotational acceleration injuries [3,4].

Patients with blast-induced traumatic brain injury (blast-TBI) endure a wide range of short-term and persistent complications and comorbid conditions after injury. Vestibulomotor deficits and gait abnormalities are noted in 98% of patients with blast-TBI during the acute phase after injury (within 72 hours), and complaints of dizziness persist in 72% into a subacute phase (<30 days after injury) and 85% into the chronic phase (>30 dpi) [5]. However, evidence for vestibulomotor abnormalities after blast exposure to rodents have been mixed. Deficits in beam balance and rotarod performance were not observed following blast injury in several studies [6,7] until blast strength was increased to 551 kilopascals (kPa)

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[8]. Therefore, we predicted that exposure to moderate primary blast injury at 427 kPa would induce vestibulomotor abnormalities.

Survivors of blast-TBI also report high rates of post-traumatic headache and post-traumatic stress disorder (PTSD) developing in the weeks to months after injury [9–14]. Chronic post-traumatic headaches significantly affect the quality-of-life of a sizeable subset, upwards of 57%, of TBI survivors [15]. In a US Army combat brigade, of the 22% of members diagnosed with mild TBI, 81% had acute post-traumatic headaches immediately following injury and 20% had chronic post-traumatic headache, when surveyed after returning from their one-year deployment [16]. Service members with a history of mild blast-TBI and persistent neurological symptoms reported more frequent headaches that had more migraine-like features, worse pain, higher rates of PTSD diagnosis, and impaired sleep with nightmares when compared to mild blast-TBI survivors without persistent neurocognitive defects [17].

Chronic post-traumatic pain and hyperalgesia is understudied following blast-TBI in rodents. The Rat Grimace Scale (RGS) has been validated in a variety of inflammatory and neuropathic pain models [18–20], but it had not been applied to blast-TBI until the current study. In similar traumatic models capable of inducing pain, Saine and colleagues [21] found elevated RGS scores following induction of intracerebral hemorrhage, a common injury secondary to TBI, while Schneider et al. [22] found facial markers of persistent pain in rats five weeks after spinal cord injury. Monitoring non-evoked markers of pain after blast-TBI with RGS facilitates assessment of post-traumatic pain in animal models.

Previous studies of chronic pain after neurotrauma, such as spinal cord injury (SCI), have causally-related pain behavior to maladaptive hyper-excitability and persistent glial inflammation in the posterior nucleus of the thalamus (PO), the primary pain relay nucleus in the sensory pain pathway [23–26]. Because we have previously demonstrated an impact of PO inflammation on pain after SCI, we predicted that pain after blast injury would also be mediated by persistent glial activation in PO.

Rodents have been exposed to complex blast conditions in shock tubes and open field explosions [27–29]. While these other rodent models of blast-TBI mirror many of the environmental conditions faced by soldiers exposed to explosive blasts, who are often in vehicles or areas with buildings that would interact with the blast wave, there is utility in focusing on the mechanism unique to blast-TBI over other TBIs: the primary blast injury, the damage caused by the rapid passage of a wave of over-and under-pressure through the skull and brain tissue. Clinical presentations and some pathological mechanisms overlap between blast-TBI and other, blunt force-induced TBIs, but there is evidence that blast exposure may cause unique brain injuries. Erickson et al. reported that patients with blast-TBI were more resistant to prophylactic treatment for chronic, migraine-like headaches when compared to patients with other types of TBI [30]. There is also a significant diagnostic overlap between mild cases of blast-TBI and PTSD [31], even when comparing reports of PTSD symptoms in blast- and other types of TBI [32]. We therefore isolated the primary blast injury by tightly physically coupling the rat head to the blast source using the Cranium-Only Blast Injury apparatus (COBIA) [33]. We applied the COBIA model to the

study several debilitating, common, and persistent comorbidities reported following blast-TBI: vestibulomotor abnormalities, chronic post-traumatic headaches, and PTSD. We hypothesize that blast-TBI caused by COBIA will cause persistent pain and anxiety-like behaviour in rats due to periventricular inflammation of central pain-processing nuclei of the thalamus, the PO.

Methods

Animals

All procedures were conducted according to Animal Welfare Act regulations and Public Health Service guidelines and approved by the University of Maryland School of Medicine Animal Care and Use Committee. A total of 42 pair-housed adult male Long-Evans rats (250–300 g; Harlan, Indianapolis, IN) and 20 adult male Wistar rats (250–325 g; Harlan, Indianapolis, IN) were used in this study. We used Wistar rats specifically for facial grimace analysis, which necessitates adequate contrast between light-coloured faces and darker eyes. No effects of strain on behavioural metrics were observed. Following sham procedure and blast injury, we tested one group of rats (Long Evans, $n=30$) for short-term vestibulomotor function, while we reserved another two groups (Long Evans, $n=12$, and Wistar strains, $n=20$) for persistent pain, hyperalgesia, and anxiety assessments following blast injury (see experimental schematic in Figure 1). Animals were randomly allocated to experimental or control groups, as described by Kim and Shin [34]. In all experiments, the investigators were blinded to animal condition. A coded key of all specimens evaluated was kept and not shared with the investigators performing the experiments until data analysis was completed. Thus, allocation, concealment, blinded conduct of the experiment, and blinded assessment of the outcomes was performed.

Blast injury

Rats were anesthetized with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) before placement in the COBIA apparatus. We traced the occipital ridge with marker on the scalp to ensure proper alignment of the occipital ridge under the blast dissipation chamber-cranium interface (BDCCI). Rats were intubated, but not yet placed on a ventilator, to allow for timely oxygen delivery if spontaneous breathing ceased following injury. No rats in the present study needed artificial ventilation. After intubation, rats were placed prone on the platform below the blast dissipation chamber. The head was aligned under the BDCCI, and a small balloon was inflated under the chin to create a seal between the scalp and the BDCCI. The cartridge was then detonated in the gun to prompt blast injury. Rats allocated to Group 1 were exposed to a blast injury at 427 kPa using the 29.5 cm blast dissipation chamber (BDC) [33] or sham procedures, while rats in Groups 2 and 3 were subjected to a 462 kPa blast with the 27 cm BDC or sham procedure. Sham animals were anesthetized and placed in the apparatus but no cartridge was detonated.

Oxygen Saturation

All rats underwent continuous pulse-oximetry with the sensor placed on the hind-limb (Mouse Ox™; STARR Life Sciences Corp., Oakmont, PA) at baseline, immediately after

intubation. Arterial oxygen saturation measurements were continuously collected for 30 minutes after the injury or sham procedure.

Vestibulomotor function tasks

Beam Walk and Balance Assessment—Starting one day before injury (baseline) and continuing on 1, 3, 7, 14, and 21 days post-injury (dpi), rats were placed at the end of a 3 cm wide wooden beam, and time to traverse the 90 cm length of the beam was recorded. The rat was timed as it walked the beam to a darkened box, while a loud noise was created behind it. The average time needed to walk the beam in three trials per testing day was used to determine an average beam walk time. Beam balance was also scored during the three beam walking trials per test day, using guidelines in Petullo et al. [35]: if a rat balances on the beam without foot slipping or wavering, it received a 0. As more limbs slip from the beam or foot faults accumulate, the score is increased. However, if the rat falls off the beam, it received the maximum score of 5.

Accelerating Rotarod—Rats were tested for motor coordination on a Rotarod (IITC Life Science, Woodland Hills, CA) set to accelerate 2 m/s from its starting speed of 4 rotations per minute (rpm) every five seconds, up to a maximum speed of 45 rpm and maximum test duration of 300 s. Latency to fall was recorded for three trials, each separated by twenty minutes, per test day, on the same testing schedule as balance beam assessments. The average latency to fall off the drum each testing day is reported.

Rearing—Also on the same testing days as other vestibulomotor tasks, we placed rats in a clear, cylindrical Plexiglas chamber (19 × 20 cm) with metal grid floor. An experimenter observed the rat for three minutes and recorded the amount of time the rat spent reared up onto its hind-limbs with its fore-limbs placed on the walls of the chamber, termed rearing time.

Anxiety metrics

Experimenters handled rats for three days before testing anxiety-like, pain, and hyperalgesia behaviors. During the fifteen minutes-long handling session per rat per day, the rats were wrapped in cloth to mimic their mild restraint conditions during the facial mechanical withdrawal assessments.

Open Field—Twenty eight days after sham or blast injury, rats from group 2 were placed in a chamber measuring 36 × 50 cm. for thirty minutes. Any-Maze video tracking software (Stoetling Co., Wood Dale, IL) was used to measure distance travelled and time spent in the 26 × 35 cm centre rectangle and outer perimeter zones.

Elevated Plus Maze—Also in the fourth week following injury (29 dpi), rats performed an elevated plus maze task, which consists of two 10 by 50 cm. platforms intersecting in the middle, one arm closed off with 30 cm. high walls and the other without walls. Each rat was placed in the centre of the plus maze facing an open arm and allowed to travel throughout the maze for five minutes. Time spent in the open arms of the maze, with the rat exposing at

least its head and body through to its fore-limbs in the open arm, was quantified using Any-Maze video tracking software, validated by an experimenter.

Light-dark box task—To assess anxiety-like behaviour, rats were placed in a two-chambered Plexiglas box on 33 or 34 dpi. One chamber was lit from above with a clear Plexiglas roof, while the other side of the chamber was dark, with an opaque lid. Rats walked freely between the chambers and the time spent in each side was measured in real-time using piezo pressure-sensitive devices in the floor of the chambers.

Assessments of pain and hyperalgesia

To study chronic pain and hyperalgesia behavior, acute injuries must first heal with time [36]. We allotted at least thirty days after injury for recovery from any acute effects of blast exposure before assessing pain and hyperalgesia.

Rat Grimace Scale scoring—Rat grimace scores were determined based on methods described by Sotocinal et al. [18]. At 30 to 32 dpi, rats were placed in a lidded Plexiglas chamber measuring 10 × 20 × 14 cm and filmed for thirty minutes. Video files were processed using Rodent Face Finder® software, graciously provided by J. Mogil, [18] to randomly select ten images from each video, discarding images in which the rat appeared to be grooming or sleeping. We then scored the images for three action units: orbital tightness, nose/cheek flatness, and ear position changes. However, because in our set-up the vibrissae could not be reliably and consistently imaged, we excluded analysis of the vibrissae from the grimace scale. In a psychometric evaluation of the Rat Grimace Scale, Oliver et al. [37] reported that the vibrissae action unit scoring was the least reliable between raters, with an intraclass correlation coefficient (ICC) of 0.52, while ICC for the other three action units was higher, ranging from 0.62 to 0.92. Due to our technical limitations and this previous finding, we determined rat grimace scores as the mean score of the three action units for each image, averaged by rat.

Orofacial mechanical withdrawal threshold measurement—We measured mechanical withdrawal thresholds, at 58–68 dpi, by restraining the rat in a cloth, leaving the head exposed. Starting with the 4.0 grams filament, we applied von Frey filaments to the centre of the whisker pad. Mechanical withdrawal thresholds measured in the whisker-pad reliably assess cutaneous hypersensitivity in rats habituated to experimenter handling [38]. We observed the rat for a withdrawal, consisting of either an abrupt head withdrawal, an attempt to bite, or attempt to swipe away the filament. We determined the average mechanical threshold using the Dixon up-and-down method [39] over ten trials.

Quantification of glial populations in thalamus

Immunohistochemistry—After behavioural assessments were complete, rats were euthanized and transcardially-perfused with phosphate buffered saline, followed by 4% paraformaldehyde (PFA). Brains were fixed in 4% PFA for two days and then sectioned on a vibratome. Sections were collected from 3.5 to 3.8 mm posterior to bregma at 50 μm thickness. Primary antibodies were directed against glial fibrillary acid protein (GFAP; mouse, 1:20,000, C9205, Sigma-Aldrich, St. Louis MO) and ionized calcium-binding

adapter molecule 1 (Iba1; rabbit, 1:20,000, 019–19741, Wako Pure Chemical Industries, Richmond VA). The GFAP antibody was conjugated to a Cy3 fluorophore. Secondary antibody for Iba1 labeling was goat anti-rabbit antibody conjugated to an Alexa Fluor 488 nm fluorophore (1:1500, 4412, Cell Signaling Technology, Danvers MA). Sections were imaged for fluorescence with an Olympus Fluoview 500 confocal microscope (Olympus Corp., Center Valley, PA).

Assessment of Fluorescence Area—After identifying our regions-of-interest in bright-field images using rat brain atlas images of the thalamus [40], we collected z-series of 5 images per ROI at 4 μ m intervals through the z-plane. Using ImageJ software [41], the VPM/VPL and PO thalamus were traced in summed zseries images. Integrated fluorescence density quantifying either the GFAP or Iba1 labeling was measured, normalized to an unlabeled region of the image stack, and averaged by rat.

Scoring Microglia Morphology—Two experimenters sorted microglia into three morphological categories: ramified, bushy, and amoeboid [42,43]. Total number of microglia in the counting frame was also quantified.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla). Results of tests performed at a single time point were compared with a Mann-Whitney U ranked sum test. Repeated measures were compared with a two-way mixed model ANOVA and p-values reported are from post-hoc tests with correction for multiple comparisons. All data, unless otherwise noted, are presented as median $\pm$ 95% confidence interval error bars.

Results

Apnea

Blast injury typically results in transient apnea [44]. To compare the immediate effects of blast injury produced by the Blast Dissipation Chamber (BDC) tubes yielding different peak over-pressures, we measured the duration of apnea that immediately followed the blast. Mortality due to blast injury occurred at a rate of 3.7% (n=1) and apnea duration values for that rat was excluded from analysis. The median apnea duration of rats exposed to blast waves using the 29.5 cm BDC with peak over-pressures of 427 kPa was 11.35 seconds (95% CI 8.5–15.5; n=14). These apnea periods were indistinguishable from those exposed to blast waves peaking at 462 kPa (27 cm BDC: median 9, 95% CI 3–22; Mann-Whitney U=64; p=0.32; Figure 2A). Because apnea durations did not differ significantly between rats exposed to blast injuries with peak over-pressures of 427 and 462 kPa, we combined data from both groups and considered both to model a similar severity of blast-TBI.

Oxygen saturation

To determine the effects of blast and apnea on respiration, we compared arterial oxygen saturation in blast animals with that in sham-treated rats. Before the blast or sham treatments, oxygen saturation did not differ significantly between blast and sham groups

(blast: $89.11 \pm 4.64\%$, 80.7 to 98.2%; sham: $89.33 \pm 5.06\%$, 82.4 to 98.4%; two-way ANOVA $t(234)=0.06$, $p=0.99$). However, 30 seconds after the blast, rats exposed to blast injury had significantly ($t(234)=7.72$, $p<0.0001$) lower oxygen saturation levels ($70.13 \pm 16.28\%$, 35.0 to 92.1%), compared to shams ($90.16 \pm 8.01\%$, 71.4 to 98.9%), and these remained depressed for at least 1 minute after the blast. The decrease in oxygen saturation was transient, as by 5 minutes post-blast there was no significant difference ($t(234)=2.07$, $p=0.30$) between blast-injured ($86.23 \pm 7.73\%$, 68.6 to 97.5%) and sham rats ($91.85 \pm 5.10\%$, 82.4 to 99.2%), and oxygen saturation levels remained stable until monitoring ceased at thirty minutes post-injury (Figure 2B). These results indicate that blast injury causes a transient depression in respiratory function that recovers within five minutes.

Vestibulomotor tests

Beam walk: Previous studies in models of blunt-force and penetrating TBI in rats have validated the beam walk task as a sensitive metric of injury severity and deficit duration during the post-injury period [45]. To assess the detrimental effects of blast injury on motor coordination, we measured the time required to walk a narrow wooden beam. Mean beam walking duration did not differ at baseline between the sham and blast groups (sham: 1.69 ± 0.36 s, 1.20 to 2.30; blast: 1.83 ± 0.33 s, 1.20 to 2.40; two-way ANOVA $t(132)=0.58$, $p=0.99$, Figure 3A). However, 24 hours post-injury, animals in the blast group traversed the beam in significantly longer time, compared to shams (blast: 3.51 ± 2.50 , 1.43 to 5.83; shams: 1.88 ± 0.70 , 1.32–3.63; $t(132)=4.76$, $p<0.0001$). The decline in beam walk performance in blast-injured animals at one day post-injury recovered by three days post-injury (sham: 1.54 ± 0.42 , 1.06 to 2.67; blast: 2.14 ± 0.65 , 1.07 to 3.33; $t(132)=2.55$, $p=0.07$). These findings suggest that blast-TBI resulted in transient impaired motor coordination and vestibular function.

Beam Balance: To further assess vestibulomotor function and balance, we scored performance on a beam balance task [35]. Beam balance scores did not differ between sham animals and blast animals at baseline (sham: 0 ± 0 ; blast: 0 ± 0 ; two-way ANOVA $t(138)=0$, $p=1.00$). However, testing one day after injury revealed a deficit in beam balance in blast-injured rats, compared to shams (blast: 0.93 ± 0.62 , 0 to 2; sham: 0 ± 0 ; $t(138)=7.24$, $p<0.0001$), that persisted for at least one week after injury (Figure 3B). However, beam balance scores of blast rats returned to sham levels by two weeks after injury (blast: 0.23 ± 0.44 , 0 to 1; sham: 0.00 ± 0.00 ; $t(138)=1.81$, $p=0.36$). After blast injury, the ability of rats to balance on the narrow beam decreases, suggesting that vestibulomotor function is transiently impaired.

Rearing: Rearing in a Plexiglas chamber measures exploratory behavior, as well as rats' motor coordination and ability to balance on hind-limbs [45]. Rats in the sham and blast groups did not differ in rearing time before the injury (sham: 54.0 ± 15.3 seconds, 31.0 to 82.0; blast: 58.1 ± 11.0 seconds, 41.0 to 78.0; two-way ANOVA, $t(138)=0.55$, $p=0.99$; Figure 3C). However, rats in the blast group spent significantly less time rearing at one day post-injury, compared to shams (blast: 28.62 ± 14.11 , 7 to 55; shams: 53.25 ± 23.17 , 18 to 105; $t(138)=3.37$, $p=0.01$). Rats with blast-TBI also spent less time rearing than shams when tested three days after injury (blast: 39.67 ± 13.16 , 22 to 71; shams: 58.27 ± 19.28 , 36 to 92;

$t(138)=2.99$, $p=0.02$). The transient difference in rearing duration in the initial days after blast injury reflects a short-lived decrease in exploratory behavior and motor coordination in blast-injured animals. However, the deficits resolve back to sham levels shortly after injury.

Accelerating rotarod: Traumatic brain injury in rodents induces balance and motor deficits that affect performance on the accelerating rotarod task, even in cases of mild brain injuries that do not significantly affect beam balance and beam walk [46]. We have previously shown that blast-TBI with the COBIA system results in balance and motor function deficits [33,47–48]. Here, we attempted to replicate the post-injury motor coordination deficit on a longer time-scale after blast injury in an accelerating rotarod task. Pre-injury baseline latencies to fall off the rotating barrel of the rotarod were not different between rats in the blast and sham groups (sham: 68.04 ± 12.28 seconds, 50.00 to 92.67; blast: 76.16 ± 21.56 seconds, 49.00 to 145.00; one-way ANOVA, $t(126)=1.60$, $p=0.22$; Figure 3D). Rotarod performance, normalized to the pre-injury baseline performance of each individual, subsequently decreased significantly in blast-TBI rats when compared to sham rats at 1, 3, 7, and 14 dpi. Like other vestibulomotor assessments performed after blast-TBI, performance on the rotarod was indistinguishable from sham by 21 dpi.

Anxiety metrics: Tracking activity in an open field arena serves two purposes: to measure anxiety-like behavior through thigmotaxis tracking and to assess locomotor function by measuring total distance traveled during the trial. Distance traveled in the open field arena did not differ between blast-injured (median 41.71m, 95% CI 34.16–57.61) and sham rats (median 43.11m, 95% CI 30.24–62.94; Mann-Whitney $U=46$; $p=0.66$), suggesting that there is no lasting locomotor deficit in an open field four weeks after injury.

Distance traveled in the open field did not differ between sham-injured and blast-TBI rats, and, likewise, time spent in the center of the open field, reflecting exploratory behavior and suppression of anxiety-like behaviors, also did not change in the injured rats. This lack of anxiety-like behavior was corroborated by the elevated plus maze and light-dark box assessments (Table 1). Three separate tests of approach-avoidance conflict behavior indicative of an anxiety-like phenotype found no effect of blast exposure three weeks after injury.

Pain metrics

Rat Grimace Scale: The facial grimace scale is widely used and validated as a sensitive, objective metric for pain in humans [49,50], and in animal models of pain [18–22, 51–53]. We have recently validated its use in chronic orofacial neuropathic pain to dissociate affective aspects of pain from sensory and reflexive measures of mechanical hyperalgesia [20]. Modified rat grimace scale (RGS) scores, representing the mean of the orbital tightening, nose and cheek flattening, and ear position change scores for each frame (representative images shown in Figure 4B) averaged for each rat, were significantly increased in blast-TBI rats compared to sham rats at four weeks after injury. The median RGS score of blast-TBI rats was 1.13 (95% CI 0.98–1.27; $n=5$), while the sham-injured rats displayed less severe markers yielding scores with a median of 0.79 (95% CI 0.58–0.98;

n=4; Mann-Whitney U=1; p=0.03; Figure 4A). These elevated rat grimace scale scores in blast-injured rats suggest that those rats experience ongoing pain.

Orofacial mechanical withdrawal threshold assessment: Post-traumatic pain and chronic migraines are associated with cutaneous allodynia of the face [54]. To determine if blast-TBI resulted in persistent hyperalgesia, we tested each rat for mechanical withdrawal thresholds, 58 to 68 days after TBI, as described in Methods. Mean thresholds for withdrawal from mechanical stimuli applied to the face were significantly decreased in animals with blast-TBI, compared to shams (Fig. 4C). Thresholds in animals with TBI (median 5.23; 95% CI 3.4–8.04; n=10) were 60% lower than those calculated from sham animals (13.12; 7.45–20.58; n=9; Mann Whitney U=7.5; p=0.001). These findings indicate that TBI results in tactile sensitization that lasts at least eight weeks after the injury.

Glial markers: We have previously shown that blast-TBI results in changes in hippocampal glia activity that is correlated with behavioral abnormalities in learning and memory tasks [47]. To assess glia in thalamic nuclei important for pain and sensory processing, we labeled thalamic sections for GFAP and Iba1 to quantify the amount of astrocytes and microglia. We focused on the PO and on the VPM, nuclei we have previously shown to be involved in the pathogenesis of chronic orofacial pain [55]. We found no significant changes in labeling for either Iba1 or GFAP in both the PO and VPM (Figure 5A, B, C).

To further test the prediction that blast-TBI results in an increase in microglia, we used stereological approaches to compare the number of Iba1-labeled microglia in blast-TBI and sham animals, which did not differ between shams and blast-TBI sections through either PO (sham: 46.61+/-5.73, 39.5 to 56.25; blast: 47.94+/-12.34, 32 to 65; Mann-Whitney U=17, p=0.94; Figure 5D) or VPM (sham: 37.72+/-5.40, 29.25 to 46; blast: 38.25+/-11.45, 24 to 53.5; Mann-Whitney U=17.5, p=0.97).

Microglia phenotypes are indicative of their inflammatory states, so we classified the Iba1-labeled microglia in PO and VPM as ramified, bushy, or amoeboid, corresponding to their transition from anti-inflammatory to pro-inflammatory [56,57]. The majority of microglia in both nuclei were ramified in appearance (sham: 80.34+/-12.86%, 57.69 to 91.80; blast: 86.08+/-8.64, 75.43 to 98.75; Figure 5E and 5F), suggesting that microglia-mediated inflammation, if present after injury, had abated by 9 weeks after injury.

Discussion

Because most studies of blast-TBI rodent models monitor outcomes for only relatively short post-injury times, we sought to determine if blast-TBI using COBIA recapitulates the polytrauma clinical triad – persistent post-concussive symptoms (PCS), chronic pain, and PTSD. Here we show that some sequelae to blast-TBI are transient, whereas others are persistent, consistent with the human condition.

Blast exposure

The low mortality produced by primary blast injury with COBIA at these blast strengths (427 to 462 kPa) is consistent with our first paper using the COBIA system in which fifty

percent mortality occurred at 515 kPa peak overpressure [33]. Other studies of primary blast injury in rodents have found mixed results of blast exposure on survivability. Two studies from the Chandra group at the University of Nebraska-Lincoln found that no rats survived exposure to blasts over 290 kPa in their head-fixed shock tube model [58,59]. However, other studies have found no or much lower levels of mortality after primary blast injuries of even higher overpressures: 0% mortality at 323 kPa [60] and 33% mortality at 400 kPa [61].

Comparatively, composite blast exposures in which the head is free to move during blast exposure have been hypothesized to induce greater levels of mortality. Again, results of blast exposures vary widely group-to-group and model-to-model. Turner and colleagues [62] exposed rats to blast at 496 kPa using a shock tube model without head fixation, resulting in 33% lethality. Another shock tube blast study at 358 kPa resulted in only 2% mortality [63]. Song et al. [64] reviewed shock tube and open field blast models in rodents and found 50% mortality at an average of 400 kPa, a similar strength to our current study. Additionally, a review of explosive blasts in Northern Ireland found that humans exposed to blasts died in 50% of incidents at 550 kPa [65]. Overall, while our blast exposures induce lower mortality than composite blast models at similar peak overpressures, they are in line with other studies of primary injury and are conceivably survivable to substantial populations exposed in the field.

Soldiers exposed to composite blasts in the field are less likely to survive a blast of 400–500 kPa, as used in this study, than a rat, owing to species differences in blast injury susceptibility [66]. Traditionally, blast models have scaled exposures to rodents in comparison to humans by either body mass ratios or pulmonary injury susceptibility because the lungs are especially vulnerable to blast injury [67–69]; however, advances in body protection in the field have reduced blast lung injuries and unveiled higher incidences of survivable blast-TBI [70,71]. A head modeling study concluded that rodents may instead be more resilient to brain injury due to blast exposure than humans [66]. While a primary blast of 427 to 462 kPa is necessary to induce persistent behavioral abnormalities in a rat model with relatively low mortality, the same blast exposure would likely be lethal to a large subpopulation of humans. In the current study, we scaled our blast exposures with behavioral outcomes after blast injury to model a moderate blast-TBI in rats.

Vestibulomotor function

Our cranium-directed primary blast-TBI model led to transient vestibulomotor deficits (Figure 3). These findings are consistent with previous reports that examined vestibulomotor functions beyond the acute stage: Cernak et al. [72] found that mice spontaneously recover rotarod performance by 21 dpi. Also, Elder et al. [73] saw no deficits in rat motor performance at 8 weeks after injury. Results from patient studies are inconsistent, with some reporting long-term impairment of vestibular and motor coordination [74,75], whereas others report transient impairments [16,76–79], consistent with our preclinical findings in rodents.

Anxiety

Blast-TBI in humans has been associated with high incidence of mood disorders, particularly PTSD, depression, and anxiety [76,80–83]. We assessed rodent behavioral analogues to anxiety in several approach-avoidance conflict tasks, and found no effect of injury in three metrics of unconditioned exploratory behavior: the open field, elevated plus maze, and light-dark box tasks (Table 1). This matches previous reports demonstrating the absence of lasting anxiety-like signs in rats exposed to a shock-tube model of blast injury [84,85].

In contrast, other studies report persistent behavioral markers of PTSD and anxiety-like conditions. However, these studies expose the animals to different sources of blast or to multiple injuries. Zuckerman et al. [86] used a flash-bang blast model in rats while awake – 11% developed PTSD-like behaviors. Also, Elder et al. [73] reported persistent anxiety-like behavior in rats exposed to three blasts, suggesting that repeated injury increases susceptibility to developing lasting anxiety. A single, cranium-directed blast injury performed under anesthesia, such as the one performed here, may be too subtle to induce lasting anxiety.

Pain and hyperalgesia

Migraine-like and tension-type headaches are common, debilitating complaints after blast-TBI [30,87,88]. Hoge et al. [13] found that persistent post-traumatic headaches (PTH) after blast-TBI were the only persistent symptom of PCS that was not correlated with PTSD symptoms, suggesting that PTH is not the result of psychological distress. While migraine-like headaches are well documented in clinical literature, there are few studies of blast-TBI-induced PTH in rodent models [89].

Through the use of a validated, experimenter manipulation-independent metric of ongoing pain (the RGS), we found that, weeks after the blast injury, rats display increased RGS scores (Fig. 4A). Grimace scores are reliable metrics of ongoing pain in several species, including humans [18–19,51,90,91]. These findings mirror RGS score increases induced in rodent models of migraine [92–95].

Cranial cutaneous hypersensitivity occurs in both migraineurs and sufferers of blunt force-induced TBI [96–99], but hyperalgesia associated with orofacial pain has not been previously examined, to our knowledge, in rodent models of blast-TBI. Here, rats subjected to blast injury displayed signs of significant and persistent mechanical hypersensitivity of the face, lasting at least two months after injury (Fig. 4C). This suggests that rats with a COBIA-induced blast-TBI are useful models for studying persistent neurotrauma-related orofacial pain and hyperalgesia and effectively recapitulates aspects of the polytrauma triad (chronic pain and TBI).

Gliosis in the thalamus

The PO and VPM thalamus are critical somatosensory nuclei implicated in central pain following injury [23–24,100–102]. Glia, as potent inflammatory mediators and sources of synaptic modulation, can mediate long-term changes in the thalamus to potentiate post-

traumatic behavioral abnormalities [103–105]. Indeed, we have demonstrated that persistent pain following spinal cord injury (SCI) is associated with persistent microglial and astroglial activation in PO [25].

Here, rats exposed to blast-TBI, and examined 9 weeks later, did not show elevated levels of astrocyte expression or reactive microglia in the PO or VPM, suggesting that the chronic pain and facial hyperalgesia is not dependent on sustained PO and VPM gliosis. Brain tissue at interfaces with fluid, such as that bordering the cerebrospinal fluid-filled ventricles or blood-filled sinuses, are particularly susceptible to primary blast injury due to the reflection of blast waves at borders of materials with differing densities [106–108]. The thalamus may be protected from the brunt of primary blast injury due to its location in the center of the brain, which may explain the lack of gliosis present after blast-TBI in the present study.

It is also possible that transient gliosis occurred at time-points earlier than those studied here. For example, after a similar mild blast injury, Perez-Polo et al. [109] found higher Iba1 labeling in the thalamus, lasting through 30 dpi. Similarly, we have previously demonstrated increased astrocyte and microglia labeling in the hippocampus 4 weeks after blast-TBI [48]. Our results suggest that glial activation in rat thalamus, which may be present at earlier time-points, subsides after several months.

Modeling human condition after blast-TBI

With the current goal of this study to validate the COBIA model of blast injury for study of chronic complications of blast-TBI, we found that our rat model of blast injury recapitulates some aspects of the polytrauma triad (TBI PCS, PTSD, and chronic pain) experienced by a large population of soldiers and veterans of recent conflicts [75,81,83,110]. Like humans exposed to mild blast-TBI, rats display vestibulomotor deficits resolving in the weeks after injury and chronic pain and facial hyperalgesia persisting at least several months. Persistent pain and hyperalgesia after blast-TBI is not mediated by innate immune cell-mediated inflammation in the sensory thalamus. The COBIA model does not recapitulate the persistent anxiety-like behavior, indicative of a PTSD-like phenotype, frequently noted after human blast-TBI. Altogether, our results suggest that this model of primary blast-TBI is suited for studies regarding persistent post-traumatic headache in the absence of PTSD.

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Figure 1.

Experimental schematic outlining group sizes and strains and behavioural testing schedule. The black arrow indicates the day of blast injury, while the grey arrows indicate days on which vestibulomotor tasks, pain and hyperalgesia, or anxiety metrics were assessed.

Figure 2.

Rats display transient periods of apnea and decreased arterial oxygen saturation immediately following COBIA blast injury. (A) Blast injuries at 427 and 462 kPa led to comparable apnea durations following blast induction (Mann–Whitney ranked-sum test, $U=64$). (B) Arterial oxygen saturation levels are transiently depressed after cranium-directed blast injury, but recover to sham levels within 5 min (sham $n=14$, blast-TBI $n=14$, two-way mixed-model ANOVA with Sidak's multiple comparisons). Data represent medians with 95% confidence intervals (CI). Asterisks signify time-points at which $p < 0.05$.

Figure 3.

Vestibulomotor function and exploratory behaviour are transiently impaired following blast injury. (A) Latency to walk the beam and foot fault observations comprised a beam walk score, which was impaired in rats with blast-TBI ($n = 12$) compared to shams only at 24 h after injury before resolving to sham ($n = 12$) performance levels. (B) Ability to balance on the beam without foot fault errors and compensatory gripping strategies were scored and found to be transiently dysfunctional in blast-TBI rats ($n = 13$), reaching sham ($n = 12$) levels 14 days post-injury (dpi). (C) Rearing in a Plexiglas chamber, like the beam walk and balance tasks, was reduced short-term in blast-TBI rats ($n = 13$), which increased to sham ($n = 12$) times by 7 dpi. (D) Latency to fall from the accelerating rotarod, normalized to baseline performances, was decreased following blast-TBI ($n = 12$) until 21 dpi (sham $n = 11$). Data points are mean $\pm$ 95% CI bars and statistical comparisons were made using a two-way mixed-model ANOVA with multiple comparisons.

Figure 4.

Metrics of hyperalgesia and spontaneous pain suggest pain symptoms persist at least 4 weeks after blast injury. (A) The median RGS scores were significantly elevated in blast-TBI rats compared to shams at 5 weeks after injury (Mann-Whitney $U=1$). (B) Representative images of screenshots used to score rat grimace scale (RGS), with each action unit area highlighted with boxes. (C) Mechanical withdrawal thresholds of the face are significantly reduced in blast-TBI rats compared to shams 4 weeks after injury (Mann-Whitney $U=7.5$). Data represented are medians and 95% CI.

Figure 5.

Persistent pain behaviours were not associated with increased glial expression in the posterior thalamus (PO) or the ventral posteromedial thalamus (VPM). (A) Integrated fluorescence densities of GFAP labelling in PO (sham: 326.1 ± 106.4 , 227.8–484.4; blast: 376.6 ± 54.47 , 249.2–569.4; $U = 12$, $p = 0.39$) and VPM (sham: 348.9 ± 154.7 , 227.9–608.7; blast: 310.2 ± 188.7 , 174.1640.4; $U = 10$, $p = 0.43$) did not differ between sham ($n = 6$) and blast-TBI ($n = 5$) rats. Note representative images of GFAP labelling inset. (B) Representative images of Iba1 labelling in PO and VPM show the microglial phenotypes. (C) Integrated fluorescence densities of Iba1 in PO (sham: 259.9 ± 28.25 , 225.6302.3; blast: 290.2 ± 58.92 , 224.3362.7; $U = 11$, $p = 0.54$) and VPM (sham: 265.4 ± 49.95 , 214.8351; blast: 299.1 ± 80.43 , 218.4419.6; $U = 12$, $p = 0.66$) did not show a difference in total fluorescent signal associated with microglial labelling in sham and blast-TBI rats (sham $n = 6$, blast-TBI $n = 5$). (D) Total microglia counts in PO and VPM are comparable in sections taken from sham and blast-TBI rats (sham $n = 6$, blast-TBI $n = 6$). Data for 5A through 5D are medians and 95% CI, tested with Mann–Whitney ranked-sum analyses. (E, F) Categorization of microglia morphology shows comparable microglia phenotype in PO (5E) and VPM (5F) after blast injury or sham treatment (sham $n = 6$, blast-TBI $n = 6$). Data for

5E and 5F are medians with 95% CI and analyzed with two-way mixed-model ANOVA tests with corrections for multiple comparisons.

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Table 1.

No lasting anxiety-like behaviors are present 3–5 weeks after blast-TBI. A: Thigmotaxis in an open field did not significantly differ between blast-TBI and sham rats. C: Rats in both groups spent comparable amounts of time in the open arms of the elevated plus maze. D: Blast-TBI rats spent similar lengths of time in the light side of a light-dark box as their sham counterparts.

Anxiety Metric (% of trial time)	Sham median ($\pm$ 95% CI, n)	Blast-TBI median ($\pm$ 95% CI, n)	Mann-Whitney U	p-value
Center of Open Field	9.55 (0.59–37.14, 6)	10.11 (6.90–12.06, 6)	17	0.94
Open arms of Elevated Plus Maze	23.87 (5.7–41.1, 9)	27.80 (15.73–33.13, 11)	17	0.82
Light side of Light-Dark Box	42.09 (17.06–51.31, 6)	25.50 (11.9–53.76, 6)	14	0.59