Title: Blast-induced mild traumatic brain injury promotes comorbid stress responses elicited by environmental cues attending blast exposure

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ABSTRACT

Repetitive mild traumatic brain injury (mTBI) has been called the “signature injury” of military Servicemembers in the Iraq and Afghanistan wars, is highly comorbid with posttraumatic stress disorder (PTSD), and a major source of morbidity among Veterans enrolled in the VA health care system. Correct attribution of adverse blast-induced outcomes to TBI vs PTSD remains a challenge, engendering added difficulty for subsequent clinical diagnosis and treatment. Preclinical research efforts using rodent models can provide needed insight into underlying mechanisms by which blast produces subsequent dysfunction, but only in so much as the animal model recapitulates the human experience. Here we sought to understand the extent to which a mouse model of blast reproduces the phenomena experienced by Servicemembers and/or those with comorbid mTBI and PTSD. Drawing upon well-established work in the chronic stress and fear learning literature, we hypothesized that environmental cues associated with blast exposure are sufficient to evoke aversive/dysphoric psychological stress and reproduce traumatic stress in addition to blast-induced brain injury. Using an electronically controlled pneumatic shock tube that models battlefield-relevant open-field blast forces generated by detonation of high explosives, we provide direct evidence that psychological stress is inherent to repetitive blast exposure, resulting in chronic aversion/dysphoria to previous blast-paired cues. Demonstrating a previously unappreciated translational aspect, this study brings into line the relevance of repetitive blast trauma in rodent models to the experience of Servicemembers and/or those with comorbid mTBI and PTSD, providing significant opportunities for translationally relevant mechanistic understanding and therapeutic development.

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of death and disability, affecting every segment of the population, with youth, elderly, and athletes being most affected. Likewise, following a traumatic event, post-traumatic stress disorder (PTSD) is common and affects 5-10% of the adult population of United States. Moreover, mild traumatic brain injury (mTBI/concussion) is labeled the "signature injury" of military Servicemembers in Operation Iraqi Freedom/Enduring Freedom/New Dawn (OIF/OEF/OND) (O'Neil et al., 2013; Tanielian, 2008), is highly comorbid with posttraumatic stress disorder (PTSD), and a major source of morbidity among Veterans enrolled in the VA health care system. Such symptoms are common, with approximately 350,000 Veteran mTBIs diagnosed since 2000 (estimated post-deployment rates of 10-25%), with an estimated PTSD comorbidity rate of 50-75% (DVBIC, 2019; Hoge et al., 2008; Tanielian, 2008). Blast exposure (via detonation of high explosives) is the primary source of mTBI (accounting for 75% of all TBIs reported by Veterans) with multiple exposures more common than a single blast exposure (Owens et al., 2008; Tanielian, 2008). Efforts to elucidate the biological processes responsible for the clinical manifestations of blast-related mTBI and PTSD are impeded by factors that include: (i) high rates of comorbidity; (ii) overlapping symptoms; and (iii) the initiating insult (i.e. blast exposure) can simultaneously induce both biomechanical/neurologic and substantial neuropsychological stress (Bryant, 2011; Hendrickson, Schindler, & Pagulayan, 2018). Indeed, physicians have wrestled with this clinical conundrum for more than 100 years since the initial descriptions of "shell shock" among World War I soldiers exposed to artillery bombardments (Jones, 2006; Jones, Fear, & Wessely, 2007). There are at least two competing hypotheses that address the apparent connection between mTBI and PTSD: 1) simultaneous exposure to TBI-causing events and PTSD-related stressors with additive/synergistic results or 2) TBI-induced compensatory changes in stress-related brain regions produce outcomes similar in nature to those seen in PTSD and/or render the brain more susceptible to subsequent psychological stressors (or vice versa – PTSD-related trauma

damages brain regions involved in post-concussive symptom outcomes). Following mTBI exposure in rodents, we and others have published evidence of post-concussive syndrome-like and PTSD-like outcomes in both rats and mice (Elder et al., 2012; Meabon et al., 2016; Perez-Garcia et al., 2018; Perez-Garcia et al., 2016; Perez-Garcia et al., 2019; Schindler et al., 2017). Anesthesia is commonly used in pre-clinical settings. The nature of the precipitating blast-induced trauma (physical and/or psychological) while animals are anesthetized has been viewed alternately as a complicating factor or as a useful reductionistic tool in working toward a better understanding of the mechanisms by which blast mTBI causes chronic PTSD-related symptoms (Hendrickson et al., 2018; Perez-Garcia et al., 2019). Translational research efforts using rodent models can provide much needed insight into underlying mechanisms by which blast exposure produces dysfunction, but only in so much as the rodent model recapitulates the human experience. Here we sought to understand the extent to which rodent models of blast exposure reproduce the phenomenon experienced by military Servicemembers and others with comorbid mTBI and PTSD.

Drawing upon fundamental concepts regarding chronic stress and Pavlovian fear learning (Bruchas, Land, & Chavkin, 2010; Chavkin, Xu, Land, Redila, & Bruchas, 2006; Heinrichs & Koob, 2004; Land et al., 2008; Li & McNally, 2014; Richter-Levin, Stork, & Schmidt, 2019; Schindler, Li, & Chavkin, 2010), we postulated that despite the animals being anesthetized during the blast exposure itself, environmental cues attending to events before and after the blast exposure are sufficient to elicit psychological stress responses with PTSD-like behavioral outcomes. We further postulated that such psychological stress induces a negative hedonic state with motivational properties that can be associated with neutral cues (odors, visual patterns, etc.) to engender subsequent avoidance and/or reexperiencing-induced stress and dysphoria to those associated cues (Bruchas et al., 2007; Chavkin et al., 2006; Edwards et al., 2013; Heinrichs & Koob, 2004; Land et al., 2008; Schindler et al., 2010). To test these ideas, we used place conditioning to measure avoidance and examined behavioral (locomotion and

mouse vocalizations) and physiological (corticosterone production) stress responses provoked by re-exposure to blast-related cues. Ultrasonic vocalizations (USVs) are thought to vary with behavioral context, with lower kHz USVs primarily seen during aversive scenarios such as restraint stress or social isolation (Bonasera et al., 2015; Grimsley et al., 2016; Simola & Granon, 2019), thereby providing a sensitive readout of the animals' affective state.

Using a well-established electronically-controlled pneumatic shock tube that models battlefield-relevant open-field blast forces generated by detonation of high explosives (Huber et al., 2016; Huber et al., 2013; Logsdon et al., 2018; Meabon et al., 2016; Schindler et al., 2017), we found that blast-induced psychological stress produces chronic aversion and dysphoria to prior blast-paired environmental cues. These results offer new insight regarding how repetitive blast exposure may give rise to PTSD-like symptoms. In addition, these findings support the idea that this animal model simultaneously provokes both neurologic and psychological insults, as in military Servicemembers with blast-related comorbid mTBI and PTSD, thus demonstrating a previously unappreciated translational aspect of repetitive blast trauma in rodent models.

MATERIALS AND METHODS

Animals and mouse model of blast overpressure:

Male C57Bl/6 mice (Jackson Laboratory) aged 3–4 months (weight 22-35 g; mean 27.0 ± 0.2 g) were used. All animal experiments were carried out in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by the VA Puget Sound Institutional Animal Care and Use Committees. The shock tube (Baker Engineering and Risk Consultants, San Antonio, TX) was designed to generate blast overpressures that mimic open field high explosive detonations encountered by military Servicemembers in combat, and the design and modeling characteristics have been described in detail elsewhere (Huber et al., 2016; Huber et al., 2013; Schindler et al., 2017). Briefly, mice were anesthetized with isoflurane (induced at 5% and maintained at 2-3%), secured against a gurney, and placed into the shock tube oriented perpendicular to the oncoming blast wave (ventral body surface toward blast) in accordance with previously established blast overpressure methods (Koliatsos et al., 2011). Sham (control) animals received anesthesia only for a duration matched to blast animals. Repeated blast/sham exposures occurred successively over the course of three days (one per day). Following exposure, mice were immediately removed from the shock tube and anesthesia was discontinued (anesthesia duration ranged from 3-4 min). Mice were then placed in a heated enclosure for observation during recovery. The blast overpressure (BOP) peak intensity (psi), initial pulse duration (ms), and impulse (psi▪ms) used were in keeping with mild to moderate blast injury (Cernak et al., 2011; Koliatsos et al., 2011) (20.23 psi +/- 0.08 psi; 5.797 ms +/- 0.017 ms; 0.037 psi*ms +/- 0.000 psi*ms) (Figure 1a). Under these experimental conditions, the overall survival rate exceeded 95%, with blast-exposed mice appearing comparable to sham-exposed mice by inspection 2 hours-post blast exposure as previously reported (Huber et al., 2016; Huber et al., 2013; Meabon et al., 2016; Schindler et al., 2017). Animals were weighed daily prior to sham/blast exposure and at 24- and 72-hours post-exposure. There were no statistically significant weight differences between 1x

sham and 3x sham-treated mice (24h post-exposure: Student's unpaired t-test, t[25]=0.355, *p*>0.05;. 72h post-exposure: Student's unpaired t-test, t[25]=0.9368, *p*>0.05), thus, 1x and 3x sham animals at each time point were pooled together for subsequent analyses.

Odorant conditioning paradigm:

See Figure 1c for experimental schematic. Animals were pre-exposed to a Plexiglas T-Maze (66 cm long × 40 cm wide × 15 cm high) for 5 min prior to Pavlovian conditioning. Next, sham/blast exposure occurred on three consecutive days. Ten minutes prior to the first exposure animals received a stainless-steel mesh tea-ball (Amazon) containing two-quarter Nestletts (PharmaServ, Framingham, MA) with 20 µl of imitation almond extract (Kroger, Cincinnati, OH) in their home cage. The Nestletts and scent were refreshed on each morning of sham/blast exposure and the tea-ball remained in place until 24 hours following the final sham/blast exposure. One month following exposure, animals were tested for odorant-conditioning in the T-Maze with a tea-ball containing two-quarter Nestlett with 20 µl imitation almond odorant cue placed in the left arm of the maze and a tea-ball containing two-quarter Nestlett with 20 ul saline placed in the opposite arm of the maze. Animals were placed in the long arm of the T-maze and given 5 min to explore. Latency to enter and time spent in each of the two distal ends of the short arms was recorded and analyzed using Anymaze. There were no statistically significant differences between 1x sham and 3x sham-treated mice (saline corner time: Student's unpaired t-test, t[9]=0.537, *p*>0.05; odor corner time: Student's unpaired t-test, t[9]=0.036, *p*>0.05*;* saline corner latency: Student's unpaired t-test, t[9]=0.712, *p*>0.05; odor corner time: Student's unpaired t-test, t[9]=1.113, *p*>0.05), thus, 1x and 3x sham animals were pooled together for subsequent analyses.

Place conditioning paradigm:

See Figure 2a for experimental schematic. A balanced-three compartment conditioning apparatus was used as described previously (Schindler et al., 2010). Animals were pre-tested by placing individual animals in the small central compartment and allowing exploration of the entire apparatus for 20 min. Time spent in each compartment was recorded and analyzed using Anymaze. Mice were randomly assigned an AM and PM box (either gray walls or vertical black and white strip walls). Next, sham/blast exposure occurred on three consecutive days. On each day of exposure, in the morning, animals were first placed in their AM-pairing chamber containing distinct visual cues for 10 minutes and then were immediately given a sham exposure. In the afternoon, animals were placed in their PM-pairing chamber containing a different set of distinct visual cues for 10 minutes and then were immediately given a blast or sham exposure (depending on injury group assignment). Place conditioning was assessed at one and three months following repetitive exposure by allowing the mice to roam freely in all three compartments. Time spent in each compartment was recorded and analyzed using Anymaze. Place conditioning scores were calculated by subtracting the time spent in the PM paired compartment from the time spent in the AM paired compartment.

Cue conditioning and re-exposure paradigm:

See Figure 3a for experimental schematic. Sham/blast exposure occurred on three consecutive days. On each day of exposure, animals were first placed in a pairing chamber containing distinct visual cues (randomly assigned to gray or black and white striped walls) for 10 minutes and then were immediately given a sham or blast exposure (depending on injury assignment). One month following repetitive exposure, the animals were re-exposed to either a neutral chamber or the chamber previously paired with blast or sham for 10 minutes, and movement (via Anymaze) and ultrasonic vocalizations were recorded. Blood was collected from the submandibular vein one day prior and 30 minutes after removal from the pairing chamber. Plasma samples were processed to assay corticosterone levels using an ELISA kit as per

manufacture protocol (Arbor Assays, Ann Arbor, MI) and fold change over baseline (blood collected prior to re-exposure) was calculated. USVs were recorded using a Petterson microphone (Norway, model M500-384) and Avisoft SASLab Lite recording software and were manually analyzed using RavenLite (Cornell lab of Ornithology).

Data analysis:

As appropriate, data were analyzed using: (*i*) two-tailed Student's t-tests; (*ii*) one-way or two-way (between/within subjects design) repeated measures analysis of variance (RM ANOVA), followed by Newman-Keuls Multiple Comparison Tests or Bonferroni Post-hoc tests, respectively. Reported *p* values denote two-tailed probabilities of $p \leq 0.05$ and non-significance (n.s.) indicates $p > 0.05$. Statistical analyses were conducted using Graph Pad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA) and SPSS software (IBM, Armonk, NY).

RESULTS

Chronic aversion to cues previously paired with blast:

Using well-established methods (Huber et al., 2016; Huber et al., 2013; Logsdon et al., 2018; Meabon et al., 2016; Schindler et al., 2017), male C57Bl/6 adult mice were exposed to one or three blast overpressures (BOPs) using a pneumatic shock tube delivering a peak static pressure of 20.23 psi +/- 0.08 psi, positive phase duration of 5.80 ms +/- 0.02 ms; 0.037 psi*ms +/- 0.000 psi*ms (Figure 1a).

A common outcome of stress exposure in mice is weight loss, thus we measured body weight at baseline, 24h, and 72h following sham or blast exposure. There were no statistically significant differences between 1x sham and 3x sham-treated mice. Thus, 1x and 3x sham animals at each time point were pooled together for subsequent analyses (see Materials and Methods for sham comparison statistics). In accordance with an acute stress response, blast exposure resulted in acute weight loss (two-way RM ANOVA: interaction effect $F[2,46]=12.95$, $p<0.0001$, Bonferroni's Multiple Comparison Test post-hoc: n=9-27). Post-hoc analyses demonstrate significant weight loss for both 1x and 3x blast animals at both 24h and 72h following injury (Figure 1b), suggesting that even a single blast exposure is able to induce weight loss starting just 24h after injury.

To further investigate potential blast-induced psychological stress we employed a conditioned odorant paradigm in mice that pairs sham or blast exposure with a neutral odorant cue (Fig. 1c). The neutral odorant cue (almond scent) was placed in the home cage five minutes prior to the first sham or blast exposure and remained in place (scent refreshed daily) until 24 hours post final sham or blast exposure. Finally, conditioning to the paired-odorant cue was assessed one month following injury. There were no statistically significant differences between 1x sham and 3x sham-treated mice. Thus, 1x and 3x sham animals at each time point were pooled together for subsequent analyses (see Materials and Methods for sham comparison statistics). The panels in Figure 1d show occupancy heat maps for each group during the post-

test (odorant is placed in left corner of T-maze) one month following the injury/odorant pairings. Mice developed a significant aversion to the odorant when subsequently presented alone in one corner of the testing chamber (left corner of T-maze) (two-way RM ANOVA: main effect of location $F[1,19]=11.83$, $p=0.003$, Bonferroni's Multiple Comparison Test post-hoc: n=5-13) (Figure 1e). Post-hoc analyses demonstrated significant aversion following only repetitive (3x, one per day) but not single (1x) blast or sham exposure. Odorant-blast pairings also increased the latency to enter the odorant corner in a blast number-dependent manner (two-way RM ANOVA: main effect of location $F[1,19]=8.798$, $p=0.008$, Bonferroni's Multiple Comparison Test post-hoc: n=5-13) (Figure 1f). These results demonstrate that while a single blast exposure is sufficient to produce acute weight loss, only repetitive blast exposures induce significant odorant aversion, suggesting repetitive blast-induced psychological stress formation at time of injury.

To assess the generality of these observations, the ability of repetitive blast exposure to induce place aversion was investigated using a modified place conditioning paradigm in mice that pairs sham and blast exposure with distinct visual cues (Fig. 2a). On each day of exposure, in the morning, animals were first placed in a pairing chamber containing distinct visual cues for 10 minutes and then were immediately given a sham exposure (e.g. anesthesia only), then in the afternoon, animals were placed in a pairing chamber containing a different set of distinct visual cues for 10 minutes and then were immediately given a blast or sham exposure. Robust conditioned aversion to the blast-paired compartment was evident one month post-blast exposure and is sustained until at least three months post-blast exposure (one-sample t-test vs. a theoretical mean of 0 (no aversion): n=12-16; sham baseline: $t[15]=0.5882$, $p=0.5651$, sham 1 month: $t[15]=0.0433$, $p=0.9660$, sham 3 months: $t[15]=0.1021$, $p=0.9200$; blast baseline: $t[11]=0.7758$, $p=0.4529$, blast 1 month: $t[11]=3.118$, $p=0.0098$, blast 3 months: $t[11]=3.235$, $p=0.0079$; (Figure 2b). Despite significant place aversion, blast exposure did not affect total distance traveled during the post-test, suggestion that aversion was not an nonspecific effect of locomotion changes (two-way RM ANOVA: interaction effect $F[2,50]=0.931$, $p=0.401$,

Bonferroni's Multiple Comparison Test post-hoc: n=12-16) (Figure 2c). These data suggest that in multiple distinct conditioned-aversion paradigms (odorant and place aversion), repetitive blast exposure produces significant aversion, suggesting psychological stress formation at the time of blast injury.

Dysphoric and aversive behavioral response to visual cues previously paired with blast.

In order to investigate whether cues previously paired with blast or sham exposure elicit distinct behavioral responses during re-exposure, we extended the modified place conditioning paradigm used above to allow for re-exposure to either a neutral visual environment or one that was previously paired with injury. On each day of exposure, animals were first placed in a pairing chamber containing distinct visual cues for 10 minutes and then were immediately given a sham or blast exposure (e.g. anesthesia only). One month following repetitive exposure, animals were re-exposed to either a neutral chamber or the chamber previously paired with injury (blast or sham) for 10 minutes, movement and ultrasonic vocalizations (USVs) were recorded, and blood was collected (pre/post re-exposure) for subsequent plasma corticosterone analysis (Figure 3a). Differential behavioral responses were demonstrated following re-exposure to neutral vs. injury-paired cues (two-way ANOVA: interaction effect $F[1,43]=9.462$, $p=0.0036$, Bonferroni's Multiple Comparison Test post-hoc: n=11-14) (Figure 3b). Post-hoc analyses demonstrated a significant decrease in distance traveled during re-exposure to blast-paired cues as compared to sham-paired cues with no difference when re-exposed to neutral cues. A corresponding relative increase in plasma corticosterone levels following re-exposure to blast but not sham paired cues was also evident (fold change over baseline (pre-exposure) level) (one-sample t-test vs. a theoretical mean of 1 (no difference in corticosterone levels pre/post re-exposure): n=3-4; sham: $t[2]=1.717$, $p=0.2282$, blast: $t[3]=4.352$, $p=0.0224$) (Figure 3c). In addition to movement, we also recorded ultrasonic vocalizations (USVs) during re-exposure as an additional read-out of affective state. Total USVs were significantly increased in blast-

exposed mice during re-exposure to the injury-paired but not neutral-paired cues (two-way ANOVA: interaction effect $F[1,23]=9.714$, $p=0.0049$, Bonferroni's Multiple Comparison Test post-hoc: n=7-9) (Figure 3d). In line with an aversive/dysphoric stress response to reminders of blast injury, low kHz USVs were specifically increased during re-exposure to the injury paired but not neutral-paired cues (two-way ANOVA: interaction effect $F[1,23]=8.820$, $p=0.0069$, Bonferroni's Multiple Comparison Test post-hoc: n=7-9) (Figure 4e). Post-hoc analyses demonstrated a significant increase in low kHz USVs during re-exposure to blast-paired cues as compared to sham-paired cues with no difference when re-exposed to neutral cues. While a significant interaction effect was also seen in the number of high kHz USVs (two-way ANOVA: interaction effect $F[1,23]=5.206$, $p=0.0321$, Bonferroni's Multiple Comparison Test post-hoc: n=7-9) (Figure 3f), post-hoc analyses did not demonstrate significant differences between injury and neutral paired cues or blast and sham exposure. Together these results suggest that environmental cues at time of blast exposure are sufficient to engender behavioral and physiological outcomes reminiscent of aversive stress and dysphoria.

Discussion

Blast-induced mTBI is currently defined as cellular and/or structural damage to the brain, which can cause symptoms that include adverse somatic, vestibular, cognitive, and affective outcomes (Tanielian, 2008; Warden, 2006). PTSD is an anxiety disorder which develops after exposure to potentially life-threatening events (such as blast) and persistent symptoms include re-experiencing, avoidance, and hyperarousal (Association, 2013; Crocq & Crocq, 2000; Richter-Levin et al., 2019). As such, blast-induced mTBI is commonly associated with the development of battlefield PTSD and the two conditions share several overlapping symptoms. Indeed, some have even postulated that postconcussive symptoms following mTBI are non-specifically related to blast, and instead better explained by psychological trauma consistent with PTSD (Bryant, 2011; Elder et al., 2012). Because battlefield blast exposures can simultaneously provoke both psychological and neurological insults, understanding the basis for chronic symptoms in dual-diagnosis (e.g. mTBI and PTSD) Veterans has remained challenging.

Translational research efforts using rodent models can provide much needed insight into underlying mechanisms by which blast exposure produces dysfunction, thought to be free from many of the confounding variables associated with human exposures in the battlefield, but only in so much as the rodent model recapitulates the human experience. Drawing from long-established chronic stress and fear learning research (Bruchas et al., 2010; Chavkin et al., 2006; Heinrichs & Koob, 2004; Land et al., 2008; Li & McNally, 2014; Richter-Levin et al., 2019; Schindler et al., 2010), the results herein strongly argue that the conditions surrounding the immediate blast exposures per se, are sufficient to drive Pavlovian fear learning and generate subsequent PTSD-like behavioral outcomes. Specifically, these data directly support the notion that exposure to environmental cues attending blast injury events give rise to Pavlovian learning-induced stress responses that elicit subsequent PTSD-like avoidance, intrusive symptoms, and mood alteration (e.g. dysphoria)(Gewirtz & Davis, 2000; Wessa & Karl, 2007). We utilized place conditioning as an objective measure of behavior and to infer a relationship

between the aversion exhibited at time of testing to previous psychological stress and dysphoria at the time of conditioning. Indeed, Land et al., (2008) operationally defined dysphoria as the emotional response to a sustained stimulus that creates aversion, and while not directly measured, dysphoria is thought to represent the underlying emotional state at time of stimulus exposure. Importantly, we demonstrate chronic effects which persist at least three months following blast exposure, an important requirement to differentiate PTSD from acute stress disorder in humans as per the Diagnostic and Statistical Manual of the American Psychological Association (DSM). While PTSD-like outcomes were only seen in animals exposed to repetitive blast, acute stress effects related to weight loss were apparent following a single blast exposure, raising the possibility that a single blast might model aspects of acute stress disorder without progressing to full PTSD-like outcomes.

Blast exposure in rodents is typically carried out under anesthetized, thus restricting opportunities for the animals to form cognitive associations with the immediate blast exposure. Under such conditions it is well-established that blast exposure produces chronic PTSD-like behavioral responses, thus suggesting that cellular/structural insults to the brain caused by blast may be sufficient to drive pathogenic processes leading to PTSD-like symptoms with limited appeal to psychological trauma and/or stress (Perez-Garcia et al., 2019). Some studies have used an additional stressor at the time of injury (e.g. dual exposure) in order to study mTBI/PTSD comorbidity (Kamnaksh et al., 2011; Klemenhagen, O'Brien, & Brody, 2013; Kwon et al., 2011; Ojo et al., 2014). The findings in this report demonstrate that repetitive blast exposure paradigms can generate traumatic psychological stress, despite anesthesia and without the requirement for an additional, experimentally administered stressor.

In a typical pre-clinical Pavlovian fear conditioning experiment, a rodent receives repeated presentations of a conditioning stimulus (CS, e.g. an environmental chamber, odor, or auditory cue) that coincide with presentation of an unconditioned stimulus (US, e.g. shock, predator odor, or social defeat). Subsequently, the animal will display a variety of conditioned

responses upon later re-exposure to the CS (e.g. avoidance, freezing, increased heart rate and blood pressure, corticosterone release, ultrasonic vocalizations). Importantly, the typical blast exposure paradigm provides opportunities for Pavlovian fear learning (even in the absence of experimenter-delivered conditioned stimuli). For example, animals must first be transported from the vivarium to a blast holding room where they stay until transferred to the blast injury room. Once transferred to the blast injury room, animals are placed in an anesthesia induction chamber, anesthetized, exposed to blast, and then allowed to recover. Overall, while the actual blast exposure under anesthesia might only last 3-5 minutes, the entire episode outside of the vivarium might last anywhere from 10 minutes to hours (depending on location of vivarium vs. blast holding and injury rooms). In the current study we exposed animals to discrete Pavlovian cues (i.e. an almond scent or a distinct visual environment), but we also postulated that additional cues available surrounding events such as transport to and wait time in the blast holding room, transport to the blast injury room, and placement in the anesthesia induction chamber might also serve as potential conditioned stimuli without intentional experimenter administration or manipulation. As such, a large literature exists outside the field of blast research suggesting that consciousness specifically during a traumatic event is not required for subsequent aversion and PTSD-like behavioral outcomes (e.g. drug-facilitated sexual assault, post-intensive care syndrome, and conditioned taste aversion to anesthetics (Jaffe, Blayney, Bedard-Gilligan, & Kaysen, 2019; Jaffe et al., 2017; Lin, Arthurs, & Reilly, 2017; Rawal, Yadav, & Kumar, 2017).

The idea that a state of unconsciousness can be used as an experimental tool to dissociate psychological from neurological/cellular trauma is inherently attractive from a reductionist standpoint, particularly when considered within the historical context of the 100 years controversy regarding the cellular/neurological versus psychological correlates of blast exposure initially described during World War I as “shell shock” (Mott, 1916; Shively & Perl, 2012). We find the implications of the current study, namely that psychological stress is inherent

to repetitive blast exposure (as it is for Servicemembers exposed to blast in the battlefield), particularly encouraging, demonstrating an important and previously unappreciated degree of translational integrity of this animal model. Indeed, using blast-paired Pavlovian cues, it is possible carry out increasingly relevant studies of re-exposure on mTBI- and PTSD-related adverse outcomes such as sleep quality, substance use and misuse, and aggression. Likewise, without the requirement of additional stress surrounding the time of injury, one can use pharmacological and behavioral interventions to study underlying mechanisms related to mTBI/PTSD-like outcomes, which will simplify experimental paradigms and better align the preclinical rodent experience with that of Servicemembers and Veterans.

In conclusion, the current study supports that animal models of repetitive blast exposure engender significant opportunities for translationally relevant mechanistic understanding and therapeutic development in relation to both mTBI- and PTSD-related adverse outcomes.

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Figure Legends

Figure 1:

Blast-induced odorant aversion. (a) Time versus pressure plot (averaged over 350 blasts) measuring the static blast overpressure (measured 5 cm above the animal). Note close correspondence with the superimposed Friedlander waveform expected from an open-field detonation of approximately 20 kg of TNT at a distance of 7.8 m. (b) Blast exposure results in acute weight loss that lasts at least three days. ***$p \leq 0.0001$: sham vs. blast. (c) Schematic of odorant-blast aversion paradigm. (d) Heat maps of odorant aversion post-test (odorant placed in left corner of T). (e) Significant aversion to repetitive blast/odor pairings one-month post-injury, with no aversion seen from sham or a single blast/odor pairing. (f) Latency to enter to odorant corner is significantly increased one-month post repetitive blast/odor pairings. Two-way RM ANOVA *post hoc* Bonferroni Multiple Comparison Test. *$p \leq 0.05$ and **$p \leq 0.001$: + odor corner vs. – odor corner, $^{\&}p \leq 0.05$ and $^{\&\&}p \leq 0.001$: sham vs blast odor corner. Error bars are mean +/- SEM.

Figure 2:

Blast-induced place aversion. (a) Schematic of blast-induced place aversion paradigm. (b) Significant aversion to blast-paired but not sham-paired cues at one month and three months post-injury. (c) No difference in locomotion during place aversion post-test across groups and timepoints. Student's one-sample t-test vs. theoretical of 0 (no aversion). Two-way ANOVA *post hoc* Bonferroni. *$p \leq 0.05$, **$p \leq 0.001$, and ***$p \leq 0.0001$: sham vs blast or neutral vs. paired. Values represent mean ± SEM.

Figure 3:

Re-exposure to blast-paired cues produces stress and dysphoria. (a) Schematic of blast-induced environmental pairing and re-exposure paradigm. (b) Significant decrease in locomotion during re-exposure to blast-paired but not sham-paired or neutral cues one-month post-injury. (c) Significant increase in plasma corticosterone following re-exposure to blast-paired but not sham-paired or neutral cues one-month post-injury. (d-f) Aversive (low kHz) ultrasonic vocalizations are significantly increased following re-exposure to blast-paired but not sham-paired or neutral cues one-month post-injury. Student's one-sample t-test vs. hypothetical mean=1.0. Two-way ANOVA *post hoc* Bonferroni. *$p \leq 0.05$, **$p \leq 0.001$, and ***$p \leq 0.0001$: sham vs blast or neutral vs. paired. Values represent mean ± SEM.

Figure 1

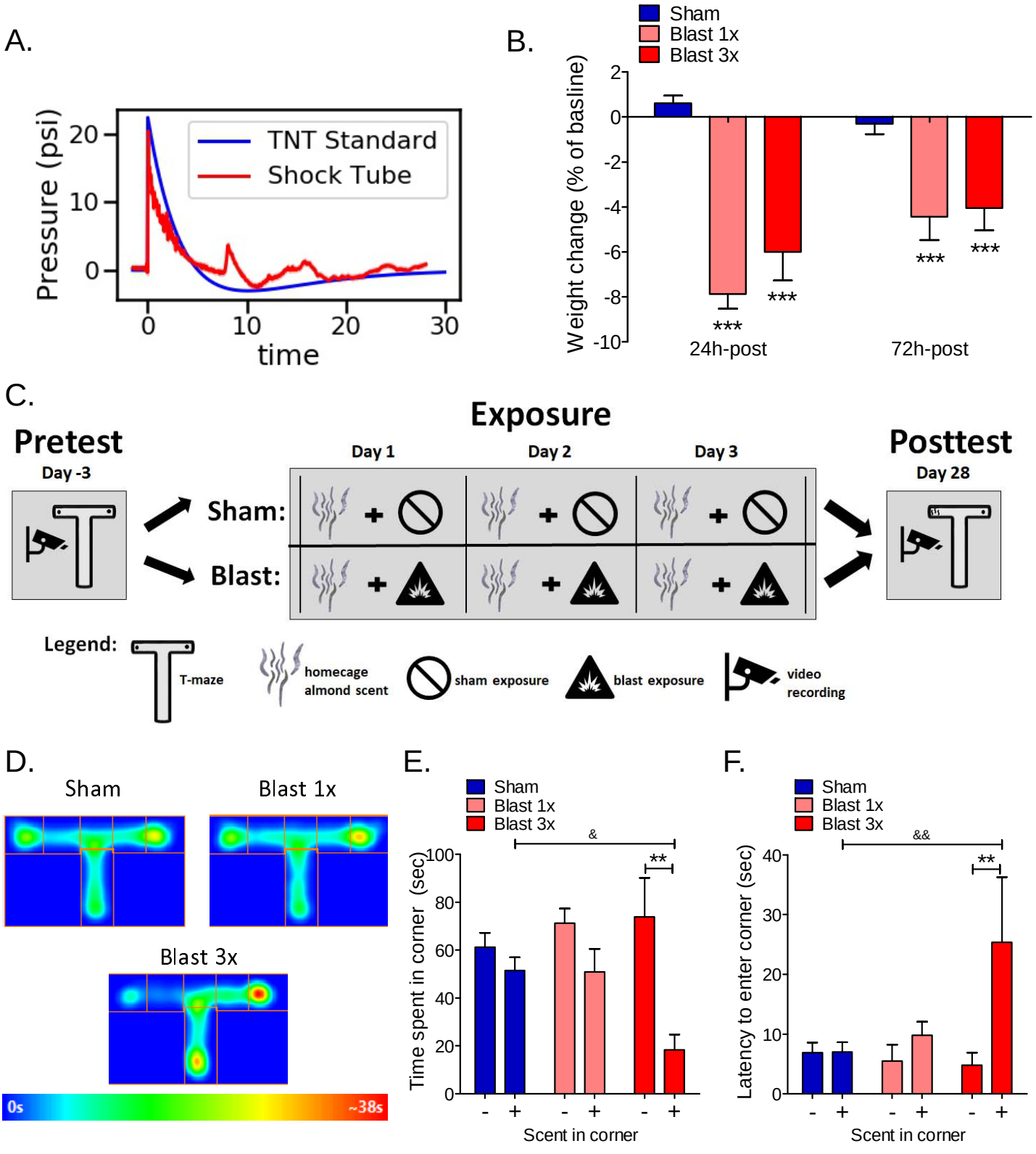
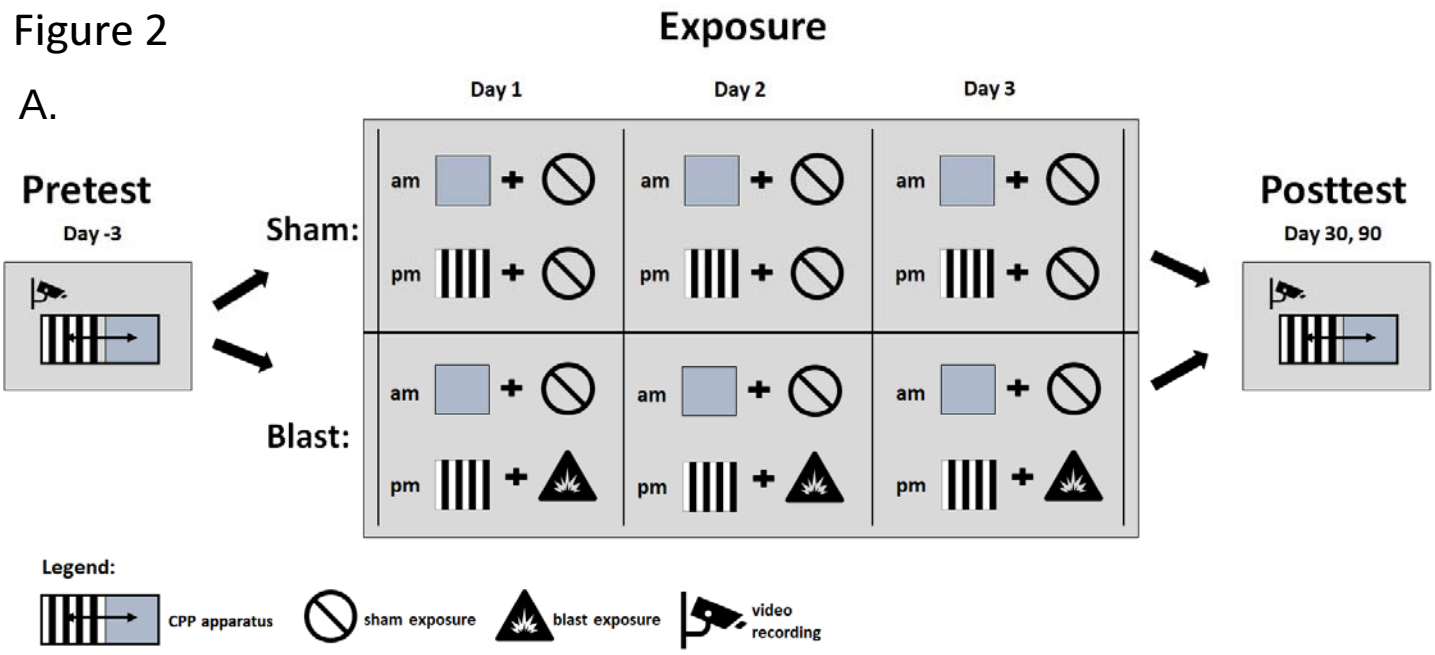
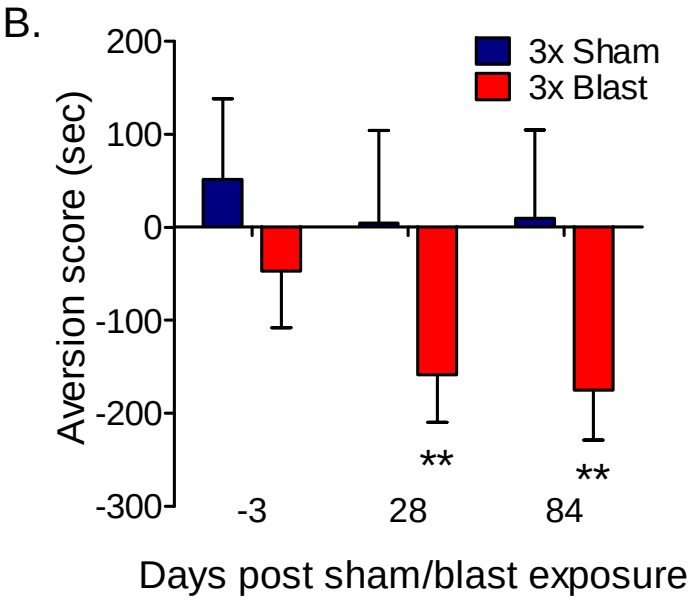


Figure 2





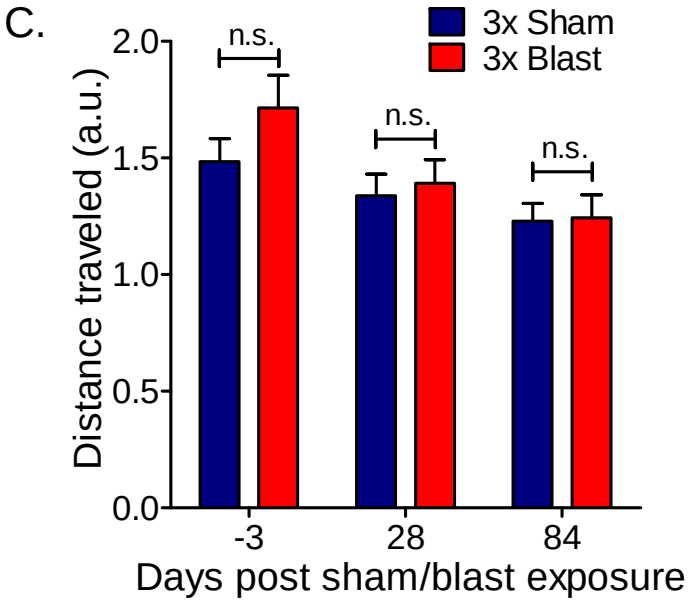


Figure 3