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Research report

Disturbances in fear extinction learning after mild traumatic brain injury in mice are accompanied by alterations in dendritic plasticity in the medial prefrontal cortex and basolateral nucleus of the amygdala

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ABSTRACT

Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) have emerged as the signature injuries of the U.S. veterans who served in Iraq and Afghanistan, and frequently co-occur in both military and civilian populations. To better understand how fear learning and underlying neural systems might be altered after mTBI, we examined the acquisition of cued fear conditioning and its extinction along with brain morphology and dendritic plasticity in a mouse model of mTBI. To induce mTBI in adult male C57BL/6J mice, a lateral fluid percussive injury (LFP 1.7) was produced using a fluid pulse of 1.7 atmosphere force to the right parietal lobe. Behavior in LFP 1.7 mice was compared to behavior in mice from two separate control groups: mice subjected to craniotomy without LFP injury (Sham) and mice that did not undergo surgery (Unoperated). Following behavioral testing, neural endpoints (dendritic structural plasticity and neuronal volume) were assessed in the basolateral nucleus of the amygdala (BLA), which plays a critical sensory role in fear learning, and medial prefrontal cortex (mPFC), responsible for executive functions and inhibition of fear behaviors. No gross motor abnormalities or increased anxiety-like behaviors were observed in LFP or Sham mice after surgery compared to Unoperated mice. We found that all mice acquired fear behavior, assessed as conditioned freezing to auditory cue in a single session of 6 trials, and acquisition was similar across treatment groups. Using a linear mixed effects analysis, we showed that fear behavior decreased overall over 6 days of extinction training with no effect of treatment group across extinction days. However, a significant interaction was demonstrated between the treatment groups during within-session freezing behavior (5 trials per day) during extinction training. Specifically, freezing behavior *increased* across within-session extinction trials in LFP 1.7 mice, whereas freezing behavior in control groups did not change on extinction test days, reflecting a dissociation between within-trial and between-trial fear extinction. Additionally, LFP mice demonstrated bilateral increases in dendritic spine density in the BLA and decreases in dendritic complexity in the PFC. The translational implications are that individuals with TBI undergoing fear extinction therapy may demonstrate within-session aberrant learning that could be targeted for more effective treatment interventions.

Abbreviations: PTSD, Post-traumatic stress disorder; mTBI, Mild traumatic brain injury; OEF, Operations Enduring Freedom; OIF, Operation Iraqi Freedom; OND, Operation New Dawn; FC, Fear conditioning; FE, Fear extinction; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; IL, infralimbic cortex; PL, prelimbic cortex; PFC, prefrontal cortex; mPFC, medial prefrontal cortex; ITC, intercalated cells of the amygdala; NAc, nucleus accumbens; LMM, linear mixed-effects.

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1. Introduction

Mild traumatic brain injury (mTBI) or concussion is a very common condition in the general population and for U.S. military personnel. For unclear reasons, the risk of developing post-traumatic stress disorder (PTSD) is approximately 2–3 times higher after trauma involving an mTBI in civilians and military personnel alike (Brassil and Salvatore, 2018; Schneiderman et al., 2008; Stein et al., 2019, 2015; Tanev et al., 2014; Van Praag et al., 2019; Vasterling et al., 2018; Yurgil et al., 2014). An estimated 12–23% of military service members experienced a TBI while deployed in these conflicts of Operations Enduring Freedom, Iraqi Freedom, and New Dawn (OEF, OIF, OND, ; O'Neil et al., 2013). PTSD itself is also common in this population, with estimates ranging from 13% to 52% (Milliken et al., 2007; Seal et al., 2007). An alarming landmark study conducted by (Hoge et al., 2008) found that 43.9% of U. S. Army soldiers returning from a deployment to Iraq with TBI and a loss of consciousness met criteria for PTSD highlighting the high prevalence of PTSD/TBI comorbidity. Supporting these concerns amongst Veterans is a study that estimated that the relative frequency of PTSD in military populations with TBI is 48.2% compared to 18.6% in civilian populations (Iljazi et al., 2020). Furthermore, PTSD symptoms are more intense in OEF/OIF Veterans with comorbid TBI compared to Veterans with PTSD only (Ragsdale et al., 2013).

The majority of reported TBI incidents among OEF/OIF/OND service members and civilians are categorized as mild in severity (Carlson et al., 2011; Rosen and Ayers, 2020). Acute cognitive, emotional and physical symptoms of mTBI include headaches, fogginess, dizziness, sleep disturbances, impaired concentration and memory, mood fluctuations, increased anxiety and/or depression, and sensitivity to sound and light (Kaplan et al., 2018; Kontos et al., 2013). While many people recover within days or weeks, others go on to experience a post-concussive syndrome that can last more than 3 months with various cognitive, emotional, and neurological symptoms. Depending on diagnostic criteria, the timing of the concussion and timing of the assessment, prevalence rates of post-concussion symptoms vary between 11% and 82% (Polinder et al., 2018). Only 27% of those with post-concussive syndrome eventually recover, illustrating how mTBI can become a chronic condition (Hiploylee et al., 2017). PTSD is often a chronic disorder resulting from physical and/or emotional trauma that presents with symptoms such as re-experiencing of trauma, avoidance of trauma cues, hypervigilance, sleep disturbances, mood changes, and impaired concentration. This entanglement of overlapping symptoms between mTBI and PTSD not only creates challenges for diagnosis and treatment (Carlson et al., 2011; Miller et al., 2015; Rosen and Ayers, 2020; Tanev et al., 2014), but also suggests that overlapping neurobiological mechanisms and alterations in neural circuits exist for both conditions (Kaplan et al., 2018; Vasterling et al., 2018).

Neuroimaging studies in individuals with PTSD have identified dysfunction in the neural circuits controlling fear and other PTSD-related emotions and behaviors. Specifically, hyper-responsivity of the amygdala and hypo-responsivity of the medial prefrontal cortex are observed in individuals with PTSD, and these abnormalities are thought to underlie aberrant fear learning in these individuals (VanElzakker et al., 2014). Traumatic brain injury, particularly when mild, causes diffuse damage to both gray matter (neuronal cell bodies) as well as white matter (axonal) integrity, and as such is difficult to observe using most current neuroimaging techniques (Kaplan et al., 2018). Interestingly, neuroimaging studies of individuals with comorbid mTBI and PTSD have identified larger amygdalae volume in individuals with comorbid mTBI and PTSD compared to individuals with mTBI only, and this morphological difference is thought to result from increased arborization and dendritic hypertrophy (Pieper et al., 2022). Rodent models can be a useful tool for teasing apart the separate and combined impacts of physical and emotional trauma on the brain. A few studies have demonstrated amygdala dendritic hypertrophy in animal models of mTBI (Hoffman et al., 2017; Ratliff et al., 2019), which could be a mechanism underlying aberrant fear learning following mTBI in humans, but these studies did not link morphological analyses with behavioral measures of fear learning. More clinical and preclinical studies are needed to delineate dendritic plasticity abnormalities in TBI/PTSD models.

Diffuse axonal injury produced by TBI disrupts brain regions and their connections via a variety of possible mechanisms including glutamatergic excitotoxicity, neuroinflammation, oxidative stress, generation of reactive oxygen species and/or and apoptosis (Kaplan et al., 2018). It is possible that brain injury could cause a "PTSD-like" syndrome after mTBI, characterized by mPFC hyporesponsivity and amygdala hyperresponsivity (VanElzakker et al., 2014). Consistent with this hypothesis is evidence that mTBI-PTSD comorbidity is accompanied by more extensive changes in white matter (axonal) integrity compared to mTBI alone (Lepage et al., 2018). Additionally, individuals with comorbid mTBI and PTSD have decreased integrity specifically of fronto-limbic pathways compared to individuals with mTBI only, and this measure was correlated with PTSD symptomatology (Santhanam et al., 2019). More research is needed to understand the mechanisms by which diffuse injuries in mTBI can result in alterations in neuroplasticity in the PFC and BLA that subserve functional increases in fear learning processes and PTSD symptomology.

One animal model relating to PTSD is that of fear conditioning (FC) and fear extinction (FE). Pavlovian FC, as well as the subsequent extinction of these conditioned responses in FE, have been established as well-validated models for measuring fear behaviors relevant to PTSD in rodents (Bowers and Ressler, 2015; LeDoux, 2014; Maren and Holmes, 2016). FC is the process by which an individual learns to associate a specific non-threatening stimulus with an aversive event such as foot shock. This leads to the individual demonstrating fear responses to the conditioned fear cue alone. FE is the process by which a conditioned fear response is weakened by repeated exposure to the conditioned cue, without the unconditioned aversive stimulus. This FC model produces cue and/or contextual fear responding that occurs in the human disorder of PTSD. PTSD-related behaviors are produced by increases in fear acquisition and increased attention to threat signals, reduced extinction of these fear responses, and greater return of fear resulting in relapse (Gonzalez and Martinez, 2014). Humans with PTSD have exaggerated psychophysiological responses in fear conditioning paradigms and PTSD is conceptually thought of as a fear memory disorder characterized by deficits in FE processes (VanElzakker et al., 2014). Additionally, exaggerated acquisition and expression of conditioned fear has been observed in military personnel with a TBI history (Glenn et al., 2017).

In our TBI model, we aimed to link fear learning behaviors with morphological analyses of dendritic plasticity in the medial prefrontal cortex (mPFC) and the basolateral amygdala (BLA), two regions crucial for mediating fear learning processes (Courtin et al., 2014; Bloodgood et al., 2018; Krabbe et al., 2018). We used the lateral fluid percussive injury (LFP) model of mTBI that produces both a focal and diffuse brain injury, mimics brain injury in humans, and has been validated in both rats and mice (Alder et al., 2011; Thompson et al., 2005). Our group has previously demonstrated altered synaptic plasticity in the basolateral nucleus of the amygdala (BLA) during expression and extinction of conditioned fear (Heinrichs et al., 2013a). Altered dendritic plasticity in fear learning regions of the PFC and BLA are hypothesized to be linked to alterations in fear learning behaviors after TBI. The current study uses LFP followed by acquisition of FC and FE to investigate the effects of mTBI on fear-related learning behaviors and on associated dendritic changes (via Golgi-Cox staining) in the mPFC and BLA of adult male mice.

2. Materials and methods

2.1. Subjects and lateral fluid percussion injury

A total of sixty-seven male C57BL/6J mice (Jackson Laboratories,

Bar Harbor, Maine) weighing 24–26 g upon arrival were housed under a 12:12 h light-dark cycle and acclimated to a humidity and temperature-controlled colony with ad libitum access to food and water for a minimum of 7 days prior to experimentation. Throughout the study, mice were housed in groups with all experimental groups represented so that experimental differences could not be attributed to cage effects. All procedures were conducted between the hours of 0800 and 1800 (lights on at 0700). All procedures were approved by the Institutional Animal Care and Use Committee of the Boston VA Healthcare System. Data were collected from three cohorts of mice. For all cohorts, mice were randomly assigned to one of three experimental groups at the time of surgery/traumatic brain injury induction: 1) naïve control mice which remained in the home cage (Unoperated; n = 17), 2) craniectomy control mice (Sham/LFP 0.0; n = 17), and 3) mice which were exposed to an LFP injury of 1.7 atmospheres of force (LFP 1.7; n = 16; Fig. 1A). Craniectomy surgery with and without LFP was targeted to the right parietal region of the cortex (Alder et al., 2011). Mice were anesthetized (isoflurane in 2–5% in oxygen) and secured in a stereotaxic apparatus. After exposing the skull, a trephine (3 mm outer diameter) was used to remove a bone disc. A sculpted Luer-loc fitting (3 mm inside diameter) was secured to the skull over the opening with surgical cement (Durelon, 3 M) to form a watertight bond. The fitting was filled with sterile saline and connected to high-pressure tubing attached to the outlet of an LFP device (Dragonfly R & D Inc., Ridgeley, WV). A 20 ms pulse of fluid was imposed onto the dura using a force intensity of 1.7 atmospheres as measured by a calibrated pressure transducer (Kistler Instrument Corp., Amherst, NY) and oscilloscope (Tektronix, Inc, Beaverton, OR). Immediately following craniectomy/LFP exposure, the Luer-loc fitting (Cole-Parmer, Vernon Hills, IL) was removed from the skull, the craniectomy opening covered with a concave polystyrene disc 3.5 mm in diameter held in place by surgical cement (Durelon, 3 M Corporate Headquarters, St. Paul, MN), and the incision closed with suture and VetBond (3 M Corporate Headquarters). Craniectomy control animals (Sham) received the craniectomy surgery together with Luer-loc attachment and pressure tubing connection with the exception that the fluid pulse was not delivered. After surgery, mice were returned to the home cage. Mice were treated with the post-operative analgesic meloxicam (5 mg/kg s.c.; Norbrook Inc. Overland Park, KS) immediately after surgical procedures and for two subsequent days.

2.2. Behavioral testing

Following 7–12 days of post-operative recovery, all mice were exposed to a battery of behavioral tests (Fig. 1B). A two-compartment light-dark task was used twice (once before fear conditioning and once after fear extinction testing) to assess general locomotor behavior and general anxiety-like behavior (Takao and Miyakawa, 2006). The apparatus used was a polycarbonate cage (51 cm long, 26 cm wide, 26 cm high) divided into two sections of equal size by a partition which provided a 2.5 cm high opening at the bottom for mice to pass freely from one side of the chamber to the other. One chamber was brightly illuminated (390 lux) and the other chamber was darkened (2 lux) by the presence of a cover opaque to light. Mice were placed into the dark side to begin the trial and allowed to move freely between the two chambers for 10 min. Activity of each mouse in the visible light compartment was tracked and analyzed using video-tracking software (EthoVision XT; Noldus Information Technology, Leesburg, Virginia). The frequency of visits and duration of time spent in the light chamber (sec), and mean velocity while in the light chamber (cm/s) were measured.

The ability to acquire conditioned fear behavior was assessed in a single day (Heinrichs et al., 2013a). Standard fear conditioning chambers (Med Associates, Inc., Fairfax, VT) consisted of electrified grid floors within sound attenuated boxes monitored using infrared cameras and were connected to a computer that conducted automated quantification of freezing (VideoFreeze software; Med Associates). The training session (27 min total) consisted of 3 habituation tone-only trials (10 kHz, 75 dB, 30 s tones; 180 s average intertrial interval (ITI)) followed by 6 fear acquisition trials in which the same tones co-terminated with a 2 s, 0.7 mA electric footshock (180 s ITI). A separate group of naive unoperated control mice (n = 17) were run concurrently and were not exposed to shocks; these mice did not exhibit increased freezing behavior across the 9 tone-only trials to which they were exposed (data not shown). Three to four days after fear conditioning, mice were tested for the extinction of conditioned fear over 6 consecutive days (Heinrichs et al., 2013b). Mice were exposed to 5 tone-only trials each day (15 min/day; 180 s ITI) for 6 days. The dependent measure for all trials (fear acquisition and extinction training) was the percent of time each mouse spent freezing during each 30 s tone presentation.

The day after fear conditioning (in between fear conditioning and fear extinction training; Fig. 1B), mice were placed in an open field chamber (46 cm long, 31 cm wide, and 26 cm high) and allowed to

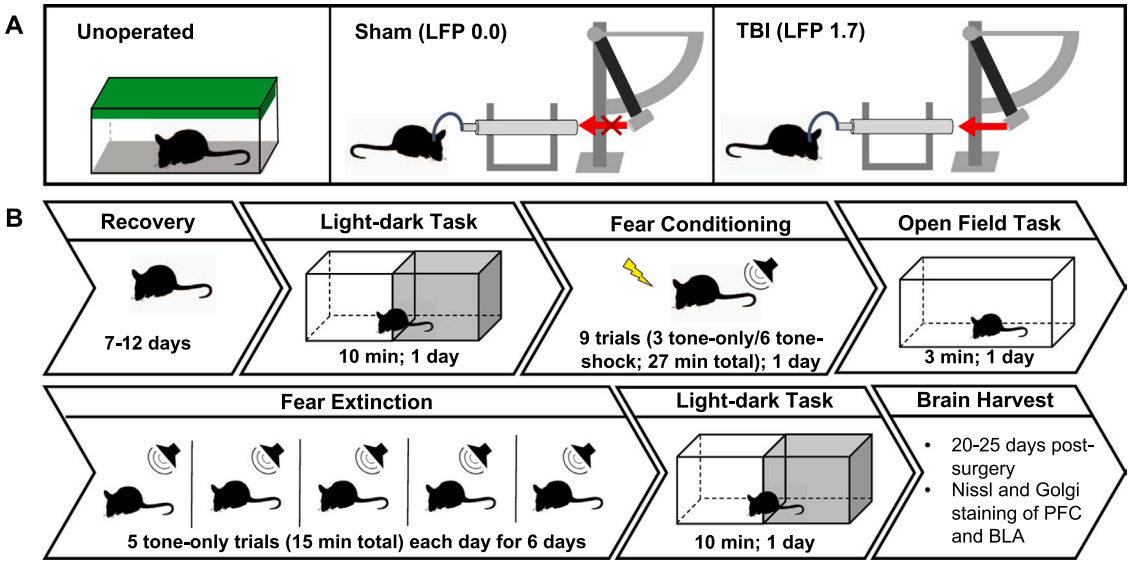


Fig. 1. Schematic of experimental design. (A) Summary of experimental groups depicting naïve controls (Unoperated), sham lateral fluid percussion (LFP 0.0) craniectomy controls surgery, and mild traumatic brain injury surgery (LFP 1.7 or 1.7 atm). (B) Summary of timeline for behavioral testing and experimental endpoints.

explore for three minutes. Locomotor activity was recorded using an overhead camera and total distance traveled (cm) was measured using video tracking software (EthoVision XT, Noldus).

2.3. Tissue processing

After all behavioral testing (20–25 days after LFP/Sham surgery), brains were harvested and processed for either histopathological and neural plasticity analyses using Nissl and Golgi-Cox staining, respectively. Mice were anesthetized deeply with isoflurane and transcardially perfused with 20 mL each of chilled heparinized phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. For Nissl staining, brains were harvested into ice-cold vials containing 4% paraformaldehyde and stored at 4 °C overnight, were then submerged in 12.5% sucrose in PBS for 24 h, and finally 25% sucrose in PBS. Brains were then frozen and 40-μm coronal sections were collected onto gelatin-coated slides using a Leica cryostat. Slides containing sections from the mPFC (from Bregma 1.94–1.78) and the BLA (from Bregma −1.46 to −1.70; Paxinos and Watson), were stained with Cresyl violet. Slides were first demyelinated in Xylenes for 5 min and ethanol (3 min each in 95%, 70% and 0% ethanol in deionized water). Slides were then incubated in a Cresyl violet solution (0.04 g cresyl violet in 300 mL buffer containing 0.1 M acetic acid and 0.1 M sodium acetate) for 8 min at 60 °C. Slides were then dehydrated, cleared in Xylenes, and coverslipped with Permount.

An Olympus BX51 microscope was used to visualize Cresyl violet stained mouse brain sections and brightfield images of both hemispheres of the mPFC and BLA were captured with HCImageLive at 10x magnification. The mPFC was identified by locating the midline and the forceps minor callosum. Fig. 2A shows the boundary of the region used for mPFC quantification. The BLA was identified by locating both sides of the external capsule, dorsal and ventral intercalated cells, and the central nucleus of the amygdala. Fig. 2C shows the boundary of the region used for BLA quantification. Neurons from both regions were manually quantified using a sampling method with the grid function in ImageJ. Specifically, neurons were counted by a single investigator blind to experimental condition within 3 random squares of equal size (in pixels) within the boundaries marked by the aforementioned anatomical landmarks for each region of interest. A neuron was counted if it had a dark cell body or distinct outline of the cell body and the nucleus was marked with a dark spot. The number of neurons in the 3 sampling squares was then averaged for each hemisphere of each coronal section. Then, neurons counts from 2 to 3 sections per brain were averaged for each mouse, for each hemisphere separately.

For Golgi Cox staining, brains were processed using the FD Rapid Golgi Stain Kit (FD NeuroTechnologies, Inc., Catonsville, MD) according to the kit directions, as we have previously described (Heinrichs et al., 2013a). After perfusion brains were removed, rinsed with water, and placed directly into a 1:1 mixture of Solution A and Solution B for 2 weeks at room temperature in the dark. Brains were then transferred to Solution C for 3–5 days at 4°C in the dark. Whole brains were then snap-frozen in 2-methylbutane chilled to − 20 °C, encased in Tissue Freezing Medium (Triangle Biomedical Sciences, In., Durham, NC, USA), coronally sectioned at 100 μm on a Leica cryostat and mounted on chrome alum gelatin coated slides. Sections were dried for several days and then underwent a stain development procedure according to the kit directions. Finally, slides were dehydrated in ethanol, cleared in xylenes and coverslipped.

Golgi-stained sections containing the mPFC and BLA were visualized with an Olympus BX51 bright field microscope interfaced with a color digital camera (MicroFire Optronics, Goleta, CA). Regions of interest were identified by anatomical markers based on a mouse brain atlas (Franklin & Paxinos 2008). Neurons were initially captured at 60x magnification with a 0.3 μm z-step, manually traced and reconstructed using Neurolucida 360, and analyses were conducted using Neurolucida Explorer (MBF Bioscience, Williston VT). For Sholl analyses, concentric circles were overlaid at 10 μm intervals outward from the cell body for a total of 9 neurons per experimental group in each hemisphere (from at least three mice/group). The complexity of dendritic arborizations was determined by the number of intersections at each distance via Sholl analysis. For spine density analyses, dendritic segments from neurons used for Sholl analysis were captured using a 100x objective with a 0.1 μm z-step. Spine density (spines/10 μm dendritic segment) was calculated at dendritic branch orders 2 and 3, and as an average of these two branch orders. Branch orders 2 and 3 were chosen for analysis based on our prior work showing decreased spine density following fear extinction (Heinrichs et al., 2013a). A total of three dendritic segments were traced at each branch order and averaged per neuron

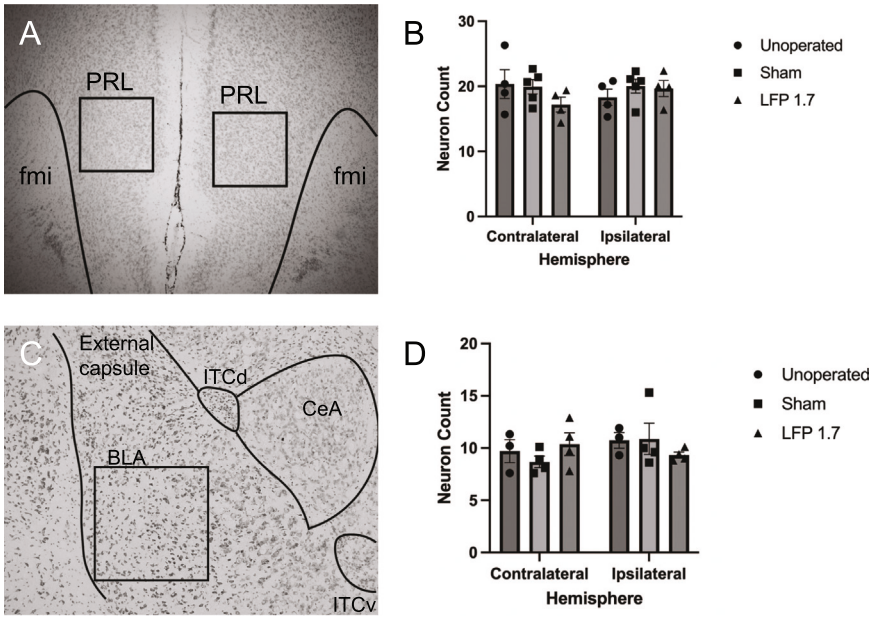


Fig. 2. Quantification of neurons in the medial prefrontal cortex (mPFC) and the basolateral nucleus of the amygdala (BLA) after TBI. (A) Representative photomicrograph of Nissl-stained coronal mouse brain section (Bregma 1.78) depicting the prelimbic cortex (PRL) region within the mPFC used for quantification at 4x magnification. Two to three sections/mouse (n = 4–5/group) were manually quantified using a sampling method where neuron counts in 3 equal sized areas (in pixels) within the PRL were averaged from each hemisphere for each coronal section. (B) Quantification of neurons in the mPFC across experimental groups. Data are expressed as average neuron count ± SEM. There was no significant effect of experimental (surgical) group in either hemisphere of the PRL (p's > 0.05). (C) Representative photomicrograph of Nissl-stained coronal section depicting the basolateral amygdala (BLA) at 10x magnification. Two to three sections/mouse (n = 4/group) were manually quantified using a sampling method where neuron counts in 3 equal sized areas (in pixels) within the BLA were averaged from each hemisphere for each coronal section. (D) Quantification of neurons in the BLA across experimental group. Data are expressed as average neuron count ± SEM. There was no significant effect of experimental (surgical) group in either hemisphere of the BLA (p's > 0.05). fmi = forceps minor collosum, CeA = central nucleus of the amygdala, ITCd = dorsal intercalated cell population, ITCv = ventral intercalated cell population.

(n = 9/hemisphere/group). Golgi analyses were performed by a single investigator blind to the experimental group.

2.4. Statistical analysis

2.4.1. Locomotor/exploratory behavior

The effects of LFP surgery and fear conditioning/extinction interventions on exploratory/locomotor behavior were evaluated in two tasks: 1) by measuring mean velocity, frequency of visits, and duration of time spent in the light compartment during two identical light-dark tasks (one before between surgery and fear exposure, and one after fear extinction; Fig. 1 and Tables 1), and 2) by measuring total distance traveled in an open field task (Fig. 1 and Table 2). All data passed tests for normality and were each analyzed by two-way ANOVA using SPSS 26 (IBM Corporation), with fear conditioning state (pre- vs. post-conditioning) and surgical group as within-subjects factors. Post-hoc analyses, when appropriate, were conducted using Tukey's HSD test.

2.4.2. Fear behavior

Because fear acquisition and extinction subjects were measured repeatedly over time and across different time scales (trials within a day versus between days) and because separate cohorts of mice were tested, we employed a linear mixed-effects (LME) model as our primary analytic approach in R (lme4 package; Bates et al., 2015). LME models can analyze data with more than one source of variation, i.e.data with both fixed and random effects. LME is particularly helpful for repeated measures designs with complex, nested experimental design structures, where data and even fixed factors are correlated. Because LME models can account for dependence/correlation of data, this statistical method is thought to be more rigorous than traditional ANOVA statistical tests (Yu et al., 2022). This approach also enables optimal control for variability between individual mouse behaviors not related to experimental condition (i.e. to control for random effects). Specifically, we aimed to control for individual differences unrelated to the treatment condition in overall freezing behavior by using a mouse subject index as a random intercept. Further, to control for between-subject variability unrelated to the treatment condition in extinction freezing across test days we used test day as a random slope. Our primary goal of model selection was to choose the simplest model that provides the best fit to the observed data and yields an estimated marginal mean for the observed responses based on the specified model (see Supplemental Fig. 1 for visual representation of the data plotted as estimated marginal means). The estimated means method predicts the behavior of the dependent variable with respect to the independent variable. In total, 3 mixed models were built: 1) for fear conditioning habituation (tone-only) trials, freezing behavior was predicted across the 3 test trials including subject index as a random intercept, 2) for fear conditioning acquisition trials (tone-shock pairings), freezing behavior was predicted across the 6 test trials including subject index as a random intercept, and finally 3) for the fear extinction trials, freezing behavior was predicted across 5 trials per day over 6 test days including subject index as a random intercept and test day as a random slope.

2.4.3. Brain parameters

Dendritic parameters (spine density and Sholl data) and neuronal count in each hemisphere were analyzed using SPSS 26 (IBM Corporation). For average spine density and neuron count counts in the mPFC and BLA, a one-way ANOVA was used to compare means across experimental groups (Unoperated, Sham, and LFP 1.7) from each hemisphere separately. Spine density by branch order was analyzed in each hemisphere with repeated measures ANOVA with branch order as a within-subjects factor and surgery group as a between-subjects factor. Sholl data were analyzed with repeated measures ANOVA with distance from the soma as a within-subjects factor and surgery group as a between-subjects factor. Post-hoc analyses, when appropriate, were conducted using Tukey's HSD test.

Table 2
Locomotion in the open field task.

Treatment Group	Total Distance Traveled (cm)
Unoperated (n = 15)	3665.84 (632.56)
Sham (n = 15)	3370.89 (577.37)
LFP 1.7 (n = 14)	3506.88 (528.71)

Data are presented as group means (SEM). Total distance traveled was not significantly impacted by surgery.

3. Results

3.1. Histology and neuron count

Neuron count in key regions of interest was quantified by a blinded manual counting of Nissl-stained cells in the mPFC (Fig. 2A) and BLA (Fig. 2C) in a 600 × 600 pixel area. Experimental group (unoperated, sham surgery, or LFP 1.7) did not have a significant effect on the neuron count in either hemisphere in either the mPFC or the BLA. Specifically, a one-way ANOVA found no significant effect of experimental group on neuron count in either the right (ipsilateral to surgery; $F_{2,12} = 0.57$, $p > 0.05$; Fig. 2B) or left (contralateral to surgery; $F_{2,12} = 1.2$, $p > 0.05$; Fig. 2B) hemisphere of the mPFC. Likewise, a one-way ANOVA revealed no significant effect of experimental group on the neuron count in either the right (ipsilateral to surgery; $F_{2,10} = 0.69$, $p > 0.05$; Fig. 2D) or left hemisphere (contralateral to surgery; $F_{2,10} = 0.94$, $p > 0.05$; Fig. 2D) of the BLA.

3.2. Exploratory/locomotor behavior

The effects of experimental groups on exploratory/locomotor behavior were evaluated in two ways: 1) by measuring total distance traveled in an open field task (Fig. 1 and Tables 1) and 2) by measuring mean velocity, frequency of visits, and duration of time spent in the light compartment during two identical light-dark tasks (one before and one after fear conditioning; see Fig. 1 and Table 2). There was no significant difference among experimental groups on total distance traveled ($p > 0.05$; Table 1), suggesting that surgery group did not affect locomotor activity. There was no significant difference among experimental groups on any measure in the light-dark task at either time point ($p > 0.05$; Table 2), suggesting that surgical group did not affect exploratory behavior. However, the number of light compartment visits were significantly different and were reduced on day after fear

Table 1
Behavior in the light-dark task.

Timepoint	Treatment Group (sample size)	Light Compartment (# of visits)	Light Compartment Duration (s)	Mean Velocity (cm/s)
Pre-Fear Conditioning	Unoperated (n = 17)	102.9 (12.2)	233.4 (33.0)	23.4 (2.2)
	Sham (n = 17)	112.3 (15.9)	190.4 (27.6)	20.1 (1.8)
	LFP 1.7 (n = 16)	93.1 (18.9)	172.1 (32.7)	18.8 (2.3)
Post-Fear Conditioning	Unoperated (n = 17)	61.5 (11.9)	372.8 (36.0)	26.1 (3.4)
	Sham (n = 17)	79.8 (15.8)	295.1 (35.4)	23.0 (2.3)
	LFP 1.7 (n = 16)	44.8 (10.3)	380.3 (46.7)	25.4 (3.2)

Data are presented as group means (SEM). There was no significant difference among groups on any measure at any timepoint in this task.

conditioning versus before FC (main effect of time point; $F_{1,47} = 10.75$, $p < 0.01$) suggesting that fear conditioning reduced exploratory behavior in the previously aversive environment.

3.3. Fear learning behavior

As mentioned in Methods, FC and FE data were examined using linear mixed effects modeling in Fig. 3 (see Section 2.4.b). This method allowed for an analysis of the effect of experimental group (Unoperated, Sham, or LFP 1.7) on the estimated percent freezing during habituation, fear acquisition and fear extinction both within session (across trials) and between sessions (across days; see Supplemental Fig. 1 for visual representation of the data plotted as estimated marginal means). This approach enables optimal control for variability between individual mouse behaviors not related to experimental condition (i.e. to control for random effects).

3.3.1. Fear acquisition: habituation

Our step-up modeling approach showed that none of the possible predictors (experimental group, trial number or their interaction) improved the model fit to predict freezing behavior (Fig. 3A and Supplemental Table 1), suggesting that the tone alone was not aversive and did not increase fear behavior in any group.

3.3.2. Fear Acquisition: Tone-Shock pairings

Freezing behavior during tone-shock pairings was significantly predicted by test trial as a factor indicating that freezing behavior had already significantly increased by the second tone-shock pairing as compared to the first trial ($\beta = 0.29$, 95% CI: [0.11–0.46], $p_{bonf} = 0.011$) and continued to the sixth trial ($\beta = 2.21$, 95% CI: [2.03–2.38], $p_{bonf} < 0.001$ (Fig. 3A and Supplemental Table 2). Critically, the inclusion of experimental group into the model was not significant (see Supplemental Table 2), suggesting that freezing behavior during acquisition of conditioned fear changed independently of surgical group assignment.

3.3.3. Fear extinction

Freezing behavior during fear extinction was significantly impacted by test day as a predictor (day 1–6; Fig. 3B and Supplemental Table 3) and there was an interaction between trial number and experimental (surgical) group (Fig. 3C, trial 1–5; and Supplemental Table 3). Specifically, extinction freezing decreased across test days with first significant effects starting at day 4 (4th day vs 1st day: $\beta = -0.44$, 95% CI: [−0.71 to −0.18], $p_{bonf} = 0.023$ and continued to the sixth day (6th vs 1st day: $\beta = -1.27$, 95% CI: [−1.56 to −0.99], $p_{bonf} < 0.001$; Fig. 3B and Supplemental Table 3). The effect of test day did not vary as a function of experimental group (i.e. no significant model fit improvement by the inclusion of interaction between day and experimental group, see Supplemental Table 3). Although there was no impact of experimental group on freezing behavior across extinction test days, there was a significant interaction between extinction trial number (within session) and experimental group (Fig. 3C), showing that the LFP 1.7 group demonstrated increased freezing behavior at test trial 5 as compared to test trial 1, whereas the unoperated group did not show a significant change in freezing behavior as compared to the sham group (LFP vs. sham: $\beta = 0.51$, 95% CI: [0.20, 0.81], $p_{bonf} = 0.023$; unoperated vs. sham: $\beta = -0.42$, 95% CI: [−0.72, −0.11], $p_{bonf} = 0.138$). In summary, fear acquisition procedures produced increases in freezing behaviors across acquisition trials for all three experimental groups. Fear extinction procedures reduced freezing behaviors across test days for all three groups. However, within-session extinction procedures, on each test day, produced *increased* freezing behavior for only the LFP 1.7 group. Interestingly, the craniotomy Sham group showed a nonsignificant trend of increased freezing within sessions during sessions 5 and 6 suggesting an impact of craniotomy on behavior. Craniotomy sham shows a pattern that is intermediate in freezing effects between the Unoperated and LFP 1.7 groups.

3.4. Dendritic plasticity

The effect of experimental (surgical) group on dendritic plasticity in fear conditioned mice was assessed in each hemisphere of the mPFC (Fig. 4) and BLA (Fig. 5) using the following parameters: average spine density (spines/10 μm dendritic segment), spine density by branch order (dendritic branches 2 and 3), and the complexity of dendritic arborization (number of intersections via Sholl analysis).

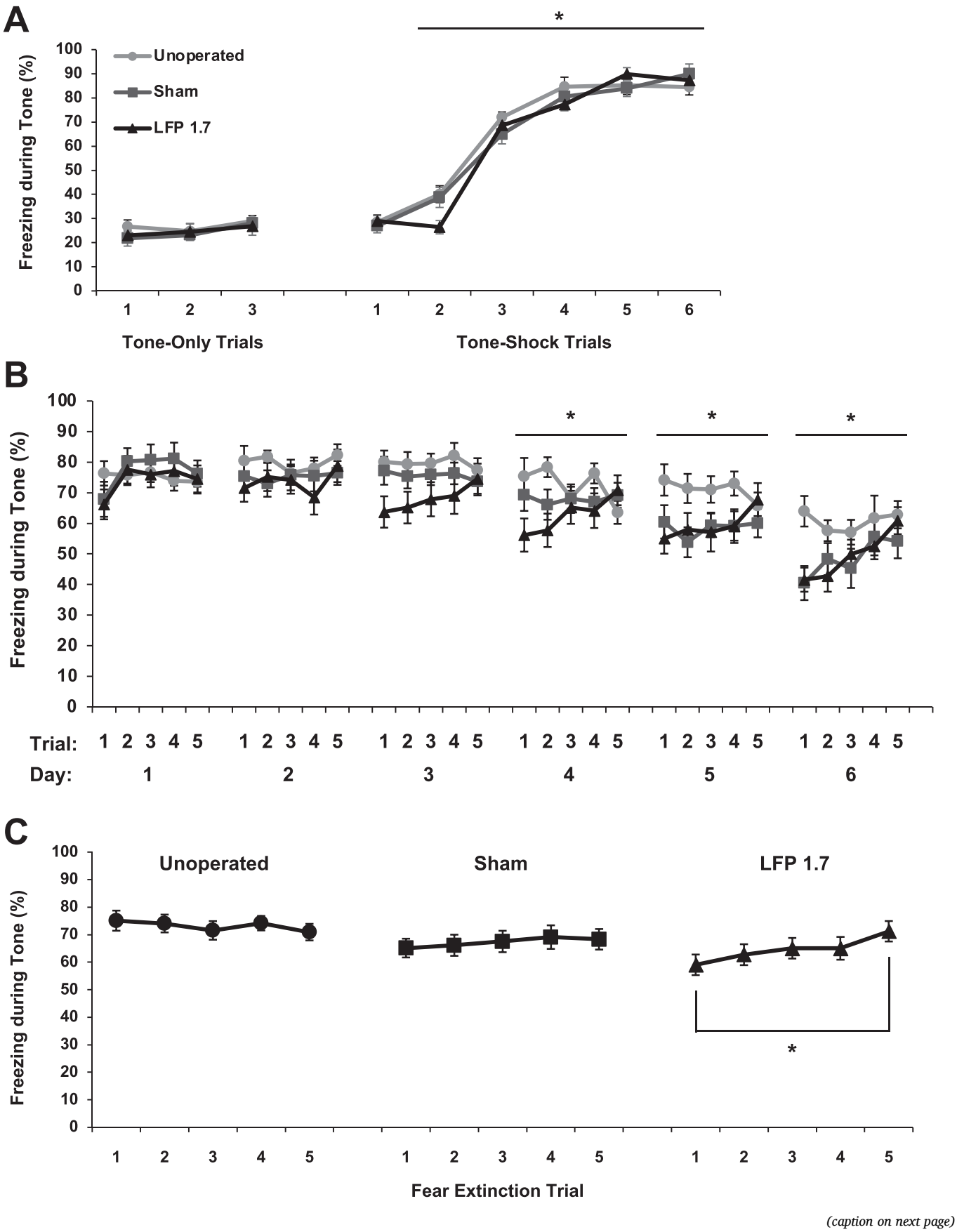
3.4.1. PFC

In the mPFC, a one-way ANOVA did not detect a significant effect of experimental group on the average spine density in either the left (contralateral) or right (ipsilateral) hemisphere ($p > 0.05$; Fig. 4A). In the ipsilateral hemisphere, there was no significant difference between spine density in intermediate dendrites at branch orders 2 and 3 (no main effect of branch order, $p > 0.05$; Fig. 4B), and experimental group did not affect spine density in this hemisphere (no significant main effect of experimental group or interaction between experimental and branch order, $p > 0.05$; Fig. 4B). In the contralateral hemisphere, spine density was significantly higher in branch order 3 compared to branch order 2 (main effect of branch order, $F_{1,24} = 5.3$, $p < 0.05$; Fig. 4D). The increase in spine density in branch order 3 appears to be mainly driven by the Sham condition, although experimental group did not significantly impact spine density in this hemisphere (no main effect of experimental group or interaction between experimental and branch order, $p > 0.05$; Fig. 4D).

Sholl analysis is a procedure that counts the number of dendritic intersections that occur at fixed distances from the soma in concentric circles, a measure of dendritic complexity. The complexity of dendritic arborizations in the mPFC was assessed by measuring the number of dendritic intersections (or arborizations) via Sholl analysis. The complexity of dendritic arborizations in the ipsilateral (right) hemisphere of the mPFC changed significantly as a function of distance from the soma ($F_{3.8,90.1} = 77.6$, $p < 0.001$) and was significantly different between experimental groups (main effect of experimental group; $F_{2,24} = 35.6$, $p < 0.05$). In this hemisphere, the impact of experimental group varied across distances from the soma (significant interaction between experimental and soma distance; $F_{25.3,90.1} = 25.3$, $p < 0.05$; Fig. 4C). Tukey's post-hoc analyses revealed that the LFP 1.7 group had significantly less intersections than the Sham group ($p < 0.05$), but that neither Sham or LFP 1.7 mice differed significantly from Unoperated mice (p's = 0.11 and 0.89, respectively). However, the main effect of group in this hemisphere does appear to be mainly driven by increased complexity in the Sham group, with an overlapping pattern observed between the LFP 1.7 and unoperated groups. Stated differently, the Sham controls had increased complexity vs. both the unoperated and LFP 1.7 groups, suggesting that it is not a neutral control condition. In the contralateral (left) hemisphere of the mPFC, a repeated measures ANOVA revealed that the complexity of dendritic arborizations changed significantly as a function of distance from soma ($F_{3.8,90.7} = 73.1$, $p < 0.001$; Fig. 4E), but did not vary significantly between experimental groups (no significantly main effect of experimental group or interaction between experimental group and distance from soma ($p > 0.05$), although in this hemisphere the LFP mice tended to have less intersections than both Unoperated and Sham mice close to the soma (50 μm or less).

3.4.2. BLA

In the BLA, experimental group significantly affected average spine density in both the left (contralateral) hemisphere ($F_{2,26} = 3.8$, $p < 0.05$) and right (ipsilateral) hemisphere ($F_{2,26} = 4.2$, $p < 0.05$; Fig. 5A). In the contralateral hemisphere, Tukey's post-hoc analyses revealed that LFP 1.7 mice had significantly higher spine density compared to Sham (craniotomy control) mice (Fig. 5A). On the ipsilateral side of injury, Tukey's post-hoc tests revealed that the LFP mice had significantly higher average spine density than Unoperated mice



(caption on next page)

Fig. 3. Percentage of time freezing is demonstrated for the three experimental groups (unoperated, sham, and LFP 1.7) during habituation, fear conditioning (acquisition) and fear extinction trials. Statistical effects were measured using 3 separate linear mixed-effects (LME) models. Panel A shows the percentage of time spent freezing during tone presentations (means ± SEM) for the experimental groups across the three habituation trials (tone-only trials on left) and during tone-shock pairings (tone-shock trials on right). Percent freezing during tone-shock pairings was significantly predicted by test trial as a factor, indicating that freezing behavior significantly increased from the first to the sixth tone-shock pairing. There was no effect of experimental (surgical group) observed during fear conditioning. In Panel B, percentage of time freezing (means ± SEM) for the experimental groups is plotted for each of the 5 sessions during the 6 consecutive days of fear extinction training. Extinction freezing decreased across test days and was significantly lower than Day 1 by Day 4. In Panel C, the percent freezing time (means ± SEM) across the 5 within-session trials (collapsed across test day of extinction training) was best fit by a 2-way interaction between experimental group and trials. Further analysis revealed significantly higher freezing during Trial 5 compared to Trial 1 only in the LFP mice. *Asterisks indicate significance at $p < 0.05$. n = 16–17/group.

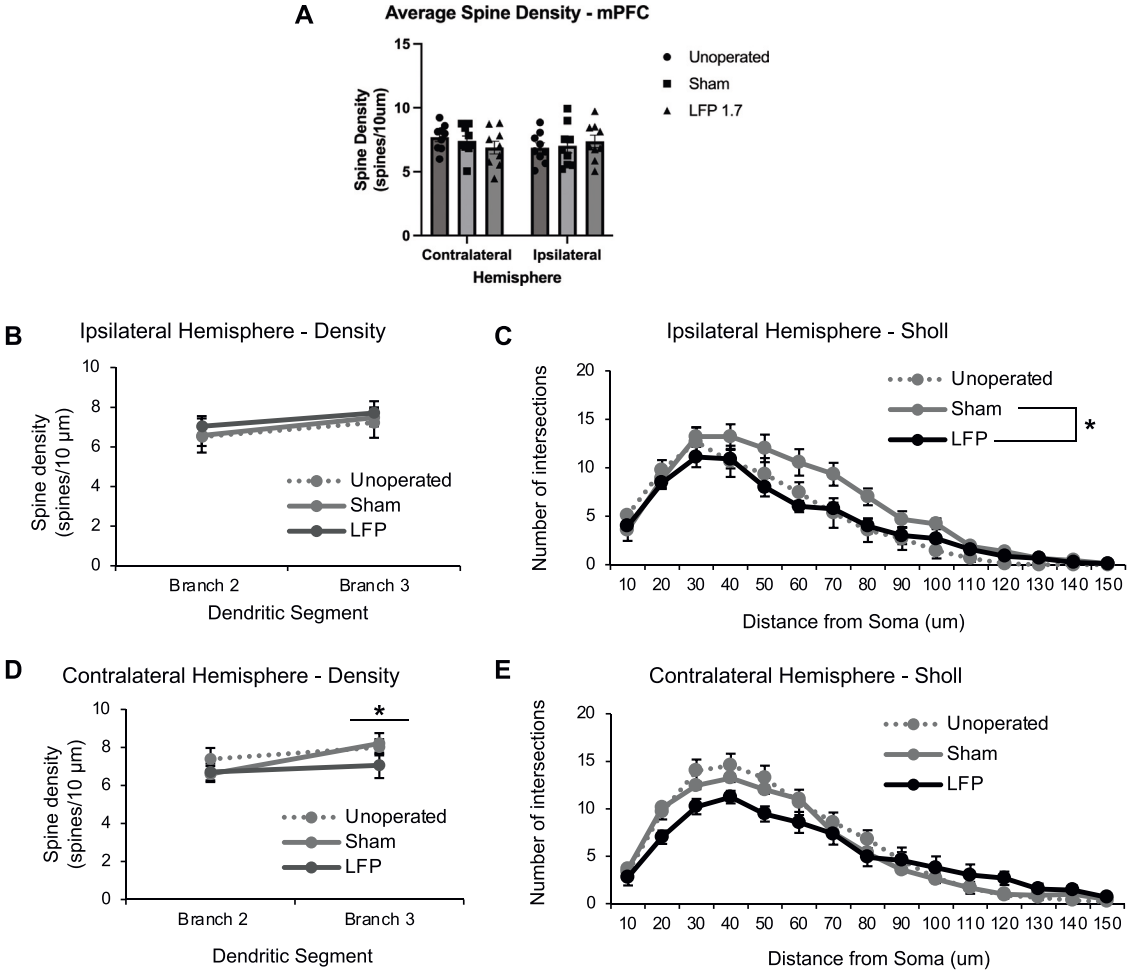


Fig. 4. Quantification of dendritic plasticity in the medial prefrontal cortex (mPFC). (A) Average spine density and individual values are plotted across experimental group in the mPFC. (B) Average spine density in the hemisphere ipsilateral to the surgical site across branch orders 2 and 3 in the mPFC. (C) Sholl analysis of mPFC dendritic branching complexity in the hemisphere ipsilateral to the surgical site. (D) Average spine density in the hemisphere contralateral to the surgical site across branch orders 2 and 3 in the mPFC. (E) Sholl analysis of mPFC dendritic branching complexity in the hemisphere contralateral to the surgical site. Data in Panels B-E are expressed as group means ± SEM. Asterisks indicate significance at $p < 0.05$.

(Fig. 5A). Spine density was also significantly higher in branch order 3 compared to branch order 2 in the ipsilateral hemisphere ($F_{1,24} = 6.05$, $p < 0.05$; Fig. 5B), an effect that was not observed in the contralateral hemisphere (Fig. 5D). There was no significant interaction between branch order and experimental group in either hemisphere of the BLA, however the increased average spine density observed in LFP mice appears to be primarily driven by branch order 2 in both hemispheres. Additionally, spine density in the ipsilateral hemisphere of Sham mice appeared slightly elevated, and the fact that Sham and LFP mice did not differ significantly in this hemisphere further suggests that craniotomy alone impacted the BLA, albeit to a lesser extent than LFP.

The complexity of dendritic arborizations in the BLA, as assessed by the number of intersections in Sholl analysis, changed significantly as a function of distance from the soma in both the right (ipsilateral to injury; $F_{18,432} = 178.6$, $p < 0.001$; Fig. 5C) and left (contralateral to injury; $F_{18,432} = 173.1$, $p < 0.001$; Fig. 5E) hemispheres, but there was no significant effect of experimental group X group by soma distance interaction on this measure of plasticity in either hemisphere ($p > 0.05$). However, in both hemispheres the Sham group demonstrates an intermediate pattern of number of dendritic intersections between the LFP and unoperated groups, further suggesting that craniotomy alone affected dendritic complexity in the BLA.

4. Discussion

In this study we examined fear learning processes in a mouse model

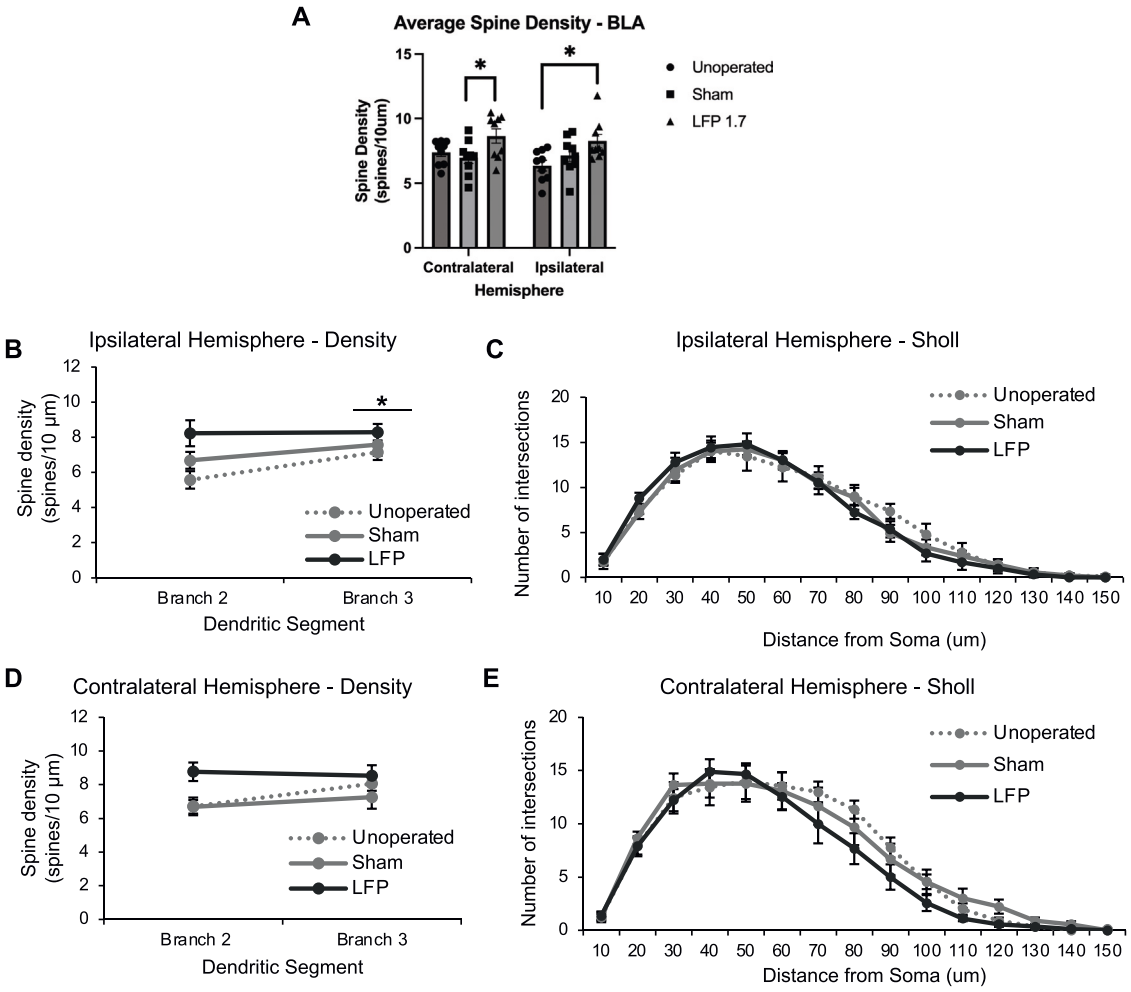


Fig. 5. Quantification of dendritic plasticity in the basolateral nucleus of the amygdala (BLA). (A) Effect of experimental group on average spine density along with individual values in the BLA. Asterisk indicates post-hoc significant group differences ($p < 0.05$). (B) Average spine density in the hemisphere ipsilateral to the surgical site across branch orders 2 and 3 in the BLA. Asterisk indicates significant effect of branch order ($p < 0.05$). (C) Sholl analysis of BLA dendritic branching complexity in the hemisphere ipsilateral to the surgical site. (D) Average spine density in the hemisphere contralateral to the surgical site across branch orders 2 and 3 in the BLA. (E) Sholl analysis of BLA dendritic branching complexity in the hemisphere contralateral to the surgical site. Data in Panels B-E are expressed as experimental group means ± SEM.

of mTBI and the resultant changes in dendritic plasticity in key areas of the mPFC and BLA. We studied the impact of fear acquisition and fear extinction in three experimental groups: Unoperated controls, Sham (craniotomy) controls, and LFP mice (1.7 mm force fluid pulse applied to the brain in right parietal cortex). Since cellular learning processes require the restructuring of neurons, we examined dendritic arborization, complexity, and density in these models. Because brain injury can impact neuronal number in diffuse regions, we also measured cell numbers in regions of interest. We measured these elements in key brain regions (mPFC and BLA) related to fear learning at 2–3 weeks post-LFP surgery. Our study revealed the following main findings: 1) During fear acquisition, there were significant and comparable increases in freezing behavior across tone-shock pairing trials across all 3 experimental groups, 2) Freezing behavior gradually decreased in all experimental groups over 6 days of extinction training, 3) During extinction training, freezing behavior significantly increased across within-session extinction trials in the LFP 1.7 group, whereas freezing behavior in control groups did not change on extinction test days. 4) In the ipsilateral hemisphere of the PFC, dendritic arborizations in the PFC were less complex in the LFP 1.7 group compared to the Sham control group, 5) In the BLA, there was a higher average spine density in the LFP 1.7 group versus control groups. 6) On some behavioral and dendritic measures, the craniotomy Sham group produced values similar to the LFP 1.7 group or intermediate between the Unoperated and LFP 1.7 groups. Even with the increases in fearfulness in the LFP 1.7 group within trials on extinction days, extinction learning did take place over the 6-day time course of the trial *reflecting a dissociation between within-trial and between-trial fear extinction learning*. TBI may impact fear extinction by producing structural and functional impairments within both the PFC and BLA. Limitations to our study are that we did not examine other fear or PTSD-like behaviors, PFC-BLA projections or PFC or BLA neuronal function.

During associative conditioning, subjects learn that a Conditioned Stimulus or CS (auditory cue in this study) predicts the occurrence of an Unconditioned Stimulus or US (footshock). This association produces Conditioned Responses such as freezing during CS presentation alone. Breaking the CS-US associative bond by presenting the CS without the US for several trials normally produces a reduction in the CR and this phenomenon is known as extinction. Fear extinction creates a new inhibitory memory that competes with the original CS-US bond (Maren, 2011). In our study, the TBI group demonstrated increased conditioned fear responses after repeated presentations of the CS within extinction training sessions. After 30 fear cue exposures over 6 days of training, the TBI group did learn to extinguish the fear memories. Alterations in processing of the fear extinction memory in the TBI group could be explained by the associated changes in dendritic plasticity. The increases

in freezing behavior during extinction training sessions in the TBI group (vs. Sham and Unoperated groups) were accompanied by increased dendritic spine density in the BLA versus both control groups. These behavioral changes in the TBI group were associated with reduced dendritic complexity in the PFC (LFP 1.7 vs. Sham only). These changes in structural plasticity in the PFC and BLA could underlie the alterations in fear extinction learning observed in the TBI group.

As mentioned, on some freezing measures and dendritic parameters, the craniotomy Sham group produced values similar to the TBI group or intermediate between the Unoperated controls and TBI groups. This is a phenomenon that has been previously demonstrated. Cole et al. (2011) demonstrated that the traditional craniotomy operation as a control produced inflammatory, morphological, and behavioral damage, which confounds interpretation of conventional experimental brain injury models. Similarly, Langaroui and colleagues (2012) presented findings demonstrating that the craniotomy control produced impairments in motor function with some deficits similar to the experimental TBI group. Further, the craniotomy control groups stimulated the transcription of several regulators of inflammation. In this study, there were similarities between inflammatory responses induced by a severe brain lesion (TBI) versus the mild brain injury from craniotomy. Corne et al. (2019) demonstrated that mild TBI and to a lesser degree craniotomy in mice induced conditioned fear responses when tested for fear extinction memory recall 6 weeks after extinction training. Changes in brain areas volumes in cortical regions in the craniotomy group were intermediate between the TBI and unoperated controls post-surgery. These studies and our findings highlight that craniotomy is not a neutral procedure and produces proinflammatory effects and affects dendritic plasticity as it alters behavior.

The basolateral amygdala (BLA) is the major input site for sensory information relating to both fear cues and threat information via the thalamus and sensory cortex. The BLA is connected to the central nucleus of the amygdala (CeA), which mediates expression of fear, and is reciprocally connected with key regions of the PFC (providing fear cue information) and ventral hippocampus (fear context information). The CeA mediates emotional, autonomic and motoric fear and stress responses through connections to nuclei in the brainstem, midbrain, and hypothalamus (Krabbe et al., 2018). Our findings of increased dendritic plasticity in the BLA may result in enhanced BLA processing of fear information in TBI subjects and could mediate the increased freezing behavior exhibited during extinction sessions. The medial PFC projects to the amygdala and inhibits fear responses (Likhtik and Paz, 2015). In fear extinction, inputs from the infralimbic cortex (IL) of the PFC to the BLA and to the intercalated gamma-aminobutyric acid or GABAergic cells (ITC) of the amygdala results in reduced outputs from the CeA to hypothalamus, locus coeruleus and periaqueductal gray regions (Maddox et al., 2019). Our findings of decreased dendritic complexity in the PFC may result in reduced processing of fear information in TBI subjects and could contribute to the increased freezing behavior exhibited during extinction sessions.

In our study, there was no observed effect of mTBI on the acquisition of cued conditioned fear. Other studies on the effects of mTBI on fear expression have produced differing results. For example, a study of mTBI in male rats showed increased expression of contextual conditioned fear which was associated with increased cell number in regions of the amygdala (Meyer et al., 2012). Likewise, Reger et al. (2012) found that LFP-induced mTBI produced greater cued and contextual conditioned fear in rats. However, using blast overpressure injury in rats, mTBI reduced the expression of fear that was conditioned in an operant task prior to mTBI exposure (Genovese et al., 2013). Similarly, Palmer et al. (2016) observed decreased expression of cued fear after LFP in male mice. In a model of LFP, acquisition of fear freezing was similar between all experimental groups during the contextual memory assessment (Fitzgerald et al., 2022). In this study sex-specific brain injury influenced freezing behavior changes over time. Finally, other studies showed that the controlled cortical impact model of mTBI did not affect the acquisition or expression conditioned fear or its extinction in mice (Sierra-Mercado et al., 2015, McAllister et al., 2015). The differences in these findings may be attributed to a variety of study design factors such as species of rodent (rats or mice), mTBI model (LFP, blast injury or CCI), area of brain injury, sex, fear conditioning and extinction methodologies and timing after injury. Overall, these factors can influence fear learning behavior and circuitry contributing to a variety of findings.

As stated, we observed a dissociation between within-session and between-session extinction of conditioned fear in the TBI group. This abnormal acquisition of fear extinction memory has been previously observed. Dissociation of within- and between-session extinction has been produced by varying the extinction protocol (Plendl and Wotjak, 2010). This last study demonstrated the independence of within-session and between-session extinction highlighting the importance of understanding both phenomena. There have been several molecular mechanisms for this effect previously reported. For example, inhibition of signaling of the phosphatase calcineurin (Almeida-Corrêa et al., 2015) or of the endocannabinoid system (Plendl and Wotjak, 2010) impairs within- but not between-session extinction of fear. Future studies using our model can better examine the molecular mechanisms for our observed findings. The endocannabinoid system mediates neural repair and neuronal survival. TBI results in endocannabinoid release that inhibit injury-induced neuroinflammation, vasoconstriction and excitotoxicity (Bales et al., 2010). Similarly, calcineurin plays a role in dendritic plasticity (Oh et al., 2015) and its expression is altered by TBI. These endogenous neuroprotectants and signaling molecules could play a role in the dendritic reorganization. More studies are needed to demonstrate the linkage to these molecular mechanisms to dendritic plasticity and fear-related behavioral changes in TBI.

In this study, we observed increased dendritic spine density in the BLA and decreases in PFC dendritic complexity in TBI mice, and these results are largely consistent with previous findings. For example, Hoffman and associates (2017) found that brain-injured rats demonstrated enhanced structural plasticity in both pyramidal and stellate BLA neuronal types. Another study using blast TBI observed increased BLA dendritic branching and density of dendritic spines in injured mice (Ratliff et al., 2019). Other studies demonstrated similar reductions in PFC dendritic spine density in pyramidal neurons after mTBI (LFP) that was associated with impairments in fear extinction (Zhao et al., 2018). Using LFP injury in rats, Zhao and coworkers found reduced overall dendritic spine density of basal and apical dendrites on layer II/III pyramidal neurons within the PFC in mTBI rats. The mTBI group also showed increased freezing behaviors during and after extinction training compared to controls. Additionally, our group demonstrated that fear conditioning alone increases structural plasticity of dendrites in the BLA and values returns to baseline levels following fear extinction training (Heinrichs et al., 2013a). These studies in concert with ours suggest that reductions in PFC spine density from TBI impair their ability to extinguish amydala-based fear responses. Conversely, fear-related learning transforms the number and shape of dendritic spines in the BLA and this process of reorganization and reconnection may be more responsible for increases in fearfulness during extinction training.

These results have potential translational impacts related to comorbid TBI and PTSD. Clinical studies have shown that extinction training does produce reductions in fear-related PTSD symptoms in TBI subjects (Sripada et al., 2013; Wolf et al., 2012). In a human fear extinction trial, Golkar and Öhman,(2012) demonstrated that immediate extinction (vs. delayed extinction) resulted in differences in fear outcomes. Delayed extinction resulted in greater reinstatement of fear responses and suggested that within-session extinction responses may be relevant to this poorer outcome. Similarly, Bluette and colleagues (2012) examined patients with PTSD in a Prolonged Exposure treatment trial, which incorporates fear extinction approaches, and examined within-session distress. More PTSD patients demonstrated unreliable changes in distress (64.7%) than reliable changes (35.3%) and the reliable changes produced less PTSD severity (re-experiencing, hyperarousal) and

depression, and better functioning. These studies highlight possible translational implications of the within-session impairments of fear extinction as demonstrated in our study.

Animal model studies in TBI have demonstrated that many compounds and therapeutics have the potential to greatly reduce post-injury behavioral sequela for individuals experiencing TBI. However, to date there are no FDA approved drugs for the treatment of TBI-induced anxiety and fear responses (Shear and Tortella, 2013). Future clinical studies should utilize both more effective extinction therapy approaches in combination with potential therapeutic compounds. In addition, clinical studies could also employ functional neuroimaging during fear extinction treatment with a focus on mPFC and amygdala regions. Such studies may provide a better mechanistic understanding of understanding of neural mechanisms in comorbid TBI and PTSD and elucidate optimal treatment approaches for these debilitating conditions.

Data Availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2023.04.001.

References

Alder, J., Fujioka, W., Lifshitz, J., Crockett, D.P., Thakker-Varia, S., 2011. Lateral fluid percussion: model of traumatic brain injury in mice. J. Vis. Exp. https://doi.org/10.3791/3063.

Almeida-Corrêa, S., Moulin, T.C., Carneiro, C.F.D., Gonçalves, M.M.C., Junqueira, L.S., Amaral, O.B., 2015. Calcineurin inhibition blocks within-, but not between-session fear extinction in mice. Learn. Mem. 22, 159–169. https://doi.org/10.1101/lm.037770.114.

Bales, J.W., Ma, X., Yan, H.Q., Jenkins, L.W., Dixon, C.E., 2010. Regional calcineurin subunit B isoform expression in rat hippocampus following a traumatic brain injury. Brain Res 1358, 211–220. https://doi.org/10.1016/j.brainres.2010.08.029.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67. https://doi.org/10.18637/jss.v067.i01.

Bloodgood, D.W., Sugam, J.A., Holmes, A., Kash, T.L., 2018. Fear extinction requires infralimbic cortex projections to the basolateral amygdala. Transl. Psychiatry. https://doi.org/10.1038/s41398-018-0106-x.

Bowers, M.E., Ressler, K.J., 2015. An overview of translationally informed treatments for posttraumatic stress disorder: animal models of Pavlovian fear conditioning to human clinical trials. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2015.06.008.

Brassil, H.E., Salvatore, A.P., 2018. The frequency of post-traumatic stress disorder symptoms in athletes with and without sports related concussion. Clin. Transl. Med 7. https://doi.org/10.1186/s40169-018-0200-y.

Carlson, K.F., Kehle, S.M., Meis, L.A., Greer, N., Macdonald, R., Rutks, I., Sayer, N.A., Dobscha, S.K., Wilt, T.J., 2011. Prevalence, assessment, and treatment of mild traumatic brain injury and posttraumatic stress disorder: a systematic review of the evidence. J. Head. Trauma Rehabil. 26, 103–115. https://doi.org/10.1097/HTR.0b013e3181e50ef1.

Cole, J.T., Yarnell, A., Kean, W.S., Gold, E., Lewis, B., Ren, M., McMullen, D.C., Jacobowitz, D.M., Pollard, H.B., O'Neill, J.T., Grunberg, N.E., Dalgard, C.L., Frank, J.A., Watson, W.D., 2011. Craniotomy: true sham for traumatic brain injury, or a sham of a sham. J. Neurotrauma 28 (3), 359–369. https://doi.org/10.1089/neu.2010.1427.

Corne, R., Leconte, C., Ouradou, M., Fassina, V., Zhu, Y., Déou, E., Besson, V., Plotkine, M., Marchand-Leroux, C., Mongeau, R., 2019. Spontaneous resurgence of conditioned fear weeks after successful extinction in brain injured mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 88, 276–286. https://doi.org/10.1016/j.pnpbp.2018.07.023.

Courtin, J., Chaudun, F., Rozeske, R.R., Karalis, N., Gonzalez-Campo, C., Wurtz, H., Abdi, A., Baufreton, J., Bienvenu, T.C., Herry, C., 2014. Prefrontal parvalbumin interneurons shape neuronal activity to drive fear expression, 2014 Jan 2 Nature 505 (7481), 92–96. https://doi.org/10.1038/nature12755.

Fitzgerald, J., Houle, S., Cotter, C., Zimomra, Z., Martens, K.M., Vonder Haar, C., Kokiko-Cochran, O.N., 2022. Lateral fluid percussion injury causes sex-specific deficits in anterograde but not retrograde memory. Front Behav. Neurosci. 16, 806598 https://doi.org/10.3389/fnbeh.2022.806598.

Genovese, R.F., Simmons, L.P., Ahlers, S.T., Maudlin-Jeronimo, E., Dave, J.R., Boutte, A. M., 2013. Effects of mild TBI from repeated blast overpressure on the expression and extinction of conditioned fear in rats. Neuroscience 254, 120–129. https://doi.org/10.1016/j.neuroscience.2013.09.021.

Glenn, D.E., Acheson, D.T., Geyer, M.A., Nievergelt, C.M., Baker, D.G., Risbrough, V.B., MRS-II Team, 2017. Fear learning alterations after traumatic brain injury and their role in development of posttraumatic stress symptoms. Depress Anxiety 34, 723–733. https://doi.org/10.1002/da.22642.

Golkar, A., Öhman, A., 2012. Fear extinction in humans: effects of acquisition-extinction delay and masked stimulus presentations. Biol. Psychol. 91 (2), 292–301. https://doi.org/10.1016/j.biopsycho.2012.07.007.

Gonzalez, P., Martinez, K.G., 2014. The role of stress and fear in the development of mental disorders. Psychiatr. Clin. North Am. 37 (4), 535–546. https://doi.org/10.1016/j.psc.2014.08.010.

Heinrichs, S.C., Leite-Morris, K.A., Guy, M.D., Goldberg, L.R., Young, A.J., Kaplan, G.B., 2013a. Dendritic structural plasticity in the basolateral amygdala after fear conditioning and its extinction in mice. Behav. Brain Res. 248, 80–84. https://doi.org/10.1016/j.bbr.2013.03.048.

Heinrichs, S.C., Leite-Morris, K.A., Rasmusson, A.M., Kaplan, G.B., 2013b. Repeated valproate treatment facilitates fear extinction under specific stimulus conditions. Neurosci. Lett. 552, 108–113. https://doi.org/10.1016/j.neulet.2013.07.035.

Hiploylee, C., Dufort, P.A., Davis, H.S., Wennberg, R.A., Tartaglia, M.C., Mikulis, D., Hazrati, L.-N., Tator, C.H., 2017. Longitudinal study of postconcussion syndrome: not everyone recovers. J. Neurotrauma 34, 1511–1523. https://doi.org/10.1089/neu.2016.4677.

Hoffman, A.N., Paode, P.R., May, H.G., Ortiz, J.B., Kemmou, S., Lifshitz, J., Conrad, C.D., Currier Thomas, T., 2017. Early and persistent dendritic hypertrophy in the basolateral amygdala following experimental diffuse traumatic brain injury. J. Neurotrauma 34, 213–219. https://doi.org/10.1089/neu.2015.4339.

Hoge, C.W., McGurk, D., Thomas, J.L., Cox, A.L., Engel, C.C., Castro, C.A., 2008. Mild traumatic brain injury in U.S. soldiers returning from Iraq. N. Engl. J. Med 358, 453–463. https://doi.org/10.1056/NEJMoa072972.

Iljazi, A., Ashina, H., Al-Khazali, H.M., Lipton, R.B., Ashina, M., Schytz, H.W., Ashina, S., 2020. Post-traumatic stress disorder after traumatic brain injury-a systematic review and meta-analysis. Neurol. Sci. 41, 2737–2746. https://doi.org/10.1007/s10072-020-04458-7.

Kaplan, G.B., Leite-Morris, K.A., Wang, L., Rumbika, K.K., Heinrichs, S.C., Zeng, X., Wu, L., Arena, D.T., Teng, Y.D., 2018. Pathophysiological bases of comorbidity: traumatic brain injury and post-traumatic stress disorder. J. Neurotrauma 35, 210–225. https://doi.org/10.1089/neu.2016.4953.

Kontos, A.P., Kotwal, R.S., Elbin, R.J., Lutz, R.H., Forsten, R.D., Benson, P.J., Guskiewicz, K.M., 2013. Residual effects of combat-related mild traumatic brain injury. J. Neurotrauma 30, 680–686. https://doi.org/10.1089/neu.2012.2506.

Krabbe, S., Gründemann, J., Lüthi, A., 2018. Amygdala inhibitory circuits regulate associative fear conditioning. Biol. Psychiatry 83 (10), 800–809. https://doi.org/10.1016/j.biopsych.2017.10.006.

LeDoux, J.E., 2014. Coming to terms with fear. Proc. Natl. Acad. Sci. USA 111, 2871–2878. https://doi.org/10.1073/pnas.1400335111.

Lepage, C., de Pierrefeu, A., Koerte, I.K., Coleman, M.J., Pasternak, O., Grant, G., Marx, C.E., Morey, R.A., Flashman, L.A., George, M.S., McAllister, T.W., Andaluz, N., Shutter, L., Coimbra, R., Zafonte, R.D., Stein, M.B., Shenton, M.E., Bouix, S., 2018. White matter abnormalities in mild traumatic brain injury with and without post-traumatic stress disorder: a subject-specific diffusion tensor imaging study. Brain Imaging Behav. 12, 870–881. https://doi.org/10.1007/s11682-017-9744-5.

Likhtik, E., Paz, R., 2015. Amygdala-prefrontal interactions in (mal)adaptive learning. Trends Neurosci. 38, 158–166. https://doi.org/10.1016/j.tins.2014.12.007.

Maddox, S.A., Hartmann, J., Ross, R.A., Ressler, K.J., 2019. Deconstructing the Gestalt: mechanisms of fear, threat, and trauma memory encoding. Neuron 102 (1), 60–74. https://doi.org/10.1016/j.neuron.2019.03.017.

Maren, S., 2011. Seeking a spotless mind: extinction, deconsolidation, and erasure of fear,emory. Neuron 70, 830–845. https://doi.org/10.1016/j.neuron.2011.04.023.

Maren, S., Holmes, A., 2016. Stress and fear extinction. Neuropsychopharmacology 41, 58–79. https://doi.org/10.1038/npp.2015.180.

McAllister, L.M., Lee, C.C.H., Milad, M.R., Eskandar, E.N., Whalen, M.J., 2015. Controlled cortical impact before or after fear conditioning does not affect fear extinction in mice. Brain Res. 1606, 133–141. https://doi.org/10.1016/j.brainres.2015.02.031.

Meyer, D.L., Davies, D.R., Barr, J.L., Manzerra, P., Forster, G.L., 2012. Mild traumatic brain injury in the rat alters neuronal number in the limbic system and increases conditioned fear and anxiety-like behaviors. Exp. Neurol. 235, 574–587. https://doi.org/10.1016/j.expneurol.2012.03.012.

Miller, S.C., Whitehead, C.R., Otte, C.N., Wells, T.S., Webb, T.S., Gore, R.K., Maynard, C., 2015. Risk for broad-spectrum neuropsychiatric disorders after mild traumatic brain injury in a cohort of US Air Force personnel. Occup. Environ. Med. 72, 560–566. https://doi.org/10.1136/oemed-2014-102646.

Milliken, C.S., Auchterlonie, J.L., Hoge, C.W., 2007. Longitudinal assessment of mental health problems among active and reserve component soldiers returning from the Iraq war. JAMA 298, 2141–2148. https://doi.org/10.1001/jama.298.18.2141.

O'Neil, M.E., Carlson, K., Storzbach, D., Brenner, L., Freeman, M., Quiñones, A., Motu'apuaka, M., Ensley, M., Kansagara, D., 2013. Complications of mild traumatic brain injury in veterans and military personnel: a systematic review (internet). Department of Veterans Affairs (US), Washington (DC). VA Evidence-based Synthesis Program Reports.

Oh, W.C., Parajuli, L.K., Zito, K., 2015. Heterosynaptic structural plasticity on local dendritic segments of hippocampal CA1 neurons. Cell Rep. 10 (2), 162–169. https://doi.org/10.1016/j.celrep.2014.12.016.

Palmer, C.P., Metheny, H.E., Elkind, J.A., Cohen, A.S., 2016. Diminished amygdala activation and behavioral threat response following traumatic brain injury. Exp. Neurol. 277, 215–226. https://doi.org/10.1016/j.expneurol.2016.01.004.

Pieper, J., Chang, D.G., Mahasin, S.Z., Swan, A.R., Quinto, A.A., Nichols, S.L., Diwakar, M., Huang, C., Swan, J., Lee, R.R., Baker, D.G., Huang, M., 2022. Brain amygdala volume increases in veterans and active-duty military personnel with combat-related posttraumatic stress disorder and mild traumatic brain injury. J. Head. Trauma Rehabil. 35, E1–E9. https://doi.org/10.1097/HTR.0000000000000492.

Plendl, W., Wotjak, C.T., 2010. Dissociation of within- and between-session extinction of conditioned fear. J. Neurosci. 30, 4990–4998. https://doi.org/10.1523/JNEUROSCI.6038-09.2010.

Polinder, S., Cnossen, M.C., Real, R.G.L., Covic, A., Gorbunova, A., Voormolen, D.C., Master, C.L., Haagsma, J.A., Diaz-Arrastia, R., von Steinbuechel, N., 2018. A multidimensional approach to post-concussion symptoms in mild traumatic brain injury. Front. Neurol. 9, 1113. https://doi.org/10.3389/fneur.2018.01113.

Ragsdale, K.A., Neer, S.M., Beidel, D.C., Frueh, B.C., Stout, J.W., 2013. Posttraumatic stress disorder in OEF/OIF veterans with and without traumatic brain injury. J. Anxiety Disord. 27, 420–426. https://doi.org/10.1016/j.janxdis.2013.04.003.

Ratliff, W.A., Mervis, R.F., Citron, B.A., Schwartz, B., Rubovitch, V., Schreiber, S., Pick, C.G., 2019. Mild blast-related TBI in a mouse model alters amygdalar neurostructure and circuitry. Exp. Neurol. 315, 9–14. https://doi.org/10.1016/j.expneurol.2019.01.020.

Reger, M.L., Poulos, A.M., Buen, F., Giza, C.C., Hovda, D.A., Fanselow, M.S., 2012. Concussive brain injury enhances fear learning and excitatory processes in the amygdala. Biol. Psychiatry 71, 335–343. https://doi.org/10.1016/j.biopsych.2011.11.007.

Rosen, V., Ayers, G., 2020. An update on the complexity and importance of accurately diagnosing post-traumatic stress disorder and comorbid traumatic brain injury, 2633105520907895 Neurosci. Insights 15. https://doi.org/10.1177/2633105520907895.

Santhanam, P., Teslovich, T., Wilson, S.H., Yeh, P.-H., Oakes, T.R., Weaver, L.K., 2019. Decreases in white matter integrity of ventro-limbic pathway linked to post-traumatic stress disorder in mild traumatic brain injury. J. Neurotrauma 36, 1093–1098. https://doi.org/10.1089/neu.2017.5541.

Schneiderman, A.I., Braver, E.R., Kang, H.K., 2008. Understanding sequelae of injury mechanisms and mild traumatic brain injury incurred during the conflicts in Iraq and Afghanistan: persistent postconcussive symptoms and posttraumatic stress disorder. Am. J. Epidemiol. 167, 1446–1452. https://doi.org/10.1093/aje/kwn068.

Seal, K.H., Bertenthal, D., Miner, C.R., Sen, S., Marmar, C., 2007. Bringing the war back home: mental health disorders among 103,788 US veterans returning from Iraq and Afghanistan seen at Department of Veterans Affairs facilities. Arch. Intern. Med. 167, 476–482. https://doi.org/10.1001/archinte.167.5.476.

Shear, D.A., Tortella, F.C., 2013. A military-centered approach to neuroprotection for traumatic brain injury. Front Neurol. 12 (4), 73. https://doi.org/10.3389/fneur.2013.00073.

Sierra-Mercado, D., McAllister, L.M., Lee, C.C., Milad, M.R., Eskandar, E.N., Whalen, M. J., 2015. Controlled cortical impact before or after fear conditioning does not affect fear extinction in mice. Brain Res. 1606, 133–141. https://doi.org/10.1016/j.brainres.2015.02.031.

Sripada, R.K., Rauch, S.A.M., Tuerk, P.W., Smith, E., Defever, A.M., Mayer, R.A., Messina, M., Venners, M., 2013. Mild traumatic brain injury and treatment response in prolonged exposure for PTSD. J. Trauma. Stress 26, 369–375. https://doi.org/10.1002/jts.21813.

Stein, M.B., Kessler, R.C., Heeringa, S.G., Jain, S., Campbell-Sills, L., Colpe, L.J., Fullerton, C.S., Nock, M.K., Sampson, N.A., Schoenbaum, M., Sun, X., Thomas, M.L., Ursano, R.J., Army, S.T.A.R.R.S., collaborators, 2015. Prospective longitudinal evaluation of the effect of deployment-acquired traumatic brain injury on posttraumatic stress and related disorders: results from the Army Study to Assess Risk and Resilience in Servicemembers (Army STARRS). Am. J. Psychiatry 172, 1101–1111. https://doi.org/10.1176/appi.ajp.2015.14121572.

Stein, M.B., Jain, S., Giacino, J.T., Levin, H., Dikmen, S., Nelson, L.D., Vassar, M.J., Okonkwo, D.O., Diaz-Arrastia, R., Robertson, C.S., Mukherjee, P., McCrea, M., Mac Donald, C.L., Yue, J.K., Yuh, E., Sun, X., Campbell-Sills, L., Temkin, N., Manley, G.T., TRACK-TBI Investigators, 2019. Risk of posttraumatic stress disorder and major depression in civilian patients after mild traumatic brain injury: a TRACK-TBI study. JAMA Psychiatry 76, 249–258. https://doi.org/10.1001/jamapsychiatry.2018.4288.

Takao, K., Miyakawa, T., 2006. Light/dark transition test for mice. J. Vis. Exp. 104. https://doi.org/10.3791/104.

Tanev, K.S., Pentel, K.Z., Kredlow, M.A., Charney, M.E., 2014. PTSD and TBI co-morbidity: scope, clinical presentation and treatment options. Brain Inj. 28, 261–270. https://doi.org/10.3109/02699052.2013.873821.

Thompson, H.J., Lifshitz, J., Marklund, N., Grady, M.S., Graham, D.I., Hovda, D.A., McIntosh, T.K., 2005. Lateral fluid percussion brain injury: a 15-year review and evaluation. J. Neurotrauma 22, 42–75. https://doi.org/10.1089/neu.2005.22.42.

Van Praag, D.L.G., Cnossen, M.C., Polinder, S., Wilson, L., Maas, A.I.R., 2019. Post-traumatic stress disorder after civilian traumatic brain injury: a systematic review and meta-analysis of prevalence rates. J. Neurotrauma 36, 3220–3232. https://doi.org/10.1089/neu.2018.5759.

VanElzakker, M.B., Dahlgren, M.K., Davis, F.C., Dubois, S., Shin, L.M., 2014. From Pavlov to PTSD: the extinction of conditioned fear in rodents, humans, and anxiety disorders. Neurobiol. Learn. Mem. 113, 3–18. https://doi.org/10.1016/.nlm.2013.11.014.

Vasterling, J.J., Jacob, S.N., Rasmusson, A., 2018. Traumatic brain injury and posttraumatic stress disorder: conceptual, diagnostic, and therapeutic considerations in the context of co-occurrence. J. Neuropsychiatry Clin. Neurosci. 30, 91–100. https://doi.org/10.1176/appi.neuropsych.17090180.

Wolf, G.K., Strom, T.Q., Kehle, S.M., Eftekhari, A., 2012. A preliminary examination of prolonged exposure therapy with Iraq and Afghanistan veterans with a diagnosis of posttraumatic stress disorder and mild to moderate traumatic brain injury. J. Head. Trauma Rehabil. 27, 26–32. https://doi.org/10.1097/HTR.0b013e31823cd01f.

Yu, X., Guindani, M., Grieco, S.F., Chen, L., Holmes, T.C., Xu, X., 2022. Beyond t test and ANOVA: applications of mixed-effects models for more rigorous statistical analysis in neuroscience research. Neuron 110, 21–35. https://doi.org/10.1016/j.neuron.2021.10.030.

Yurgil, K.A., Barkauskas, D.A., Vasterling, J.J., Nievergelt, C.M., Larson, G.E., Schork, N. J., Litz, B.T., Nash, W.P., Baker, D.G., Marine Resiliency Study Team, 2014. Association between traumatic brain injury and risk of posttraumatic stress disorder in active-duty Marines. JAMA Psychiatry 71, 149–157. https://doi.org/10.1001/jamapsychiatry.2013.3080.

Zhao, J., Huynh, J., Hylin, M.J., O'Malley, J.J., Perez, A., Moore, A.N., Dash, P.K., 2018. Mild traumatic brain injury reduces spine density of projection neurons in the medial prefrontal cortex and impairs extinction of contextual fear memory. J. Neurotrauma. https://doi.org/10.1089/neu.2016.4898.