PRE-CLINICAL MODELS OF PTSD

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PRE-CLINICAL MODELS OF PTSD

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EDITORIAL
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Editorial: Pre-clinical Models of PTSD

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Keywords: PTSD, animal models, pre-clinical/preclinical, traumatic stress, comorbidity

Editorial on the Research Topic

Pre-clinical Models of PTSD

This editorial outlines the *Research Topic: Pre-clinical Models of PTSD* that includes 16 publications: 11 original research articles, 2 reviews, 2 mini-reviews, and a perspective that all integrate rodent models of posttraumatic stress disorder (PTSD) in the conceptual framework of the publications. By using rodent models of traumatic stress exposure and/or measuring PTSD-specific physiologic characteristic and PTSD-like behaviors, the articles explore both established and novel brain mechanisms implicated in PTSD. The manuscripts span neurochemical and anatomical levels, sex-dependent differences, relationships to comorbid conditions, and new rodent models of PTSD-like effects.

Similarly to other brain based pathologies, pre-clinical models of PTSD are critical for the ability to mechanistically explore the effects that trauma have on brain structure and function, yielding considerable insight into the neurobiological processes that likely underlie PTSD phenotypes. This *Research Topic* contains manuscripts that continue this exploration, including studies that focus on brain catecholamine signaling in well-characterized and novel neurocircuitry implicated in PTSD. Papers by Chaby et al., Deslauriers et al., and Liu et al. examine aspects of catecholamine signaling as a key system relevant to the expression of PTSD-like behaviors with potential relevance to PTSD treatment. Manuscripts by Piggott et al. and Miles and Maren, and others investigate specific brain regions that likely play an important role in PTSD, including prefrontal cortex and amygdala, as well as brain regions, that have been less studied in this context, namely the striatum and bed nucleus of the stria terminalis. This subgroup of papers that explore involvement of brain mechanisms, often linked with reward, provides conceptual bridge for the understanding of the highly prevalent association of PTSD with substance use disorders, a comorbidity that is reviewed by Gisquet-Verrier and Le Dorze.

Beyond direct mechanistic studies, PTSD-related animal models provide a means to study additional risk and protective factors that can modify the development of psychopathology following exposure to traumatic stress. This *Research Topic* includes a number of studies on the mitigating role of sex and developmental factors in expression of PTSD following trauma. For example, Asok et al. show that fear generalization is sex-dependent, Colom-Lapetina et al. illustrate a sex-dependent diversity in behavioral response to trauma, and Nahvi et al. provide evidence that females show anxiety- and depression-like behaviors akin to males, but have a unique neuro-mechanism underlying these behaviors. Interestingly, Chen et al. using two animal models of PTSD report that while fear extinction learning deficits manifest when trauma occurs in adulthood, adolescent animals might be resilient to similar trauma.

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Together these studies contribute to the growing body of work addressing the need to understand factors, such as sex and age that contribute to PTSD, highlighting the need to use multiple models of PTSD to define specific aspects or uncover unique characteristics associated with traumatic stress exposure.

Historical evidence shows that with new models new candidate mechanisms come into the investigative focus. The *Pre-clinical Models of PTSD Research Topic* embraces several manuscripts that involve new models and/or investigate new mechanisms potentially relevant to PTSD. For instance, Paredes and Morilak review the use of extinction learning as a model of exposure therapy in rodents, Conoscenti and Fanselow discuss key aspects that differentiate animal models in the stress literature, and Pinna uses a novel social-isolation model to determine if a biomarker, allopregnanolone in this case, can be identified for PTSD. Furthermore, this *Research Topic* delves into novel mechanisms, such as those explored by Moshfegh et al. involving inflammatory response to stress, or the effects of fear extinction learning on conditioned cardiovascular response by Swiercz et al. It also includes papers that investigate neurotherapeutics for PTSD, such as a study by Shallcross et al. that studied cannabidiol and a mGlu5 positive allosteric modulator as potentially useful agents with unique effects on stress and fear-related behaviors. These novel models and mechanisms continue to expand our understanding of PTSD and emphasizing the importance of scientific and conceptual diversity in PTSD research.

The collection of manuscripts in *Pre-clinical Models of PTSD* focus on the use of animal models to advance understanding of PTSD-related pathophysiology, including mechanisms targeting aspects of the brain, the body, and individual behavior. Moreover, factors affecting vulnerability and resilience, comorbidities, and treatment outcomes are considered in this *Research Topic*. Finally, while recognizing that PTSD is a uniquely human disorder, the use of appropriate animal models of PTSD and traumatic stress exposure and measurement of defining characteristics of PTSD remain critical components of the research endeavor to understand the complex neurobiology of PTSD.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Effects of Trauma in Adulthood and Adolescence on Fear Extinction and Extinction Retention: Advancing Animal Models of Posttraumatic Stress Disorder

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Evidence for and against adolescent vulnerability to posttraumatic stress disorder (PTSD) is mounting, but this evidence is largely qualitative, retrospective, or complicated by variation in prior stress exposure and trauma context. Here, we examine the effects of development on trauma vulnerability using adult post-natal (PN) day 61, early adolescent (PN23) and mid adolescence (PN34) rats and two types of trauma: an established animal model of PTSD, single prolonged stress (SPS), and a novel composite model—SPS predation (SPSp) version. We demonstrate that early and mid adolescent rats are capable of fear conditioning and fear extinction, as well as extinction retention. Our results also demonstrate that both types of trauma induced a deficit in the retention of fear extinction in adulthood, a hallmark of PTSD, but not after early or mid adolescence trauma, suggesting that adolescence might convey resilience to SPS and SPSp traumas. Across all three life stages, the effects of SPS exposure and a novel predation trauma model, SPSp, had similar effects on behavior suggesting that trauma type did not affect the likelihood of developing PTSD-like symptoms, and that SPSp is a predation-based trauma model worth exploring.

Keywords: adolescence, single prolonged stress, PTSD, predation stress, developmental stress

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Highlights

- Posttraumatic stress disorder (PTSD) risk is mediated by trauma type and age at exposure.
- We compare an established and novel composite trauma model in adolescence and adulthood.
- Both models induced extinction retention deficits in adulthood, but not adolescence.
- Adolescents had intact extinction and extinction retention after trauma, suggesting resilience.

Abbreviations: BDNF, brain-derived neurotrophic factor; GR, glucocorticoid receptor; PFC, prefrontal cortex; PTSD, posttraumatic stress disorder; SPS, single prolonged stress; SPSp, single prolonged stress-predation.

INTRODUCTION

It is commonly asserted in the literature that children and adolescents are at higher risk for trauma related psychopathology (Pynoos et al., 1987; North et al., 1994); however, the empirical/mechanistic evidence for this assertion is sparse and often contradictory (Green et al., 1991; Shannon et al., 1994; Tottenham and Gabard-Durnam, 2017; reviewed in Chaby et al., 2017). Indeed, increased vulnerability to posttraumatic stress disorder (PTSD) following trauma in adolescence has been supported by some (Green et al., 1991) but not other studies (McFarlane, 1987; Lonigan et al., 1991). These differences stem partly from challenges inherent in the study of clinical populations, such as variation between participants in trauma type, timing, duration and prior stress history (van der Kolk, 1985; Davidson and Smith, 1990; Bokszczanin, 2007; reviewed in Schwarz and Perry, 1994). While some studies examined the relationship between trauma type and PTSD symptom profile (Kelley et al., 2009; Price et al., 2013), the categorization of trauma type is hard as it depends on individual experiences, culture and individual history, that can also interact with the age at exposure (McCloskey and Walker, 2000; Avital and Richter-Levin, 2005; Ricon et al., 2012). Animal studies that systematically evaluate the effect of various trauma types across ontogeny can help to overcome some of these challenges by elucidating aspects of the traumatic response that are common across traumas.

With respect to the risk conveyed by the developmental stage itself, adolescents may have differential sensitivity to trauma because of states of maturation of brain tissues, developmental plasticity, immature capacity for emotion regulation, and age-specific differential cognitive coping skills (Spear, 2000; Crews et al., 2007; Semple et al., 2013). One of the ways developmental processes can alter vulnerability to post traumatic psychopathology, is via altered fear associated learning and extinction, which has been shown to be affected in both PTSD patients and animal models of PTSD in adulthood (Liberzon et al., 1997, 1999). In fact, it has been reported that contextually learned fear expression is temporarily suppressed in adolescence, a phenomenon modulated by developmental changes in brain derived neurotrophic factor (BDNF; Pattwell et al., 2011; Dincheva et al., 2014). In addition, trauma may also have differential effects across ontogeny on extinction retention, a capacity that is deficient in PTSD patients. Extinction retention has been linked to activation of the ventromedial prefrontal cortex (PFC) and the hippocampus in adults, areas that still undergo development in adolescence (Meaney et al., 1985; Sowell et al., 1999), and that show reduced activity in PTSD patients (Milad et al., 2007, 2009).

In the present study, we aimed to test the effects of trauma exposure in adult and adolescent rats using two different models of trauma. First, we used a well established trauma model, single prolonged stress (SPS), which has been used for nearly two decades to model PTSD-specific deficits in extinction retention (Knox et al., 2012; reviewed in Yamamoto et al., 2009). Second, we employed a novel predation model that used similar to SPS temporal characteristics. Predator models have been popular due to their face validity, and they had been shown to induce both overlapping and distinct symptoms of PTSD compared with SPS (Daskalakis et al., 2013; Deslauriers et al., 2017). For example, both predation models and SPS can induce heightened glucocorticoid receptor (GR) receptor levels, startle responsivity, anxiety-like behavior and pro-inflammatory cytokine levels (Khan and Liberzon, 2004; Yamamoto et al., 2009; Elharrar et al., 2013; Zoladz and Diamond, 2013; Lin et al., 2016; reviewed in Deslauriers et al., 2017). Yet, to the authors knowledge, SPS has been shown to result in PTSD-like sleep disturbances, extinction retention deficits and anhedonia (Perrine et al., 2016; Nedelcovych et al., 2015; Vanderheyden et al., 2015), while predation models have been shown to induce avoidance of trauma reminders and decreased dendritic length and spine density in the hippocampus (Cohen et al., 2011, 2014). Using SPS temporal characteristics (single prolonged exposure to multiple stressors, followed by a sensitization period) we created a novel model, SPS-predation (SPSp), to investigate potential effects of trauma type and developmental timing on fear learning and extinction retention. Given distinct psychopathological effects of trauma type at different developmental timepoints (McCloskey and Walker, 2000; Kelley et al., 2009), we predicted that the trauma models would have distinct effects on fear learning processes across three developmental stages, but that early and mid adolescents would have enhanced vulnerability to both trauma types because of immature neural networks for processing extinction retention. In addition, we further examined the validity of the SPSp model by testing their startle response, a response shown to be heightened in PTSD patients (Shalev et al., 2000) and in animals exposed to SPS (Khan and Liberzon, 2004).

MATERIALS AND METHODS

Animals and Housing

Male Sprague-Dawley rats were obtained as adults (post-natal day [PN] 61, n = 48), mid adolescents (PN 34, n = 24) and early adolescents (PN 23, n = 24), from Charles River (Kingston, NY, USA). To describe developmental stages, we used nomenclature from Cicchetti (2016), which reflect sex organ maturation. The upper limit of the phase is defined by the emergence of sexual behavior. The second phase, mid adolescence (also called mid puberty), encompasses widespread maturation of the sex organs. The lower limit, termed early adolescence (also called early puberty), ends prior to the onset of most maturation processes in the sex organs (Spear, 2000; Cicchetti, 2016).

Following arrival, all rats were pair housed and allowed to acclimate for 4 days at the Veterinary Medical Unit of the Ann Arbor Veterans Affairs Medical Center. Rats were maintained on a 12:12 h light/dark cycle (lights on at 6 am), at 20–22°C, and 50% humidity. Rats were given *ad libitum* access to water and chow (25% protein, Laboratory Rodent Diet 5001, LabDiet). All procedures and protocols were approved by the Ann Arbor Veteran Affairs Institutional Animal Care and Use Committee (protocol #1312-004) and were in accordance with the National Institute of Health standard for the treatment of animals.

Trauma Models

Single Prolonged Stress

In the SPS procedure, rats were exposed to three stressors in the first day (Liberzon et al., 1997) that target the hypothalamic-pituitary-adrenal axis via psychological, physiological and direct pituitary routs. Briefly, rats were restrained for 2 h, were exposed to a 20 min forced swim (68 × 56 × 45 cm container with water kept at 23–24°C) and, after a 15 min recuperation, were finally exposed to ether vapors in a desiccator until they lost consciousness and were fully anesthetized. Animals were then individually housed in clean cages and left undisturbed for 7 days, the time required for PTSD-like phenotype to develop (Liberzon et al., 1999; Knox et al., 2012). Control rats were also single housed starting on the day that SPS rats were exposed to trauma and left undisturbed for 7 days.

Single Prolonged Stress Predator Version

Similar to SPS, the SPSp, encompasses three stressors that are ecologically relevant to rodents. Animals were left in their home cages and exposed to a scent of a predator, fox urine (Tink's Fox-P®), for 2 h. The scent was sprayed onto cotton balls and encased in a small ventilated plastic container, which was removed at the end of the 2 h (Chaby et al., 2015b). Following scent exposure, rats were exposed to a recording of predatory feline calls for 20 min in their home cages (Chaby et al., 2015a,b, 2016). Finally, animals were placed in an open arena (92 × 92 × 63 cm) for 5 min with a hawk shaped kite hanging overhead, which was lowered towards the rats to mimic approach (Chaby et al., 2015b). Rats were then transferred to clean cages, individually housed and left undisturbed for 7 days. Control rats were also single housed starting on the day that SPSp rats were exposed to trauma and left undisturbed for 7 days.

Fear Conditioning, Fear Extinction, Extinction Recall

Rats first learned a fear association of a tone followed by a foot shock, which was extinguished the next day when animals were presented with the tone alone. On the last day, rats were again presented with tones alone to evaluate retention of extinction.

In detail, eight sound-attenuating boxes housed individual experimental chambers measuring 30 × 24 × 21 cm (MED Associates, St. Albans, VT, USA), which were connected to interfaces that controlled the experimental contingencies determined using MedPC software. The floor of each chamber was made of stainless steel rods measuring 4 mm in diameter and placed 1.5 cm apart from each other. The floor was connected to a shock source, which delivered a 1 s 1 mA shock unconditioned stimulus. The chambers were mounted with a speaker that delivered a 10 s 1 kHz 80 dB acoustic tone, which was used as the conditioned stimulus. Each chamber was also equipped with a 15 W light, and a fan that provided a 65 dB white noise.

Two distinct contexts with different auditory, visual, tactile and olfactory cues were used. Context A: lidded black boxes were used to transport the animals from their home cage to the testing chambers. Ammonium hydroxide (1%) was used as the scent cue and a room red light was used as the visual cue. The doors of the sound-attenuating boxes were left open, and the chamber lights and fans were left off. Context B: lidded white boxes with clean bedding were used to transport animals from their home cages to the testing chambers. Acetic acid (1%) was used as the scent cue, chamber lights as the visual cue, and chamber fans as the auditory cue. The doors of the sound-attenuating boxes were left closed.

For fear conditioning, context A was used. Baseline movement was recorded for 3 min before the animals were presented with five tones as the conditioned stimulus that co-terminated with a foot shock as unconditioned stimulus. For fear extinction and extinction recall, context B was used. Rats were left for 3 min in the chamber before being presented with 30 or 10 conditioned stimulus tones, respectively. Tones lasted 10 s and time between each conditioned stimulus was 1 min. For all three tests, animals were transported to the testing room and left to acclimate for 10 min in their transport boxes before testing. Transport boxes and testing chambers were cleaned with running tap water and dried between animals.

To minimize disturbance, animal behavior was recorded with an overhead camera on top of each chamber and the experimenter was not present in the room during testing. Percent freezing (total time between tones × 100/time freezing between tones) was analyzed using the Any-Maze Software (Stoelting, Co., Wood Dale, IL, USA). Freezing was defined as immobility, lasting longer than 1 s. Freezing behavior during a conditioned stimulus presentation and the following inter-trial interval was considered a single trial.

Acoustic Startle

The startle chambers were 30 × 30 × 30 cm and housed a cylindrical tube 20 cm in length and 10 cm in diameter. The chamber was equipped with a 15 W light and speakers that delivered the acoustic bursts. Rats were allowed to acclimate to the acoustic chamber for 5 min. Thirty acoustic bursts (108 dB, 50 ms, 30 s intervals) were then delivered for 15 min and startle response (V_{max} = startle amplitude) was recorded and analyzed by an automated hardware/software package (San Diego Instruments, San Diego, CA, USA).

Experiment 1: Trauma in Adulthood, Mid-puberty, or Early-Puberty and Susceptibility to Reduced Fear Extinction and Retention

To determine whether developmental stage affects fear associated learning after exposure to SPS, three different studies were run where rats at three different developmental stages were used: the first study used adult (PN61, n = 24), the second used mid adolescent (PN34, n = 24) and the third used early adolescent (PN23, n = 24) male rats. To evaluate whether effects on fear associated learning are SPS specific, we exposed animals in the above-mentioned developmental stages to the SPSp model. Thus, each study comprised of 24 rats of the same age group, and included the SPS, SPSp or control groups, resulting in eight rats per group.

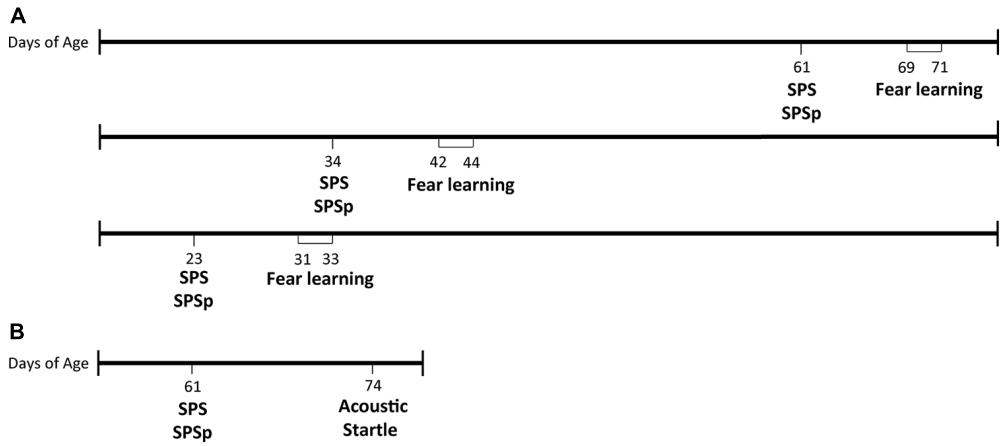

FIGURE 1 | Timeline of procedures. **(A)** Experiment 1. **(B)** Experiment 2.

Four days after arrival, rats underwent SPS, SPSp, or, if assigned to an unstressed control group, were transferred to clean cages and singly housed for the remainder of the study. After 7 days, all rats underwent 3 days of testing: fear conditioning, extinction, and extinction recall (timeline in **Figure 1A**).

To eliminate the possibility that a group difference is falsely identified, we tested a separate cohort of adult animals ($n = 8$ per group) on the same paradigm mentioned above.

Experiment 2: SPS and SPSp Modeling the PTSD Symptom of Acoustic Startle Response

Our laboratory previously reported that exposure to SPS enhances startle response (Khan and Liberzon, 2004), a phenotype also present in patients with PTSD. To determine whether effects of SPSp on startle response are similar to those resulting from SPS, we exposed 24 PN61 male rats to the SPS, SPSp or control procedures, and tested them on the acoustic startle test 13 days later (timeline in **Figure 1B**). Rats were single housed on the first day of SPS, SPSp or control procedures and remained so for the rest of the study.

Statistical Analysis

Experiment 1: for all studies, fear learning data were analyzed with a repeated measures ANOVA with treatment (SPS, SPSp or control) as the between groups factor and trial (baseline, tone 1, tone 2, etc.) as a within groups factor. If a main effect of treatment was found, a *post hoc* least significant difference (LSD) analysis was used to compare marginal means. If an interaction effect was found, one-way ANOVAs were conducted to compare cell means. Rats that did not acquire conditioning (percent freezing remained below 30% for all fear conditioning trials; one rat in the adult Control group, two rats in the late adolescent control group, and one rat in the late adolescent SPS group), did not show recall of conditioning (percent freezing remained below 30% in the first five fear extinction trials; one rat in the late adolescent SPS group and three in the late adolescent SPSp group) were removed; and rats that showed freezing 2 standard deviations above or below the mean in half or more of the tones in one session were also removed (one rat in ER in the Control late adolescent group was removed). For fear extinction, percent freezing across three trials were averaged into a block, resulting in 10 blocks.

Experiment 2: startle amplitude across three acoustic bursts were averaged into a block, resulting in 10 acoustic burst blocks. A repeated measures ANOVA was conducted with treatment (SPS, SPSp or control) as the between subjects factor and acoustic burst (burst block 1, burst block 2... burst block 10) as a within subjects factor. If a main effect of treatment was found, a *post hoc* LSD analysis was used to compare marginal means. If an interaction effect was found, one-way ANOVAs were conducted to compare cell means.

RESULTS

Experiment 1: Trauma in Early-Puberty, Mid-puberty, or Adulthood and Susceptibility to Reduced Fear Extinction and Retention

Adult Rats: (Figure 2A)

Fear conditioning: all animals showed increasing freezing through consecutive trials ($F = 87.05$, $p < 0.001$). There was no main effect of trauma exposure ($F = 1.896$, $p = 0.176$). An interaction effect was found ($F = 3.938$, $p < 0.001$) such that, at trial 3, control animals showed lower freezing compared to rats exposed to SPS ($p = 0.007$) and SPSp ($p = 0.001$). In other words, though all groups acquired fear conditioning, animals that experienced SPS and SPSp learned conditioning faster compared to control rats.

Fear extinction: all rats showed normal fear learning effects and extinction acquisition ($F = 9.386$, $p < 0.001$). There was a main effect of trauma exposure ($F = 12.276$, $p < 0.001$), such that control rats showed overall lower freezing compared to those that

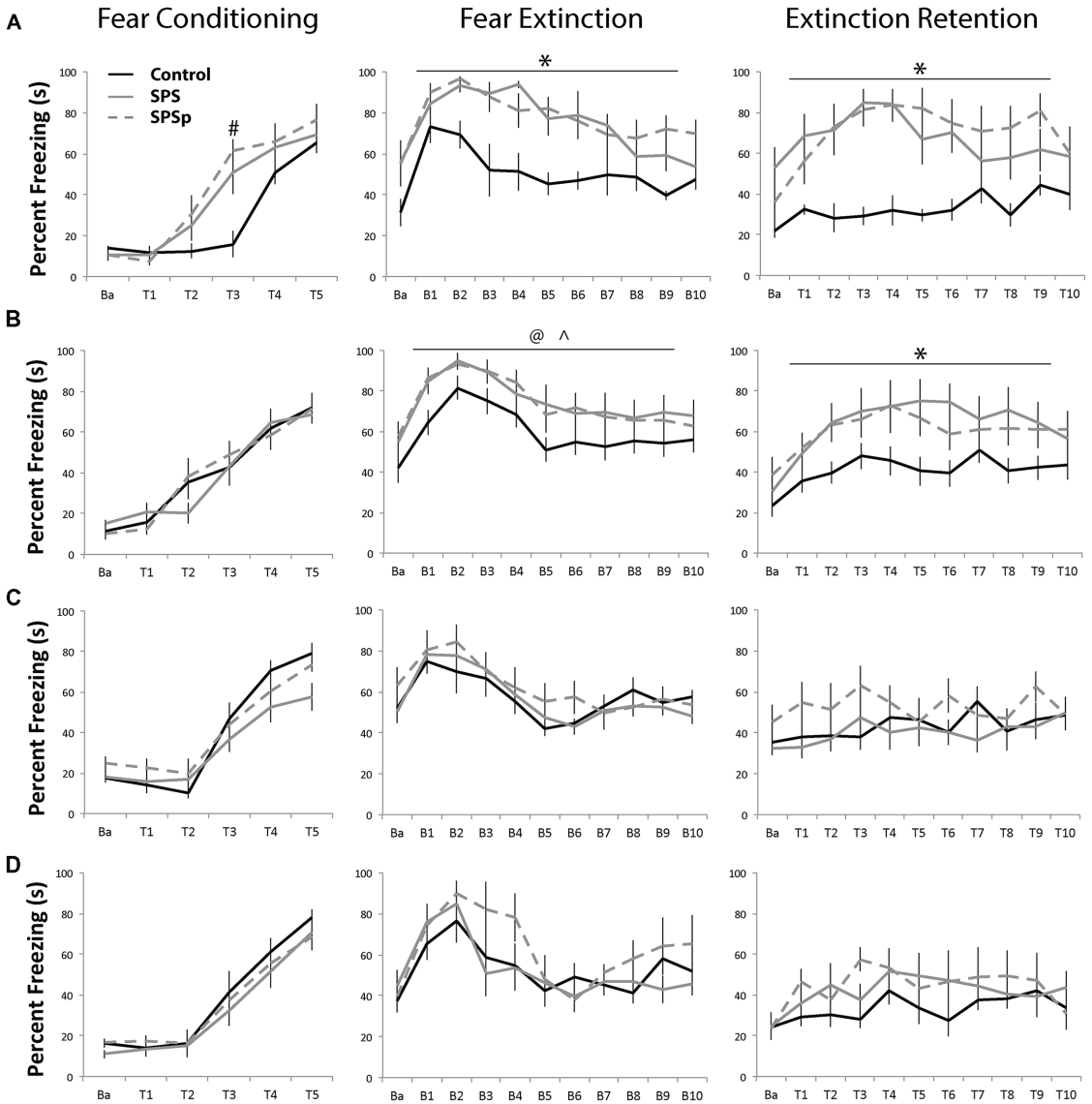

FIGURE 2 | (A,B) Adults: all adult rats acquired fear conditioning and learned fear extinction equally. Compared with control rats, rats that experienced single prolonged stress (SPS) and single prolonged stress-predation (SPSp) showed higher freezing, and higher fear trace and slower extinction learning; the experience of the trauma models also resulted in extinction retention deficits. **(C)** Early and **(D)** Mid Adolescents: all adolescent rats learned fear conditioning and extinction, and showed no extinction retention deficits. Ba: baseline; T: trial; B: block of three trials; #indicates different from controls, $p < 0.05$; *indicates different from controls, $p < 0.05$; ^indicates SPSp different from controls, $p < 0.05$; @indicates SPS different from controls, $p = 0.06$.

experienced SPS ($p = 0.001$) or SPSp ($p < 0.001$). No difference between the SPS and SPSp groups ($p = 0.646$) and no interaction effects were found ($F = 0.900$, $p = 0.0587$).

Extinction recall: main effects of trial ($F = 4.789$, $p < 0.001$) and trauma exposure ($F = 9.269$, $p = 0.001$) were found; control rats showed overall lower freezing compared to those that experienced SPS ($p = 0.002$) or SPSp ($p = 0.001$). A significant interaction was also found between trial and treatment ($F = 2.086$, $p = 0.006$). While control rats showed constant low freezing levels, those that experienced SPS or SPSp exhibited heightened freezing in response to the initial tones and later within session extinction.

Adult Rats, Experiment 2: (Figure 2B)

Fear conditioning: rats showed increasing freezing through consecutive trials ($F = 61.299$, $p < 0.001$). No effects of treatment ($F = 0.023$, $p = 0.977$) or interaction ($F = 0.984$, $p = 0.461$) were found.

Fear extinction: all rats showed recall of fear learning and extinction acquisition ($F = 14.124$, $p < 0.001$). A trend level effect of trauma exposure ($F = 2.946$, $p = 0.067$) was present, indicating that control rats showed overall lower freezing during the extinction session as compared to SPSp animals ($p = 0.038$), and a trend towards lower freezing compared to SPS animals ($p = 0.064$). No interaction effect was found ($F = 0.418$, $p = 0.999$).

Extinction recall: a main effect of trial ($F = 9.875$, $p < 0.001$), and a trend level effect of treatment ($F = 3.234$, $p = 0.053$) was found; control animals froze significantly less than rats that experienced SPS ($p = 0.040$) or SPSp ($p = 0.037$). No interaction effects were found ($F = 0.927$, $p = 0.552$).

Mid-Adolescence Rats: (Figure 2C)

Fear conditioning: across the five tone-shock presentations, all animals increased freezing over time ($F = 76.512$, $p = 0.000$). No effect of trauma exposure ($F = 0.418$, $p = 0.664$) or interaction ($F = 0.301$, $p = 0.907$) were found.

Fear extinction: all rats showed normal fear learning and acquisition of extinction ($F = 10.680$, $p < 0.001$). No main effect of trauma exposure ($F = 1.027$, $p = 0.379$) or interactions ($F = 1.026$, $p = 0.434$) were found.

Extinction recall: a main effect of trial was found ($F = 3.214$, $p = 0.001$) that indicated normal extinction retention. No main effect of trauma exposure ($F = 0.867$, $p = 0.439$) or interaction ($F = 0.946$, $p = 0.530$) were found.

Early Adolescence Rats: (Figure 2D)

Fear conditioning: a main effect of trial was found ($F = 78.087$, $p < 0.001$); as rats progressed through the tones paired with foot shocks, all acquired conditioning by showing more freezing. No main effect of trauma exposure ($F = 1.379$, $p = 0.276$) or interaction between trauma exposure and trials ($F = 1.685$, $p = 0.095$) on fear learning were found.

Fear extinction: a main effect of trials was found ($F = 12.215$, $p < 0.001$) where animals showed more freezing in the early trials, indicating learned fear, and less freezing in the later trials, indicating acquisition of extinction. No main effect of trauma exposure ($F = 0.375$, $p = 0.692$) or interaction effects ($F = 0.697$, $p = 0.827$) were found.

Extinction recall: no effects of trials ($F = 1.431$, $p = 0.168$), trauma exposure ($F = 1.351$, $p = 0.281$), or interaction ($F = 1.029$, $p = 0.430$) were found.

Experiment 2: SPS and SPSp Modeling the PTSD Symptom of Enhanced Fear-Potentiated Startle Response (Figure 3)

Acoustic startle: as expected, all rats showed more startle amplitude in the early trials ($F = 5.651$, $p < 0.001$). A main effect ($F = 6.936$, $p = 0.003$) of trauma exposure was found, where control animals showed similar startle amplitude compared to SPSp rats ($p = 0.699$), and overall lower startle amplitude compared to SPS rats ($p = 0.001$). No interaction effect was found ($F = 1.430$, $p = 0.115$).

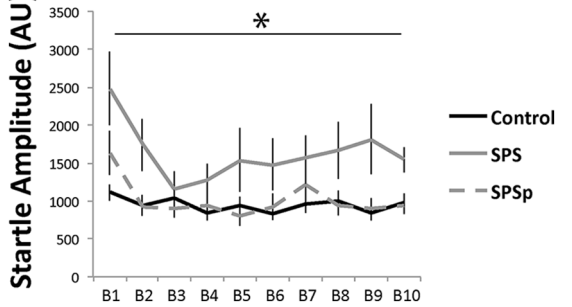

FIGURE 3 | Rats that experienced SPS showed increased startle responses compared to those that experienced SPSp or no trauma; B: block of three acoustic bursts *indicates $p < 0.05$.

DISCUSSION

Here, we compared the effects of exposure to two trauma models, a well-established PTSD model (SPS) and a novel predation version (SPSp), on fear associated learning in adult, mid adolescent, and early adolescent rodents. Our results demonstrated that a deficit in extinction retention, a hallmark of PTSD seen both in PTSD patients (Milad et al., 2008, 2009) and PTSD rodent models (Knox et al., 2012, 2016; George et al., 2015), was induced by exposure to two types of trauma in adulthood, but not in early or mid adolescence.

Across the three life stages studied, exposure to a novel SPSp paradigm reliably replicated previously documented effects of SPS on fear learning processes (Yamamoto et al., 2009; Knox et al., 2012; George et al., 2015), suggesting that the predation-based trauma model had convergent validity with SPS in this aspect, and that the structural aspects of the SPS such as prolong stressor exposure followed by sensitization period, rather than the specific types of stressors, might be the most pertinent to the development of PTSD-like extinction retention deficits. In addition, our findings show that early and mid adolescent animals were capable of fear conditioning and extinction, as well as of extinction recall, contrasting with previous indications that adolescent rats have impaired extinction learning and extinction retention (McCallum et al., 2010; Baker et al., 2014). When the effects of the two trauma models were compared in adults for a second symptom of PTSD, however, SPS accurately modeled increased fear-potentiated startle (concurrent with prior findings, Khan and Liberzon, 2004), while SPSp did not, suggesting that the effects of trauma type may differ across PTSD symptoms (Butler et al., 1990; Shalev et al., 2000).

There are two potential explanations for deficits in extinction retention after SPS and SPSp in adulthood, but not early or mid adolescents. First, adolescents might be less vulnerable to effects of SPS/SPSp trauma compared with adults. Alternatively, the effects of trauma may manifest differently in adolescence compared with adulthood, or may be delayed and appear only in adulthood (Gluckman et al., 2005; Pattwell et al., 2011). For the first possibility, there is evidence from human literature that support (Green et al., 1991; Liu et al., 2006) and contradict (McFarlane, 1987; Davidson and Smith, 1990) this assertion. If indeed adolescence does not convey increased risk for PTSD, as our data suggests, higher rates of PTSD in adolescents (e.g., Green et al., 1991; Liu et al., 2006), seen in humans, might not reflect common developmental differences, but rather psychological factors, such as poor social support or limited independence to escape adverse conditions, not readily modeled

in rodent studies. Supporting this, child abusers are typically in a child's social network (up to 90%), and risk of recurrent abuse ranges from 9% to 85%, suggesting ongoing adverse conditions may be commonplace after abuse, resulting in distinct effects compared with isolated traumatic incidents (in the United States, Howard, 2000; Hindley et al., 2006; U.S. Department of Health and Human Services, Administration for Children and Families, Administration on Children, Youth and Families, Children's Bureau, 2017).

Neurobiologically, adolescence can convey differential responsivity to trauma exposure via a number of development-specific mechanisms. In adolescence, the retention of fear extinction information may be less susceptible to trauma because of: (i) protective effects of heightened plasticity, which may facilitate discounting of old information (i.e., fear conditioning) in favor of new information (i.e., fear extinction; Cicchetti, 2016; Panchanathan and Frankenhuis, 2016); or (ii) developmental differences in neural networks recruited for fear extinction and retention. Developmental plasticity is facilitated by BDNF, which peaks in adolescence, and enhances synaptic plasticity, memory formation, and the growth and survival of new neurons (Katoh-Semba et al., 1997; Bekinschtein et al., 2008; Cicchetti, 2016). In adult rats, administration of BNDF to the medial PF (mPFC) can rescue extinction retention deficits (Kabir et al., 2013). This suggests that higher levels of BDNF in adolescence may enhance extinction retention, despite immaturity of the mPFC and hippocampus, attenuating the behavioral effects of trauma. The PFC is very sensitive to stress (Arnsten, 2009; Somerville et al., 2010), but adolescent neural networks have yet to incorporate the role of the PFC, and instead rely on faster developing regions to perform similar cognitive tasks (Spear, 2000, 2013; Tottenham and Galván, 2016). If so, adolescents may be less sensitive to cognitive effects of trauma because they can more effectively compensate for decreases in PFC function, for example during extinction retention. Adolescents also undergo synaptic and receptor density increases, followed by pruning (Cicchetti, 2016). For example, GR binding peaks in the hippocampus during the transition from early to mid adolescence (at 35 days of age in rats; Spear, 2000; Lupien et al., 2009), and adolescent GR levels may be resistant to upward regulation (reviewed in Spear, 2000), a change has been linked to extinction retention deficits in adults (Knox et al., 2012). Thus, adolescents may not exhibit extinction retention deficits following trauma due to lesser GR density changes or GR changes that represent a smaller percentage change compared with adults.

Overall, we found that two different rodent models, an established PTSD model, SPS, and a novel model, SPSp, both replicated adult extinction retention deficits found in PTSD patients (Garfinkel et al., 2014), suggesting that extinction retention deficits might represent PTSD-like phenotype that is not dependent on trauma type. Thus, SPS and SPSp could be valuable models of deficits in extinction retention following trauma in adulthood. Yet, only SPS increased acoustic startle response, suggesting that trauma type may have differential effects on mechanisms underlying distinct PTSD symptoms. To fully ascertain the utility of the SPSp model, future investigations will need to assess further behavioral and physiological symptoms of PTSD following SPSp exposure. Despite the novelty of the SPSp model, following SPSp and the established SPS model, adults exhibited PTSD-like extinction retention deficits, whereas adolescent rats showed no such deficits following either trauma type, suggesting that adolescents may be resilient to cognitive effects of these types of trauma exposure.

DEFINITIONS

Extinction—the ability to extinguish fear responses when presented with a cue that was previously negatively reinforced, but is no longer associated with a negative stimulus.
Extinction retention—the ability to recall and apply extinction to attenuate fear responses upon re-exposure to the extinction context.

DATA AVAILABILITY

The raw data supporting the conclusions to the present manuscript will be provided to any qualified researcher upon request.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the study. SN, CC and LC conducted the study. CC analyzed the data. CC, LC and IL wrote the manuscript.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Extinction of Fear Memory Attenuates Conditioned Cardiovascular Fear Reactivity

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Post-traumatic stress disorder (PTSD) is characterized by a heightened emotional and physiological state and an impaired ability to suppress or extinguish traumatic fear memories. Exaggerated physiological responses may contribute to increased cardiovascular disease (CVD) risk in this population, but whether treatment for PTSD can offset CVD risk remains unknown. To further evaluate physiological correlates of fear learning, we used a novel pre-clinical conditioned cardiovascular testing paradigm and examined the effects of Pavlovian fear conditioning and extinction training on mean arterial pressure (MAP) and heart rate (HR) responses. We hypothesized that a fear conditioned cardiovascular response could be detected in a novel context and attenuated by extinction training. In a novel context, fear conditioned mice exhibited marginal increases in MAP (∼3 mmHg) and decreases in HR (∼20 bpm) during CS presentation. In a home cage context, the CS elicited significant increases in both HR (100 bpm) and MAP (20 mmHg). Following extinction training, the MAP response was suppressed while CS-dependent HR responses were variable. These pre-clinical data suggest that extinction learning attenuates the acute MAP responses to conditioned stimuli over time, and that MAP and HR responses may extinguish at different rates. These results suggest that in mouse models of fear learning, conditioned cardiovascular responses are modified by extinction training. Understanding these processes in pre-clinical disease models and in humans with PTSD may be important for identifying interventions that facilitate fear extinction and attenuate hyper-physiological responses, potentially leading to improvements in the efficacy of exposure therapy and PTSD–CVD comorbidity outcomes.

Keywords: PTSD, fear memory, Pavlovian fear conditioning, cardiovascular disease, extinction, physiological hyperarousal

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder in which over-generalization of fear and impaired fear extinction recall can lead to a permanent state of hyperarousal and emotional numbing that can negatively impact daily life (Desmedt et al., 2015). PTSD is in part characterized by an inability to adequately suppress fear responses under safe conditions (Jovanovic et al., 2010; Sijbrandij et al., 2013) and is often accompanied by exaggerated physiological symptoms [e.g., increased heart rate (HR), blood pressure, and sympathetic drive]

(American Psychiatric Association, 2013; Edmondson et al., 2013; Vaccarino et al., 2013; Park et al., 2017). Enhanced acute stress responses may contribute to increased rates of acute cardiac events and cardiovascular disease (CVD) risk in PTSD patients (Edmondson et al., 2013; Edmondson and von Känel, 2017; Myers, 2017), however, the underlying physiological mechanisms remain unclear. Further examination of altered cardiovascular reactivity that occurs in states of fear is therefore required to better understand the link between PTSD and CVD.

Classical rodent and human models of fear conditioning are commonly used to study both the expression and extinction of learned fear, providing practical methods to identify PTSD biomarkers and prevention strategies. For example, extinction training is believed to be analogous to exposure therapy, which is one of the most effective therapeutic treatments for PTSD, phobias, and anxiety disorders (Morrison and Ressler, 2014). Extinction training results in the formation of a new extinction memory, and a gradual reduction of conditioned response (Quirk, 2002). Extinction recall occurs when the extinction memory is retrieved and expressed at a later time (Quirk et al., 2000; Milad et al., 2009). Due to its potential as a therapeutic target, many efforts have recently been made to discover treatments that may strengthen or facilitate extinction recall (Bukalo et al., 2014; Bowers and Ressler, 2015). Critical to these efforts is the ability to accurately measure extinction-specific changes to conditioned responses.

While most assessments of extinction in animal models rely entirely on changes in freezing behavior, conditioned cardiovascular responses have previously been shown to serve as important physiological correlates of fear learning (Gaburro et al., 2011). For example, rodent studies using radiotelemetry demonstrate that HR (Tovote et al., 2005b), HR variability (Stiedl et al., 2009), and blood pressure (Hsu et al., 2012) are reliable indicators of fear memory acquisition that can be used to distinguish between non-specific and associative threat responses. However, few studies have examined the effects of fear extinction on cue-dependent conditioned cardiovascular responses. Within these studies, the focus has primarily been on reductions in HR reactivity (Stiedl, 1999; Stiedl et al., 2009; Camp et al., 2012; Hager et al., 2014).

Under certain conditions, re-exposure to a conditioned stimulus causes co-activation of the sympathetic and parasympathetic branches of the autonomic nervous system. Blockade of sympathetic outflow with propranolol decreases fear-associated tachycardia following auditory fear conditioning, while atropine enhances it (Iwata and LeDoux, 1988). Similar results have also been reported in contextual models of fear conditioning (Carrive, 2006). These findings suggest that the conditioned cardiovascular response consists of activation of the sympathetic nervous system (SNS), which is partially buffered by simultaneous activation of the parasympathetic nervous system (PSNS). Through cardiac nerves and circulating adrenal catecholamines, sympathetic activation results in an increased HR. Mean arterial pressure (MAP) also increases in response to sympathetically mediated blood vessel constriction (Baudrie et al., 2001). Parasympathetic activation, on the other hand, simultaneously acts to lower HR via cholinergic modulation of sinoatrial node activity. Given that blood pressure and HR are under autonomic regulation, but with distinct temporal and network control (Tovote et al., 2005a), both parameters should be considered during assessment of fear expression and extinction recall. To date, the effects of extinction training on conditioned blood pressure responses have not been directly tested.

Here we developed a novel conditioned cardiovascular response behavioral paradigm to examine the effects of extinction training on cue-dependent blood pressure and HR responses. We hypothesized that a fear conditioned cardiovascular response could be detected in a novel context and attenuated by extinction training. Closely evaluating cardiovascular reactivity to conditioned fear may contribute to a better understanding of the hyper-physiological responses in PTSD and associated CVD risk. Physiological measures of inhibitory learning could also lead to more accurate assessments of extinction efficiency in animal models. The objectives of this study were to examine the real-time behavioral and cardiovascular responses to fear conditioning and extinction using a mouse model, and to examine the effects of extinction training on MAP and HR responses during extinction recall.

MATERIALS AND METHODS

Animals

Adult male (3–4 months old) C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME, United States) were used for all experiments. The C57BL/6 strain is a commonly used inbred strain that has been shown to extinguish fear responses well in comparison to other strains (Hefner et al., 2008; Camp et al., 2012). Mice were housed individually in temperature and humidity-controlled polyethylene cages on a 12 h light/dark cycle. Animals were supplied with water and food *ad libitum* for the duration of the experiments. All procedures were approved by the Institutional Animal Care and Use Committee at The George Washington University and were in compliance with National Institutes of Health guidelines.

Radiotelemetry

Telemeter Implantation, Data Collection and Analysis

Animals were anesthetized with an IP injection of ketamine/xylazine and maintenance of anesthesia was assessed with toe pinch. HDX-11 transmitters [Data Sciences International (DSI), St. Paul, MN, United States] were implanted subcutaneously, with a blood pressure transducer inserted into the carotid artery. Animals were allowed to recover for 14 days before beginning behavioral experiments. Blood pressure signals were sampled at a rate of 500 Hz. Blood pressure and activity data were continuously collected during 24 h baseline measurements, fear conditioning, extinction training, and cardiovascular response tests. Blood pressure data were analyzed using Ponemah software version 6.3 (DSI). Baseline day, night, and 24 h averages were calculated from 12 h epochs corresponding with the light/dark cycle. HR was derived from the blood pressure channel.

Behavioral Experiments

Fear Conditioning

For 2 days prior to fear conditioning, animals were exposed to the chamber to habituate them to handling and context. Auditory fear conditioning was performed in conditioning test cages (7″ × 7″D12″H; model H10-11M-TC) equipped with overhead cameras and grid shock floors (H10-11M-TC-SF). Test cages were enclosed in sound attenuating isolation cubicles (Model H10-24T; Coulbourn Instruments, Holliston, MA, United States). Fear conditioned animals received both the conditioned stimulus and unconditioned stimulus (CS-US group), and were presented with CS-US pairings of a 30 s auditory cue (6 kHz, 75 db) co-terminating with a mild footshock (0.5 s, 0.5 mA). There was a 3 min 30 s inter-trial interval between each pairing. Control (CS group) animals were exposed to the CS under fear conditioning conditions but never received a footshock. Fear conditioning test cages were cleaned thoroughly with 70% ethanol before each session. After conditioning, animals were returned to the home cage for 24 h before extinction training.

Extinction Training

Two rounds of extinction training were performed in modified test cages to distinguish them from the fear conditioning context. The shock grid was replaced with a clear plexiglass floor and the clear chamber walls were covered with paper. The chambers were wiped down with water and peppermint soap before each extinction session. Extinction training occurred 24 and 48-h following fear conditioning (**Figure 1A**). A 5 min pre-CS period preceded the first tone presentation in each test. Extinction sessions consisted of either 30 conditioned stimulus tone trials in CS and CS-US groups, or 35 trials in the Extinction (Ext) group. Each trial lasted 30 s and was followed by a 30 s inter-trial interval. The No Extinction (No Ext) control group was placed into the modified context for the same duration, but was not exposed to the conditioned stimulus during extinction training. A non-conditioned (No US) control group was not included in the home cage extinction experiments based on previous studies showing that the auditory stimulus would evoke only mild, transient cardiovascular effects that do not differ significantly from baseline values (Tovote et al., 2005a). The percentage of time spent freezing was calculated using Freezeframe 3.32 (Coulbourn Instruments). All behavioral experiments occurred during the light phase (7am–7pm).

Cardiovascular Response Tests

The conditioned cardiovascular response was measured in the home cage 24-h after fear conditioning (Cardiovascular Response Test 1), 24-h after the first extinction session (Cardiovascular Response Test 2), and 1-h after the second extinction session (Cardiovascular Response Test 3) (**Figure 2**). The home cage was placed in a sound attenuating chamber, and a speaker was positioned on top of the cage. Mice were left undisturbed for 1 h before remotely initiating a 4 CS memory test to determine the effects of extinction training on the conditioned cardiovascular response during extinction recall.

Statistical Analysis

Prism 6.0 (Graphpad Software Inc., La Jolla, CA, United States) was used for statistical evaluation of mouse data. Data are presented as the mean ± SEM, with p-values <0.05 considered statistically significant. Analysis of variance (ANOVA) for repeated measures was used for statistical analysis followed by Bonferroni tests for *post hoc* comparison.

RESULTS

Behavioral and Cardiovascular Responses During Extinction Training in a Novel Context

To examine the cardiovascular responses to conditioned fear during extinction training, two groups of animals were equipped with radiotelemeters and were either exposed to a fear conditioning protocol (CS-US group) or exposed to five auditory cues without footshocks (CS group). Behavioral and cardiovascular responses (freezing behavior, MAP, and HR) were simultaneously monitored in the extinction context during two consecutive days of extinction training in the CS-US and CS groups (**Figure 1A**).

Freezing Behavior

As expected, a progressive increase in freezing response to CS exposure was observed only in the CS-US animals (**Figure 1B**). Simultaneous behavioral and cardiovascular responses to extinction trials across 2 days are shown in **Figures 1C–K**. The average of the first 4 CS (CS1–4) presentations on Day 1 and the last 4 CS presentations on Day 2 (CS27–30) were taken as measures of acquisition and extinction, respectively (Yang et al., 2016). On Day 1 of extinction training, the CS-US group exhibited increased freezing throughout the 30 CS presentations (**Figure 1C**). Fear acquisition was demonstrated in the CS-US animals compared to the CS by group differences in freezing during the first 4 CS presentations (71% ± 4 vs. 10% ± 3, $p < 0.05$) (**Figure 1E**). CS-US animals showed a significant reduction in freezing from Day 1–2, with a significant group x time interaction [$F(1,17) = 20.68$, $p = 0.0003$] (**Figure 1E**).

Mean Arterial Pressure and HR in Novel Context

At baseline on Day 1 of extinction training, MAP and HR were similar between groups (**Figures 1F,G**). In response to the first CS, a trend for a small increase in MAP (3 mmHg), which remained elevated throughout the extinction session and a corresponding bradycardic HR response were observed in the CS-US group compared to control. Overall, both groups showed a slow reduction in HR throughout the extinction session. Following the last CS presentation, the CS-US group displayed a rapid increase in HR corresponding with the cessation of freezing. There was a significant group by time interaction [$F(48,864) = 5.032$, $p < 0.0001$], with group differences in HR throughout the No CS period (**Figure 1G**).

On Day 2 of extinction training, in response to CS, there was a similar trend for a small transient increase in MAP and bradycardic response within the CS-US animals only during CS

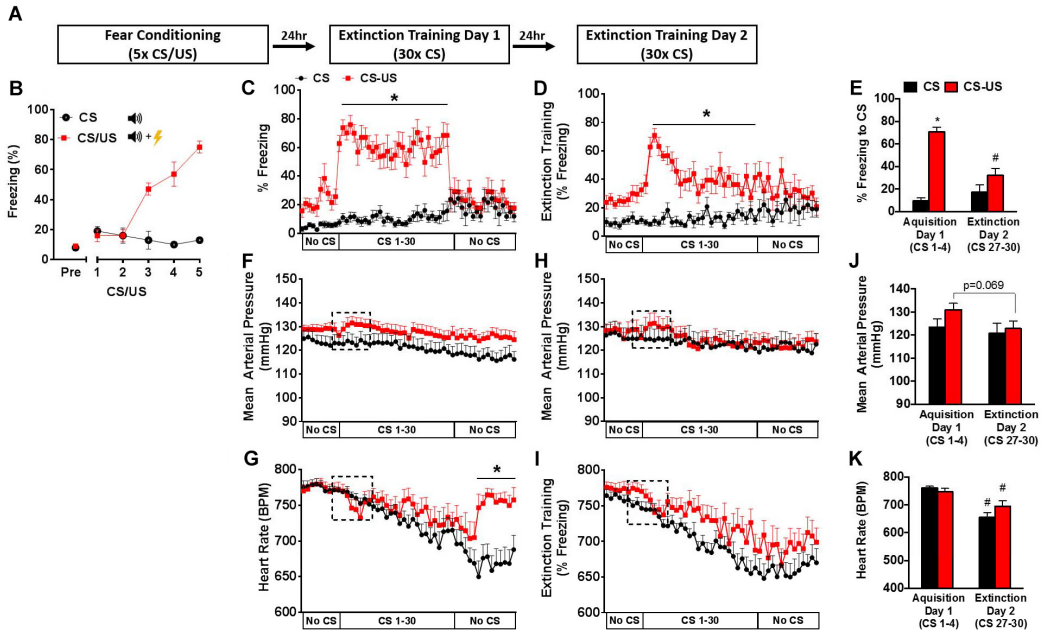

FIGURE 1 | Behavioral and cardiovascular changes during extinction training. Schematic of fear conditioning and 2-day extinction protocol **(A)**. Freezing behavior during fear conditioning **(B)**. Freezing behavior **(C,D)**, mean arterial pressure **(F,H)**, and heart rate **(G,I)** during 2-day extinction training protocol. Average freezing behavior **(E)**, mean arterial pressure **(J)**, and heart rate **(K)** during CS 1–4 (Day 1), and CS 27–30 (Day 2) in the extinction context. Error bars indicate standard error of the mean (n = 9–11 per group, $^{*}p < 0.05$ CS vs. CS-US; $^{\#}p < 0.05$ Day 1 vs. Day 2).

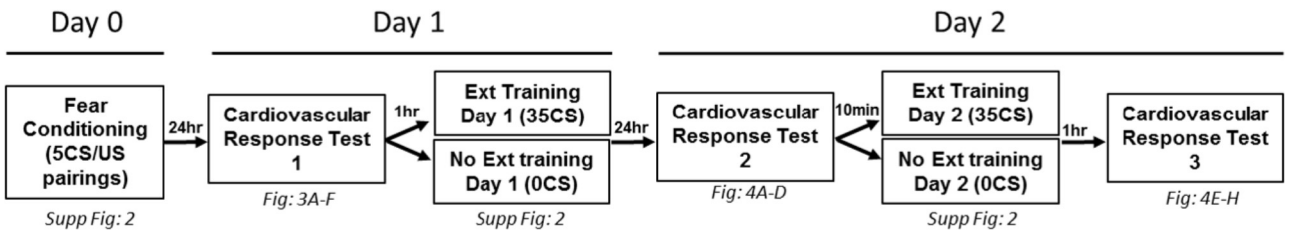

FIGURE 2 | Schematic of fear conditioning and testing protocol for home cage extinction studies. All animals were fear conditioned on Day 0. Conditioned cardiovascular responses were measured in the home cage 24 h later (Cardiovascular Response Test 1). Animals were then placed into a novel context and exposed to either 35 CS (Ext group) or no CS (No Ext group) for the first extinction session. On Day 2, a home cage test (Cardiovascular Response Test 2) was conducted prior to the second extinction session. The Ext group was then exposed to another 35CS extinction session. One hour later, all animals were tested in the home cage (Cardiovascular Response Test 3).

1–4. For the remainder of the session, MAP was similar between groups while HR slowly declined (−100 bpm). Unlike Day 1 of extinction, the CS-US group did not exhibit the sharp increase in HR during the No CS period. MAP responses were then evaluated across days between extinction sessions (**Figure 1J**). When comparing the first 4 CSs (Day 1 of extinction) and the last 4 CSs (Day 2 of extinction), repeated measures-ANOVA revealed a main effect of time, [$F(1,18) = 14.28$, $p = 0.0014$], and a trend for group by time interaction [$F(1,18) = 3.736$, $p = 0.0691$]. There was also a reduction in HR in response to CS from Day 1 of extinction to Day 2 of extinction in CS-US animals (**Figure 1K**). However, because a comparison of HR between groups revealed a significant group by time interaction [$F(1,18) = 5.936$, $p = 0.0254$] with a main effect of time [$F(1,18) = 52.52$, $p < 0.0001$] but not group [$F(1,18) = 0.3778$, $p = 0.5465$], this reduction cannot be attributed solely to extinction learning and is more likely a result of within session HR recovery.

In addition, both groups displayed a slow, gradual recovery of HR throughout each session, but neither MAP nor HR returned to resting baseline levels by the end of the test on either day. This suggests that handling and novel context exposure contribute to the elevations of MAP and HR regardless of whether or not the animals were fear conditioned. Furthermore, because these HR elevations are similar at the beginning of both days of extinction training, the effects of habituation appear to be minimal. These findings are consistent with previous studies showing that novel environments can induce HR elevations in mice despite previous habituation (Liu et al., 2013). Because elevated baseline cardiovascular measures could potentially mask cardiovascular adjustments caused by the conditioned stimulus,

we next examined the effects of extinction training on fear conditioned (CS-US) mouse cardiovascular reactivity in a home cage environment.

CS-Dependent Conditioned Cardiovascular Responses in the Home Cage

Mean Arterial Pressure and HR Response (Cardiovascular Response Test 1)

Conditioned physiological responses are highly dependent on the resting physiological state, which in part influences the cardiovascular response to conditioned stimuli. Therefore, in order to examine both the conditioned cardiovascular responses and the effects of extinction, cardiovascular response tests were conducted in the home cage environment (Stiedl et al., 2004). Two groups of mice (No Ext and Ext) were equipped with radiotelemeters and all animals were fear conditioned as previously described (**Figure 2**). 24 h after fear conditioning (prior to extinction training), mice were exposed to 4 CS trials in the home cage (Cardiovascular Response Test 1). Baseline activity levels, blood pressure and HR during the pre-CS period were significantly lower than in the extinction context in both groups (**Supplementary Figure S1**), while Pre-CS cardiovascular baselines were similar to mean 12-h baselines during the light cycle (**Supplementary Table S1**). As shown in **Figure 3A**, a two-phase pressor response was observed during the first CS presentation of the 4 CS test in both groups. This consisted of a rapid rise (general arousal) of approximately 10 mmHg within the first 10 s of the CS, followed by a slower, steady increase which has previously been attributed to associative learning (Tovote et al., 2005a; **Figure 3B**). Subsequent CS presentations also coincided with a rapid rise in MAP. Peak MAP (No Ext 122 ± 5; Ext 124 ± 3 mmHg) were reached within the first 3 s of the second CS. MAP averaged over the 4 CSs was significantly increased from Pre-CS baselines in both No Ext and Ext groups confirming a strong conditioned CS-dependent MAP pressor response in these animals (**Figure 3C**). An ANOVA comparing the 4 CS MAP revealed no significant main effect of group [$F(1,13) = 1.212$, $p = 0.2908$] or group by time interaction [$F(1,13) = 0.7181$, $p = 0.4121$], yet did reveal a significant main effect of time [$F(1,13) = 33.38$, $p < 0.0001$]. As shown in **Figures 3D–F**, there was an overall significant increase (∼100 bpm) in HR over the 4 CS trials in both groups relative to pre-CS baseline. An ANOVA revealed a main effect of time [$F(1,11) = 10.59$, $p = 0.0077$], with no group by time interaction [$F(1,11) = 0.01081$, $p = 0.9190$] and no main effect of group [$F(1,11) = 0.6541$, $p = 0.4358$]. In summary, these data demonstrate a consistent CS-dependent home cage pressor response that was accompanied by an overall increase in HR 24 h following fear conditioning.

Extinction of CS-Dependent Cardiovascular Responses

Mean Arterial Pressure and HR Response (Cardiovascular Response Test 2)

The two groups of animals subsequently went through either an Ext or No Ext training protocol as shown in **Figure 2**. Following extinction training, animals were tested at two time points in order to examine (A) long-term retention of the CS-dependent cardiovascular response and (B) within-session cumulative effects of additional extinction trials. To examine the long-term retention of extinction learning during the CS-dependent cardiovascular response, a 4-tone cardiovascular response test (#2) was conducted 24 h following extinction training. As shown in **Figures 4A,C**, 5 min pre-CS baseline MAP and HR were similar between Ext and No Ext groups (MAP: 103 ± 6 vs. 108 ± 4 mmHg; HR: 522 ± 49 vs. 515 ± 28 bpm) and within the range of normal daytime averages (**Supplementary Table S1**). Similar to the initial CS response in **Figure 3A**, the No Ext group displayed a biphasic pressure increase that was characterized by a rapid increase in MAP (∼10 mmHg) within the first 10 s, followed by a slower increase that persisted until the end of the first CS. In these animals, peak MAP (132 mmHg) was again reached during the first 3 s of the second CS presentation (**Figure 4A**). An ANOVA comparing the 4 CS MAP revealed a significant group by time interaction [$F(1,13) = 5.164$, $p = 0.0407$] (**Figure 4B**). *Post hoc* tests revealed that MAP in the No-Ext group significantly increased from baseline, while MAP in the Ext group did not. In animals that underwent extinction training (Ext group), the initial rise in blood pressure of ∼10 mmHg was observed, however, the second phase was distinctively absent (**Figure 4A**). Despite a trend for a change in HR increase to CS in the No Ext group (**Figure 4C**), HR changes were highly variable (**Figure 4C**). An ANOVA comparing the 4 CS HR revealed no significant group by time interaction [$F(1,13) = 2.463$, $p = 0.1406$] (**Figure 4D**). These results demonstrate that 24 h after training, the conditioned MAP response is significantly blunted in the Ext group while the HR response to CS is not significantly different between groups, and thus may track closely to the extinguished freezing behavior.

Mean Arterial Pressure and HR Response (Cardiovascular Response Test 3)

To further evaluate the effects of extinction on the conditioned cardiovascular response, the Ext group was exposed to a second day of extinction training, which resulted in an extinction effect as determined by freezing responses to CS (**Supplementary Figure S2**). All animals were tested 1 h later in their home cage for CS-dependent cardiovascular responses. Consistent with the two previous cardiovascular response tests, a two-phase blood pressure response was observed in the No Ext animals and the peak MAP (122 ± 7) was reached within the first 3 s of the second CS (**Figure 4E**). In the Ext group a small, slow increase in MAP was observed throughout the test (∼5 mmHg) but this increase did not appear to be associated with CS onset. Peak MAP in the Ext group was significantly lower than that of the No Ext group (106 mmHg ± 3 vs. 122 ± 7, $p < 0.05$) (**Figure 4E**). Moreover, both phases of the MAP response were absent in the Ext animals. An ANOVA comparing the 4 CS MAP revealed a significant group by time interaction [$F(2,16) = 4.409$, $p = 0.0298$] (**Figure 4F**). Similar comparisons of HR indicate no group by time interaction [$F(1,12) = 1.504$, $p = 0.2436$] (**Figure 4H**). Peak HR were not significantly different between No Ext (555 ± 61) and Ext (523 ± 28) groups

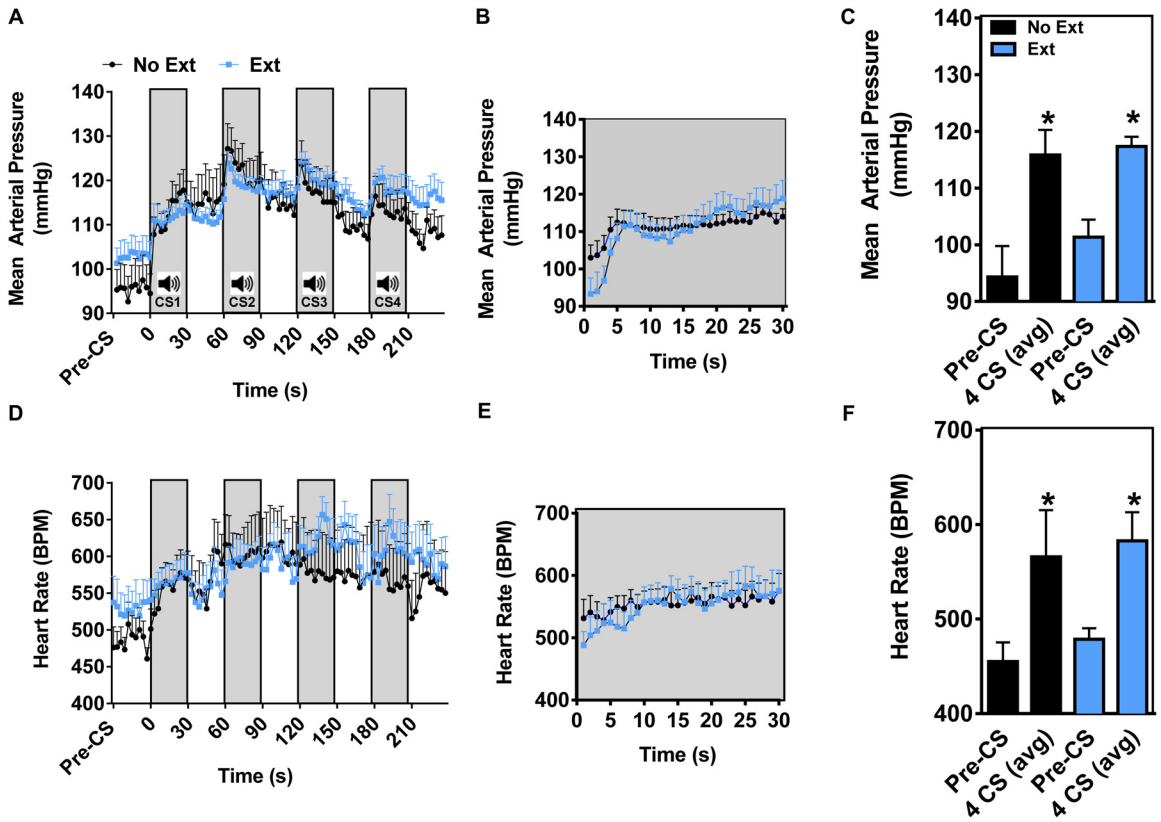

FIGURE 3 | Pre-extinction training CS-dependent cardiovascular response test 1 (home cage). Mean arterial pressure **(A)**, and heart rate **(D)** collected during 4 CS presentations and averaged every 3 s. **(B,E)** Depict second-by-second fluctuations in MAP and HR during the first CS presentation. Average MAP **(C)** and HR **(F)** during the 5 min pre-CS period, and over 4 CS presentations (n = 6–9 per group, $^*p < 0.05$ pre-CS vs. 4 CS avg).

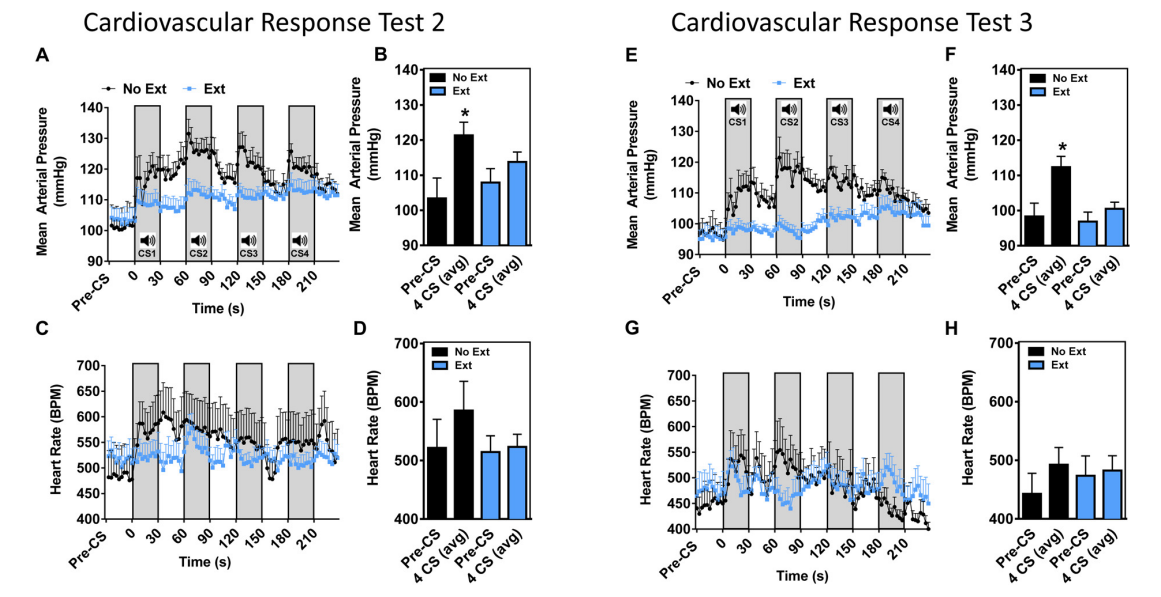

FIGURE 4 | Post-extinction training CS-dependent cardiovascular response tests 2 and 3 (home cage). Mean arterial pressure **(A)** and heart rate **(C)** collected during cardiovascular response test 2. Average MAP **(B)** and HR **(D)** during 4 CS presentations. Mean arterial pressure **(E)**, and heart rate **(G)** collected during cardiovascular response test 3. Average MAP **(F)** and HR **(H)** during 4 CS presentations (n = 6–9 per group, $^*p < 0.05$ pre-CS vs. 4 CS avg).

(**Figure 4G**) and remained within the range of normal resting HRs (**Supplementary Table S1**).

DISCUSSION

The current findings demonstrate that recall of consolidated extinction memories can modulate the conditioned cardiovascular response, which is influenced by context-dependent differences in blood pressure and HR sensitivity. Alterations of the conditioned MAP response may serve as a novel index in the evaluation of extinction efficiency and may aid in identifying the hyper-physiological underpinnings of PTSD and co-morbid CVD-PTSD.

Conditioned HR (Fitzgerald and Martin, 1971) and BP (Iwata and LeDoux, 1988) during CS presentation have previously been reported in the conditioning context, which elicits contextual and non-specific stress related alterations in cardiovascular activity. Furthermore, freezing behavior and locomotor activity are closely linked to changes in blood pressure and HR (Vliet et al., 2003) and can potentially confound conditioned cardiovascular responses. To minimize these disruptions, conditioned cardiovascular responses are often evaluated in the resting or home cage environment (Iwata and LeDoux, 1988; Stiedl et al., 2004, 2009; Camp et al., 2012). In the present study, we predicted that testing in a novel context would reduce contextual fear and minimize non-specific stress enough to observe HR and MAP responses to CS between conditioned (CS-US) and control (CS) groups. Additionally, we hypothesized that blood pressure and HR responses in the extinction context would decrease across days as a result of extinction training.

During the first extinction session in a novel context (Day 1), freezing in response to CS confirmed fear acquisition in conditioned animals, while the magnitude of the cardiovascular response was not significantly different from controls. Throughout the CS period on Days 1 and 2 of extinction, MAP remained high while HR gradually fell in both groups over the CS period, indicating a within-session habituation effect on HR but not MAP. While the relatively small conditioned cardiovascular response limits the interpretation of these findings, it is possible that the small elevation of MAP in the CS-US group on Day 1 was sustained by repeated CS presentation and extinguished by repeated CS exposure, while HR increases were offset by reductions in physical activity during times of freezing.

During the second extinction session in a novel context (Day 2), there was trend for a reduction in MAP in the CS-US group only, while HR was significantly reduced in both groups. These data may suggest an extinction-specific reduction of blood pressure response that was distinct from a generalized habituation-like effect on HR. Interestingly, following cessation of CS presentation during extinction training on Day 1, CS-US animals displayed a rapid increase in HR. While this may have been a consequence of increased locomotion due to an immediate reduction in freezing, there were no overall differences in activity levels between groups during this time period. Because conditioned HR responses are controlled by activation of both the sympathetic and parasympathetic components of the autonomic nervous system (Iwata and LeDoux, 1988; Carrive, 2006), and freezing behavior is accompanied by parasympathetically driven HR deceleration (Roelofs, 2017), this increase in HR may be mediated in part by parasympathetic withdrawal. Similarly, on both day 1 and 2 in the novel context we observed a initial brief decelerative HR response upon CS onset (**Figures 1G,I**), which is likely parasympathetically mediated (Iwata and LeDoux, 1988). This deceleration in HR is consistent with prior human fear research using HR (typically associated with fear responding) (Lang et al., 2011; Sege et al., 2017). Taken together, these findings suggest that CS exposure in a novel context may elicit MAP and HR responses at different rates, with changes in autonomic regulation of HR first emerging upon CS onset (deceleration) and again when the conditioned stressor is removed (acceleration).

To further investigate extinction-dependent responses in our mouse model, while minimizing context-enhanced basal cardiovascular effects (see **Supplementary Figure S1**), we next sought to determine whether repeated CS exposure could extinguish conditioned cardiovascular responses when measured in the home cage. As opposed to previous studies using an extended single CS (Stiedl, 1999; Stiedl et al., 2004, 2007, 2009; Tovote et al., 2005a), we evaluated the cardiovascular response using 4 CS presentations. A 4 CS memory test was used based on the following considerations: (1) the cumulative effect of multiple CS presentations could be determined by using shorter CS presentations spaced with inter-trial intervals; (2) the test duration would be long enough to encompass both the fast, sympathetic-mediated vasoconstriction response (Baudrie et al., 1997), and the slower humoral-mediated responses (Tovote et al., 2005a); (3) the low number of CS presentations would minimize extinction in the No Ext group caused by multiple testing sessions.

In the home cage context, both groups of mice showed stable resting MAP and HR during the pre-CS period. Prior to extinction training and 24-h post-fear conditioning, MAP increased across the 4 CS trials in a CS-dependent manner. This increase reached ∼20 mm Hg and was characterized by two phases of rise. The initial, rapid increase is thought to result from general arousal and lasts for approximately 5 s. The second phase of the conditioned blood pressure response, which has been shown to result from associative fear learning in mice, allows for distinction between general arousal and associative memory (Tovote et al., 2005a). While HR was increased in both groups across CS trials, the magnitude of these responses was markedly less than previously described (Stiedl and Spiess, 1997). Variations in conditioning protocols and testing procedures likely account for these differences (Stiedl and Spiess, 1997; Stiedl, 1999; Stiedl et al., 2004, 2007; Tovote et al., 2005a). Based on the clear presence of an associative blood pressure response in this experiment, and the ability of a 30 CS extinction protocol to reduce long-term fear expression (Marvar et al., 2014), we reasoned that the MAP response would be suppressed during extinction recall.

To evaluate the cardiovascular response during long-term extinction memory recall, animals were re-tested 24 h after extinction training in the home cage. The No Ext group exhibited a CS-dependent MAP increase across 4 CS home cage trials, while extinguished animals did not. Furthermore, the general

arousal phase of the MAP response was present, while the associative phase was absent in the Ext group. These data suggest that extinguished animals respond with an acute, generalized arousal similar to non-extinguished animals, while the associative component of the blood pressure response can be modified by non-reinforced CS exposure.

Despite the extinction-dependent reduction in MAP response, there was no significant difference in HR response between groups. Although we observed a trend for increased HR in the No Ext across the 4 CS presentations, it was not significantly different from the pre-CS period at this time point. We attribute the lack of a conditioned HR response to the increased variability and small conditioned HR response observed in this model. Either the fear conditioning protocol did not result in a strong enough conditioned HR response to detect the effects of extinction between Ext and No Ext groups, or the HR response was so sensitive to CS presentation in the home cage that it was quickly extinguished in both groups following the first home cage test. In either case, the results of this study point to MAP as a reliable index of extinction in mouse models of fear conditioning. Taken together, the MAP and HR data from these experiments show that that the conditioned blood pressure response is significantly blunted by extinction training while the HR response did not allow for distinction between extinguished and non-extinguished control animals.

Short-term effects of an additional extinction training session were also evaluated during the final home cage test. Consistent with the two previous cardiovascular response tests, a two-phase blood pressure response was observed in the No Ext animals with peak MAP values occurring within the first 3 s of the second CS (**Figure 4E**). There were no significant HR responses in either group at this timepoint. The results of this extinction test confirm a reduction in the MAP response in the Ext group and show minimal change of HR in response to CS in either group. Interestingly, conditioned HR responses in our mouse model in the home cage were not as robust as those previously reported by other groups (Stiedl and Spiess, 1997; Tovote et al., 2005a). As a result, HR increases in resting animals were seemingly extinguished during home cage testing. Future studies will need to address the effects of conditioned stimulus intensity and duration on HR responses.

In summary, the present study demonstrates for the first time that extinction training attenuates the acute blood pressure responses evoked by conditioned stimuli and that in this behavioral paradigm, MAP was a more reliable measure of conditioning and extinction than HR. Moreover, this reduction of the fear-induced cardiovascular response appears to be independent of activity-related behavioral changes. While there is some evidence to suggest that extinction training can attenuate cardiac responses in humans (Panitz et al., 2015), future studies are required to determine the impact of extinction-based therapeutic interventions on cardiovascular reactivity in PTSD patients. Such studies may potentially lead to improvements in extinction-based therapies and PTSD–CVD comorbidity outcomes.

AUTHOR CONTRIBUTIONS

PM and APS contributed to the conception of the work, data analysis, results interpretation, drafting, revision and final approval of the article. AVS and JP contributed to data analysis, results interpretation, revision and final approval of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00276/full#supplementary-material

FIGURE S1 | Baseline cardiovascular measures in training and home cage contexts. Activity level **(A)**, mean arterial pressure **(B)**, diastolic pressure **(C)**, systolic pressure **(D)**, and heart rate **(E)** over the 5 min pre-CS period in each context (n = 9–11 per group. *$p < 0.05$ Training vs. Home Cage).

FIGURE S2 | Freezing behavior during fear conditioning **(A)**, extinction training Day 1 **(B)**, and extinction training Day 2 **(C)**. All animals received a footshock and CS during fear conditioning (n = 6–9 per group).

TABLE S1 | Baseline day/night (12 h) mean arterial pressure (MAP), heart rate (HR), and activity levels in mice prior to fear conditioning.

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Single Prolonged Stress as a Prospective Model for Posttraumatic Stress Disorder in Females

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Sex plays an important role in susceptibility to stress triggered disorders. Posttraumatic Stress disorder (PTSD), a debilitating psychiatric disorder developed after exposure to a traumatic event, is two times more prevalent in women than men. However, the vast majority of animal models of PTSD, including single prolonged stress (SPS), were performed mostly with males. Here, we evaluated SPS as an appropriate PTSD model for females in terms of anxiety, depressive symptoms and changes in gene expression in the noradrenergic system in the brain. In addition, we examined intranasal neuropeptide Y (NPY) as a possible treatment in females. Female rats were subjected to SPS and given either intranasal NPY or vehicle in two separate experiments. In the first experiment, stressed females were compared to unstressed controls on forced swim test (FST) and for levels of expression of several genes in the locus coeruleus (LC) 12 days after SPS exposure. Using a separate cohort of animals, experiment two examined stressed females and unstressed controls on the elevated plus maze (EPM) and LC gene expression 7 days after SPS stressors. SPS led to increased anxiety-like behavior on EPM and depressive-like behavior on FST. Following FST, the rats displayed elevated tyrosine hydroxylase (TH), CRHR1 and Y1R mRNA levels in the LC, consistent with increased activation of the noradrenergic system. The expression level of these mRNAs was unchanged following EPM, except Y1R. Intranasal NPY at the doses shown to be effective in males, did not prevent development of depressive or anxiety-like behavior or molecular changes in the LC. The results indicate that while SPS could be an appropriate PTSD model for females, sex differences, such as response to NPY, are important to consider.

Keywords: anxiety, CRH, depression, females, locus coeruleus, mRNA, neuropeptide Y, tyrosine hydroxylase

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INTRODUCTION

Sex differences are prevalent in neuropsychiatric disorders. Men tend to be more susceptible to attention-deficit hyperactivity disorder and substance abuse while depression, eating and anxiety disorders are more prevalent in women (Hudson et al., 2007; Marcus et al., 2008; Bangasser and Valentino, 2014; Kucharska, 2017; Green et al., 2018). Moreover, women are at nearly double the risk for developing symptoms of posttraumatic stress disorder (PTSD) compared to men (Ditlevsen and Elklit, 2010). PTSD is a disabling, long-lasting, and difficult-to-treat neuropsychiatric disorder that develops in a subset of individuals

after exposure to traumatic stress. Core symptomology of patients with PTSD are hyperarousal behavior, avoidance, re-experiencing of the trauma, and negative changes in cognition or mood (Friedman, 2013). It is often co-morbid with depression, drug abuse, alcoholism, increased risk of suicide, and marked psychosocial and occupational impairments.

Changes in the hypothalamus-pituitary-adrenal (HPA) axis and its regulators glucocorticoid receptor and FKBP5 and the noradrenergic system are strongly associated with PTSD symptoms (O'Donnell et al., 2004; Yehuda, 2005; Hendrickson and Raskind, 2016). The HPA axis is activated during stress releasing CRH from the hypothalamus to act on the anterior pituitary, which stimulates ACTH synthesis and release. ACTH then acts on the adrenal gland to synthesize and release glucocorticoids into circulation.

The catecholaminergic system, both peripherally and centrally, play key roles in responding to stress. Patients with PTSD have exaggerated noradrenergic activity, with elevated norepinephrine (NE) in the cerebral spinal fluid (CSF) correlating to PTSD severity (Geracioti et al., 2001). The locus coeruleus (LC), with its widespread afferents, is the major source of NE in the brain (Valentino and Van Bockstaele, 2008). In addition to responding to stress, the LC/NE regulates arousal, memory consolidation, emotion, and cognition, among other neurologic processes (Southwick et al., 1999; Kobayashi, 2001; Takeuchi et al., 2016).

A number of animal models of PTSD have been proposed and were used primarily with males (Cohen et al., 2012; Daskalakis et al., 2013; Goswami et al., 2013; Whitaker et al., 2014; Deslauriers et al., 2018). The single prolonged stress (SPS) paradigm is one of the best models for eliciting PTSD related symptoms in rodents such as anxiety, depression, hyperarousal, reduced social behavior, impaired fear extinction and cognition, as well as molecular changes in the HPA axis and NE system and evaluating possible pharmacologic interventions (Liberzon et al., 1997; Yamamoto et al., 2008, 2009; Eagle et al., 2013; Sabban et al., 2015b; Souza et al., 2017; Lisieski et al., 2018). However, few studies with SPS have included females (Keller et al., 2015; Pooley et al., 2018a,b).

Previous studies indicate that neuropeptide Y (NPY) has promise to provide therapeutic relief of PTSD symptoms in males (Serova et al., 2013, 2017; Laukova et al., 2014; Sabban et al., 2015a,b; Schmeltzer et al., 2016; Sabban and Serova, 2018; Sayed et al., 2018). NPY may be an important potential pharmacologic therapy in addition to the SSRIs paroxetine and sertraline, the only two drugs FDA approved for PTSD treatment which are not sufficiently effective (Alexander, 2012; Krystal et al., 2017). NPY is one of the most abundant endogenous peptides. It is involved in regulating many systems throughout the body including sleep, appetite, memory, anxiety, fear, and stress (Brothers and Wahlestedt, 2010; Reichmann and Holzer, 2016; Tasan et al., 2016; Kautz et al., 2017). Considerable evidence from humans and animals demonstrate an association between NPY and stress resilience (Morales-Medina et al., 2010; Thorsell, 2010; Enman et al., 2015; Kautz et al., 2017). Studies in soldiers revealed higher plasma NPY is associated with increased positive coping (Morgan et al., 2000; Yehuda et al., 2006). PTSD is associated with low plasma NPY levels and the severity of symptoms is negatively correlated with CSF NPY levels (Rasmusson et al., 2000; Morgan et al., 2003; Sah et al., 2009, 2014). NPY is proposed to provide resilience by controlling pro-stress transmitters CRH and NE (Kask et al., 2002; Heilig, 2004; Thorsell, 2010; Sah and Geracioti, 2013; Sabban et al., 2015a).

Selective delivery of NPY to the brain by intranasal administration is effective at early intervention and reversal of PTSD symptoms in male rats while avoiding peripheral side effects (Serova et al., 2013; Laukova et al., 2014; Sabban et al., 2015a; Camp et al., 2018). A recent clinical trial demonstrated high-dose intranasal NPY can reduce self-reported anxiety levels in PTSD patients (Sayed et al., 2018).

In this article, our primary goal was to evaluate SPS as an appropriate model for PTSD in female rodents in terms of anxiety, depressive symptoms and changes of gene expression in the LC. Our secondary aim was the assessment of intranasal NPY as a potential early intervention for females.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the PHS policy and NIH Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the New York Medical College's Institutional Animal Care and Use Committee (IACUC). The approved protocol number is 33-1-0517. Female Sprague-Dawley (SD) rats were purchased from Charles River (Wilmington, MA, USA). Animals were maintained on a 12-h light/dark cycle at 22°C with food and water *ad libitum*. They were housed four per cage for at least a week prior to the experiment.

Single Prolonged Stress (SPS)

SPS was performed between 9 am and 2 pm as previously described (Serova et al., 2013). The rats were immobilized for 2 h on a metal board by taping the limbs with surgical tape and restricting the motion of their head. Then, they were immediately subjected to force swim in a plexiglass cylinder (50 cm height, 24 cm diameter; Stoelting, Wood Dale, IL, USA) filled two-thirds with 24°C fresh water. They were dried and allowed to recuperate for 15 min and then exposed to ether vapor in a bell jar until loss of consciousness. Afterwards, all animals were housed two per cage and left undisturbed until experimental testing.

Intranasal NPY Administration

Rats were administered a single intranasal infusion of NPY (NeoBioSci, Cambridge, MA, USA) freshly dissolved in water, or equal volume of water (vehicle) while the animals were under the influence of ether (the last SPS stressor). A pipetteman with disposable plastic tip was used to infuse 10 μl into each nostril. Care was taken to avoid contact with the intranasal mucosa. Following intranasal administration, the head of the animal was held in a tilted back position for approximately 15 s to prevent loss of solution from the nostrils.

Experiment 1: Forced Swim Test (FST) for Depressive Symptoms

The experimental design is shown in **Figure 1A**. Forty-eight 7-week-old, naturally cycling female SD rats (150–175 g) underwent a 12-day acclimation period and were randomly assigned to experimental or control groups (n = 16). Thirty-two animals underwent SPS and were infused with 150 μg intranasal NPY (n = 16) or vehicle (n = 16) immediately afterwards while still under the influence of ether. After the 12-day consolidation period, they were tested on the forced swim test (FST) together with an unstressed control group. The animals were euthanized 30 min after the FST, vaginal smears were collected and the LC region of the brain (9.2–10.4 mM posterior to the Bregma) was isolated and frozen immediately in liquid nitrogen for molecular analysis.

Experiment 2: EPM for Anxiety Symptoms

The experimental design is shown in **Figure 1B**. Thirty-six female 7-week-old, naturally cycling SD rats (150–175 g) arrived at the animal facility and had a 1-week acclimation period. Twenty-four animals underwent SPS and were infused with 300 μg intranasal NPY (n = 12) or vehicle (n = 12) immediately afterwards. Rats were left undisturbed for 1 week and then tested on the elevated plus maze (EPM) together with unstressed controls (n = 12). The animals were euthanized immediately afterwards. Vaginal smears and LC punches were collected as described above.

Forced Swim Test (FST)

FST was performed in plexiglass cylinders filled to two-thirds with 24°C fresh water for 5 min and behavior during the forced swim was videotaped as described by Serova et al. (2013). Time spent swimming, defined as movement of the forelimbs and hind limbs, and the time spent immobile when the animal showed no movement, or only movements needed to keep its head above the water was scored by a trained individual blinded to the experimental groups.

Elevated Plus Maze (EPM)

Anxiety-like behavior was tested on the EPM as previously described (Serova et al., 2013). The apparatus (Stoelting, Wood Dale, IL, USA) 50 cm above ground level had four cross shaped platforms, two platforms with a 2-cm-high plexiglass wall were open while the other two platforms with 40-cm-high opaque walls on sides were closed. Arms of the same type were located opposite each other. Experiments were performed in a room with dim light. Animals were given 30 min to acclimate to the room prior to each experiment. Every rat was placed on the central platform with their head towards an open arm and allowed 5 min to explore the maze. The maze was cleaned with 70% ethanol between animals. The test was recorded using a tracking software (Viewer 3.0) Biobserve and the following measurements were taken; open arm (OA) and closed arm (CA) entries; total entries into all arms; duration of exploration in open and closed arms; time and frequency of risk assessment; number of head dips; and total distance traveled. Arm entry is defined as entering an arm with all four paws. Time in the arms was calculated as percent of the total time of the test. Percent of entries was calculated as percent of total open or CA entries to the total of all arm entries. Risk assessment is assessed by the rat poking its head or trunk into an OA while its hind quarters were located in one of the

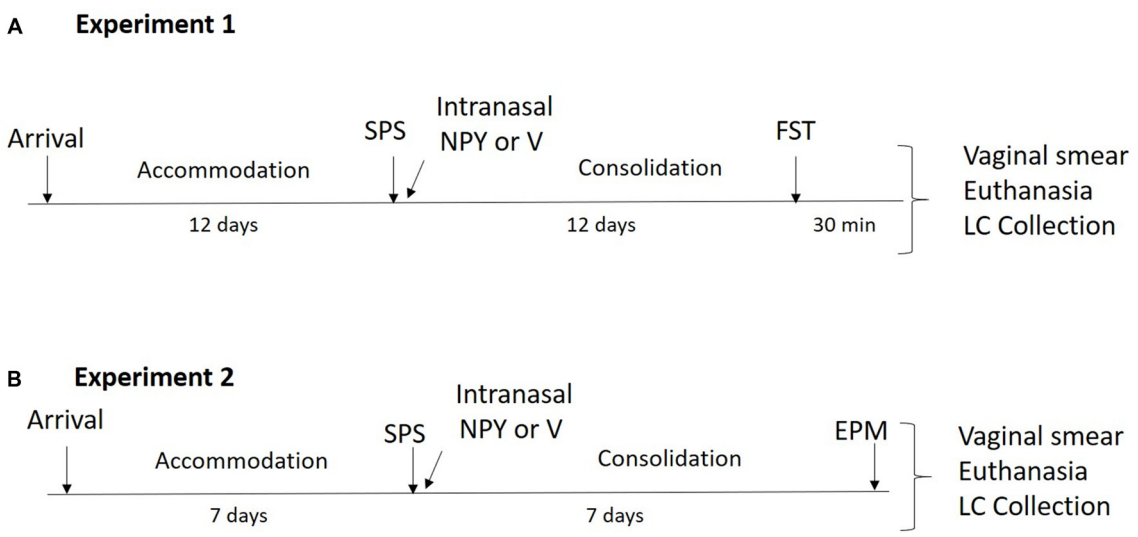

FIGURE 1 | Design of the experiments. **(A)** In experiment 1, animals underwent a 12-day accommodation period upon arrival after which they were exposed to single prolonged stress (SPS) stressors and were administered 150 μg of intranasal neuropeptide Y (NPY) or vehicle while under the influence of ether. After a 12-day consolidation period, forced swim test (FST) was performed and vaginal smears, euthanasia and locus coeruleus (LC) collection occurred 30 min later. **(B)** In experiment 2, animals had a 7-day accommodation period after which they underwent SPS stressors and were administered 300 μg of intranasal NPY or vehicle while under the influence of ether. After a 7-day consolidation period, elevated plus maze (EPM) was performed and immediately afterwards vaginal smears, euthanasia and LC collection occurred.

CA (Augustsson et al., 2005). We calculated anxiety index as 1 − [(time spent in OA/total time on the maze)/2 + (number of entries to the OA/total exploration on the maze)/2] (Cohen et al., 2012).

Real-Time Polymerase Chain Reaction (PCR)

Total RNA from LC was isolated using STAT 60 (Tel-Test Inc., Friendswood, TX, USA). and concentration was quantified using Nano Drop 2000 (Thermo Fisher Scientific, Pittsburgh, PA, USA). Reverse transcription of RNA (500 ng) was performed with the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol, using an oligo dT primer. For quantitative Real-Time Polymerase Chain Reaction (PCR), 2 μl of cDNA product was mixed with 12.5 μl of FastStart Universal SYBR Green Master Rox (Roche Diagnostics, Indianapolis, IN, USA) and 1 μl of the following primer pair sets: rat tyrosine hydroxylase (TH; forward 5′-CCGGTCTACTGTCCGC CCGT-3′, reverse 5′-TCATGGCAGCAGTCCGGCTC-3′), GAPDH (forward 5′-TGGACCACCCAGCCCAGCAAG-3′, reverse 5′-GGCCCCTCCTGTTGTTATGGGGT-3′), CRHR1 (Qiagen, cat. no. PPR44886F), NPY receptor 1 (Y1R; cat no. PPR6253024), or NPY receptor 2 (Y2R; cat no. PPR06816A) to a final volume of 25 μl, and analyzed on an ABI7900HT Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA, USA). Each gene was normalized to GAPDH mRNA levels and expressed as the relative fold change vs. unstressed control, calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

Vaginal Smears

Vaginal smears were prepared according to the procedure outlined in McLean et al. (2012). In brief, a vaginal lavage was performed using distilled water and the collected specimen were allowed to dry on a glass slide. The slides were stained with 0.1% crystal violet and observed under a light microscope at 10× and 40× objectives to determine the phase of the estrus cycle by a trained researcher blind to the experiment conditions.

Statistical Analysis

Data analysis was performed in Prizm 8 (GraphPad) software. Normality test was done using D'Agoustino and Pearson Omnibus. Data were analyzed by planned comparison *t*-test to evaluate the primary and secondary aims of this study. Outliers were removed when they were greater than two standard deviations away from the mean. Values at $p \leq 0.05$ were considered significant.

RESULTS

Experiment 1: FST for Depressive Symptoms

Development of immobility during the forced swim portion of SPS stressors, as well as depressive-like behavior and molecular changes in the LC 12 days after SPS were evaluated in females (**Figure 2**). The animals became progressively more immobile in each 5 min block during the 20 min forced swim after a 2 h immobilization. Animals in the 2nd block spent significantly more time immobile than the 1st block ($t_{(62)} = 7.3$, $p < 0.0001$), spending almost 4× more time floating. The duration immobile also increased in the 3rd block compared to the 2nd ($t_{(62)} = 4.9$, $p < 0.0001$) and the 4th block compared to the 3rd ($t_{(62)} = 2.5$, $p = 0.01$; **Figure 2A**).

In the 5 min FST, the SPS/V group spent more time immobile than controls ($t_{(24)} = 2.1$, $p = 0.05$). Early intervention administration of intranasal NPY did not prevent development of this depressive-like behavior (**Figure 2B**).

Thirty minutes following FST, animals were euthanized and quantitative RT-PCR performed to determine expression levels of several genes involved in mediating the response of the LC/NE system to stress. A *t*-test revealed that mRNA levels of TH, the rate-limiting enzyme in NE biosynthesis, was changed with higher levels in the SPS/V than in controls ($t_{(23)} = 2.8$, $p = 0.011$). Intranasal NPY did not attenuate TH mRNA levels in the LC (**Figure 2C**).

The mRNA levels for CRHR1, the CRH receptor subtype expressed in the LC, was significantly elevated in SPS/V animals as compared to controls ($t_{(23)} = 4.9$, $p < 0.0001$). However, there were no differences between the SPS/V and SPS/NPY groups (**Figure 2D**).

Since the response to NPY differed from that in males (Sabban et al., 2015a), we assessed gene expression of the Y1 and Y2 receptor subtypes (**Figure 2E**). There were significant changes in the levels of Y1R mRNA between the SPS/V and control groups ($t_{(26)} = 2.4$, $p = 0.02$), but not Y2R mRNA (data not shown). Y1R mRNA levels were elevated in the SPS treated animals and were not reduced by early intervention with intranasal NPY.

Experiment 2: EPM for Anxiety Symptoms

We examined anxiety behavior on the EPM and molecular changes in the LC 7 days after SPS in females (**Figure 3**). A *t*-test revealed significant differences in the percent of entries into the OA ($t_{(18)} = 2.8$, $p = 0.01$), with the SPS/V females entering the OA less frequently than unstressed controls. The mean of OA entries for SPS/NPY animals did not differ from the SPS/V group (**Figure 3A**). There were significant differences in the percent duration in OA between the SPS/V and unstressed control groups ($t_{(18)} = 2.7$, $p = 0.02$). SPS/V animals spent less time in the OA than controls, however intranasal NPY did not attenuate this behavior (**Figure 3B**). There were significant differences in anxiety index between the control and SPS/V groups. SPS/V females had a higher anxiety index than controls ($t_{(18)} = 2.9$, $p = 0.01$), but the SPS/NPY group did not differ significantly from SPS/V animals (**Figure 3C**).

There were significant differences in the frequency of unprotected head dips with SPS/V females having fewer unprotected head dips than controls ($t_{(18)} = 3.6$, $p = 0.002$). While there was a tendency for SPS/NPY animals to have more OA head dips than SPS/V, there were no significant difference between these groups (**Figure 3D**). There were significant differences in the total duration of risk assessment between the SPS/V and

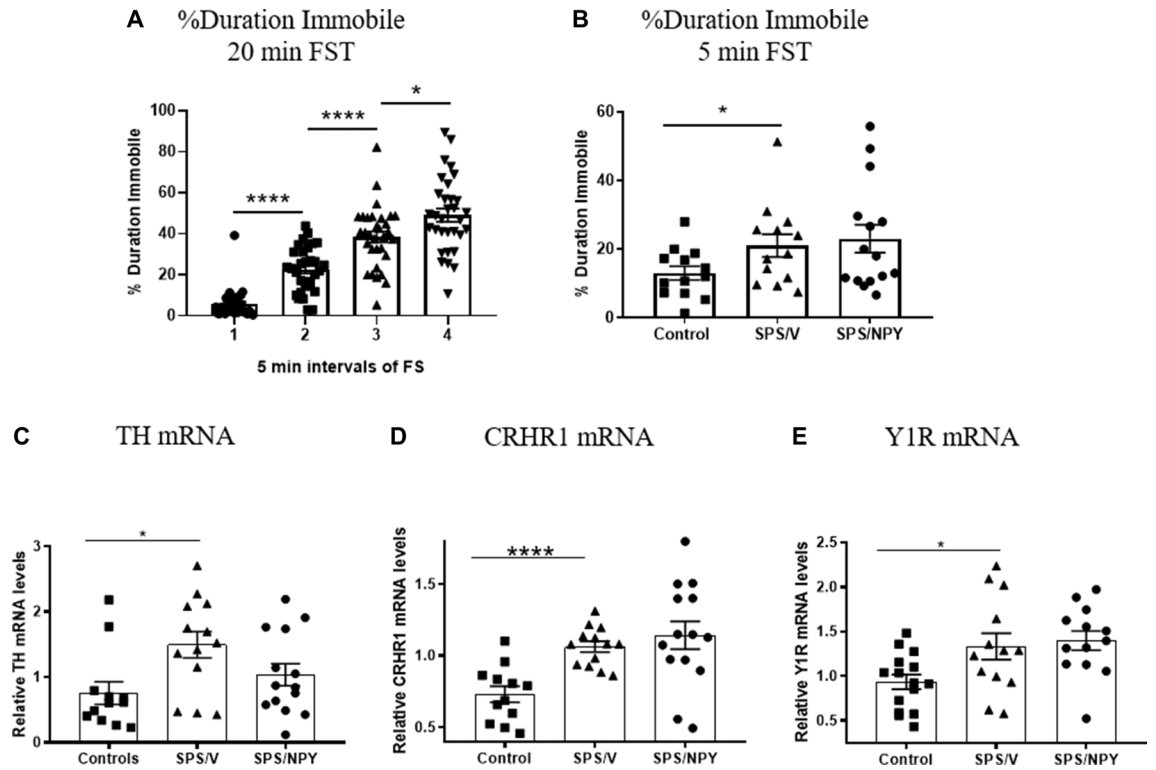

FIGURE 2 | Effect of SPS on response to FST. Female rats were unstressed (Control), or exposed to SPS and while still under the influence of ether (the last stressor) administered 150 μg/rat of NPY; (SPS/NPY) or vehicle (SPS/V). The forced swim portion of the SPS stressors was recorded and immobility scored in 5 min intervals **(A)**. Twelve days later they tested for **(B)** immobility time on the FST and changes in mRNA levels of: **(C)** tyrosine hydroxylase (TH); **(D)** CRHR1; **(E)** NPY receptor 1 (Y1R) in the LC 30 min later. Means ± SEM are shown. Each point represents values for an individual animal. Planned comparisons *t*-test determined differences between groups. $^{*}p < 0.05$; $^{****}p < 0.0001$.

control groups ($t_{(18)}$ = 3.7, p = 0.002). SPS/V females spent less time engaging in risk assessment behavior than control females while there was no difference with SPS/NPY animals (**Figure 3E**).

Furthermore, there were significant differences in total distance traveled (track length) between the SPS/V and control groups ($t_{(18)}$ = 2.5, p = 0.02). Comparison between the means determined that SPS/V females traveled less distance during the EPM than control females. NPY treatment did not improve this behavior (**Figure 3F**).

Immediately following EPM, the LC was isolated and we performed quantitative RT-PCR to determine expression levels of several genes involved in mediating the response of the LC/NE system to stress. There were no observed significance differences among the groups in the mRNA levels of TH ($t_{(19)}$ = 1.4, p = 0.17) and CRHR1 ($t_{(21)}$ = 2.0, p = 0.06). However, Y1R levels differed with the SPS/V expressing elevated levels of Y1R mRNA compared to controls ($t_{(17)}$ = 2.6, p = 0.02; **Figures 3G–I**).

DISCUSSION

The findings indicate that SPS could be an appropriate model for PTSD and associated disorders in females. SPS stressors triggered development of a number of PTSD-associated symptoms previously observed in males. During the SPS, the females exhibited progressively increasing immobility on the forced swim portion. SPS elicited elevated depressive-like behavior or passive coping strategy on FST, as well as anxiety and decreased risk assessment on EPM in females. Additionally, gene expression of TH, CRHR1, and Y1R in the LC/NE system was elevated following the FST and Y1R gene expression was elevated following EPM in the SPS-treated animals. However, intranasal NPY at concentrations effective for males had at most a marginal effect on these parameters in females.

SPS as PTSD Model in Females

One of the important findings of this study is that animals subjected to SPS stressors exhibited elevated depressive-like behavior, or a passive coping strategy, and anxiety a week or more afterwards. Additionally, the animals demonstrated a continual increase in immobility throughout the 20 min forced swim stressor of the SPS. After each 5 min interval of force swim, the animals exhibited more floating behavior than the previous interval. The increased behavioral despair, as indicated by the immobility, suggests the prior immobilization stress and/or the forced swim stress appropriately creates a traumatic experience for the females.

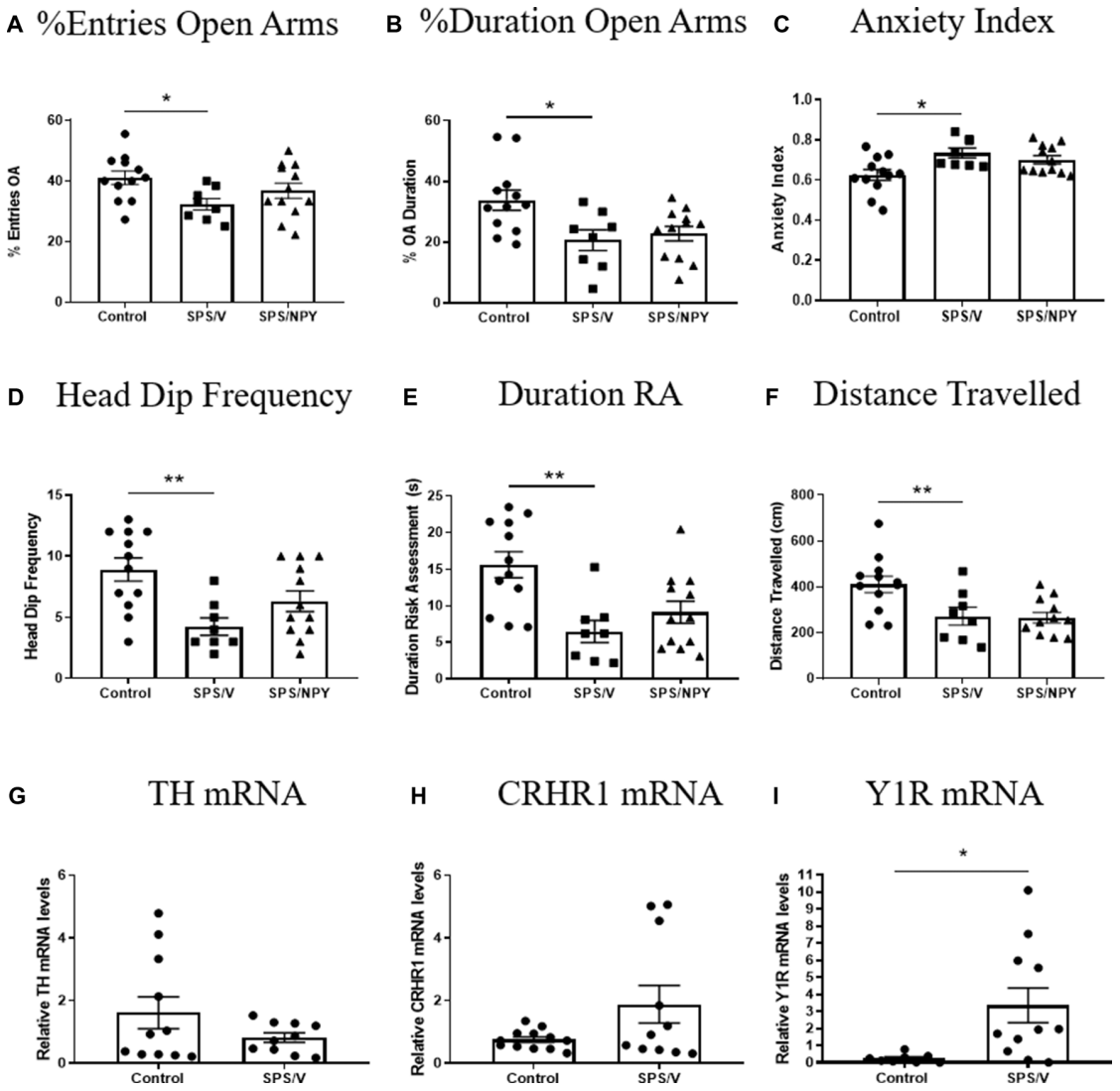

FIGURE 3 | Effect of SPS on anxiety. Female rats were unstressed (Control), or exposed to SPS and while still under the influence of ether (the last stressor) administered 300 μg/rat of NPY (SPS/NPY) or vehicle (SPS/V). Seven days later they were tested on the EPM for: **(A)** entries into the open arms (OA); **(B)** duration in the OA; **(C)** anxiety index; **(D)** frequency of unprotected head dips; **(E)** total duration of risk assessment; **(F)** distance traveled; changes in mRNA levels of: **(G)** TH, **(H)** CRHR1, **(I)** Y1R in the LC immediately after EPM. Means ± SEM are shown. Each point represents values for an individual animal. Planned comparisons *t*-test determined differences between groups. $^{*}p < 0.05$; $^{**}p < 0.01$.

This is the first study to evaluate depressive-like behavior on the FST after exposure to SPS in females. SPS/V females demonstrated more depressive-like behavior than unstressed controls 12 days after SPS. Recent discussions posit that the FST measures coping strategies and adaptation to stress rather than depression. In this theory, animals adapt to the forced swim stress by switching from active coping (swimming and climbing) to passive coping (floating; de Kloet and Molendijk, 2016). The FST has been implicated in many animal models of different psychiatric illnesses, suggesting depression might not be the sole behavioral condition involved (Commons et al., 2017). Nevertheless, our results add to growing evidence suggesting SPS elicits a depressive phenotype in females.

Previously, SPS was reported to trigger depressive measures of anhedonia in females, as assessed by reduced sucrose preference, and social interaction (Pooley et al., 2018b). Additionally, the dexamethasone suppression test showed decreased suppression of plasma corticosterone in females 1 week after SPS stressors (Pooley et al., 2018a,b). These measures, in conjunction with the results from this study, indicate that SPS is successful at eliciting a depressive phenotype.

Across all EPM behavioral measures, the stressed females showed more anxious behavior than unstressed controls 1 week after exposure to SPS stressors. SPS/V females spent less time and had fewer entries into the OA, more time and fewer entries into the CA, and higher anxiety index in the EPM than

controls. Thus, the data suggests SPS successfully elicits anxiety in females as previously observed by Fan et al. (2013). Several other measures on the EPM altered in males by SPS were also observed in females, including reduced risk assessment, head dips, and locomotion (track length).

The reduced locomotion likely does not account for the increased anxiety on the EPM. In males, we have previously shown that NPY infusion alleviates anxiety as measured by the percent of open and CA entries and duration and anxiety index, however the track length (or distance traveled) remains unchanged as compared to animals infused with vehicle and lower than unstressed controls (Serova et al., 2013, 2014). Furthermore, the total arm entry between the three groups here did not differ significantly demonstrating changes in locomotion did not influence the number of arm entries.

Other models of PTSD have also been shown to elicit increased anxiety in females. Female rodents exposed to a cat (predator stressed) or cat or fox odor (predator scent) for 10 min exhibited anxiety-like behavior on the EPM 1 week later (Adamec et al., 2006; Mazor et al., 2009). However, these models are highly dependent on odor. Females have a more sensitive sense of smell than males even in humans (Bengtsson et al., 2001; Brand and Millot, 2001; Kass et al., 2017), and smell is a particularly prominent sensory feature in rodents. In contrast, SPS does not include an element of odor but nevertheless elicited many features of anxiety and may be more easily translatable to humans.

Several other behavioral phenotypes were also previously assessed in females following SPS with mixed results. These included: fear conditioning, hyperarousal and social interaction. The effect of fear conditioning is unclear. In one study, females exhibited more contextual freezing as compared to unstressed controls 2 weeks after SPS stressors (Fan et al., 2013). In contrast, Keller et al. (2015) indicated that SPS may not be appropriate for females since, in contrast to males, they did not display enhanced recall of extinguished fear 1 week after SPS. However, darting response (as opposed to freezing) during fear conditioning may be a more appropriate behavioral measure for females (Gruene et al., 2015).

The acoustic startle response was not changed in females 7 or 10 days after SPS indicating it did not trigger hyperarousal (Pooley et al., 2018a,b). SPS affected social interaction in females, but was dependent on housing of the animals (Pooley et al., 2018b).

Further support for SPS as an appropriate model for PTSD in females comes from the molecular changes seen here in the LC. In response to stress, hypothalamic and extrahypothalamic CRH is released and binds to CRHR1 in the LC, causing a more tonic phase in these cells and consequential NE release, increased vigilance, hyperarousal and anxiety (Valentino and Foote, 1987, 1988; Van Bockstaele et al., 1996; Page and Abercrombie, 1999; Valentino and Van Bockstaele, 2008). In fact, anxiety symptoms are prevented when the LC is inhibited optogenetically suggesting its crucial role (McCall et al., 2015).

There were pronounced changes in LC mRNA levels in SPS-treated animals after the FST, but only Y1R mRNA levels differed after the EPM. Thirty minutes following FST, there was an elevation of mRNA levels of TH, the rate limiting enzyme for NE biosynthesis, in the SPS/V group compared to previously unstressed controls, as observed in males (Serova et al., 2013). Given that the LC is the primary source of NE in the forebrain and the sole source of NE in the cortex and hippocampus, this likely leads to enhanced noradrenergic activity. In addition to TH, there was elevated LC gene expression of CRHR1 and Y1R in the females after the FST.

The changes in mRNA levels of Y1R in the LC following FST and EPM between unstressed controls and the SPS/V group suggest the involvement of Y1R and the NPY system in the female stress reaction. Y1R knockout ($Y1R^{-/-}$) female mice exhibited anxiolytic or decreased depressive-like behavior 1 week after forced swim or EPM stress, as compared to Y1R wildtype controls exposed to the same stress (Painsipp et al., 2010). On the Morris water maze, females containing $Y1R^{-/-}$ Y5R-expressing neurons perform better than controls (Longo et al., 2014). Furthermore after 1-h restraint, Y1R levels are increased 6 h later in the PVN and amygdala in males (Mele et al., 2004). Thus, the regulation of NPY and its receptors is implicated in orchestrating an appropriate stress response.

The same changes following FST in LC mRNA levels of TH and CRHR1 between stressed and unstressed females were not seen following the EPM. A limitation of this study is that all the animals underwent the stress of the behavioral testing. It is unclear if the mRNA levels seen are due to the SPS stressors or the behavioral experiment, as there were no stressed controls not exposed to behavioral testing in this study. Further studies with stressed controls are needed in order to ascertain if the molecular changes in the LC occur as a result of SPS. The stronger molecular changes in the FST experiment may be attributed to sensitivity of a reexperiencing effect, one of the main components of PTSD. The LC is highly implicated in the re-experiencing reaction and can contribute to larger NE responses (Elzinga and Bremner, 2002; George et al., 2013). Thus, the SPS-treated animals would have a surge of LC activation following a re-experiencing event, such as the FST.

Other molecular changes have been shown previously in females 1 week after SPS, further suggesting the strength of this model. Females subjected to SPS had increased cFos expression in the mPFC and amygdala, as compared to unstressed controls (Pooley et al., 2018a). Additionally, upregulation of GR in the hippocampus was found 1 week after SPS stressors (Keller et al., 2015). Interestingly, SPS stressors, but not predator stress, decreased GR expression in the PVN (Pooley et al., 2018a).

There is substantial evidence pointing to the conclusion that SPS produces significant and sufficient stress in females to elicit behaviors associated with PTSD. The impact of the stress is seen as early as in the forced swim portion of the SPS, with females exhibiting progressively more helplessness or passive coping behaviors. While this stress sufficiently provokes PTSD behavior, comparison to vehicle-treated males from one of our previous studies suggest it might be less severe for females. After 10 min forced swim during the SPS stressors, males spent over 90 percent of the time immobile compared to less than

50% of the time spent immobile by females. Throughout the 20 min forced swim after the 2 h immobilization, it appears females spent less time immobile in each 5 min block than males. The suggested differences could indicate that the SPS immobilization and/or forced swim stressors are milder for females. During the 5 min FST 1 week or more after SPS, it appears stressed females and unstressed controls spent less time immobile in FST, as compared to males. (Serova et al., 2013). Therefore, adjustments to the SPS paradigm might be needed to extend the same degree of stress males experience to females.

Early Intervention With Neuropeptide Y in Females

The action of NPY is mediated by a family of G-protein coupled receptors including Y1R, Y2R, Y4R and Y5R. Y1R, a postsynaptic receptor, has been shown to be important in mediating the anxiolytic effects of NPY (Theisen et al., 2018). The Y2 receptor, located both pre- and post-synaptically, appears to be important in modulating activity of LC neurons (Kask et al., 1998). In this study, we observed increased Y1R, but not Y2R, gene expression in the SPS-treated female animals.

While our previous studies demonstrated the effectiveness of intranasal NPY to prevent development of PTSD-associated behaviors and molecular changes for male rats (Serova et al., 2013, 2017; Sabban et al., 2015a,b; Tasan et al., 2016), it appears that intranasal NPY may not be an effective treatment for females at the same dosage. NPY tended to reduce the increase in TH mRNA levels in the SPS/NPY group, but it was not significantly different from the SPS/V group. In the second experiment, a higher NPY dose was chosen to evaluate an observable therapeutic effect at higher concentrations. With the higher dose, there tended to be a slight improvement on some of the EPM measures, such as OA and CA entries, anxiety index, and frequency of head dips. Further studies are needed to determine if higher doses of NPY might be required for females.

NPY has been proposed to antagonize the actions of CRH (Schmeltzer et al., 2016). Given that females have increased sensitivity to CRH in the LC, a larger dose of NPY might be needed to antagonize its effect than in males (Heilig, 2004; Valentino and Bangasser, 2016; Bangasser et al., 2018). This increased sensitivity to CRH may be due to decreased internalization of the CRHR1 during stress, increased LC dendrite density, and increased CRHR-G_s coupling at baseline in cortical tissue and the LC, as compared to males (Bangasser et al., 2010, 2013). The molecular signaling and trafficking of the CRHR1 provides evidence of an impaired adaptation to stress in females.

With females, it is important to take into account the estrus cycle and influence of sex hormones. Estrogen has a protective effect on many of the symptoms of PTSD in rodents and humans (Contreras et al., 2000; Marcondes et al., 2001; Almeida et al., 2005; Serova et al., 2005; Milad et al., 2009; Kornstein et al., 2010; Young and Korszun, 2010; Bryant et al., 2011; Glover et al., 2012; Newhouse and Albert, 2015; Molina-Jímenez et al., 2017). Additionally, fluctuations in NPY receptors due to the estrous cycle have been reported, with increased hypothalamic Y1R expression in proestrus females compared to other phases of the estrous cycle (Martini et al., 2011). Estrogen response elements flank the Y1R gene and changes the gene expression as estradiol levels fluctuate (Eva et al., 2006). Furthermore, estrogen increases NPY-immunostaining neurons and NPY release in the hippocampus (Velíšková et al., 2015).

Here, vaginal smears were taken at sacrifice. However, only a limited number of animals were in each phase of the estrous cycle within each group. Future research conducted with females would require a larger cohort to accurately analyze SPS effects and NPY treatment on behavior in a phase-controlled manner.

LIMITATIONS

There are some limitations to note regarding this study. The difference in acclimation and consolidation periods between the two experiments, due to uncontrollable circumstances, may complicate the interpretation of the results. The lack of stressed controls not exposed to behavioral testing limits the interpretation of the molecular changes post-SPS, as the behavioral testing could induce molecular changes itself. Furthermore, the lack of unstressed female control animals administered NPY serves as a limitation to the understanding of the behavioral and molecular effect intranasally administered NPY can have in an unstressed, female animal. In males, there was no effect of NPY in unstressed controls.

CONCLUSION

Overall the results indicate that SPS, perhaps with some modifications, could be an appropriate model for PTSD and associated disorders in females. The diversity of symptoms makes finding a complete model for PTSD difficult. PTSD can manifest as depressive symptoms, anxiety, hyperarousal, social apathy, impaired cognition and altered mood. SPS in female rats elicits depressive symptoms, anxiety, altered social interaction, impaired cognitive processes as shown by fear conditioning, and ensuing molecular changes. Thus, it appears that SPS may provide a broad spectrum of PTSD impairments in female rodents, although they did not benefit from intranasal NPY treatment at the same doses given to males. As this is one of few articles investigating females post-SPS, there is a dire need to continue research efforts in this area.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

ES conceived and supervised the study, all authors participated in the study design. CN, RN and LS performed the experiments.

RN, CN, LS performed the data analysis. RN, CN wrote the manuscript. All authors read and approved the manuscript.

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Post Traumatic Stress Disorder and Substance Use Disorder as Two Pathologies Affecting Memory Reactivation: Implications for New Therapeutic Approaches

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In the present review, we provide evidence indicating that although post traumatic stress disorder (PTSD) and substance use disorder (SUD) are two distinct pathologies with very different impacts on people affected by these chronic illnesses, they share numerous common characteristics, present high rates of co-morbidity, and may result from common physiological dysfunctions. We propose that these pathologies result from hyper reactivity to reminders, and thus should be considered as two disorders of memory, treated as such. We review the different possibilities to intervene on pathological memories such as extinction therapy and reconsolidation blockade. We also introduce new therapeutic avenues directly indicate by our recent proposal to replace the consolidation/reconsolidation hypothesis by the integration concept. State dependency and emotional remodeling are two innovative treatments that have already provided encouraging results. In summary, this review shows that the discovery of reactivation-dependent memory malleability has open new therapeutic avenues based on the reprocessing of pathological memories, which constitute promising approaches to treat PTSD and SUD.

Keywords: post-traumatic stress disorder (PTSD), memory reactivation, reconsolidation blockade, state dependency, memory integration

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PTSD AND SUD: TWO PATHOLOGIES SHARING COMMON CHARACTERISTICS

Post traumatic stress disorder (PTSD) and substance use disorders (SUDs) are two complex and specific pathologies, which, however, share many properties in common. Both are chronic and relapsing disorders, the origins of which are very well known, an aspect that is quite unusual for psychiatric disorders. These pathologies result from exposures to opposite extreme and out of the norm events, which can be schematically outlined as very negative (trauma) or very positive (drug of abuse). Only a portion of the exposed individuals (around 8%–35%; Kessler et al., 1995) are vulnerable and develop the pathology. Both disorders share some similar symptoms, including anxiety, sleep problems, hyper arousal, social isolation, and emotional numbing. They also share common risk factors, such as previous stressful life events, negative affect, having previously had another psychiatric disorder, and might be related to similar genetic susceptibility concerning the D2 receptor (Enman et al., 2015). PTSD and SUD both involve deregulations of

brain reward circuitry (Schultz, 2001; Elman et al., 2005; Pierce and Kumaresan, 2006; Hopper et al., 2008) and present with very high levels of comorbidity (around 40%; Stewart et al., 1998). There is no specific treatment for these pathologies that has demonstrated its efficacy over a long period of time. Finally, another important common characteristic of these two pathologies must be emphasized: their sensitivity to cues associated with the source of the pathology i.e., the trauma or the drug. In both situations, patients tend to avoid exposure to these cues, known to elicit intrusive flashbacks of trauma in PTSD and drug craving in SUD, which may precipitate relapse of the associated pathology, even after remission or abstinence for long period of time. All these similarities provide compelling evidence emphasizing the central role that trauma and drug reminders may have for both pathologies and strengthen the hypothesis that PTSD and SUD could possibly result from common physiological dysfunctions due to exposure to extreme conditions.

PTSD AND SUD: TWO PATHOLOGIES BASED ON COMMON PHYSIOLOGICAL DYSFUNCTIONS

Traumatic events and drug experiences generate some of the most enduring forms of memories, which have the salient characteristics of being easily and vividly retrieved. As a consequence, rather than stress or reward pathologies, PTSD and SUD should instead be considered as memory pathologies (Gisquet-Verrier, 2009; Gisquet-Verrier et al., 2017). There are abundant data suggesting that both may originate from a hyper reactivity to reminders. In PTSD patients, the susceptibility to trauma reminders leads to frequent re-experiencing of the trauma accompanied by vivid emotional responses, which maintain anxiety responsible for arousal, sleep disorder, social isolation, etc. and sustain the pathology over time. In SUD patients, the hyper reactivity to drug taking reminders induces frequent and intense drug craving, responsible for the urge of drug taking, accounting for chronic relapses that characterize this pathology. Interestingly, extended evidence coming from cerebral imagery indicates that exposure to trauma or drug reminders activate similar brain circuitry involving among other areas, the amygdala, ventral striatum, ventro-tegmental area, as well as the prefrontal cortex (PFC; Rauch et al., 2006; Bremner, 2007; Carrión et al., 2010; Jovanovic et al., 2013; Johnson et al., 2013; Jasinska et al., 2014; Lowen et al., 2015).

Accordingly, although PTSD and SUD are obviously two different pathologies with different characteristics and consequences, we proposed that they rely on common physiological processes, the disruption of which could account for a hypersensitivity to reminders restricted to the drug and trauma related memories (Gisquet-Verrier, 2009). Such a view, which could well account for the strong comorbidity between PTSD and SUD, led us to consider the findings discovered by Tassin (2008), who demonstrated that mice repeatedly exposed to various drugs of abuse exhibited large increases of noradrenaline (NA) and serotonin (5-HT) release within the prelimbic part (PL) of the medial PFC (mPFC), as well as large increases in locomotor behavior, in response to a drug activating these systems (Lanteri et al., 2008, 2014). To account for these behavioral and neurochemical sensitizations, these authors proposed that after repeated drug injections, the reciprocal control exerted by noradrenergic and serotonergic systems was disrupted, leading to an uncoupling of monoaminergic systems, accounting for their increased release (Tassin, 2008; see **Figure 1**). They further indicated that, through projections to the ventral tegmental area (VTA) and the nucleus accumbens (NAc), the neurochemical sensitization could be responsible for the locomotor sensitization also observed in these mice (Pierce and Kalivas, 1997; Steketee and Kalivas, 2011).

We considered these results as potential support for our views since memory reactivation has been shown to depend on the integrity of the PFC and to require activation of the noradrenergic system (Devauges and Sara, 1991; Botreau et al., 2004; Sara, 2009). It was thus important to determine whether similar results could be obtained in rodents exposed to a PTSD model. In a series of experiments, we recently explored this hypothesis using the single-prolonged stress (SPS) procedure (Liberzon et al., 1997; Lisieski et al., 2018), a PTSD model known to provide a behavioral phenotype resembling PTSD (Toledano et al., 2013; Enman et al., 2015; Le Dorze and Gisquet-Verrier, 2016a), including the fact that it only affects a subset of the exposed population (Toledano and Gisquet-Verrier, 2014; Le Dorze and Gisquet-Verrier, 2016b).

We demonstrated that, similar to mice, rats repeatedly exposed to amphetamine injections, as well as SPS vulnerable rats, developed long lasting behavioral sensitization (Toledano et al., 2013; Toledano and Gisquet-Verrier, 2014, 2016; Le Dorze and Gisquet-Verrier, 2016b). More recently, we showed that trauma vulnerable rats further exhibited a noradrenergic sensitization. Increases of NA releases in these rats were obtained, not only in response to an amphetamine injection known to stimulate the noradrenergic system, but also after a short exposure to a trauma reminder cue (Le Dorze et al., 2018), a finding previously obtained in rats repeatedly exposed to amphetamine injections (Toledano and Gisquet-Verrier, 2016). These findings strongly suggest trauma or drug experience involved similar physiological disruptions, resulting from exposure to extreme conditions. We proposed that exposures to special homeostatic challenges, such as severe trauma or drugs of abuse, intensely activate the noradrenergic and the serotonergic systems. According to Tassin, these exaggerated activations could break the inhibitory control that the noradrenergic and serotonergic neurons exert on one another in vulnerable individuals, leading to an uncoupling of monoaminergic systems (Lanteri et al., 2008). As a result, subsequent exposures to a reminder in these individuals will trigger a large increase in noradrenergic release within the PFC responsible for memory reactivation (see **Figure 1**). The implicit or explicit reactivation induces intrusive flashbacks of trauma in PTSD patients and intense craving followed by drug seeking in SUD patients, as well as to increased risks of relapse in both populations.

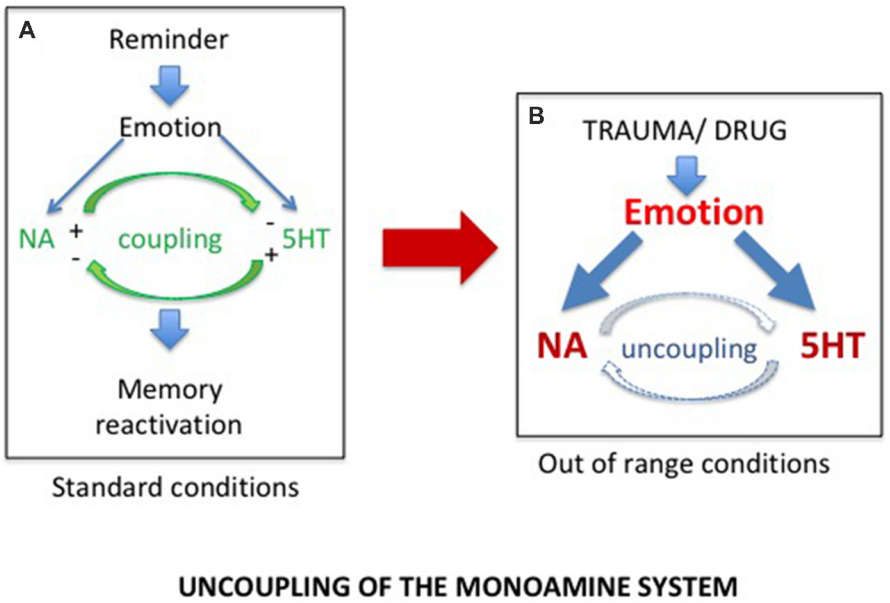

FIGURE 1 | (A) In normal conditions, exposure to a reminder induces self-regulated noradrenergic (NA) and serotonergic (5HT) releases required for memory reactivation. **(B)** Exposure to extreme conditions, such as trauma or drug of abuse experiences, induces dramatic increases in NA and 5HT release, responsible for the uncoupling of the monoamine systems.

HOW TO TREAT PTSD AND SUD CONSIDERED AS MEMORY PATHOLOGIES?

Considering that PTSD and SUD are two pathologies of memory, relying on a common physiological dysfunction, necessarily has consequences for the therapeutic approaches used to treat them. The first strategy is a direct consequence of our uncoupling hypothesis and has just began to be explored. The second group of treatments corresponds to those classically used to weaken memory, which have been adapted for therapy. We will see that despite the fact that the homology between SUD and PTSD has never actually been proposed, the way these pathologies are treated is very similar. Finally, the third approach corresponds to new treatments arising from the integration concept that we recently introduced as an alternative to the consolidation/reconsolidation view (Gisquet-Verrier and Riccio, 2018).

Recoupling the Monoaminergic System

Considering that SUD and PTSD result from the uncoupling of the monoaminergic systems, the first approach to consider, is to correct the disruption induced by the trauma or the drugs of abuse, hence to "recouple" these systems. Indeed, it has recently been shown that delivering a combination of two blocking agents, prazosin, an antagonist of α1b-adrenergic receptors, and cyproheptadine, an antagonist of 5-HT2A receptors in alcohol dependent mice was able not only to block behavioral sensitization to amphetamine, but also to reverse their alcohol preference (Trovero et al., 2016; see **Figure 2**). These authors are currently investigating this approach in a clinical trial conducted on alcoholic patients. It should be noted that, when given separately, both prazozin and cyproheptadine have been shown to reduce nightmares associated with posttraumatic stress disorder (Gupta et al., 1998; Raskind et al., 2003; El-Solh, 2018). However, it will be of interest to investigate with a preclinical approach the efficacy of their combination.

Modifying Pathological Memories

While PTSD and SUD are not generally considered to result from the same physiological dysfunction, each of them has frequently been considered to rely on abnormal learning and memory processes (Everitt, 2014; Dunbar and Taylor, 2017; Walsh et al., 2018). Accordingly, approaches consisting in decreasing the strength of these memories by extinction procedures, eliminating the pathological memories by reconsolidation blockades, or even reducing the propensity for drug/trauma associated-cues to elicit memory reactivation by emotional remodeling have been used for both PTSD and SUD.

Prolonged Exposure Therapy: An Extinction Procedure

There is extended evidence showing that exposure to reminders can evoke re-experiencing, craving and relapse. Reducing the impact of these reminders has thus been used in specific exposure therapy programs for both PTSD and SUD. Extinction corresponds to a learning process leading to progressive weakening of a learned response, due to the withdrawal of the reinforcement. Extinction serves as the basis of prolonged exposure therapy for PTSD patients, the aim of which is to reduce the emotional reactivity to trauma reminders *via* sustained imaginal and real exposure (e.g., Foa et al., 2007; see **Figure 3**).

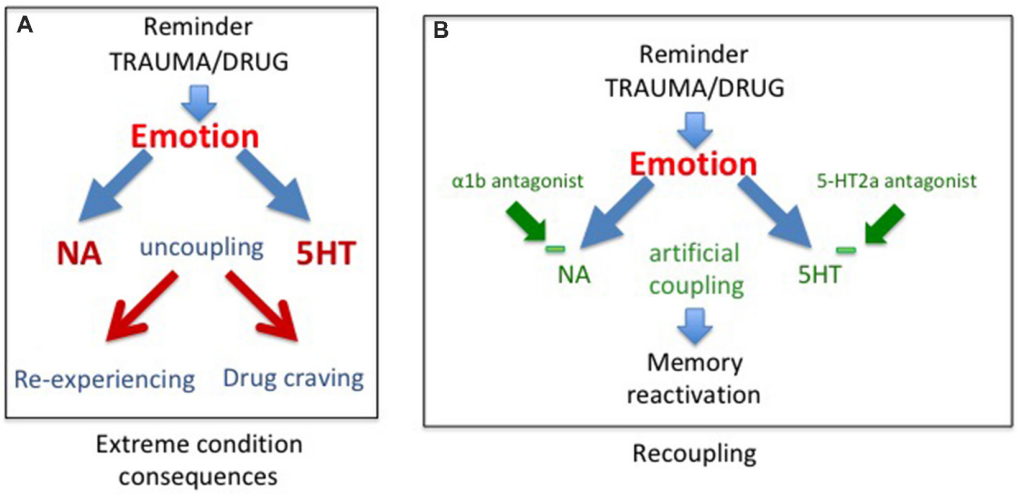

FIGURE 2 | (A) As a result of uncoupling, exposure to trauma/drug reminders induces increased release of NA and serotonin, responsible for abnormal memory reactivations in the form of re-experiencing in post traumatic stress disorder (PTSD) and drug craving in substance use disorder (SUD) patients. **(B)** The combined action of alpha1b and 5-HT2A receptor antagonists, by reducing excessive prefrontal noradrenergic and serotonergic releases, artificially re-couples the monoaminergic systems, normalizing memory reactivation.

This form of psychotherapy, considered as an effective treatment for PTSD (Powers et al., 2010), has been extensively used, whether associated or not with a pharmacological treatment. Prolonged exposure therapy has also been considered as an effective treatment for co-occurring PTSD and SUDs (Powers et al., 2010; Mills et al., 2016). The memory retrieval-extinction procedure has also been thought of as a promising nonpharmacological method for decreasing drug craving and relapse in SUD patients. Extinction of the drug-associated cues through repeated non-reinforced presentations has also been used to diminish the impact of drug reminders on relapse to drug addiction in preclinical studies, especially when delivered in combination with d-cycloserine, a treatment known to enhance extinction (Davis, 2002; Lee et al., 2006b). A retrieval-extinction paradigm administered to heroin addicts has been shown to significantly reduce subsequent cue-induced craving (Xue et al., 2012). However, extinction learning has a number of important limitations, the most important of which is the contextual specificity of extinction learning (Bouton, 2002). Despite the undeniable success of cue exposure therapy, the long-term efficacy of this treatment is still highly questionable because extinction learning does not does not eliminate fear responses but rather creates new learning that inhibits activation of the original memory and thus is subject to relapse even after long periods of remission (Conklin and Tiffany, 2002; Foa, 2011; Myers and Carlezon, 2012).

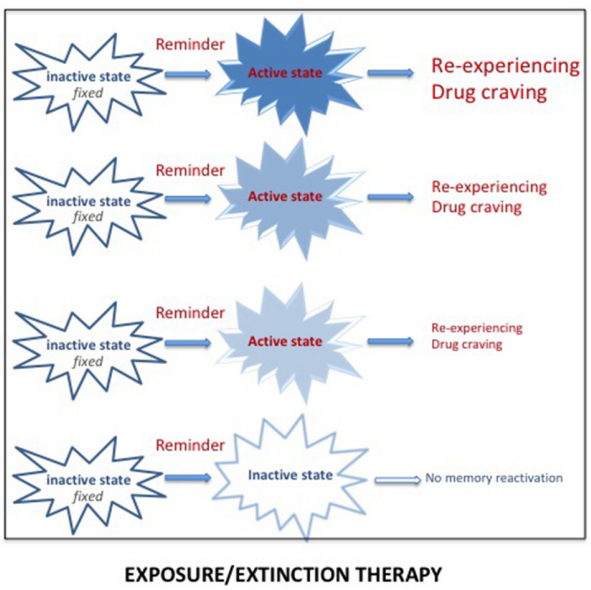

FIGURE 3 | Reactivation of a pathological memory (trauma/drug) triggered by reminders (i.e., cues associated with the original memory) places the memory in an active state and induces vivid and intense remembering, taking the form of re-experiencing (for PTSD) or drug craving (for SUD). Repeated exposure to real or imaginal reminders can progressively reduce the intensity and the vividness of the memory, leading to a new memory through extinction processes. However, since this new memory competes with the original memory, spontaneous recoveries of the original memory are frequently observed.

Reconsolidation Blockade

The best way to treat SUD and PTSD would be to eliminate the pathological memory. Since 2000, a new approach targeting memory reconsolidation suggested that this is a possibility (Nader et al., 2000). Earlier research had demonstrated retrograde amnesia for newly acquired information, i.e., a time-dependent performance disruption induced by severe

treatments such as electroconvulsive shocks, hypothermia, anesthetics or protein synthesis inhibitors. This finding led to the view that memories are not fixed immediately but undergo a consolidation period during which the memory is fragile, sustained by slow processes leading to the progressive stabilization of the memory (McGaugh, 2000). It was initially considered that once stored, however, the memory remained in that state permanently. However, numerous findings showed that memories can also be disrupted by amnesic treatments delivered shortly their reactivation/retrieval. This has been interpreted as demonstrating that reactivated memories re-enter a state of lability, and must be re-stabilized through a protein dependent process, termed reconsolidation, similar to the one engaged during the original consolidation (Nader et al., 2000; Nader and Hardt, 2009). According to that view, it was possible to disrupt remote memories, even long after their initial formation. Since that time, the opportunity to eliminate pathological memories through reconsolidation blockade has been extensively exploited in preclinical and clinical studies, for both PTSD and SUD.

Delivering treatments known to affect reconsolidation processes shortly after the reactivation of a memory, by preventing its re-stabilization, is intended to result in subsequent amnesia for that memory (see **Figure 4**). On a theoretical level, the idea has many advantages since the treatments are known to affect only the reactivated memory and not others, even closely related memories (Debiec et al., 2006).

Numerous preclinical studies have shown that treatments presumed to interfere with reconsolidation given at memory reactivation can result in a loss of the initial memory concerning either drugs of abuse (Lee et al., 2006a; Robinson and Franklin, 2007; Fricks-Gleason and Marshall, 2008) or trauma memories (Debiec and Ledoux, 2004; Muravieva and Alberini, 2010; Schneider et al., 2014). However, most of the studies, which conclusively demonstrated the possibility of inducing amnesia by the use of reconsolidation blockade, have been performed on animals, with treatments that are too toxic to be used in humans. The only one that has been tested in humans is the β-adrenoceptor antagonist, propranolol. This drug has been shown to produce retrograde amnesia when delivered shortly after training or after memory reactivation (Przybyslawski et al., 1999; Lonergan et al., 2013). As such, propranolol has been suspected of interfering with consolidation/reconsolidation processes, through mechanisms that have still not been elucidated. Particular attention has been paid to the therapeutic potential of this reconsolidation blockade in PTSD. However, despite the considerable attention to its therapeutic potential in PTSD (Lonergan et al., 2013; Brunet et al., 2018), the drug's impact on patients is not always effective and mixed findings on propranolol and reconsolidation have been reported (Giustino et al., 2016). These findings weaken the possibility that propranolol may serve to permanently abolish trauma memories.

Preclinical studies in animal models have also demonstrated the possibility of using reconsolidation blockade to weaken or even erase drug memories (Miller and Marshall, 2005; Milton et al., 2008; Barak et al., 2013). More recently, reconsolidation blockade has been explored as a therapeutic strategy to addicted patients (Dunbar and Taylor, 2017). However, although application of reconsolidation blockade treatments has also produced mixed outcomes in SUD populations, it continues to be further investigated (Exton-McGuinness and Milton, 2018).

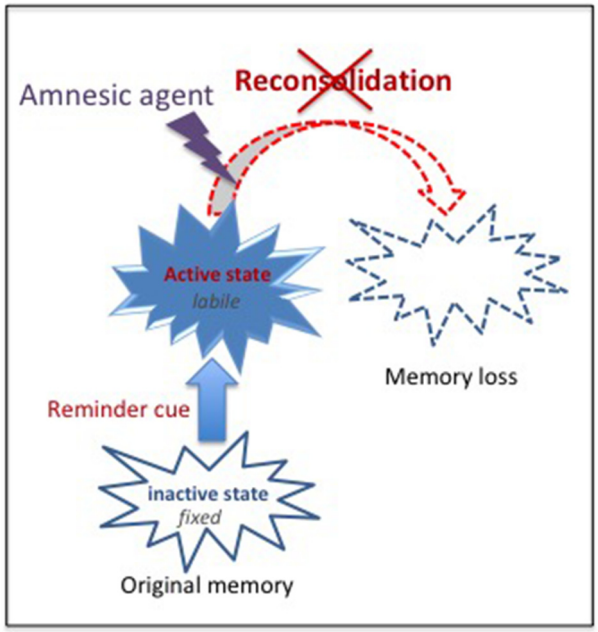

FIGURE 4 | Reconsolidation blockade. According to the consolidation/reconsolidation hypothesis, reactivation destabilizes memories, re-inducing a state of lability during which they are susceptible again to amnesic treatments. Delivering an amnesic treatment at that time, is proposed to interfere with the reconsolidation processes required to re-stabilize the memory, and thus lead to a loss of the original memory.

Retrieval-Dependent Approaches

Recent advances have proposed new therapeutic approaches based upon disruption of reconsolidation by behavioral interference, rather than pharmacologic blockade. It has been shown in rats and in humans that extinction learning delivered after the reactivation of a fear memory prevents the return of fear frequently obtained after extinction-based therapy (Monfils et al., 2009; Schiller et al., 2010; but see Luyten and Beckers, 2017). Similar results have been reproduced in rodent models of addiction and in human substance users (Cofresí et al., 2017; Germeroth et al., 2017). These studies have been analyzed as a demonstration that reconsolidation does not only support restabilization of memory but can also be used to update memory with new information. Such a view has been adopted in some recent studies with the aim of overwriting naturalistic maladaptive memories associated with substance use and trauma-related disorders, by the use of counterconditioning introduced after memory reactivation (Das et al., 2015; Walsh et al., 2018). Up to now, these attempts have provided interesting results but no decisive outcomes.

New Therapeutic Approaches Provided by the Integration Concept

The Integration Concept as an Alternative to the Consolidation/Reconsolidation Hypothesis

However, the consolidation hypothesis is unable to account for some results and especially, why the "amnesia," resulting from treatments supposed to prevent a normal functioning of the consolidation/reconsolidation processes, can be abolished either spontaneously or by pretest procedures such as delivering a reminder which can be a contextual cue, the reinforcer, and even the amnesic treatment itself (see Gisquet-Verrier and Riccio, 2018). These results demonstrate that retrograde amnesia is not due to an encoding disruption but to retrieval difficulties, a view proposed a long time ago (Miller and Springer, 1973; Miller and Matzel, 2006) but never fully considered by others. We proposed an alternative view, the integration concept, which is able to account for the variety of results obtained. According to our view, active memories (a state obtained shortly after training or memory reactivation; Lewis, 1979) are not fragile (i.e., cannot be erased) but they are malleable (i.e., can be modified) and can therefore integrate new information, including the new state induced by amnesic treatments (see **Figure 5**). Hence, the impairment detected at the time of testing is not due to a disruption of the fixation process, as proposed by the consolidation hypothesis, but results from retrieval difficulties due to the absence of a determinant cue: the internal state provided by the amnesic treatment[1] which has been integrated into the initial memory. We have presented numerous examples provided by the literature indicating that the integration of that state within the initial memory disrupts the optimal functioning of the retrieval processes. However, the fact that re-introducing the drug state before testing abolishes retrograde amnesia, shows that the disruption results from retrieval difficulties due to the absence of that state, which became a determinant aspect (internal state) of the memory, a phenomenon known as state-dependency. Memory malleability, which is the main characteristic of active memories (Lewis, 1979; Gisquet-Verrier and Riccio, 2012), allows integration of new information, a process through which memories can be rapidly updated and modified. Depending on the information content, integration may update (new information), strengthen (supplementary information), weaken (interfering information) or even distort (false information) initial memory (Gisquet-Verrier and Riccio, 2018). Accordingly, integration of new information can induce changes of the memory content, even long after the events took place. Such a view opens the way to new therapeutic approaches for pathological memories. From a theoretical point of view, there are two different possibilities for new information delivered to reduce the impact of undesirable memories. First, by modifying the internal state of the subject during the reactivation of a remote memory, a state dependency procedure by which a memory can be made inaccessible, such as for retrograde amnesia. Second, by delivering a pharmacological treatment that reduces the emotional response before reactivating the pathological memory, a procedure termed emotional remodeling, which could allow the integration of a reduced emotional value within that memory.

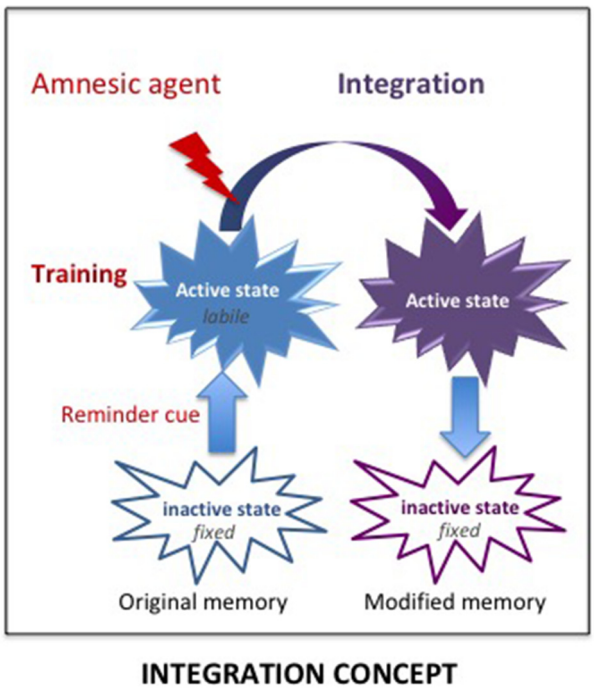

FIGURE 5 | The Integration concept. According to the integration concept, the amnesic treatment delivered shortly after training induces a new internal state, which is integrated within the original memory that has been made malleable by the reminder-induced reactivation. As a result, the memory now includes the internal state provided by the amnesic agent. The lack of this determinant cue at the time of retrieval can disrupt the retrieval efficacy, leading to an apparent loss of memory, which is based on retrieval impairment.

State Dependency

State-dependency is a very well-known phenomenon, accounting for retrieval difficulties occurring when the retention of information is tested in a state different from the one prevailing during the acquisition of that information (Overton et al., 1964; Koek, 2011). State dependency is a very general phenomenon, largely neglected, which is certainly a major source of retrieval variability (see **Figure 6**). It has been demonstrated in various circumstances with cues affecting either the internal (drug, mood) or external (environmental context) state (For a review, see Radulovic et al., 2017). Most, if not all of the psychoactive drugs such as amphetamine, cocaine, and alcohol can induce state dependency. Retrieval disruption can also be obtained by drugs which severely modifies the internal state such as lithium chloride and chemotherapy (Zarrindast et al., 2006; Gisquet-Verrier et al., 2015; Lindner et al., 2017). State dependency can also be obtained from changes concerning the surrounding environment, mood or states of consciousness

[1] Amnesic treatments are always severe treatments which deeply affect the internal state, even when delivered within specific brain structures (Gisquet-Verrier et al., 2015). Accordingly, the internal state is an important aspect of the memory, the absence of which can induce retrieval difficulties.

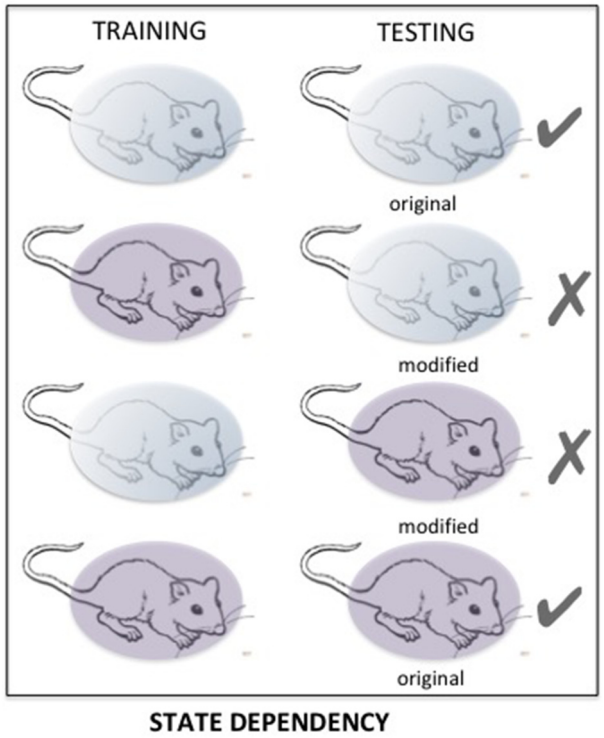

FIGURE 6 | The matching of states between training and testing determines the quality of the retention performance. Changes in important aspects of that state may induce a performance disruption resulting from impaired retrieval. Here, «state» refers to internal/external contexts, which can be naturally or intentionally modified.

(Radulovic et al., 2017). Most of the time, state dependency results in moderate memory disruptions. However, depending on the conditions, the disruption can be stronger and can even lead to complete amnesia. For instance, state dependency can explain amnesia resulting from sexual assault ("date rape") following unintended consumption of drugs such as gamma-hydroxybutyrate (GHB; Schwartz et al., 2000; Johansson et al., 2014). State dependency has also been thought to be responsible for dissociative amnesia such as those depicted in some individuals exposed to psychological trauma (Radulovic et al., 2018). Originally, it was thought that state-dependency only resulted from an alteration of the normal state at the time when the events took place. However, recent evidence showed that state dependency can also be obtained for remote memory. Under these conditions, the remote memory must be reactivated either while the subject is under the modified state (Sierra et al., 2013), or just before introducing changes of the internal state (Gisquet-Verrier et al., 2015). The possibility of disrupting retrieval, by introducing a state dependency long after training, opens new therapeutic avenues, which have not yet been explored.

Emotional Remodeling: Integration of New Information

The integration concept (Gisquet-Verrier and Riccio, 2018) provides another therapeutic approach consisting of integrating a reduced emotional component within the pathological memory. Up to now, preclinical studies showed that different types of information could be introduced while the memory is in an active state. For instance, remote memories can integrate new contextual information (Boller and Rovee-Collier, 1992; Briggs and Riccio, 2008), or a new relationship between cues (Tronel et al., 2005). There is also evidence suggesting that the emotional component of active declarative memories can be modified (Arminjon et al., 2015). Hence, we propose that preventing the occurrence of strong emotional responses elicited by the reactivation of a pathological memory, by a prior administration of a pharmacological treatment known for its relaxing properties, could allow the integration of a reduced emotional component within that pathological memory (see **Figure 7**). We have termed this procedure *emotional remodeling*. Interestingly, this can be achieved with propranolol, known to lower heart rate and blood pressure, but also to have anxiolytic properties (Turner and Granville-Grossman, 1965; Steenen et al., 2016). We recently delivered this β-adrenoceptor antagonist treatment to a cocaine-user patient before eliciting the reactivation of his drug memories by drug reminders. Several repeated pairings between reduced anxiety and reactivation of drug memories, associated with a cognitive behavioral therapy, have been able to reduce and then abolish drug taking and craving over a very long period of time (Chopin et al., 2016). Emotional remodeling can thus explain the effects obtained by propranolol in PTSD and SUD patient. However, according to our view, other treatments

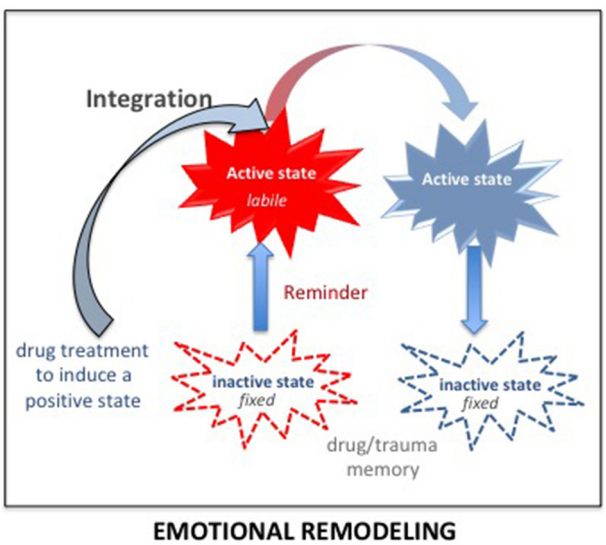

FIGURE 7 | According to the integration concept, any salient information present during memory reactivation (including just before and after) is integrated within the original memory, modifying its original content. Delivering a drug inducing a positive state before the reactivation of a pathological memory (trauma/drug) strongly reduces the emotional component of the reactivated memory. Due to the malleability of the reactivated memory, this reduced emotional value will be integrated into the original memory. By doing so several times, the trauma/drug memory will progressively lose its pathologic characteristics and reach the status of an ordinary memory. This procedure has been termed: emotional remodeling.

might be much more effective. In rats, using our SPS model, we showed that a single injection of d-amphetamine (known to induce a positive mood in human; Kirkpatrick et al., 2016) delivered 30 days after the trauma, durably abolished most of the SPS-induced effects. While amphetamine *per se* did not modify the behavior of non-traumatized or resilient rats, trauma susceptible rats treated with amphetamine no longer differed from controls in the symptom tests, (Toledano and Gisquet-Verrier, 2014). These results can be related to the "amphetamine narcosis," a procedure used during the Algerian conflict in 1960, consisting of a combination of a barbiturate and amphetamine, delivered just before the reactivation of the trauma memory, which produced successful results in PTSD patients (Delay, 1949; Crocq, 1999). Amphetamine, categorized as an agonist replacement therapy, has shown efficacy in reducing cocaine intake in human addicts in multiple clinical trials (e.g., Rush and Stoops, 2012). In the 1980s, a form of amphetamine, the *3,4-methylenedioxy-methamphetamine* (MDMA), or ecstasy, a synthetic drug producing feelings of increased energy, pleasure, emotional warmth, was used as an adjunct for psychotherapy by a number of therapists in California (USA) for treatment-resistant PTSD patients (Parrott, 2007). However, MDMA-assisted psychotherapy was abandoned when the use of MDMA became illegal. Interestingly, MDMA-assisted psychotherapy has recently been re-introduced in the United States (Amoroso and Workman, 2016; Mithoefer et al., 2018). It is emphasized that the treatment, delivered together with specialized psychotherapy support, appears to facilitate the recall of traumatic memories without the patient feeling overwhelmed by the negative affect that usually accompanies such memories (Sessa, 2017). Since MDMA treatment needs to be delivered just two or three times, it is not considered likely to prime a drug dependency. These treatments have been considered to strengthen the relationship of trust between the patient and the therapist but might be rather viewed as effective drugs to induce an emotional remodeling.

Since amphetamine and MDMA are known to produce oxytocin release, a neuropeptide which increases social approach and adaptation by attenuating anxiety and stress, and globally contributes to promote "trusting behavior" (Baumgartner et al., 2008), we tested the effects of oxytocin in our previously used PTSD rodent model. In this experiment, 1 month after SPS, rats received two remodeling sessions, involving an intraventricular infusion of oxytocin, before a re-exposure to a SPS-related cue. Our results indicated that 83% of SPS-vulnerable rats treated with oxytocin showed a complete remission of PTSD-like symptoms, with no relapse up to 1 month after the treatment. In addition, we showed that oxytocin-based emotional remodeling durably reversed the neural consequences of SPS, suggesting that this treatment represents a promising approach to treat fear memory disorders (Le Dorze et al. submitted). Interestingly, the ability of oxytocin to attenuate drug seeking and craving has been recently pointed out (Sarnyai and Kovács, 2014). After drug self-administration learning in rats followed by extinction, oxytocin combined with drug-cue presentations has been reported to attenuate drug seeking during a reinstatement test. The ability of oxytocin to attenuate drug seeking and craving has been reported with various drugs of abuse, including methamphetamine, ethanol, heroin, morphine and cocaine (Sarnyai and Kovács, 2014; Leong et al., 2017).

Unlike prolonged exposure therapy, which gives rise to an extinguished memory that competes with the original memory to control behavior, emotional remodeling is supposed to modify the original memory. Hence, any new reminders encountered after the treatment will induce the reactivation of the modified memory, suggesting that the effect of emotional remodeling could be permanent.

Other procedures, presented above in the *Retrieval-dependent approach* section, such as the multisensory disgust-based counterconditioning procedure which have recently been used to re-write alcohol cue-reward associations in maladaptive reward memories (Das et al., 2015; Hon et al., 2016; Goltseker et al., 2017) could advantageously be analyzed as cases of emotional remodeling.

CONCLUSION

Despite disparities based on differing theoretical backgrounds, a wealth of evidence shows that the malleability of reactivated memory has opened up new therapeutic avenues, based on the possibility of permanent modification of long-term memories. This has become an invaluable resource to find common psychotherapeutic strategies to treat pathological memories such as PTSD and SUD in the context of reactivation-dependent memory malleability. The procedure requires, first, placing patients in safe and secure conditions in order to enhance the therapeutic alliance (an effect that can be strengthened by the use of a pharmacological drug enhancing the sense of emotional well-being). Second, by exposing participants to trauma/drug reminders to reactivate the related pathological memory, in order render it malleable. The role of cognitively based therapy, frequently associated with this procedure, is to maintain the memory in an active state in the presence of new information. It must be emphasized that such a scheme not only corresponds to reconsolidation or integration-based treatments but also to others such as *eye movement desensitization and reprocessing* (EMDR), *Neuro-Linguistic Programming* (NLP) and even psychoanalysis. All of these have already been considered to be effective for PTSD and SUD pathologies.

Up to now, it was not clear by which mechanisms these therapies were able to treat patients. The integration concept allows us to propose that all of them may act in the same way, which is to introduce a reduced emotional response within the pathological memory, thereby reducing its disruptive consequences. All these therapies for psychiatric disorders, based upon reactivation-dependent memory malleability, are simple, inexpensive, and easy to arrange. Since these approaches have already provided promising results, they should be considered more seriously for clinical application in the near future for a number of other pathologies, such as phobias, feeding disorders, anxiety, etc.

By questioning the interpretation of numerous well-established aspects of memory processes, the integration concept adds to our understanding of the dynamic and flexible

aspects of memory and by doing so opens new research approaches to treating various psychopathologies.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

PG-V and CL wrote the article and approved it for publication.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Cognitive Flexibility Training Improves Extinction Retention Memory and Enhances Cortical Dopamine With and Without Traumatic Stress Exposure

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Stress exposure can cause lasting changes in cognition, but certain individual traits, such as cognitive flexibility, have been shown to reduce the degree, duration, or severity of cognitive changes following stress. Both stress and cognitive flexibility training affect decision making by modulating monoamine signaling. Here, we test the role cognitive flexibility training, and high vs. low cognitive flexibility at the individual level, in attenuating stress-induced changes in memory and monoamine levels using the single prolonged stress (SPS) rodent model of traumatic stress in male Sprague-Dawley rats. Exposure to SPS can heighten fear responses to conditioned cues (i.e., freezing) after a fear association has been extinguished, referred to as a deficit in extinction retention. This deficit is thought to reflect an impairment in context processing that is characteristic of posttraumatic stress disorder (PTSD). During a cognitive flexibility training we assessed individual variability in cognitive skills and conditioned rats to discriminately use cues in their environment. We found that cognitive flexibility training, alone or followed by SPS exposure, accelerated extinction learning and decreased fear responses over time during extinction retention testing, compared with rats not given cognitive flexibility training. These findings suggest that cognitive flexibility training may improve context processing in individuals with and without traumatic stress exposure. Individual performance during the reversal phase of the cognitive flexibility training predicted subsequent context processing; individuals with high reversal performance exhibited a faster decrease in freezing responses during extinction retention testing. Thus, high reversal performance predicted enhanced retention of extinction learning over time and suggests that cognitive flexibility training may be a strategy to promote context processing. In a brain region vital for maintaining cognitive flexibility and fear suppression, the prelimbic cortex (PLC), cognitive flexibility training also lastingly enhanced dopamine (DA) and norepinephrine (NE) levels, in animals with and without traumatic stress exposure. In contrast, cognitive

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Abbreviations: PTSD, posttraumatic stress disorder; SPS, single prolonged stress; DA, dopamine; NE, norepinephrine; DOPAC, 3,4-Dihydroxyphenylacetic acid; HVA, homovanillic acid; 3MT, 3-Methoxytyramine.

flexibility training prior to traumatic stress exposure decreased levels of DA and its metabolites in the striatum, a region mediating reflexive decision making. Overall, our results suggest that cognitive flexibility training can provide lasting benefits by enhancing extinction retention, a hallmark cognitive effect of trauma, and prelimbic DA, which can maintain flexibility across changing contexts.

Keywords: cognitive flexibility, dopamine, norepinephrine, single prolonged stress, trauma, PTSD

HIGHLIGHTS

- Extinction retention after trauma was enhanced by prior cognitive flexibility training.
- Cognitive flexibility training may rescue cognitive deficits in PTSD.
- Individuals with high reversal learning performance had greater extinction retention.
- Cognitive flexibility training increased dopamine in the prelimbic cortex.
- Cognitive flexibility training buffered the effects of stress on striatal dopamine.

INTRODUCTION

Maintaining cognitive flexibility, i.e., the capacity to shift behavioral strategies in a changing environment, is critical to an individual's ability to update environmental representations (reviewed in Kehagia et al., 2010). Low cognitive flexibility can be precipitated by stress exposure, and variability in cognitive flexibility is high across and within species (Laughlin et al., 2011; Miyake and Friedman, 2012). In humans, retrospective clinical studies have shown that individuals with low cognitive flexibility exhibit increased psychopathology severity or progression, and deficits in cognitive flexibility have been characterized in affective, anxiety, and neurodegenerative disorders (Chamberlain et al., 2006; Dickstein et al., 2007; Tchanturia et al., 2012, 2013; Brockmeyer et al., 2014). For example, individuals with low cognitive flexibility have higher levels of posttraumatic stress disorder (PTSD) symptoms and less posttraumatic growth and optimism (Keith et al., 2015). Conversely, individuals with heightened cognitive flexibility may have enhanced resilience to change and self-efficacy (Kim and Omizo, 2005; Genet and Siemer, 2011; Mealer et al., 2012; Romero-Martínez et al., 2013). Cognitive flexibility can be heightened through interventions in childhood or adulthood (Masley et al., 2009; Moore and Malinowski, 2009; Genet and Siemer, 2011; Lewis-Morrarty et al., 2012). Investigating whether enhancement in cognitive flexibility, through cognitive training prior to trauma exposure, can reduce PTSD symptoms following trauma, could advance discussions of interventions for resilience and recovery from traumatic experiences.

Cognitive flexibility training paradigms often incorporate multiple aspects of cognitive flexibility, which all require "letting go" of an old association and acquisition of a new association, and extensive efforts have been made to identify psychological and neuropharmacological mechanisms underpinning aspects of cognitive flexibility (Kehagia et al., 2010). Two distinct aspects of cognitive flexibility are reversal learning, when reinforcement is shifted from a familiar, previously rewarded to cue to a familiar cue that was not previously rewarded, and attention shifting, where novel cues are presented and an individual forms an association with a new unconditioned cue (Birrell and Brown, 2000; Klanker et al., 2013). Animals undergoing reversal learning exhibit greater and more extended dopamine (DA) release in the medial PFC, compared with associative learning, but no difference in norepinephrine (NE) output (van der Meulen et al., 2007). Yet, tonic elevation of NE in the mPFC can enhance reversal learning and attention-shifting (Lapiz and Morilak, 2006). Thus, behaviorally distinct mechanistic aspects of cognitive flexibility share common regional specificity, including corticostriatal circuitry, and reliance on monoamine signaling (Logue and Gould, 2014), perhaps because of shared need for extinction of a familiar association and acquisition of a novel association.

Research in rodents and humans suggests that cognitive flexibility is mediated by reciprocal interactions between the striatum and PFC (reviewed in Klanker et al., 2013). For example, increased DA activity in the striatum can decrease DA in the PFC and limit PFC afferent input into the striatum (Roberts et al., 1994; Strafella et al., 2001; Goto and Grace, 2005). Striatal DA activity regulates inhibitory input, which acts on corticostriatal circuitry to affect cognitive flexibility (Logue and Gould, 2014). Similarly, PFC α_2-adrenergic receptor binding density has a linear relationship with perseverative errors during a set shifting task (Arnsten et al., 1999). However, excess extracellular NE binds to α_1-adrenergic receptors, which can broadly impair executive functions through dysregulation in cortical circuits (Arnsten et al., 1999; Carr et al., 2007; Arnsten, 2015; Luo et al., 2014, 2015). Severe, trauma-like stress can dampen mPFC activation and prefrontal glutamate levels (Knox et al., 2010; Perrine et al., 2016), but may enhance striatal activity and monoamine release (Abercrombie et al., 1989; Jastreboff et al., 2011; Nikolova et al., 2012). Together these reciprocal changes might suggest that, stress exposure can prompt a transition from reflective, flexible responding, mediated by the PFC to compulsive, reflexive responses mediated by the striatum (Keller et al., 1983; Sinha et al., 2005; reviewed in Arnsten et al., 2015).

Individual characteristics, including stress history, cause variation in aspects of cognitive flexibility, and the monoamine regulation of cognitive performance (Dias-Ferreira et al., 2009; Laughlin et al., 2011; Naegeli et al., 2013). Animal models that use longitudinal stress manipulations have been essential for

the understanding of intersections between mechanisms that maintain cognitive flexibility and effects of trauma. For example, exposure to a rodent model of PTSD called single prolonged stress (SPS) can increase perseverative errors during reversal learning and never-reinforced errors during attention-shifting as well as dampen striatal DA signaling (Eagle et al., 2013; Enman et al., 2015; George et al., 2015). Exposure to SPS can also cause a cognitive deficit in rodents similar to that detected in PTSD patients, in which recall of fear extinction is impaired, called an extinction retention deficit (Milad et al., 2008, 2009; Knox et al., 2012, 2016; Perrine et al., 2016). Extinction retention can be impaired by reduced mPFC activation, loss of dopaminergic neurons in the mPFC, or disrupted DA signaling (Espejo, 2003; Mueller et al., 2010; reviewed in Greco and Liberzon, 2016). Indeed, rats exposed to SPS show reduced DA receptor density, DA levels, and DA metabolites in the striatum (Enman et al., 2015; Perrine et al., 2016). Conversely, completing reversal learning tasks can increase DA efflux in the mPFC (van der Meulen et al., 2007), while attention shifting tasks can increase DA efflux in the mPFC and dorsal striatum (Stefani and Moghaddam, 2006).

Given that cognitive flexibility can increase resilience and lessen PTSD symptom severity, it may mitigate effects of trauma by interacting with mechanisms directly dysregulated in PTSD (Goto and Grace, 2005; Moore and Malinowski, 2009; Lewis-Morrarty et al., 2012). Here, we investigate whether exposure to cognitive flexibility training can attenuate the effects of trauma on extinction retention of conditioned fear learning. To do this, we used SPS as a rodent model of traumatic stress that has been shown to diminish both extinction retention and cognitive flexibility performance (Knox et al., 2012, 2016; George et al., 2015). To address interacting mechanistic effects of cognitive flexibility training and stress exposure, we investigate corticostriatal monoamine and metabolite levels and their relationship with individual cognitive flexibility performance. We hypothesized that cognitive flexibility training would enhance resilience to the effects of trauma on behavior and catecholamine signaling.

MATERIALS AND METHODS

Subjects and Housing

Male Sprague-Dawley rats (n = 36) were obtained at 40 days of age from Charles River Laboratories (Kingston, NY, USA). Upon arrival, rats were pair housed and randomly assigned to one of four possible treatments, with/without cognitive flexibility training and with/without exposure to SPS. Animals were housed in standard microisolator, plastic cages (20 × 26 × 45 cm) with wood chip bedding replaced weekly, and were given 5 days to acclimate following transport before experimental procedures began. A timeline of all procedures is depicted in **Figure 1**. Standard rat chow (LabDiet® 5001, 23% protein) and tap water were available *ad libitum*, except prior to behavioral testing procedures that were rewarded, in these cases rats were food deprived for 2 h beforehand. Rats were kept at 20–22°C and 50% relative humidity on a 12:12 light/dark cycle. To control for circadian rhythms, tests were started a minimum of 3 h after the beginning of the dark cycle and completed within 4 h of the start of the test. Control rats and cognitive flexibility training rats received a weekly handling and weighing session until SPS procedures, to ensure that rats habituated to handling and maintained healthy weight. Following SPS, all rats were singly housed and were handled only for fear learning procedures described below (Liberzon et al., 1999 and Knox et al., 2012). Testing order was pseudo-randomized; and treatment groups were evenly distributed during the first and last hours of the testing. Equipment was sprayed with 70% ethanol in water solution and wiped clean between all trials and subjects. Experiments were approved by the VA Ann Arbor Healthcare System Institutional Animal Care and Use Committee (#1312-004).

Cognitive Flexibility Training

A group of 18 rats were exposed to cognitive flexibility procedures based on Birrell and Brown (2000); one rat was removed because it would not consistently sample available food bowls. To ensure that potential effects of the cognitive flexibility training would persist, there was a 2-week delay following training, before a subset of eight rats were exposed to SPS as described below. The remaining nine rats exposed to cognitive flexibility training served as an unstressed control group. An additional 18 rats, not exposed to cognitive flexibility training, were maintained in standard housing conditions as controls until 115 days of age, when nine rats were exposed to SPS, and the remaining nine were used as unstressed controls.

Cognitive Flexibility Apparatus

The plastic testing apparatus (45 × 63 × 36 cm) contained a removable divider and two ceramic bowls, each in a corner

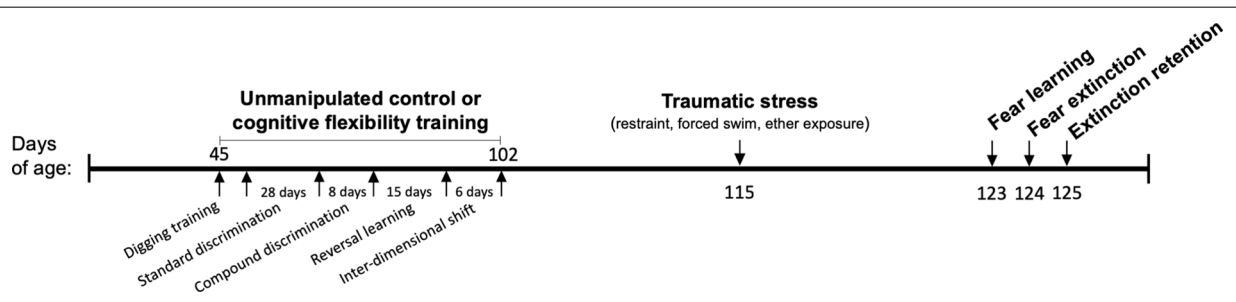

FIGURE 1 | Timeline of procedures; DA, dopamine; NE, norepinephrine.

TABLE 1 | Cues used for cognitive flexibility tasks.

Odor pairs	Digging substrate pairs
Coriander, Cumin	Paper bedding, Clay
Cinnamon, Turmeric	Coconut husks, Corncob
Mustard, Fenugreek	Cellulose fiber, Confetti crinkles bedding

opposite the start area (bowl diameter 7 cm, depth 4 cm). Both bowls contained 40 mL of digging substrate, in one bowl the substrate concealed a Honey Nut Cheerio®. Stock digging substrates contained 5.5 L of bedding mixed with 70 mL of an added scent cue (**Table 1**) and 10 Cheerios ground into powder, to balance reward scent cues. A removable divider separated rats from the bowls at the beginning of each trial, and was replaced after an error to prevent access to the rewarded bowl. The bowls used in the in-cage digging training and the cognitive flexibility training were identical.

Digging Training

Laboratory rats can be trained to dig for food reward in the substrates listed in **Table 2**. To train for digging behavior, rats were individually presented with a bowl containing one-quarter of a Cheerio in clean cages identical to their home cage for 10 min on five occasion following 2 h of food deprivation. Digging bowls were identical to the cognitive flexibility training bowls. In the first session, the reward was presented in the empty bowl. The second session was identical to the first except that 10 ml of aspen bedding was added to the bowl, such that the Cheerio reward was still visible but partially obscured by bedding. An additional 10 ml of aspen bedding was added in each of the three subsequent Cheerio presentations, completely covering the reward, such that on the final exposure the bowl contained the same amount of digging substrate as in the cognitive flexibility trials (40 mL total).

Cognitive Flexibility Training

A trial was initiated 20 s after the rat was placed into the "start area" of the arena by raising the removable divider to give the rat access to the reward bowl. In the first four trials, rats could dig in both bowls, but only one was rewarded. In subsequent trials, after a rat selected a bowl by inserting a nose or paw into the bowl, a plastic insert was immediately added to separate the animal from accessing the other chamber containing the unselected bowl. A trial was recorded as correct if the rat inserted its nose or paw into the rewarded bowl first. After a bowl was selected, the removable divider was inserted to prevent access to the unselected bowl, and the rat was allowed 20 s with the selected bowl to either consume the reward or reinforce the error. Rats were then transferred from the maze into a holding chamber for a 20 s intertrial-interval and the bowls were reset. If a rat did not select a bowl in 5 min, the animal was transferred from the maze into a holding chamber and the bowls were reset. The side of the arena containing the rewarded bowl was randomized, and bowls could be distinguished by multiple cues types. Each session contained four trials, sessions were conducted once each day during the four tasks comprising the cognitive flexibility training: a standard discrimination task (28 sessions), a compound discrimination task (eight sessions), a reversal learning task (15 sessions), and an inter-dimensional shift task (six sessions).

In the standard discrimination task, one cue type distinguished the rewarded and unrewarded bowl (associative learning; **Table 1**). For the compound discrimination task, a second cue type was introduced, but the cues from the standard discrimination task remained constant for eight additional training sessions (associative learning with distracting cues). For the reversal task, the context cues were not changed but the food-paired cue was switched, within the relevant cue type, such that the previously incorrect cue became the relevant cue. For the inter-dimensional shift task, the cues in the relevant and irrelevant cue types were all changed (a total change in all cues), and a novel cue from the previously-relevant cue type signaled the reward location. To maximize contrast between digging substrate textures and reduce the degrees of freedom, cues were always used in pairs; for example, if clay contained the rewarded stimulus, the unrewarded stimulus was always paper bedding, and vice versa (Birrell and Brown, 2000). The order of the tasks was always the same, but the cues were equally represented within groups and counterbalanced between groups so that an equal number of rats from the SPS and unstressed group were exposed to each cue pairing.

Single Prolonged Stress

The SPS rodent model of traumatic stress exposure was used here because it has been used for two decades to model PTSD-specific traits (Liberzon et al., 1997; Khan and Liberzon, 2004; reviewed in Lisieski et al., 2018). Similar to PTSD patients, exposure to SPS can increase GR receptor levels, startle responsivity, anxiety-like behavior, pro-inflammatory cytokine levels, sleep disturbances, anhedonia, and cause extinction retention deficits (Khan and Liberzon, 2004; Yamamoto et al., 2009; Nedelcovych et al., 2015; Vanderheyden et al., 2015; Lin et al., 2016; reviewed in Deslauriers et al., 2018). In the SPS model, rats are exposed to three stressors in succession (lasting approximately 3 h in total), followed by social isolation for 7 days, procedures described in

TABLE 2 | Descriptions of cognitive flexibility tasks.

Task	Description	Example cue reward pairing
Standard discrimination	Associative learning between a food reward and a cue, with only one cue type	Rewarded bowl—cinnamon Unrewarded bowl—turmeric (aspen bedding digging substrate)
Compound discrimination	Associative learning between a food reward and a cue, with two cue types: one relevant and one irrelevant cue type	Rewarded bowl—cinnamon (+ paper bedding or clay digging substrate) Unrewarded bowl—turmeric (+ paper bedding or clay digging substrate)
Reversal learning	The relevant cue is switched, but is within the same cue type	Rewarded bowl—turmeric (+ paper bedding or clay digging substrate) Unrewarded bowl—cinnamon (+ paper bedding or clay digging substrate)
Inter-dimensional shift	All available cues change; the relevant cue changes, within the same cue type	Rewarded bowl—mustard (+ cellulose or confetti digging substrate) Unrewarded bowl—fenugreek (+ cellulose or confetti digging substrate)

Khan and Liberzon (2004) and Knox et al. (2012). Rats were first restrained for 2 h, then underwent 20 min of forced swim in cold water (23–24°C), in a 68 × 56 × 45 cm opaque plastic container. After swimming, rats were dried and given 15 min to recuperate. Next, rats were exposed to ether vapors in a desiccator until loss of consciousness, as determined by lack of a paw withdrawal or toe pinch reflex response. Animals were then individually-housed in clean cages and left undisturbed for 7 days, the delay required for a PTSD-like phenotype to develop (Liberzon et al., 1999; Knox et al., 2012). Prior research has demonstrated that SPS-induced neuroendocrine effects, including HPA negative feedback and glucocorticoid receptor mRNA expression, are only evident after SPS following a 7-day quiescent period (Liberzon et al., 1997, 1999), reflecting the 30-day post-trauma delay required before PTSD can be diagnosed in humans (reviewed in Cahill and Pontoski, 2005). Thus, it is often included in the SPS model to isolate rodents for a 7-day quiescent period to prevent social buffering, and to integrate lasting effects of the trauma exposure (Knox et al., 2012; Chen et al., 2018; George et al., 2018). As brief periods of isolation in adulthood can also have neuroendocrine effects, control rats were also isolated to account for potential effects of housing (Raz and Berger, 2010).

Fear Learning

After the 7-day quiescent period following SPS, rats were trained to associate a tone with a shock across five shock-tone pairings in a fear conditioning chamber (day 1: fear conditioning). Then, in a novel context, rats were repeatedly presented with the same tone until their fear responses to the tone were extinguished (day 2: fear extinction). Finally, rats were returned to the second context and re-exposed to the tone to determine if they retained the information that the tone was not paired with the shock in the second context (day 3: extinction retention). The fear conditioning context was distinguished from the second context using visual, olfactory, and tactile cues (additional details in **Supplementary Methods**). Freezing responses were quantified as a proxy of fear (Bouton and Bolles, 1980; Knox et al., 2012); freezing was defined as immobility, lasting longer than 1 s, but allowing for small pendulum-like head movements with all four feet and the body immobile (and without vibrissae flicking), as this is also suggested to be a fear behavior in rats (Kolpakov et al., 1977) and other small mammals (Halpin, 1983; Ayon et al., 2017). To minimize disturbance, the experimenter was not in the room during testing; trials were video recorded and freezing behavior was measured by analysists blind to treatment. Freezing behavior was measured in temporal blocks defined by each stimulus presentation, then percent time freezing was calculated as (time freezing in stimulus block/total time in stimulus block) × 100.

Fear Learning: Shock Reactivity

To determine if the cognitive flexibility treatment affected pain sensitivity, behavioral response to the five shocks administered during fear conditioning was rated by two independent analysts blind to treatment conditions, using video recordings to allow for later analysis. Shock responses were rated on a 5-point scale, modified from Menard et al. (2004): (1) flinch involving only the head or forepaw; (2) whole body flinch, with or without ambulation; (3) whole body flinch and/or jump (all four feet in the air), followed by ambulation, or a jump without ambulation; (4) whole body flinch, followed by running; and (5) whole body jump (all four feet in the air), followed by running.

Neurochemical Analysis With High-Pressure Liquid Chromatography (HPLC)

On the day following extinction retention procedures, brains were harvested following rapid decapitation and flash frozen for later processing. To obtain brain region tissue punches, brains were thawed at −20°C for 10 min. Brains were then sliced using a chilled stainless steel rat brain matrix, resulting 2 mm sections were mounted on dry ice. Using a 1.5 mm biopsy punch, bilateral tissue punches were obtained from the prelimbic cortex (PLC), infralimbic cortex, and dorsal anterior striatum, in accordance with the Paxinos and Watson Rat Brain Atlas. Tissue punches were transferred to microcentrifuge tubes and frozen at −80°C for subsequent analysis.

Tissue punches were suspended in 50 μL of 0.2 N $HClO_4$, then sonically disrupted and centrifuged at 4°C and 12,300 rotations per minute for 10 min. Then, a 25 μL aliquot of the resulting supernatant was obtained from each sample, and monoamine analysis was performed on a Dionex Ultimate 3,000 high-pressure liquid chromatography (HPLC) system (Thermoscientific, Waltham, MA, USA), equipped with an autosampler maintained at 4°C, which autoinjected 10 μL of sample into a 100 μL sample loop on a C18-RP (2 μL diameter) column maintained at 25°C. Thermoscientific TEST Mobile Phase flowed in the column at a rate of 0.6 mL/min, and contained acetonitrile, phosphate buffer, and an ion-pairing reagent; coulometric electrochemical detection was achieved with a dual electrode cell set at −175 mV (reference) and 300 mV (working). Chromatograms were analyzed using Dionex Chromeleon software (version 7); a detection threshold was set at three times the average height of four solvent peaks (neurochemicals below this threshold were omitted from further analysis). Absolute values of monoamines (DA, NE) and monoamine metabolites (DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 3MT, 3-methoxytyramine) were determined by comparison with five dilutions of external standard (Sigma-Aldrich, St. Louis, MO, USA) run in parallel and in duplicate, once at the beginning and once at the end of each run. Monoamine and metabolite levels were corrected for frozen tissue weight to obtain total concentration, expressed as ng neurochemical/mg tissue weight.

Data Analysis

Percent freezing data during fear learning (five trials) were analyzed with a repeated measures analysis of variance (R-ANOVA) test, with cognitive flexibility treatment/SPS condition as fixed effects. Fear extinction (30 trials) and extinction retention (10 trials), due to their length, were separated into

an early phase (first half of trials) and late phase (second half of trials). Each phase was analyzed with a R-ANOVA test, with cognitive flexibility treatment and SPS condition as fixed effects. If an interaction effect was detected, a groupwise analysis was conducted comparing each group directly. Three rats were removed because they did not consistently show freezing behavior [two rats from the control group and one from the group exposed to cognitive flexibility and SPS, resulting in final group sizes of control (7), cognitive flexibility (7), cognitive flexibility and SPS (7), and SPS (9)]. Shock responses across the 5 shock-tone pairings during fear conditioning were also evaluated with a R-ANOVA. To evaluate the relationship between performance during the cognitive flexibility training and subsequent fear behavior during the extinction retention task, we used a R-ANOVA with cognitive flexibility performance as a fixed effect. Rats in the cognitive flexibility training were grouped with a median split for total percent correct during the cognitive flexibility training, to sort rats into high performers and low performers. For the group level neurochemical analysis, univariate general linear models were used with SPS and cognitive flexibility as fixed factors. If an interaction effect was detected, univariate general linear models were used to compare each group. If analytes were below threshold for detection for a tissue sample, that sample was excluded from analysis of that neurochemical for that region [ILC: 4 for NE, PLC: 7 for DA (a maximum of three per group, in the control group); striatum: one rat for DA, one rat HVA, three rats for 3MT]. In the ILC, nearly all rats were below threshold for DA, DOPAC, HVA, and 3MT, so these neurochemicals, and DOPAC:DA, were not analyzed for the ILC. For striatal NE, rats across all groups (21 total) were below the threshold for detection, so NE was not analyzed for the striatum. Analyses were run using IBM® SPSS® Statistics V. 24; values are reported as means ± standard error.

RESULTS

Fear Learning

Fear Conditioning

Neither cognitive flexibility nor SPS affected freezing behavior during fear conditioning, and no interactions were detected ($p > 0.05$, **Figure 2**). Similarly, neither manipulation affected responsivity to the shock ($p > 0.05$, **Supplementary Figure S1**).

Fear Extinction Learning

Given prior evidence that SPS can have distinct effects on freezing behavior during the early and late phases of extinction testing, the first and second halves of the extinction testing were analyzed separately (Knox et al., 2012, 2016; Perrine et al., 2016). During the first half of extinction learning, cognitive flexibility enhanced extinction learning (main effect: $F_{(1,26)} = 6.27$, $p = 0.02$) whereas traumatic stress exposure decreased extinction learning over time (SPS × time effect: $F_{(1,26)} = 2.19$, $p < 0.01$). A groupwise analysis revealed that in unstressed rats, cognitive flexibility enhanced extinction learning ($F_{(1,15)} = 11.82$, $p = 0.01$, **Figure 2A**) and in trauma-exposed rats, prior cognitive flexibility training increased extinction learning in the early phase of the extinction trials compared with rats exposed to trauma alone ($F_{(1,13)} = 4.34$, $p = 0.05$, **Figure 2B**). Additionally, traumatic stress (SPS) exposure

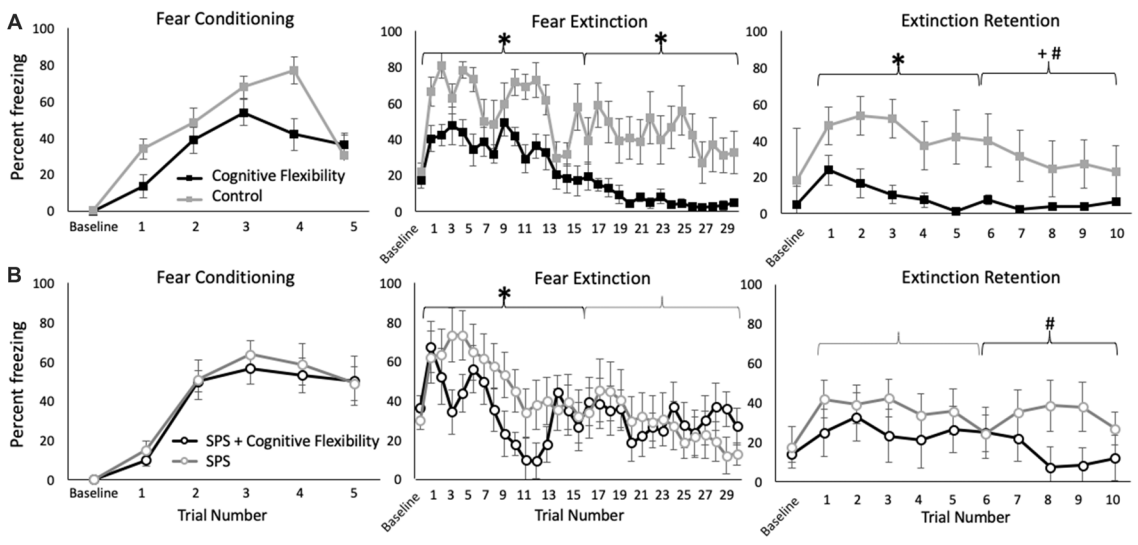

FIGURE 2 | Effects of cognitive flexibility training on freezing during fear conditioning, fear extinction, and extinction retention testing in rats without **(A)** or with **(B)** prior exposure to single prolonged stress (SPS). Animals with cognitive flexibility training are indicated with black lines, animals with SPS are indicated with open circles. During fear conditioning, no group differences were detected. **(A)** In unstressed rats, cognitive flexibility training (black) enhanced extinction learning during the first and second half of the fear extinction learning trials (*$p = 0.01$, $p = 0.04$, respectively). Flexibility training also enhanced the retention of contextual information during extinction retention testing (*$p < 0.05$). **(B)** In rats exposed to traumatic stress, cognitive flexibility training increased extinction learning during the first half of extinction learning (*$p = 0.05$), and enhanced the rate of freezing attenuation during the second half of the extinction retention trials (treatment × time effect, #$p = 0.03$).

decreased the rate of extinction learning (SPS vs. control rats, both without cognitive flexibility training, SPS × time effect: $F_{(1,15)} = 1.94$, $p = 0.03$).

In the second half of extinction learning, cognitive flexibility enhanced extinction learning (main effect: $F_{(1,26)} = 3.94$, $p = 0.05$). There was a trend-level interaction between traumatic stress and cognitive flexibility training ($F_{(1,26)} = 2.98$, $p = 0.09$). A groupwise analysis revealed that in unstressed rats, cognitive flexibility enhanced extinction learning during the second half of the extinction testing ($F_{(1,15)} = 6.11$, $p = 0.04$, **Figure 2A**). In rats exposed to traumatic stress, cognitive flexibility training did not affect extinction learning in the late phase ($F_{(1,13)} = 0.10$, $p = 0.76$, **Figure 2B**). SPS exposure alone did not affect the second half of extinction learning trials (SPS vs. control rats, both without cognitive flexibility training, $F_{(1,15)} = 0.08$, $p = 0.78$).

Extinction Retention Testing

In the first half of the extinction retention testing, cognitive flexibility training enhanced the retention of contextual cues from fear extinction training (main effect: $F_{(1,26)} = 7.26$, $p = 0.01$). No other effects were detected in the first half of extinction retention testing. In the second half of extinction retention testing, cognitive flexibility training also enhanced retention (main effect: $F_{(1,26)} = 5.88$, $p = 0.02$) and this effect interacted with SPS exposure (SPS × cognitive flexibility × time effect: $F_{(1,26)} = 5.47$, $p = 0.03$). A groupwise analysis revealed that cognitive flexibility enhanced extinction retention under control conditions, in the absence of prior traumatic stress ($F_{(1,15)} = 9.72$, $p = 0.01$, **Figure 2A**). For rats that were exposed to traumatic stress, prior cognitive flexibility training enhanced extinction recall over time (cognitive flex × time: $F_{(1,13)} = 2.97$, $p = 0.03$, **Figure 2B**). Cognitive flexibility exposed rats with and without subsequent trauma exposure did not differ in either phase of the extinction retention testing (respectively, $F_{(1,13)} = 2.63$, $p = 0.16$, $F_{(1,13)} = 3.53$, $p = 0.09$). Traumatic stress (SPS) exposure attenuated the rate of change in freezing behavior in the second half of the extinction retention testing, which is congruent with prior findings that SPS impairs extinction retention (SPS vs. control rats, both without cognitive flexibility training, $F_{(1,15)} = 2.87$, $p = 0.03$).

When the results were examined for individual performance during the cognitive flexibility phases, rats with high performance during the reversal learning task demonstrated a greater rate of extinction retention during the extinction retention testing [**Figure 3**, reversal performance (percent of trials correct) × time: $F_{(1,13)} = 1.93$, $p = 0.05$] but did not have a main effect on freezing during extinction retention (effect of reversal performance: $F_{(1,13)} = 0.02$, $p = 0.89$). Performance during the other cognitive flexibility testing phases did not predict extinction retention ($p > 0.05$). This analysis included all rats exposed to the cognitive flexibility training, with and without subsequent exposure to SPS, based on our previous conclusion that cognitive flexibility affected fear learning behavior with or without trauma exposure and because of the variance required to detect individual level performance effects. Cognitive flexibility performance data are provided in **Supplementary Figure S2**.

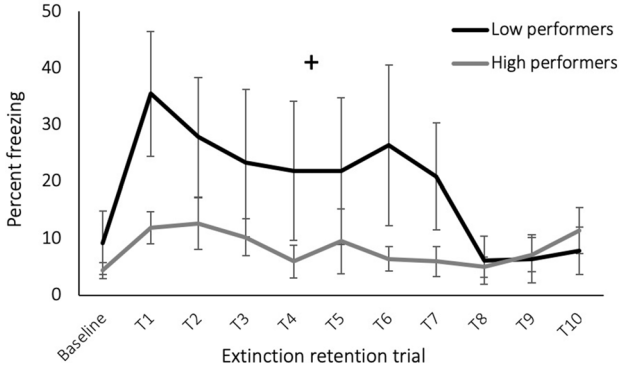

FIGURE 3 | Freezing during extinction retention testing, groups separated by performance during reversal learning task of cognitive flexibility training. Rats with low reversal performance exhibited an increase in freezing across the trials (+ time × performance effect: $p = 0.05$).

High-Pressure Liquid Chromatography (HPLC) Results

We analyzed levels of monoamines (DA and NE), and DA metabolites (DOPAC, 3MT, HVA), to understand monoamine signaling in brain regions regulating cognitive flexibility, fear learning processes, and aberrant fear responses in PTSD (Rauch et al., 2006; Milad et al., 2007, 2009; Klanker et al., 2013). Additional details for the results highlighted below, as well as for the infralimbic cortex which exhibited no treatment effects, are provided in **Supplementary Table S1**.

Prelimbic Cortex Monoamines and Metabolites

Cognitive flexibility training elevated levels of DA and NE in the PLC (**Figure 4**; main effect across all groups, with and without exposure to SPS; DA: $F_{(1,26)} = 5.49$, $p = 0.03$, NE: $F_{(1,26)} = 6.87$, $p = 0.01$, no interactions were detected). As levels of DA metabolites are inherently low in the PLC, there were too many samples that did not reach minimum threshold to allow for reliable measurement of DA metabolites, such that tissue concentrations could not be estimated for the PLC.

Striatum Monoamines and Metabolites

In the striatum, there was an interaction between cognitive flexibility training and traumatic stress (SPS) for the DA metabolite 3MT ($F_{(1,26)} = 5.09$, $p = 0.03$), and levels of the 3MT metabolite were lower in animals exposed to both SPS and cognitive flexibility training compared with animals exposed to SPS alone ($F_{(1,15)} = 6.83$, $p = 0.02$), but cognitive flexibility treatment did not have a detectable effect on 3MT in the absence of SPS ($F_{(1,13)} = 0.19$, $p = 0.67$). Similarly, at the level of a trend there was an interaction between cognitive flexibility training and SPS exposure for the DA metabolite HVA ($F_{(1,26)} = 2.90$, $p = 0.10$), and HVA metabolite levels were lower in animals exposed to both SPS and cognitive flexibility training compared with animals exposed to SPS alone ($F_{(1,15)} = 6.94$, $p = 0.02$), but cognitive flexibility treatment did not have a detectable effect on HVA in the absence of SPS ($F_{(1,13)} = 0.02$, $p = 0.90$).

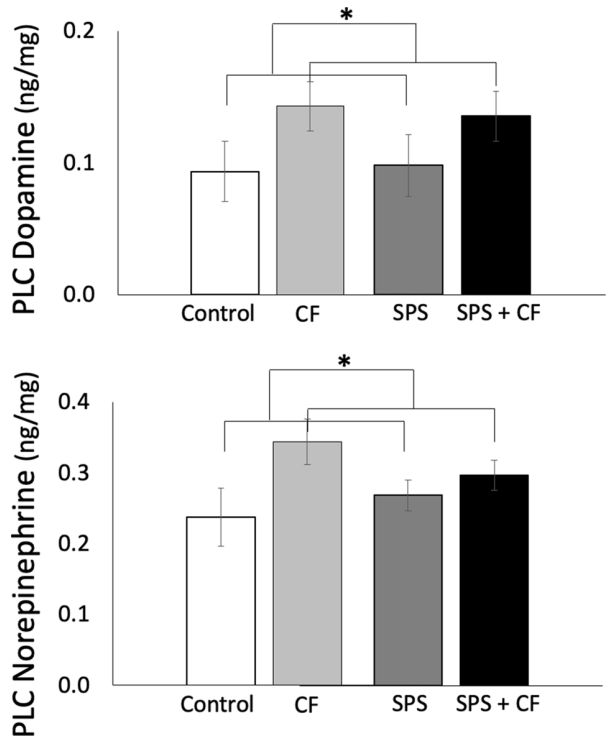

FIGURE 4 | Exposure to cognitive flexibility treatment enhanced dopamine (DA; top panel) and NE (bottom panel) levels in the prelimbic cortex (PLC), detected as a main effect across animals that were and were not exposed to SPS, $^{*}p < 0.05$.

At the level of a trend, cognitive flexibility treatment lowered DOPAC levels in a comparison across all four groups ($F_{(1,26)} = 3.58$, $p < 0.07$), and, as with prior DA metabolites, DOPAC levels were lower in animals exposed to both SPS and cognitive flexibility training compared with animals exposed to SPS alone ($F_{(1,15)} = 7.06$, $p = 0.02$), but cognitive flexibility treatment alone did not have a detectable effect on DOPAC ($F_{(1,13)} = 0.12$, $p = 0.73$). Together, decreases in the DA metabolites DOPAC, HVA, and 3MT suggest that cognitive flexibility treatment buffered the effects of SPS on dopaminergic function in the striatum.

Because of *a priori* hypotheses and effects detected in DA metabolites, we compared DA levels in rats exposed to SPS alone or the cognitive flexibility followed by SPS. We found that DA levels were lower in animals exposed to both SPS and cognitive flexibility training compared with animals exposed to SPS alone (**Figure 5**; $F_{(1,15)} = 5.04$, $p = 0.04$). The ratio of a metabolite to its neurotransmitter can be determined as an indicator of turnover, thus tissue concentrations of DOPAC to DA were assessed as an estimate of DA turnover (Karolewicz et al., 2001; Cox et al., 2011). We found no effects of cognitive flexibility or SPS exposure on DOPAC:DA (main effect: $F_{(1,26)} = 0.08$, $p = 0.78$, $F_{(1,26)} = 0.34$, $p = 0.57$, respectively, no interaction detected, data in **Supplementary Table S2**).

DISCUSSION

Cognitive flexibility represents a unique cognitive ability that is linked to resilience and the ability to deal with unpredictable change (Kehagia et al., 2010). Cognitive flexibility varies across species, and has been proposed to increase with social complexity (Bond et al., 2007), foraging demands (Day et al., 1999), and environmental complexity and unpredictability (Belanger and Willis, 1996; Wright et al., 2010). Cognitive flexibility is highly variable within species, and highly flexible individuals also appear to be resilient to challenging conditions (Genet and Siemer, 2011; Laughlin et al., 2011; Miyake and Friedman, 2012; Romero-Martínez et al., 2013). Yet, to our knowledge, this is the first study to manipulate cognitive flexibility in the context of resilience to trauma. We examined whether cognitive flexibility training could buffer effects of traumatic stress on extinction retention, a hallmark deficit of PTSD, and corticostriatal monoamine signaling that maintains cognitive flexibility (Milad et al., 2008, 2009). We found that cognitive

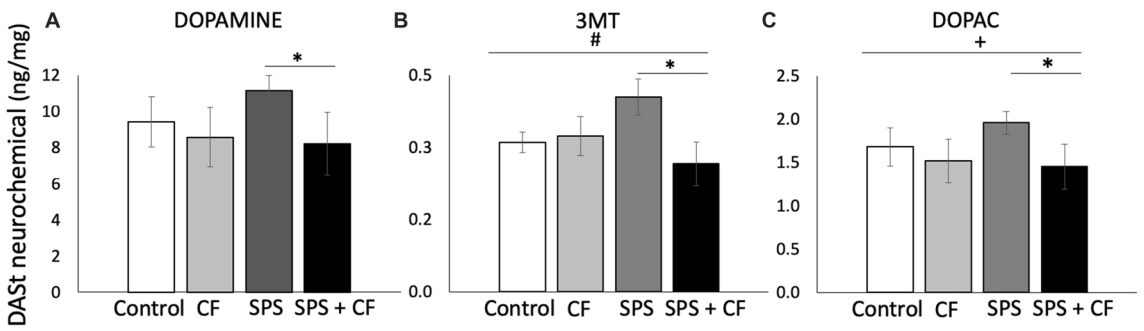

FIGURE 5 | DA and its metabolites in the dorsal anterior striatum are affected by cognitive flexibility treatment. **(A)** DA levels were lower in animals exposed to both SPS and cognitive flexibility training compared with animals exposed to SPS alone (* indicates $p = 0.04$). **(B)** For the DA metabolite 3MT, there was an interaction between cognitive flexibility training and SPS exposure ($^{\#}p = 0.03$), and levels of the 3MT metabolite were lower in animals exposed to both SPS and cognitive flexibility training compared with animals exposed to SPS alone ($^{*}p = 0.02$). Similarly, **(C)** at the level of a trend there was effect of cognitive flexibility training across all groups on the DA metabolite DOPAC ($^{+}p = 0.07$), and DOPAC metabolite levels were lower in animals exposed to both SPS and cognitive flexibility training compared with animals exposed to SPS alone ($^{*}p = 0.02$).

flexibility training enhanced extinction learning and can provide extinction retention benefits that remain after stress exposure. These benefits may be due to enhanced context processing skills conveyed by cognitive flexibility training that facilitate the recall of cues distinguishing the fear context from the safe context, or through an enhanced ability to discount old information from the fear context and acquire updated information from the safety context. To understand how individual differences in cognitive flexibility performance predict extinction retention ability, we compared performance during each cognitive flexibility training phase with extinction retention fear responses. We found that reversal learning ability predicts higher extinction retention, which prior evidence suggests is compromised by trauma (Milad et al., 2008, 2009; Knox et al., 2012, 2016; Perrine et al., 2016). Thus, reversal learning ability may facilitate prediction of individuals vulnerable to extinction retention deficits.

The precise mechanisms of cognitive flexibility in the context of trauma reactivity are underexplored. Prior research has demonstrated that both systemic increases in extracellular DA, *via* blockade of DA transporters, and moderate pharmacological increases in NE can have beneficial effects on cognitive flexibility, the current results expand on these prior findings to indicate that cognitive flexibility training can promote endogenous DA and NE increases within the PLC that may enhance context processing after trauma (Marek and Aghajanian, 1999; Volkow et al., 2002; Clatworthy et al., 2009; reviewed in Levrier et al., 2016). Moderate NE increases in the mPFC can facilitate cognitive flexibility by binding to α_2-adrenergic receptors, however, excess NE binds to α_1-adrenergic receptors, which can impair numerous executive functions (Arnsten et al., 1999; Carr et al., 2007). Both cognitive flexibility training and pharmacological blockade of α_1-adrenergic receptors may modulate NE and DA-induced effects on postsynaptic excitatory currents to enhance decision making processes and fear extinction learning (Marek and Aghajanian, 1999; Knauber and Müller, 2000; Bernardi and Lattal, 2010; Schwager et al., 2014).

We found that cognitive flexibility training interacted with traumatic stress exposure to result in decreased striatal DA and DA metabolites, suggesting that cognitive flexibility training shapes corticostriatal monoamine signaling to modify trauma processing and cognitive processes during fear extinction learning and retention. These findings, in conjunction with an absence of change in the DOPAC to DA ratio, suggest that the entire DA system is upregulated with the overall DA metabolic rate remaining unchanged. The PLC and striatal DA results demonstrate region-specific dopaminergic changes resulting from combined cognitive flexibility training and traumatic stress; the region-specific DA patterns in the striatum are distinct from the behavioral results and PLC DA, which both emphasized the robustness of the effects of cognitive flexibility training with or without traumatic stress exposure. Thus, these results highlight the distinct roles of the PLC and striatum in regulating extinction learning and retention following traumatic stress. Given that cognitive flexibility performance is facilitated by systemic blockade of β-adrenergic receptors and striatal DA receptor availability (D2), but can be impaired by infusion of a DA receptor agonist into the striatum (Volkow et al., 1998; Beversdorf et al., 1999; Goto and Grace, 2005; Alexander et al., 2007), downstream effects of cognitive flexibility training on monoamine signaling may include changes in DA and NE receptor distribution. Overall, changes in striatal DA signaling can reciprocally modulate prefrontal monoamine signaling (reviewed in Klanker et al., 2013). Over-expression of striatal DA receptors can decrease PFC DA turnover and cause learning and memory deficits (Kellendonk et al., 2006; Bach et al., 2008). Further, suppression of tonic striatal DA release enhances signaling from the PFC to the nucleus accumbens in the striatum, whereas enhanced DA release shifts striatal signaling to hippocampal inputs (Goto and Grace, 2005). Overall, our results suggest that cognitive flexibility training can increase prefrontal DA, without increasing DA in the striatum, potentially to prioritize flexible decision making over "reflexive" decision making. Conversely, traumatic stress exposure does not affect prefrontal DA, but appears to increase striatal DA, potentially resulting in "reflexive" behavior. When cognitive flexibility training occurs prior to traumatic stress exposure, cognitive flexibility training attenuates effects of traumatic stress on DA in the striatum while retaining prefrontal DA and NE changes precipitated by cognitive flexibility training in isolation. Thus, the current results emphasize the importance of monoamine signaling in PLC and striatum in maintaining cognitive flexibility, and suggest that further elucidation of downstream effects of cognitive flexibility training on corticostriatal signaling could provide valuable insights into mechanisms maintaining cognitive flexibility.

Cognitive flexibility is deficient in numerous pathologies, including anorexia, bipolar disorder, and obsessive compulsive disorder (Chamberlain et al., 2006; Dickstein et al., 2007; Tchanturia et al., 2012, 2013). In a study isolating the effects of cognitive flexibility training on anorexia, Brockmeyer et al. (2014) found that cognitive flexibility training improved cognitive flexibility performance (set-shifting), which improved perceived coping with stress. The cognitive flexibility training model in Brockmeyer et al. (2014) utilized set-shifting training; the current results indicate that a cognitive flexibility training adapted to include reversal learning could further enhance coping and could have applications for the treatment of stress-linked psychopathologies. For the treatment of PTSD, current interventions leverage computer based cognitive skill trainings to supplement more conventional treatments (Rizzo et al., 2012; Bomyea et al., 2015; Khanna et al., 2015). Further, trauma-exposed individuals that show improvement in cognitive flexibility following a month of cognitive training exhibit clinical improvement and attenuated PTSD symptoms 6 months post-trauma, compared with trauma-exposed individuals that completed control trainings (Ben-Zion et al., 2018). The cognitive training model used in Ben-Zion et al. (2018) included one aspect of cognitive flexibility, set-shifting, as well as other complex cognitive skill trainings. Our data indicate that a cognitive flexibility training model

that incorporates reversal learning could enhance existing post-trauma interventions and PTSD treatments, and may be beneficial for vulnerable populations with a high risk of encountering trauma.

A limitation of the current design is that assessment of monoamine levels occurred *ex vivo*, after all groups had completed fear learning assessments. Enman et al. (2015) found that SPS exposure can decrease levels of striatal DA and DA metabolites, DOPAC and HVA. Here, we did not find an effect of SPS on striatal DA, in the absence of an interactive effect with cognitive flexibility training. However, it has been shown that electric shock and fear learning procedures can enhance levels of striatal DA and DA metabolites in rats (Abercrombie et al., 1989; reviewed in Pezze and Feldon, 2004). Thus, a decrease in striatal DA induced by SPS could have been masked by subsequent fear learning procedures, emphasizing the role of striatal DA in aversive learning processes (Fadok et al., 2009). Overall, investigation of temporal monoamine changes following cognitive flexibility treatment, as well as investigation of downstream effects of changes in monoamine levels, could advance understanding of mechanisms that maintain cognitive flexibility. Further, additional control groups accounting for effects of novelty and cognitive stimulation, or the assessment of specific phases of the cognitive flexibility training, could help isolate the effects of cognitive flexibility. Additionally, although females were not studied here, evidence of sex differences in trauma reactions is robust (reviewed in Shansky, 2015; Bangasser and Wicks, 2017). Future studies elucidating the mechanism by which cognitive flexibility buffers effects of trauma should investigate sex-specific effects as well as effects on suites of psychopathological symptoms that have been characterized across the sexes. Although a main effect of SPS was not detected in the current experiment, effects of SPS on extinction retention freezing behavior were detected over time, and extensive prior evidence demonstrates that SPS (and PTSD) impair extinction retention (Milad et al., 2008, 2009; Knox et al., 2012, 2016; Chen et al., 2018). Thus, the authors feel the results demonstrate that cognitive flexibility training has the potential to be a meaningful non-invasive strategy to enhance wellbeing in the context of trauma and vulnerable populations.

Overall, our results demonstrate that cognitive flexibility training enhances extinction retention, a hallmark of PTSD, and PLC DA with or without subsequent traumatic stress exposure. Further, the current findings advance understanding of the role of monoamine signaling in cognitive flexibility by demonstrating that cognitive flexibility training can increase prelimbic DA and NE, and these effects are sustained after trauma exposure. Elucidating the mechanism by which cognitive flexibility modulates corticostriatal monoamine signaling could provide additional insights for the design of pharmacological interventions to mitigate adverse effects of trauma exposure. Given that individuals with high cognitive flexibility show reduced PTSD symptom severity and greater posttraumatic growth (Keith et al., 2015), cognitive flexibility training may have potential as an intervention for vulnerable populations or to supplement existing PTSD treatments.

DATA AVAILABILITY

The raw data supporting the conclusions in the present manuscript will be provided to any researcher upon request.

AUTHOR CONTRIBUTIONS

LC and IL contributed to the design of the study. LC, KK and ML conducted the study. LC analyzed the data. LC, IL and SP wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00024/full#supplementary-material

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Norepinephrine Induces PTSD-Like Memory Impairments *via* Regulation of the β-Adrenoceptor-cAMP/PKA and CaMK II/PKC Systems in the Basolateral Amygdala

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Glucocorticoids (GCs) can modulate the memory enhancement process during stressful events, and this modulation requires arousal-induced norepinephrine (NE) activation in the basolateral amygdale (BLA). Our previous study found that an intrahippocampal infusion of propranolol dose-dependently induced post-traumatic stress disorder (PTSD)-like memory impairments. To explore the role of the noradrenergic system of the BLA in PTSD-like memory impairment, we injected various doses of NE into the BLA. We found that only a specific quantity of NE (0.3 μg) could induce PTSD-like memory impairments, accompanied by a reduction in phosphorylation of GluR1 at Ser845 and Ser831. Moreover, this phenomenon could be blocked by a protein kinase A (PKA) inhibitor or calcium/calmodulin-dependent protein kinase II (CaMK II) inhibitor. These findings demonstrate that NE could induce PTSD-like memory impairments *via* regulation of the β-adrenoceptor receptor (β-AR)-3′,5′-cyclic monophosphate (cAMP)/PKA and CaMK II/PKC signaling pathways.

Keywords: PTSD, fear conditioning, basolateral amygdala, norepinephrine, AMPA, cAMP/PKA, CaMK II/PKC

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a serious anxiety disorder that usually follows a life-threatening traumatic event. The primary diagnostic criteria are re-experiencing of the trauma, autonomic reactivity to response, avoidance of trauma-related cues and elevated arousal (Long et al., 2013). A core symptom of PTSD is dysregulated fear response that is characterized by an over-generalization of fear and in tandem an inability to inhibit fear responses in the presence of safety (Jovanovic et al., 2012). Kaouane et al. (2012) developed an animal behavioral model that infusion of glucocorticoids (GCs) into the hippocampus in the predicting-context group conditioned with a high-intensity shock induced PTSD-like memory impairments, which were manifested as decreased freezing to the correct predictor and generalized fear responses to the cues that were normally not a relevant predictor of the threat. This animal model evaluates the ability of subjects to restrict fear responses to the appropriate predictor of a threatening stimulus, innovatively induced the core symptoms of PTSD; however, the neurobiological mechanisms underlying pathological PTSD-like memory impairment remain unclear.

GCs, steroid hormones from the adrenal cortex during stressful events, can modulate the memory enhancement process following an inverted-U shape dose-response relationship (Roozendaal, 2000; Sandi and Pinelo-Nava, 2007; Barsegyan et al., 2014; Deppermann et al., 2014; Finsterwald and Alberini, 2014). Additionally, this modulation requires arousal-induced norepinephrine (NE) activation in the basolateral amygdale (BLA; Roozendaal et al., 2004, 2006a,c), which is a key component of the neuronal circuits mediating emotional arousal and stress hormone effects on cognitive functions (McGaugh, 2004; Huff et al., 2006; Ehrlich et al., 2009; McIntyre et al., 2012). Evidence, including anatomical (Pikkarainen et al., 1999), behavioral (Roozendaal, 2000; Roozendaal et al., 2006c; McReynolds et al., 2010, 2014), electrophysiological (Akirav and Richter-Levin, 1999; Almaguer-Melian et al., 2003; Vouimba and Richter-Levin, 2013), and optogenetic data (Nabavi et al., 2014; Redondo et al., 2014; Tanaka et al., 2014; Rei et al., 2015), has shown that the amygdala modulates hippocampal memory storage. One of our recent studies found that an intrahippocampal infusion of propranolol dose-dependently induced PTSD-like memory impairments, demonstrating that the NE system in the hippocampus was involved in the formation of PTSD-like memory impairment (Zhu et al., 2018). However, whether NE within the BLA is a key factor in PTSD-like memory impairments remains unclear.

Numerous studies have demonstrated that NE activates adenosine 3′,5′-cyclic monophosphate (cAMP), cAMP-dependent protein kinase A (PKA; Roozendaal et al., 2002) and calcium/calmodulin-dependent protein kinase II (CaMK II) *via* β-adrenoceptor receptors (β-ARs) in the emotional memory (Hu et al., 2007). In contrast, other findings have shown that PKA activation, but not CaMK II activation, in the BLA is critical for cocaine memory restabilization processes (Arguello et al., 2014). Therefore, whether NE activates the β-AR-cAMP/PKA or CaMK II/PKC signaling pathway in PTSD-like memory impairments within the BLA remains to be elucidated.

Simultaneously, NE acting through β-ARs has powerful effects on the induction of long-term potentiation (LTP), which is one type of synaptic plasticity that has been linked to memory storage (Roozendaal et al., 2006b; O'Dell et al., 2010). Synaptic insertion of GluR1 subunit-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type receptors (AMPARs) appears to have a critical role in the synaptic strengthening observed during LTP induction (Lee et al., 2003; Hu et al., 2007). AMPARs are ionotropic glutamate receptors generated by the combination of four subunit proteins known as GluR1, GluR2, GluR3, and GluR4 (Traynelis et al., 2010). Prior findings showed that LTP was reduced in GluR1 gene inactivation mice and phosphomutant mice with knock-in mutations of the GluR1 phosphorylation sites (Jensen et al., 2003; Hu et al., 2007). Consequently, phosphorylation of AMPA receptor GluR1 subunits has a key role in β-AR-mediated enhancement of both LTP and behavioral learning. Furthermore, GluR1 subunits have many phosphorylation sites in the intracellular C-terminal domain; the central role of phosphorylation of Ser831 and Ser845 sites has been well elucidated. Ser831 is phosphorylated by PKC as well as other kinases such as CaMK II, whereas Ser845 is phosphorylated by PKA (Jensen et al., 2003; Hu et al., 2007; O'Dell et al., 2010, 2015). GluR1 Ser831 phosphorylation potentiates single-channel conductance (Derkach, 2003), and GluR1 Ser845 phosphorylation increases the channel open probability (Banke et al., 2000).

Thus, changes in GluR1 Ser845 and Ser831 phosphorylation provide an indicator of synaptic plasticity. The aim of this study was to investigate whether NE influenced PTSD-like memory impairments *via* regulation of the β-AR-cAMP/PKA or CaMK II/PKC signaling pathway. We also observed the phosphorylation changes of Ser845 and Ser831 in GluR1.

MATERIALS AND METHODS

Subjects

All male Sprague-Dawley rats (280–320 g) were purchased from the Experimental Animal Center at Sun Yat-sen University. Rats were individually on a 12-h light-dark cycle with *ad libitum* access to food and water. All behavioral experiments were performed during the light cycle between 9 AM and 3 PM. All procedures were approved by the Institutional Animal Care and Use Committee of the Zhongshan School of Medicine, Sun Yat-sen University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

The animals were adapted to the vivarium for at least 1 week before surgery. After each rat was fully anesthetized with sodium pentobarbital (50 mg/kg of body weight, i.p.), the skull was fixed to a stereotaxic frame (RWD, Shenzhen, China), and stainless-steel guide cannulas were implanted bilaterally with the cannula tips 2 mm above the BLA [coordinates: anteroposterior (AP), −2.8 mm from Bregma; mediolateral (ML), ±5.0 mm from midline; dorsoventral (DV), −6.5 mm from skull surface] according to the atlas of Paxinos and Watson (2007). The cannulas and two anchoring screws were affixed to the skull with dental cement. Stylets were inserted into the cannulas to maintain patency and were removed only for the infusion of drugs. The animals were allowed to recover for a minimum of 7 days before training and were handled for 2 min per day during this recovery period to accustom them to the infusion procedures.

Fear Conditioning

After the handling days were completed, all rats were habituated in the acclimation chamber (Context A: an opaque PVC chamber, W × L × H: 30 cm × 24 cm × 21 cm, an opaque PVC floor, a brightness of 100 lux) for 4 min without shock exposure. The acclimation chamber was also cleaned with 4% acetic acid before each trial. Two days later, animals were trained in a fear conditioning chamber (Context B: a transparent Plexiglas chamber, W × L × H: 28 cm × 21 cm × 22 cm, a brightness of 60 lux) that contained a floor with 18 stainless steel rods and was connected to a shock generator and sound generator (Coulbourn Instruments, Allentown, PA, USA) developed in-house. The conditioning chamber was also cleaned with 70%

ethanol before each trial. During training, each rat was placed into context B. After 110 s of free exploration, the rat was exposed to two footshocks (1.4 mA, 110 intertrial interval) that lasted for 3 s. After a 20 s delay, the rat received two tone cues (65 dB, 1 kHz, 30 s intertrial interval) that lasted for 15 s. After 20 s, the animals were placed back into its home cage. Twenty-four hours later, animals were tested in context A with cue trials (65 dB, 1 kHz, 2 kHz, or white noise) alone for 2 min. Two hours later, they were retested in context B for 2 min without the cues. Control groups were habituated to the training apparatus. The shock intensity (1.4 mA) was selected based on previous experiments conducted in our laboratory. In this case, animals will identify the conditioning context but not the cue as the right predictor of the shock. Freezing behavior was analyzed with a software program (Graphic State, Coulbourn Instruments, Allentown, PA, USA).

Drug and Infusion Procedures

All drug solutions were freshly prepared on the experimental days. For the first experiment, the nonspecific β-AR antagonist DL-propranolol (0.5 μg/0.2 μl per hemisphere; Sigma Aldrich, St. Louis, MO, USA) either alone or together with NE (0.3 μg/0.2 μl per hemisphere; Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.9% saline and administered into the BLA immediately after conditioning with 1.4 mA. For the second experiment, different doses of NE (0.1, 0.3 or 1.0 μg/0.2 μl per hemisphere; Sigma Aldrich) were dissolved in 0.9% saline and administered into the BLA immediately after conditioning with 1.4 mA. These doses were selected on the basis of previous research (Quirarte et al., 1997; Banke et al., 2000; Barsegyan et al., 2014). For the last experiment, the selective PKA inhibitor Rp-cAMPS (4.0 μg/0.2 μl per hemisphere; Sigma Aldrich, St. Louis, MO, USA) or the selective CaMK II inhibitor KN-93 (5.0 μg/0.5 μl per hemisphere; Sigma Aldrich, St. Louis, MO, USA) was dissolved in saline and infused into the BLA 10 min before fear conditioning training. NE (0.3 μg/0.2 μl per hemisphere) was administered to the BLA immediately after conditioning with 1.4 mA. These doses were selected on the basis of previous research (Roozendaal et al., 2002; Arguello et al., 2014).

In each experiment, the injection needle protruded 2 mm beyond the tip of the cannulas, and a 0.2 μl or 0.5 μl injection volume was infused over a period of 1 min by an automated syringe pump CMA402 (CMA Microdialysis BA, Solna, Sweden). All drug solutions were prepared freshly before each experiment, and the infusion procedures used were identical to those described above. The injection cannulas were left in place for 1 min after drug infusion to maximize diffusion and to prevent backflow of the drug into the cannulas.

Western Blot Analysis

After rats were sacrificed, brains were immediately frozen, and the BLA was microdissected using a 1 mm section rat brain matrix and frozen in liquid nitrogen prior to storage at −80°C. The supernatant was then assayed for total protein concentration using the BCA Protein Assay Kit. Tissue homogenate samples were resolved in 8% SDS-polyacrylamide gels, blotted electrophoretically onto PVDF membranes, blocked at room temperature for 1 h in PBS buffer containing 5% nonfat milk, and then blotted overnight at 4°C with antibodies to GluR1 (1:1,000; Millipore, Temecula, CA, USA), phosphorylated GluR1 at Ser831 and Ser845 (1:1,000; Millipore), and GAPDH (1/1,000; Cell Signaling Technology, Danvers, MA, USA). Then, the membranes were incubated with HRP-conjugated secondary antibody for 1 h. The densitometric analysis of Western blot was performed using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA), and data analysis was performed by Image Lab version 5.2.1 (Bio-Rad, Hercules, CA, USA).

Histology

After the testing sessions, each rat was deeply anesthetized with a moderate dose of sodium pentobarbital and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde. The animal was then decapitated, and its brain was removed from the skull and placed in 4% paraformaldehyde. After 7 days, the brains were sliced at 40 μm thickness and stained with thionin. The sites of microinjections were verified according to the atlas of Paxinos and Watson (2007). Rats with injection needle placements outside the BLA or with extensive tissue damage at the injection needle tips were excluded from analysis.

Data Analysis

All data are expressed as the mean ± SEM. One-way ANOVA (*post hoc* Fisher's least significant difference) and two-way ANOVA with Fisher's PLSD *post hoc* tests were used when appropriate. Analyses were conducted using SPSS 20.0 software. $P < 0.05$ was chosen as the criterion for statistical significance.

RESULTS

Histology

Figure 1 illustrates the schematic (part **A**) and actual (part **B**) results of the corresponding sections taken from the rat brain atlas of Paxinos and Watson (2007).

Intra-BLA Infusion of NE Induced Dose-Dependent PTSD-Like Memory Impairments

In Experiment 1, we first tested whether NE-induced effects within the BLA had a similar dose-dependent effect as the GC-induced effects on PTSD-like memory impairment, we tested the effects of NE immediately after conditioning by microinfusing different doses (0.1, 0.3, and 1.0 μg) into the BLA of rats administered the high-intensity shock. As shown in **Figure 2A**, one-way ANOVA indicated that the 0.3 and 1.0 μg NE groups both showed impaired contextual fear conditioning, and the 0.1 μg NE group showed enhanced contextual and cue fear conditioning (context: $F_{(3,18)} = 18.476$, $P < 0.001$; cue: $F_{(3,18)} = 14.929$, $P < 0.001$). Fisher's *post hoc* analysis confirmed that the 0.3 and 1.0 μg NE groups exhibited significantly decreased freezing time in the contextual memory retention test (0.3 μg NE: $P < 0.001$; 1.0 μg NE: $P < 0.001$). Fisher's *post hoc* analysis also revealed that the 0.1 μg and 0.3 μg NE groups exhibited significantly increased freezing time in the contextual memory retention test (0.1 μg NE: $P = 0.001$;

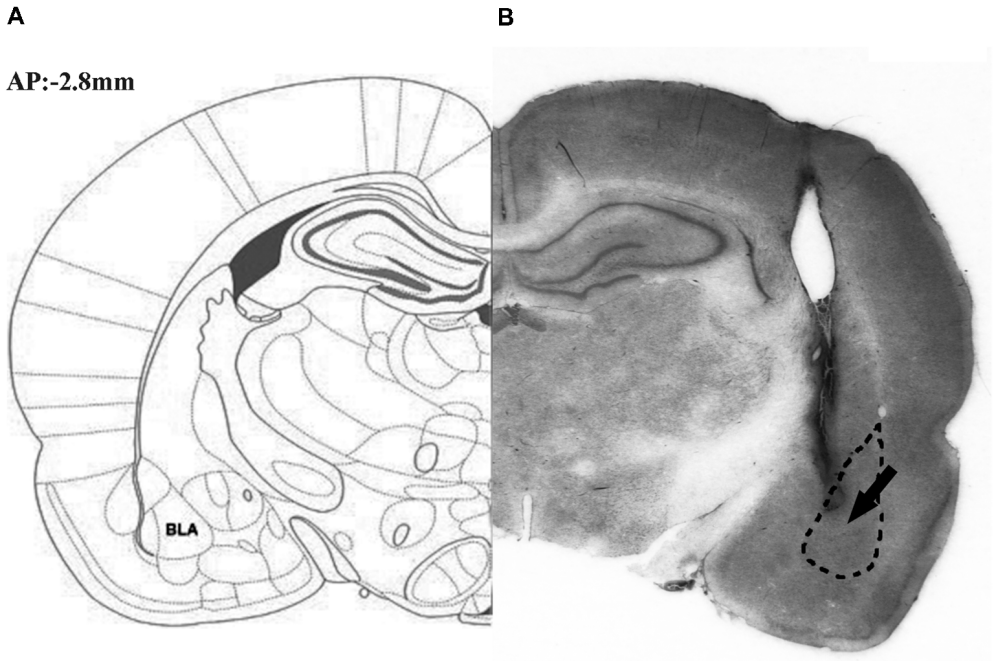

FIGURE 1 | Representative photomicrograph illustrating the location of microinjection into the basolateral amygdale (BLA). The schematic (part **A**) and actual (part **B**) results on corresponding section taken from the rat brain atlas of Paxinos and Watson (2007), arrow points to needle tip.

0.3 μg NE: $P < 0.001$). We found that 0.3 and 1.0 μg NE can both impair contextual fear conditioning, and thus, we tested the generalization of fear responses to different cues. Only the 0.3 μg NE group of rats exhibited generalization of fear responses to different cues, as enhanced 1 kHz and 2 kHz cue fear conditioning was observed ($F_{(2,11)} = 37.485$, $P < 0.001$; **Figure 2B**).

Then, to investigate whether the effects of NE on memory was mediated by beta receptors or not, effective dose of NE with or without propranolol was microinfused into the BLA immediately after conditioning with a high-intensity shock (1.4 mA). As shown in **Figure 2C**, one-way ANOVA indicated that rats in the NE and propranolol groups both had impaired contextual fear conditioning; however, the NE + propranolol groups showed enhanced contextual and cue fear memories (context: $F_{(3,17)} = 6.882$, $P = 0.003$; cue: $F_{(3,17)} = 13.459$, $P < 0.001$). Fisher's *post hoc* analysis confirmed that the NE and propranolol groups exhibited significantly decreased freezing time in the contextual memory retention test (NE: $P = 0.008$; propranolol: $P = 0.008$). Meanwhile, Fisher's *post hoc* analysis also revealed that the propranolol and NE + propranolol groups exhibited significantly increased freezing time in the cue memory retention test (propranolol: $P < 0.001$; NE + propranolol: $P = 0.006$). Interestingly, when NE and propranolol were simultaneously injected into the BLA immediately after conditioning with the high-intensity shock (1.4 mA), freezing time increased in the contextual and cue memories retention test, indicating that this treatment enhanced contextual and cue fear memories.

Altogether, these results indicated that NE has a dose-dependent effect on PTSD-like memory impairments and that only the moderate dose (0.3 μg) of NE could induce PTSD-like memory impairments.

NE Induced PTSD-Like Memory Impairments *via* Downregulation of AMPA Receptor Phosphorylation

NE is mediated by mechanisms involving activation of β-ARs in the BLA, and the β-AR is directly linked to AMPAR functional modulation and trafficking to synaptic sites. GluR1 Ser831 and Ser845 phosphorylation sites have been proposed to play a key role in AMPAR trafficking and synaptic plasticity. GluR1 Ser831 is phosphorylated by PKC as well as other kinases, such as CaMK II, whereas Ser845 is phosphorylated by PKA. To further characterize the signaling events that lead to NE-induced PTSD-like memory impairments, we observed the phosphorylation changes of Ser845 and Ser831 in GluR1. As shown in **Figure 3A**, Ser831 phosphorylation of GluR1 was decreased in the NE and propranolol groups but was increased in the NE + propranolol group of rats (GluR1 Ser831: $F_{(3,12)} = 21.291$, $P < 0.001$; NE, $P = 0.023$; propranolol, $P = 0.003$; NE + propranolol, $P = 0.004$, LSD-t after one-way ANOVA). Ser845 phosphorylation of GluR1 was decreased in the NE and propranolol groups but was increased in the NE + propranolol group of rats (GluR1 Ser845: $F_{(3,12)} = 28.524$, $P < 0.001$; NE, $P = 0.036$; propranolol, $P = 0.001$; NE + propranolol, $P = 0.001$, LSD-t after one-way ANOVA). However, GluR1 was not altered in these three groups ($F_{(3,12)} = 0.337$, $P = 0.779$, one-way ANOVA).

Meanwhile, as shown in **Figure 3B**, Ser831 phosphorylation of GluR1 was decreased in the 0.3 μg and 1.0 μg NE groups

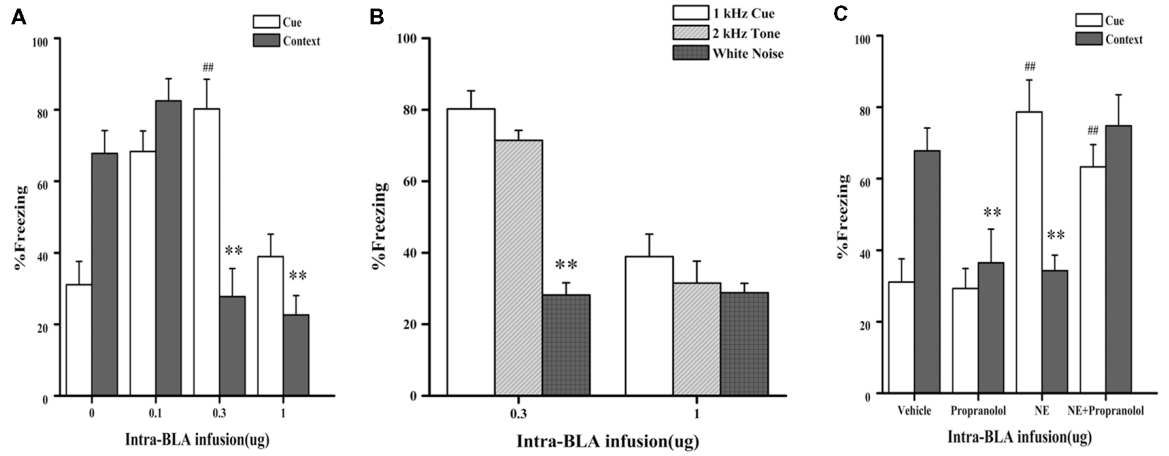

FIGURE 2 | Intra-BLA infusion of norepinephrine (NE) induced a dose-dependent post-traumatic stress disorder (PTSD)-like memory impairments. **(A)** NE (0.3 μg/0.2 μl) administered into the BLA immediately after fear conditioning impaired retention of contextual fear memory and enhanced retention of cue fear memory. Concurrent infusion of β-adrenoceptor (β-AR) antagonist DL-propranolol (0.5 μg/0.2 μl) blocked this NE-induced memory impairments. Results represent mean ± SEM. $^{**}P < 0.01$, $^{\#\#}P < 0.01$ compared with vehicle. **(B)** NE (0.1, 0.3, or 1.0 μg/0.2 μl) administered into the BLA immediately after fear conditioning dose-dependent impaired retention of contextual fear memory. Results represent mean ± SEM. $^{**}P < 0.01$, $^{\#\#}P < 0.01$ compared with vehicle. **(C)** NE (0.3 μg/0.2 μl) induced PTSD-like memory impairments, which increased the response to 2 kHz tone but not to white noise. Results represent mean ± SEM. $^{**}P < 0.01$ vs. 1 kHz cue.

but increased in the 0.1 μg NE group ($F_{(3,12)}$ = 27.479, $P < 0.001$; 0.1 μg NE, $P = 0.003$; 0.3 μg NE, $P = 0.003$; 1.0 μg NE, $P = 0.001$, LSD-t after one-way ANOVA). Ser845 phosphorylation of GluR1 was decreased in the 0.3 μg NE group ($F_{(3,12)}$ = 27.838, $P < 0.001$; 0.3 μg NE, $P < 0.001$, LSD-t after one-way ANOVA). GluR1 was not altered in these three groups ($F_{(3,12)}$ = 0.59, $P = 0.633$, one-way ANOVA). Thus, phosphorylation of both Ser845 and Ser831 was decreased in the NE-induced PTSD-like memory impairment.

NE Induced PTSD-Like Memory Impairments *via* Regulation of the cAMP/PKA and CaMK/PKC Signaling Pathways

As mentioned above, NE induces PTSD-like memory impairments along with decreased Ser845 and Ser831 phosphorylation of GluR1. Ser831 is phosphorylated by PKC as well as other kinases, such as CaMK II, whereas Ser845 is phosphorylated by PKA. Thus, to determine whether administration of NE within the BLA can activate the cAMP/PKA or CaMK II/PKC signaling pathway in PTSD-like memory impairments, we inhibited the cAMP/PKA or CaMK II/PKC pathway. As shown in **Figures 4Aa,b**, intra-BLA infusion of the PKA inhibitor Rp-cAMPS (4.0 μg) 10 min before fear conditioning blocked PTSD-like memory impairments induced by immediate post-training intra-BLA infusions of NE. The two-way ANOVA for percent freezing time during the contextual and cue memory retention test revealed a significant PKA inhibitor effect in the contextual memory retention test (context: $F_{(1,22)}$ = 0.758, $P = 0.395$; cue: $F_{(1,22)}$ = 12.541, $P = 0.002$), a significant effect of NE in the contextual and cue memory retention test (context: $F_{(1,22)}$ = 0.020, $P = 0.890$; cue: $F_{1,22}$ = 19.605, $P < 0.001$), and a significant interaction between these two factors in the contextual and cue memory retention test (context: $F_{(1,22)}$ = 25.607, $P < 0.001$; $F_{(1,22)}$ = 11.988, $P = 0.003$). Rp-cAMPS not only induced contextual memory retention impairment when administered alone but also blocked PTSD-like memory impairments induced by immediate post-training intra-BLA infusion of NE (context: $F_{(3,19)}$ = 8.533, $P = 0.001$; saline + NE vs. Rp-cAMPS + NE, $P = 0.005$, LSD-t after one-way ANOVA; cue: $F_{(3,19)}$ = 15.15, $P < 0.001$; saline + NE vs. Rp-cAMPS + NE, $P < 0.001$, LSD-t after one-way ANOVA).

Meanwhile, as shown in **Figures 4Ba,b**, intra-BLA infusion of the CaMK II inhibitor KN-93 (0.5 μg) 10 min before fear conditioning blocked PTSD-like memory impairments induced by immediate post-training intra-BLA infusions of NE. The two-way ANOVA for percent freezing time during the contextual and cue memory retention test revealed a significant CaMK II inhibitor effect in contextual memory retention test (context: $F_{(1,24)}$ = 3.036, $P = 0.096$; cue: KN-93: $F_{(1,24)}$ = 21.788, $P < 0.001$), a significant effect of NE in the contextual and cue memory retention test (context: NE: $F_{(1,24)}$ = 4.727, $P = 0.041$; cue: $F_{(1,24)}$ = 14.160, $P = 0.001$), and a significant interaction between these two factors in the contextual and cue memory retention test (context: $F_{(1,24)}$ = 8.535, $P = 0.008$; $F_{(1,24)}$ = 19.372, $P < 0.001$). KN-93 not only induced contextual memory retention impairment when administered alone but also blocked PTSD-like memory impairments induced by immediate post-training intra-BLA infusion of NE (context: $F_{(3,21)}$ = 4.708, $P = 0.011$; saline + NE vs. KN-93 + NE, $P = 0.386$, LSD-t after one-way ANOVA; cue: $F_{(3,21)}$ = 18.786, $P < 0.001$; saline + NE vs. KN-93 + NE, $P < 0.001$, LSD-t after one-way ANOVA).

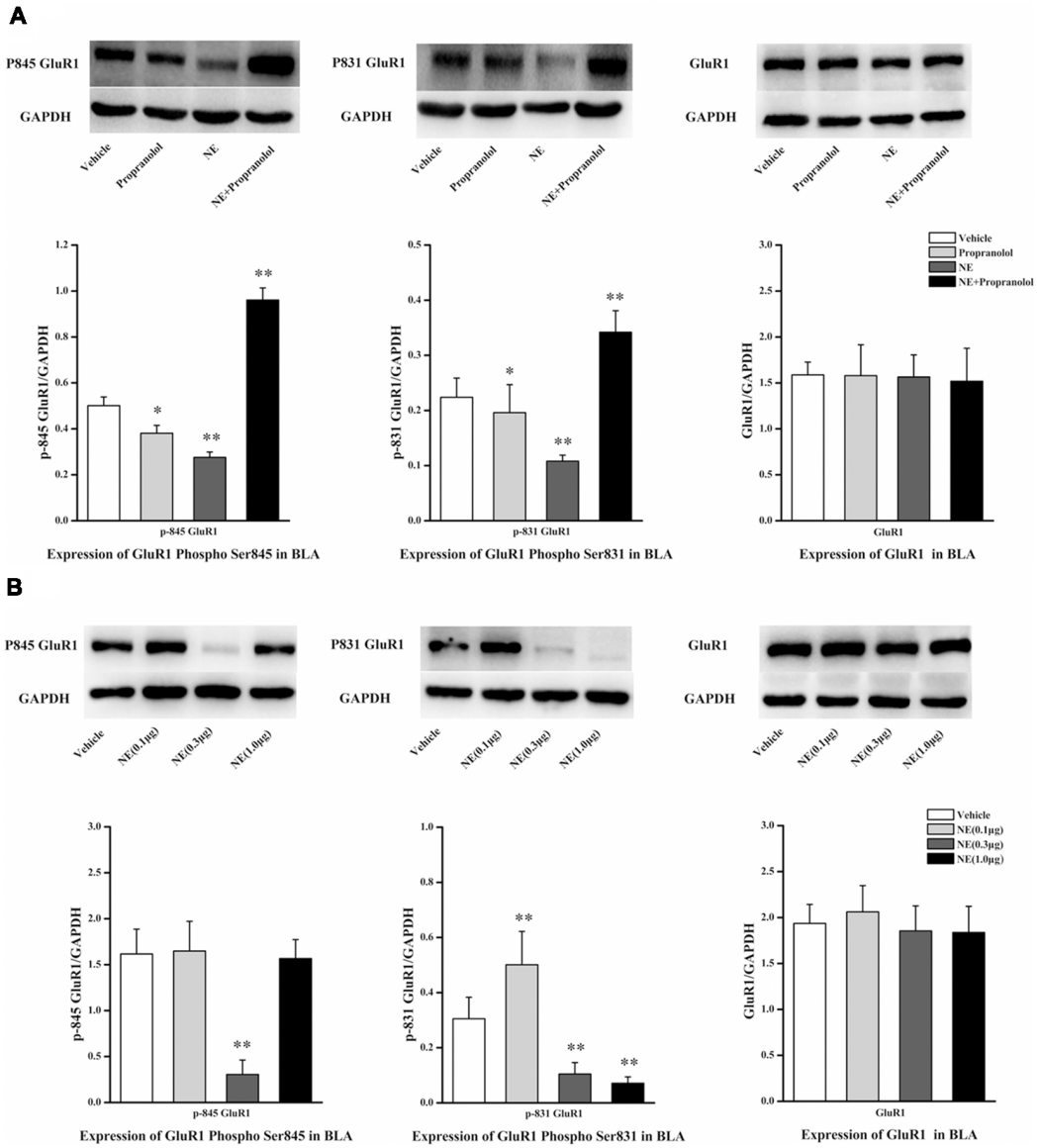

FIGURE 3 | NE induced PTSD-like memory impairments *via* down-regulation the expression of Ser845 and Ser831 phosphorylation of GluR1. **(A)** Representative immunoblots showing the effect of the bilateral intra-BLA infusion of NE (0.3 μg/0.2 μl) or propranolol (5 μg/0.2 μl) immediately after fear conditioning induced change in phospho-GluR1 and total GluR1 levels. **(B)** Representative immunoblots showing the effect of the bilateral intra-BLA infusion of NE with the different doses of NE (0.1, 0.3, or 1.0 μg/0.2 μl) immediately after training induced change in phospho-GluR1 and total GluR1 levels. All results represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. vehicle group.

We next tested the effect of inhibition of the cAMP/PKA or CaMK II/PKC signaling pathway in PTSD-like memory impairments on the phosphorylation changes of Ser845 and Ser831 of GluR1. We found that Ser831 phosphorylation of GluR1 was decreased in the saline + NE and Rp-cAMPS + vehicle groups (GluR1 Ser831: $F_{(3,12)} = 3.69$, $P = 0.043$; saline + NE, $P = 0.011$; Rp-cAMPS + vehicle, $P = 0.026$, LSD-t after one-way ANOVA; **Figure 5A**). Ser845 phosphorylation of GluR1 was decreased in the saline + NE and Rp-cAMPS + vehicle groups (GluR1 Ser845: $F_{(3,12)} = 9.73$, $P = 0.002$; saline + NE, $P = 0.002$; Rp-cAMPS + vehicle, $P = 0.011$, LSD-t after one-way ANOVA). However, GluR1 was not altered in these three groups ($F_{(3,12)} = 0.302$, $P = 0.823$, one-way ANOVA).

Additionally, Ser831 phosphorylation of GluR1 was decreased in the saline + NE, KN-93 + vehicle and KN-93 + NE groups compared with the saline + vehicle group (GluR1 Ser831: $F_{(3,12)} = 4.879$, $P = 0.019$; saline + NE, $P = 0.008$; KN-93 + vehicle, $P = 0.005$; KN-93 + NE, $P = 0.030$, LSD-t after one-way ANOVA; **Figure 5B**). Ser845 phosphorylation of GluR1 was decreased in the saline + NE and KN-93 + vehicle groups (GluR1 $F_{(3,12)} = 12.465$, $P = 0.001$; saline + NE, $P < 0.001$; KN-93 + vehicle, $P = 0.003$,

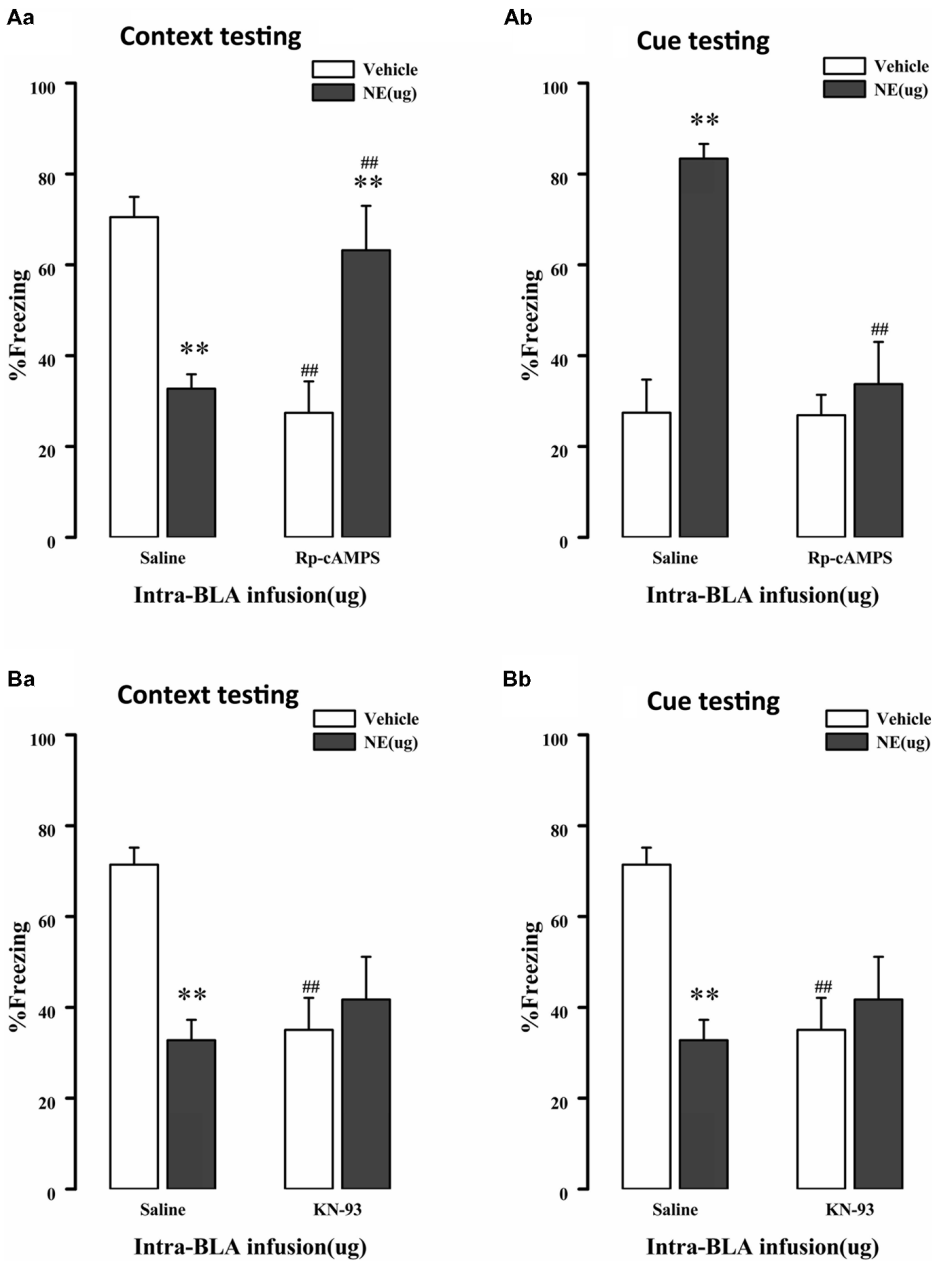

FIGURE 4 | NE induced PTSD-like memory impairments *via* regulation 3′,5′-cyclic monophosphate (cAMP)/protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CaMK II)/PKC signal pathway. **(A)** The PKA inhibitor Rp-cAMPS (4.0 μg/0.2 μl) administered into the BLA 10 min before fear conditioning blocked the impairments of retention of contextual **(Aa)** and cue **(Ab)** fear memory induced by immediately post-training intra-BLA infusions of NE (0.3 μg/0.2 μl). **(B)** The CaMKII inhibitor KN-93 (5.0 μg/0.5 μl) administered into the BLA 10 min before fear conditioning also blocked the impairments of retention of contextual **(Ba)** and cue **(Bb)** fear memory induced by immediately post-training intra-BLA infusions of NE (0.3 μg/0.2 μl). All results represent mean ± SEM. $^{**}P < 0.01$ compared with the corresponding vehicle group, $^{\#\#}P < 0.01$ compared with the corresponding saline group.

LSD-t after one-way ANOVA). However, GluR1 was not altered in these three groups ($F_{(3,12)}$ = 0.067, P = 0.977, one-way ANOVA).

Together, these results showed that intra-BLA infusion of KN-93 (0.5 μg) or Rp-cAMPS (4.0 μg) 10 min before fear conditioning can block PTSD-like memory impairments induced by immediate post-training intra-BLA infusions of NE, which can also lead to phosphorylation changes in the Ser845 and Ser831 sites of GluR1.

DISCUSSION

In PTSD patients, a core symptom is an excessive generalization of fear; patients show a strong response not only to a previous

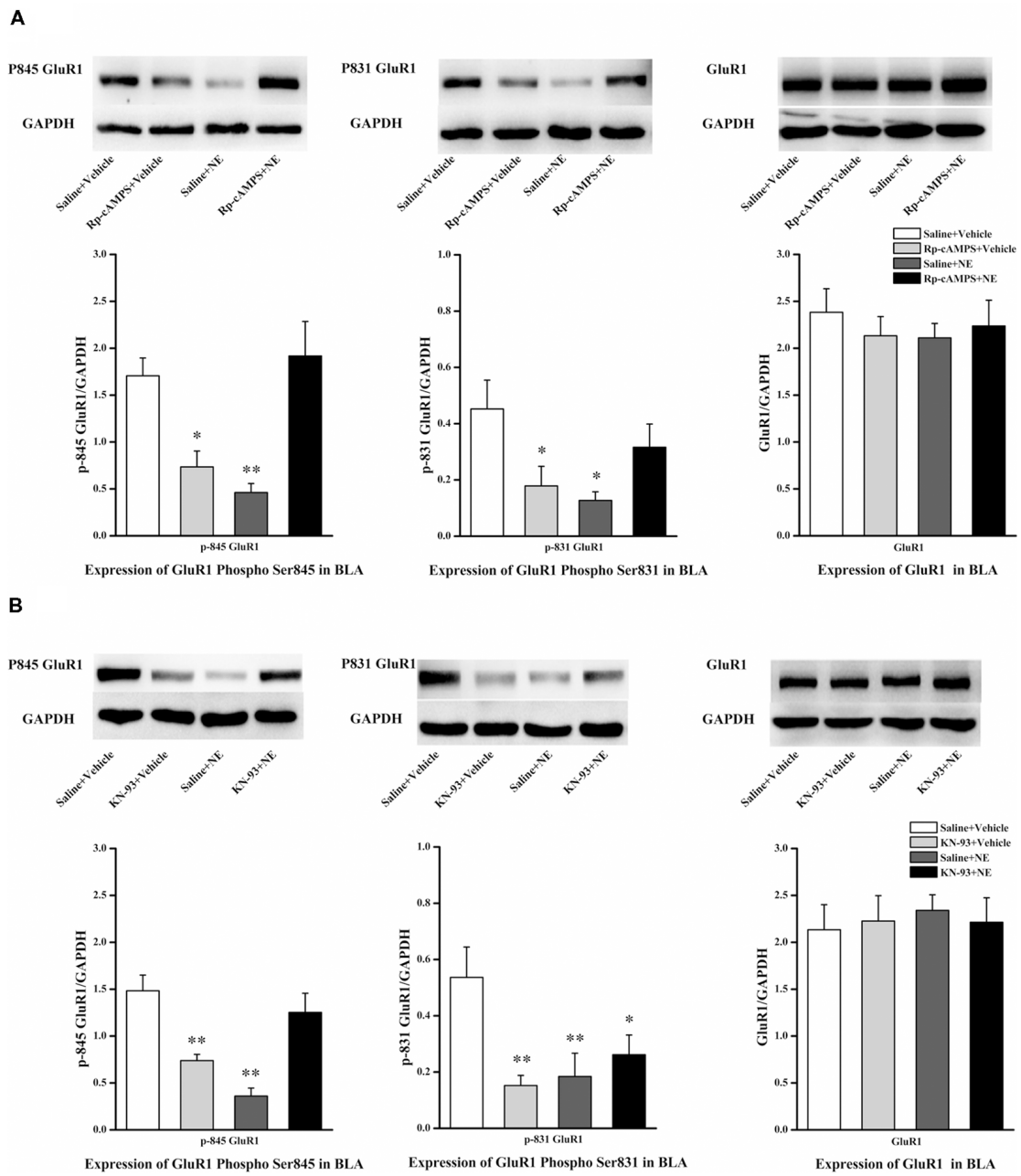

FIGURE 5 | Inhibition cAMP/PKA or CaMK II /PKC signal pathway can down-regulate the expression of Ser845 and Ser831 phosphorylation of GluR1. Representative immunoblots showing the effect of the bilateral intra-BLA infusion of Rp-cAMPS (4.0 μg/0.2 μl; **A**) or KN-93 (5.0 μg/0.5 μl; **B**) 10 min before fear conditioning induced change in phospho-GluR1 and total GluR1 levels. All results represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. vehicle group.

learned fearful cue but also cues that signal safety. This memory disturbances for the core traumatic event and peritraumatic cues, contributes to the intrusive recollection of traumatic event. In this study, we investigated whether NE was a key factor in such PTSD-like memory impairments, as well as the possible neurobiological mechanism. Researcher interest in this issue stems from recent reports showing that GCs could induce PTSD-like memory impairments (Kaouane et al., 2012). This animal model of PTSD-like memory impairment enables us to evaluate the ability of the individuals to restrict fear responses to the appropriate predictor of the threatening stimulus, instead of simply observe the freezing response to context or cue.

Evidence showed that GCs rely on NE activation in the BLA to affect memory consolidation (Banke et al., 2000; Roozendaal et al., 2006c). In this study, we found that

only 0.3 μg NE microinfused into the BLA immediately after fear conditioning could induce PTSD-like memory impairments and simultaneously reduce GluR1 Ser845 and Ser831 phosphorylation. Furthermore, NE had a dose-dependent effect similar to that of GCs on PTSD-like memory impairments. We also found that intra-BLA infusion of a PKA inhibitor or CaMK II inhibitor before fear conditioning could block PTSD-like memory impairments induced by immediate post-training intra-BLA infusions of NE. Therefore, our findings suggested that the BLA noradrenergic system is involved in mediating PTSD-like memory impairments *via* regulation of the β-AR-cAMP/PKA and CaMK II/PKC signaling pathways.

Numerous studies using different experimental paradigms have shown that the amygdala is a key brain structure in modulation of the stress response and fear memory (Ehrlich et al., 2009; Hermans et al., 2014; Aubry et al., 2016). Emotionally arousing experiences are known to be associated with elevated levels of NE (Hatfield et al., 1999; McGaugh and Roozendaal, 2002), and BLA is a critical site of NE action (Roozendaal et al., 2008; Mueller and Cahill, 2010). We found that the β-AR antagonist propranolol or NE microinfused into the BLA immediately after fear conditioning could impair contextual and cue fear memory, which is consistent with previous evidence (Bush et al., 2010; Barsegyan et al., 2014; Zhou et al., 2015). Interestingly, when NE and propranolol were injected together into the BLA immediately after fear conditioning, contextual and cue fear memory was enhanced, one possible reason is that a low dose of propranolol only partly blocked the effect of NE-induced memory impairments. Because higher intensity of noradrenergic transmission required higher doses of the β-ARs antagonist than other tasks (Debiec and Ledoux, 2004). Another possibility is the involvement of α_2-adrenoceptor. The α_2 adrenoceptor is predominantly located on presynaptic noradrenergic terminals and its activation inhibits NE release (Langer, 1974; Talley et al., 1996). Ferry and McGaugh (2008) showed that post-training intra-BLA infusions of a selective α_2 adrenoceptor agonist induced a dose-dependent impairment of retention of inhibitory avoidance. While the NE release onto β-AR is required for induction and maintenance of LTP (Roozendaal et al., 2006b; O'Dell et al., 2010), leading to retention enhancement. Further study using particularly β-ARs agonists is needed to clarify this issue.

Furthermore, we found that NE had a dose-dependent effect on PTSD-like memory impairments, and only a moderate dose of NE could induce PTSD-like memory impairments—low doses of NE enhanced contextual and cue fear memories, and high doses of NE impaired contextual fear memory. These findings are consistent with previous evidence showing that NE produced dose-dependent enhancement or impairment of memory in other experimental paradigms (Roozendaal et al., 2008; Li et al., 2011). NE or a β-ARs agonist infused into the BLA immediately post-training enhances the retention of emotionally arousing training experiences (Barsegyan et al., 2014), by regulating neural plasticity and information storage processes in other brain regions, including hippocampus (Atucha et al., 2017). However, direct effect of NE infusion into the hippocampus seems controversial. Atsak et al. (2012) indicated that post-training NE infusions into the dorsal hippocampus during retention of auditory fear conditioning has no significant effect on fear response. Our previous work (Zhu et al., 2018) suggested that intra-hippocampus infusion of propranolol, not NE, dose-dependently induced PTSD-like memory impairments. In addition, the PTSD-like memory impairment model used in this study can be regarded as the impairment of memory accuracy. Our results suggested that post-training NE infusions into the BLA decrease the accuracy of the fear memory, inconsistent with some others. This discrepancy may prominently due to our different experimental design. For example, Barsegyan et al. (2014) employed an object-in-context recognition memory design, had no emotional arousing in their study. Additionally, there are two factors in this fear conditioning, including context and cue, but only context in Atucha' work (Atucha et al., 2017), and BLA is known for its crucial effect in cue fear memory. Moreover, the footshock in this work is 1.4 mA, much stronger than 0.6 mA they used, may induce more release of stress hormones, including GC, NE, etc. Moreover, another interesting finding is that the enhancement of irrelative cue retention performed earlier than impairment of context memory as the dose increases. It has long been known that BLA is crucially involved in the formation of cue fear memory (Ehrlich et al., 2009), thus changes may first show in cue memory. Since the memory specificity impaired after NE infusion, animals could not restrict fear responses to the appropriate predictor-context, but present higher freezing in cue test. NE regulates synaptic plasticity and glutamatergic excitatory post-synaptic currents (EPSCs; Almaguer-Melian et al., 2005; Walling et al., 2016) through several pathways that are regulated by phosphorylation of the AMPARs (Esteban et al., 2003; Oh et al., 2006). GluR1 Ser831 and Ser845 phosphorylation sites have been proposed to play a key role in AMPAR trafficking and synaptic plasticity. Moreover, prior findings indicated that NE signaling induces phosphorylation of the Ser845 and Ser831 sites of GluR1 in the emotional regulation of learning and memory (Banke et al., 2000; Derkach, 2003; Hu et al., 2007). However, how this regulation occurs in PTSD-like memory impairments is unknown. Our results showed that Ser831 and Ser845 phosphorylation of GluR1 was decreased by post-training intra-BLA infusions of 0.3 μg NE, and the phosphorylation decrease at the Ser831 or Ser845 site was driven by β-AR inhibition and infusion of high doses of NE, while phosphorylation increased at low doses, which was consistent with behavioral experiments indicating that there is an association between NE and AMPAR signaling pathways in the regulation of GluR1 Ser831 and Ser845 phosphorylation. This finding reveals that NE induces PTSD-like memory impairments *via* downregulation of AMPA receptor phosphorylation. In other words, this finding provides a potential explanation for the regulation of AMPA receptor trafficking and might offer a potentially beneficial treatment for PTSD.

It is well established that signaling molecules such as PKA, mitogen-activated protein kinase (MAPK), Ca^{2+}/CaMKII have also been implicated in the maintenance of LTP and

consolidation of fear memory in the amygdala (Toyoda et al., 2011). Indeed, cAMP/PKA signaling plays a critical role in presynaptically expressed LTP (Castillo et al., 2002; Young and Thomas, 2014). Increased PKA activity leads to phosphorylation of GluR1 on Ser845, increasing the channel open probability (Banke et al., 2000). While the increased CaMKII activity leads to phosphorylation of Ser831, potentiating single-channel conductance (Derkach, 2003). Furthermore, we found that intra-BLA infusion of KN-93 or Rp-cAMPS 10 min before fear conditioning blocked PTSD-like memory impairments induced by immediate post-training intra-BLA infusions of NE. This finding provided direct evidence for NE-induced PTSD-like memory impairments *via* regulation of the cAMP/PKA and CaMK II/PKC signaling pathways. Simultaneously, we also found Ser831 and Ser845 phosphorylation of GluR1 decreased due to pre-training intra-BLA infusions of the PKA inhibitor or CaMK II inhibitor alone. However, Ser831 and Ser845 phosphorylation of GluR1 was not significantly changed by pre-training intra-BLA infusions of Rp-cAMPS and post-training infusions of NE. These findings indicated that NE activation of β-ARs enhances behavioral memory *via* the cAMP/PKA and CaMK II/PKC signaling pathways (Hu et al., 2007; Zhou et al., 2013). However, besides activates PKA and CaMK II, other molecules including NMDA receptor, has been suggested to be the downstream targets of NE (Huang et al., 1993; Raman et al., 1996), which preferentially couple to phosphatases at lower levels of activation while activating kinases at higher levels. Vanhoose and Winder (2003) reported that a saturating dose of NMDA induces dephosphorylation of Ser845. Therefore, the changes in phosphorylation levels found in this study may be caused by a combination of factors. In future studies, it will be necessary to determine whether NMDAR involved in the modulation of NE induced PTSD-like memory impairment.

In conclusion, our findings reveal that NE regulates the β-AR-cAMP/PKA and CaMK II/PKC signaling pathways, leading to PTSD-like memory impairments.

AUTHOR CONTRIBUTIONS

HZ and LX contributed to the conception of the work. X-HL and R-TZ designed and collected the data. X-HL, R-TZ, BH, Y-WS and X-GW collected and analyzed the data. XL wrote the article. All authors discussed the results and commented on the manuscript. All authors approved the final version of the manuscript.

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Behavioral Diversity Across Classic Rodent Models Is Sex-Dependent

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Symptoms of trauma and stressor related disorders such as post-traumatic stress disorder (PTSD) often develop well after the traumatic experience has occurred, and so identifying early predictors of risk or resilience is important for the implementation of interventional therapies. For example, passive coping strategies such as tonic immobility and peritraumatic dissociation during the trauma itself are risk factors for the developments of PTSD, especially in women. However, discrete, sex-specific coping responses that predict later outcomes in animal models have not been rigorously defined. Recently, we identified an active, escape-like response exhibited primarily by a subset of female rats in a classic auditory fear conditioning task ("darting"). Here, we asked whether darting during conditioning predicted active responding in a single forced swim (SFS) session to study the potential for darting to reflect a trait-like behavioral strategy that translated across stress models. Male and female Sprague-Dawley (SD) rats were tested in auditory fear conditioning acquisition and memory tests to identify Darters, and then a 15-min SFS 2 weeks later. We observed a significant effect of sex in conditioned freezing behavior, with males exhibiting greater freezing than females across conditioning and testing trials in comparison to females. However, females demonstrated higher velocities in response to shock presentations, and were more likely to exhibit darting behavior in response to the conditioned stimulus (CS). In SFS measures, females engaged in active behaviors such as climbing, head shaking, and diving in greater proportions than males, while males spent more time immobile throughout testing. Despite females exhibiting a more diverse behavioral repertoire in both tests, Darters did not differ from Non-darters in any SFS measure. These results suggest that the propensity to dart does not reflect a simple hyperactivity, and that despite conceptual overlap across the two tests (inescapable stress exposure and the ability to measure active vs. passive coping), the behavioral strategies engaged by an individual animal in each are likely driven by discrete mechanisms. We discuss potential challenges in interpretation of standard behavioral outcomes in classic models across the sexes, and consider the potential need for novel models that better tap into motivational states in females.

Keywords: sex differences, fear conditioning, forced swim, darting, coping

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INTRODUCTION

Selection of an appropriate response to threatening stimuli is a highly conserved evolutionary mechanism that can prove advantageous to the survival of a species. However, these responses can threaten survival when they become maladaptive (McEwen and Stellar, 1993; Commons et al., 2017). Post-traumatic stress disorder (PTSD) is characterized by heightened, unremitting fear

responses following exposure to a traumatic event. However, not all individuals who are exposed to trauma will go on to develop PTSD (Yehuda and LeDoux, 2007). The fact that symptoms of PTSD do not present themselves until months after the trauma has passed means that careful attention should be devoted to the delineation of markers that may serve to identify populations that are at significant risk. While a great deal of work has been devoted to identifying biological risk factors (such as being female), little is known as to how these relate to the selection of threat responses that contribute to the development of PTSD (Becker et al., 2007; Dai et al., 2017). The observation that certain peri-traumatic risk factors such as tonic immobility or peritraumatic dissociation correlate strongly with subsequent development of PTSD suggest that more efficient therapeutic interventions may arise from the study of how specific behavioral manifestations during trauma may be indicative of susceptibility or resilience to stress-induced pathology (Kassam-Adams et al., 2012; Thomas et al., 2012; Möller et al., 2017).

PTSD is characterized by intrusive memories and frequent re-experiencing of the traumatic episode (Clohessy and Ehlers, 1999). Pavlovian models of fear conditioning allow researchers to probe the neural basis of fear learning and memory by assessing behavioral responses to cues that have been associated with aversive outcomes (Schafe et al., 2001). Patients with PTSD exhibit potentiated acquisition of conditioned fear, as well as deficits in extinction learning (Milad et al., 2009; VanElzakker et al., 2014). Therefore, Pavlovian fear conditioning is a useful tool to study key neural circuitry that may go awry in patients suffering from PTSD (Phelps and LeDoux, 2005; Sijbrandij et al., 2013). In rodents, the amount of time spent freezing during the presentation of a conditioned stimulus (CS) is interpreted as an index of the strength of the association created, as well as the intensity of the fear itself (Fanselow, 1980). However, these models operate under the assumption that freezing is the only way in which fear can be expressed and quantified. Recent experiments from our lab characterized a novel, escape-like, active fear response known as "darting" that occurs primarily in a subpopulation of females during CS tone presentations (Gruene et al., 2015). Animals that darted during fear conditioning exhibited enhanced extinction retention, suggesting that engaging in a more diverse behavioral repertoire during aversive learning can promote cognitive flexibility in the future. In another study, we similarly found that in a forced swim test (FST), females are more likely to engage active coping strategies (Colom-Lapetina et al., 2017). Altogether these findings suggest that there are sex-specific, adaptive responses to threatening stimuli that are the result of individual variability within subsets of populations, and that these differences may help to identify potential markers of resilience. However, whether active responding during fear conditioning reflects a broad propensity towards active coping that translates across the two models has not been tested. The purpose of the current work, therefore, was to investigate the possibility that darting represents a sex-specific predictor of active coping in other forms of inescapable stress.

MATERIALS AND METHODS

Subjects

Young adult (∼8–9 weeks at time of arrival) male (n = 23) and females (n = 22) Sprague-Dawley (SD) rats weighing 275–300 g and 225–250 g, respectively were housed in pairs at the Nightingale Animal facility at Northeastern University. Subjects were kept on a 12:12 light:dark cycle with access to food and water *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals and approved by the Northeastern University Institutional Animal Care and Use Committee. Experimenters were male and female.

Behavioral Testing

Apparatus and Stimuli

Seven to 10 days after arrival at the vivarium, rats underwent fear conditioning trials as described in Gruene et al. (2015) in one of four identical chambers. Chambers are made with aluminum and Plexiglass walls (Rat Test Cage, Coulbourn Instruments, Allentown, PA, USA). A shock generator (Model H13–15; Coulbourn Instruments) was attached to the metal stainless steel rod flooring and the chambers were lit with a small house light. Each chamber was enclosed in sound-isolation cubicle (Model H10–24A; Coulbourn Instruments) and an infrared digital camera in each cage recorded behavioral testing from above. Once each trial was completed, chamber grid floors, trays, and walls were thoroughly cleaned with ethanol in preparation for the next session.

Fear Conditioning and Memory Test

On day 1, rats were allowed to explore cage for 4 min before five tone (CS) presentations (habituation), followed by seven conditioning trials [CS-unconditioned stimulus (US) pairings] on day 1. The CS was a 30 s, 5 kHz, 80 dB SPL sine wvave tone, which co-terminated with a 0.5 s, 0.7 mA footshock (US) during conditioning trials. On day 2, rats were placed back in fear conditioning apparatus and exposed to two CS-only presentations to assess successful acquisition of conditioned fear. Time freezing and velocity traces during both trials were recorded using Ethovision software (Noldus) and analyzed using custom Python code[1].

Single Forced Swim Session

All procedures were conducted during the animal's light cycle, under standard lighting. Animals were returned to facilities immediately following Fear conditioning paradigm and allowed to reacclimate to facilities for 14 days prior to single forced swim (SFS). At approximately 1100, rats were transferred from facility and placed in different room from testing and animal facilities for 1 h before forced swim. Subjects were placed individually in a plexiglass cylinder measuring 50 cm high and 20 cm wide that was filled with clean tap water (25 ± 2°C) to a height of 32 cm. Animals were subjected to a 15-min swim period. Once the session ended, animals were removed from the tank and carefully dried for 30 min under a lamp

[1] https://github.com/annajxli/scaredy-rat

in home cages before being returned to animal facilities. We tested four animals per day, counterbalanced for sex. Forced swim sessions were recorded with a built-in camera on an iMac computer and Noldus Ethovision XT software was used to score total time of immobility. Ethovision detection settings were calibrated to match those of experimenter scores. Subjects were considered immobile when floating and displaying minimal limb movements necessary to keep heads above water. Head shaking, climbing, and diving behavior were quantified *via* video hand-scoring by experimenters, blind to the animal's experimental group. Head shaking was described as short, vigorous bouts of head shaking and climbing was considered when rats approached walls of cylinders and displayed vertical limb movement (Colom-Lapetina et al., 2017).

Statistical Analysis

Behavioral data were analyzed using two-way ANOVAs with corrected *post hoc* tests for multi-trial or block behavioral tests (Fear conditioning, Forced Swim). Paired *t*-tests were used for single comparison tests (time spent diving), and chi-square tests to assess sex differences in subpopulations of Darters and Divers.

RESULTS

Fear Conditioning Behavior

Fear conditioning data are shown in **Figure 1A**. Freezing at baseline (BL) was low in both sexes, but freezing increased as tone-shock pairs progressed (main effect of trial $F_{(7,301)} = 51.6$; $p < 0.0001$), suggesting that both males and females learned the tone-shock association (**Figure 1A**). We also observed a main effect of sex ($F_{(1,43)} = 4.3$; $p = 0.04$), suggesting that overall, females froze less than males across trials. The next day, animals were presented with a two-tone test in a new context to evaluate consolidation. Again, both sexes increased freezing to the tone compared to BL (**Figure 1B**; main effect of trial ($F_{(2,86)} = 113.5$; $p < 0.0001$), and males froze more than females (main effect of sex $F_{(1,43)} = 10.1$; $p = 0.003$). We also evaluated the shock response across fear conditioning trials, and found that females responded with a greater velocity than males did (**Figure 1C**; main effect of sex: $F_{(1,44)} = 14.6$; $p = 0.0004$), consistent with our previous findings (Gruene et al., 2015). We then characterized males and females as "Darters" or "Non-darters" based on criteria set in Gruene et al. (2015). As we previously observed, a greater proportion of females qualified as Darters compared to males (**Figure 1D**; 32% vs. 9%, respectively. Chi square = 3.76; $p = 0.053$). We attribute the near-significance of this analysis to a potential slight underpowering of cohorts, but note that with larger cohorts, we have previously observed statistically significant sex differences in Darter populations (Gruene et al., 2015). To confirm that Darters and Non-Darters can be differentiated by behavior during the CS, we evaluated the maximum velocity reached by each animal during CS 3–7 (**Figure 1E**). A one-way ANOVA revealed a significant effect of group ($F_{(2,42)} = 26.1$; $p < 0.0001$) and *post hoc* tests revealed that Darters were significantly different from both Males and Non-darters (both comparisons $p < 0.0001$). Darters and Non-Darters did not differ in pre-shock locomotor activity (276.9 cm vs. 277.6 cm, respectively; $p = 0.99$, data not shown). Together, these data demonstrate that females exhibit slightly less conditioned freezing than males, but also exhibit more exaggerated shock responses and are more likely to engage in active conditioned responding, supporting our previous findings (Gruene et al., 2015).

Forced Swim Behavior

Two weeks after fear conditioning, all animals were exposed to a 15-min single SFS. We first evaluated immobility across three 5-min blocks of the SFS using a two-way ANOVA with factors of time and sex (**Figure 2A**). In both sexes, immobility increased with time [main effect of time ($F_{(2,86)} = 172.6$; $p < 0.0001$)]. However, females exhibited less immobility than males [main effect of sex ($F_{(1,43)} = 10.53$; $p = 0.002$)]. Adjusted Bonferroni *post hoc* tests revealed significant sex differences during time blocks 2 ($p = 0.002$) and 3 ($p = 0.02$), but not 1 ($p = 0.14$). Analysis of climbing behavior (**Figure 2B**) revealed a significant sex × time interaction ($F_{(2,126)} = 3.98$; $p = 0.02$). Adjusted Bonferroni *post hoc* tests revealed significant sex differences during time block 1 ($p = 0.002$), but not 2 ($p = 0.99$) or 3 ($p = 0.99$), suggesting that animals of both sexes exhibit climbing primarily during the first 5 min, but females engage in more climbing than males. We next evaluated head shakes in 2.5 min blocks (**Figure 2C**), because our video scorers noticed a reliable increase in head shaking during the second half of the first 5 min block, followed by a decrease over the remaining 10 min. We found significant main effects of time ($F_{(5,215)} = 10.52$; $p < 0.0001$) and sex ($F_{(1,43)} = 4.15$; $p = 0.047$). Adjusted Bonferroni *post hoc* tests revealed significant sex differences during time block 2 only ($p = 0.04$). Finally, we examined diving behavior. Like darting, diving is a discrete behavior that is exhibited by a subset of animals and may signify an alternate coping strategy. As with darting, we observed diving in a greater proportion of females compared to males (**Figure 2D**; 82% vs. 57%, respectively). Chi-square analysis revealed a near-significant sex difference (chi-sq = 3.4, $p = 0.067$). As above, we attribute the near significance of this analysis to slightly under-powering our *n*'s. Evaluation of time spent diving among animals that did engage in diving ("Divers") suggested that while there was variability in both males and females, there were no overall sex differences (**Figure 2E**; unpaired *t*-test: $t = 0.68$, $df = 29$; $p = 0.5$). Together, these results suggest that in the SFS, females are more likely to engage in a more diverse set of behavioral strategies than males, who are more likely to engage in immobility.

To determine whether these diverse SFS behaviors in females are related to darting during fear conditioning—in other words, whether behavioral diversity is a "trait" that can be observed across models—we next evaluated SFS behavior in females who were categorized as "Darters" or "Non-Darters" based on the occurrence of darting during fear conditioning. Seven females qualified as Darters. Darting during fear conditioning did not predict time spent immobile, climbing, or head shaking [**Figures 3A–C**; no main effects of darting on immobility ($F_{(1,21)} = 0.93$, $p = 0.35$), climbing ($F_{(1,20)} = 0.38$; $p = 0.54$), or head shaking ($F_{(1,20)} = 0.12$; $p = 0.73$]. Of the four females

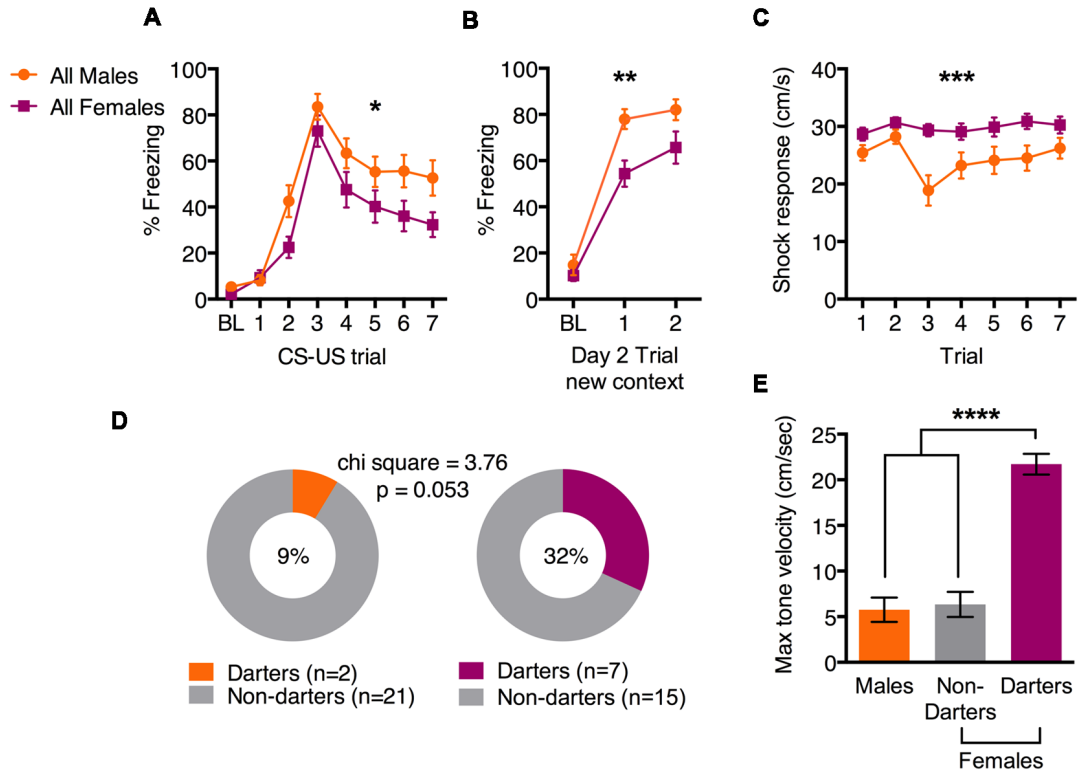

FIGURE 1 | Sex differences in active and passive behavior during fear conditioning. **(A)** Females froze less than males during fear conditioning tone presentations. **(B)** Females froze less than males during a tone test in a new context 24 h after fear conditioning. **(C)** Females exhibited greater shock responses than males during fear conditioning, as measured by the maximum velocity reached. **(D)** Proportions of male and female cohorts that exhibited darting behavior. **(E)** Maximum tone velocity differed between female Darters and both males and female Non-Darters. *$p < 0.05$ main effect of sex; **$p < 0.01$ main effect of sex; ***$p < 0.001$ main effect of sex; ****$p < 0.0001$ adjusted *post hoc*, Darters vs. both males and female Non-darters.

who did not engage in diving, two each were Darters and Non-Darters. A comparison of time spent diving between Darters and Non-Darters was not significant (**Figure 3D**; $t = 1.68$, $df = 16$; $p = 0.11$). However, we note that our "Diving Darter" n is fairly low ($n = 5$), and that this difference may have reached significance with more statistical power. In that case, diving and darting may represent common strategies across fear conditioning and SFS.

DISCUSSION

Our goals for the present study were to examine the relationships between individual behavioral strategies across classic behavioral paradigms. For decades, auditory fear conditioning and the FST have been used in laboratories across the world to study how the brain processes and responds to aversive experiences. However, the vast majority of studies using these models have been conducted in male rodents, and whether the standard behavioral metrics associated with each (e.g., freezing or immobility) sufficiently map onto the same motivational or emotional states in females has not been rigorously investigated (Shansky, 2015). Because stress-related disorders like PTSD are more prevalent in women (Breslau, 2009), it is imperative that we assess the validity our behavioral models in both sexes. A closer, more nuanced look at the range of behaviors an animal can exhibit in each may provide better insight into the core aspects of these diseases in both men and women.

Along these lines, we have recently found that females, but not males, will exhibit an active conditioned response during auditory fear conditioning ("darting"). This finding was important for several reasons. First, it demonstrates that measures of freezing alone are inadequate for assessing learning in classic Pavlovian models. Second, darting during conditioning was associated with enhanced extinction retention, suggesting that darting may reflect an alternate behavioral strategy that predicts long-term adaptive outcomes. Because we only observe darting in about 30%–40% of females, Darters may represent a discrete subpopulation of animals that are generally prone to active responding in stressful situations. The work here therefore represents our first attempt to identify additional behavioral patterns that can be predicted by darting. To test this idea, we identified Darters based on behavior during fear conditioning, and asked whether they were more likely to exhibit active responses during the SFS.

As we observed previously, more females than males exhibited darting during fear conditioning in the current study. Females also exhibited less conditioned freezing but greater shock

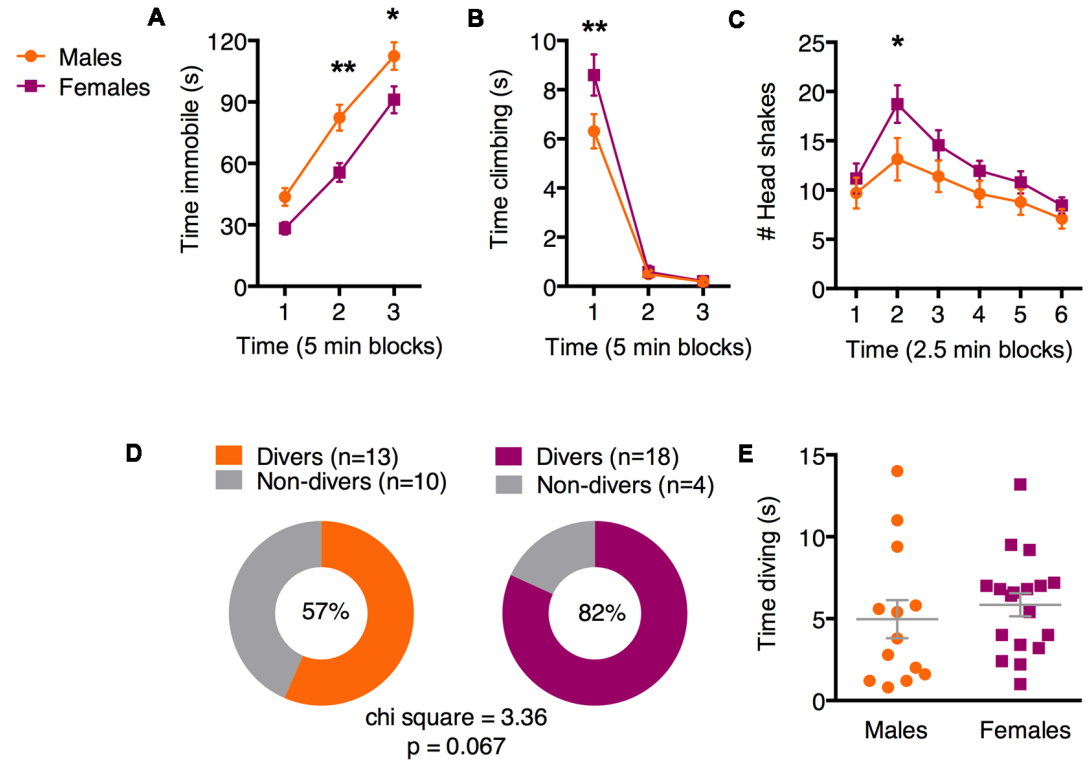

FIGURE 2 | Sex differences in active and passive behavior during the forced swim test (FST). **(A)** Females exhibited less immobility than males in the last two single forced swim (SFS) time bins. **(B)** Females spent more time climbing than males during the first 5 min of the SFS. **(C)** Females exhibited more head shaking than males during early SFS time points. **(D)** Proportions of male and female cohorts that exhibited diving behavior. **(E)** Among Divers, males and females did not differ in time spent diving. $^{*}p < 0.05$ adjusted *post hoc* males vs. females; $^{**}p < 0.01$ adjusted *post hoc* males vs. females.

responsivity than males, again supporting our previous findings. Importantly, the shock response data suggest that lower freezing in females is not due to lower perceived pain. In the SFS, we also observed more diverse behavioral patterns in females, who were more likely to exhibit climbing, head-shaking, and diving compared to males. These data are consistent with our previous findings (Colom-Lapetina et al., 2017), but do not map onto all studies of sex differences in the 2-day FST. For example, Kokras et al. (2017) have found that males are more likely to exhibit head shaking than females, and Rincón-Cortés and Grace (2017) recently reported more immobility in females compared to males. A comprehensive review of sex differences in the FST (Kokras et al., 2015) shows that there is no clear consensus as to the directionality of how males and females differ in immobility, swimming, and climbing. Important considerations that may contribute to these discrepancies include animal strain, vivarium light/dark cycle, water temperature, duration of the test, and disparities in scoring protocols across laboratories. Our primary goal here was not to examine sex differences in each test, but to identify behavioral measures that tracked across both tests, based on an animal's propensity to dart during fear conditioning. However, we found that SFS measures were not different between females that qualified as Darters vs. Non-darters. To our knowledge, this is the first examination of non-freezing conditioned responses as a potential predictor of alternate coping strategies.

Other groups have attempted to categorize individual differences among cohorts in order to identify adaptive vs. maladaptive behavioral profiles and predictors. For example, a recent study divided male rats into "active" or "passive" coping groups based on behavior after a chronic social defeat experience (Grafe et al., 2018). In a subsequent FST, passive responders exhibited more immobility and less time swimming compared to non-defeated animals, which were statistically comparable to active responders. Unfortunately, female animals were not examined in this study. In another report, assessment of struggling behavior when briefly restrained on their backs (Back Test) allowed animals to be classified into "passive," "active," or "variable" coping groups. In males, these groups differed in behaviorally in the FST as well as in several physiological measures of stress, such as fecal corticosterone levels and cardiovascular activation (Hawley et al., 2010). However, although females could also be separated by passive, active, and variable coping, their classification was not predictive of behavioral differences in measures of diving in the FST, or of rearing and grooming in the Dry Land Maze task (Kent et al., 2017).

Our data suggest that in both auditory fear conditioning and the SFS, female SD rats exhibit greater behavioral diversity than

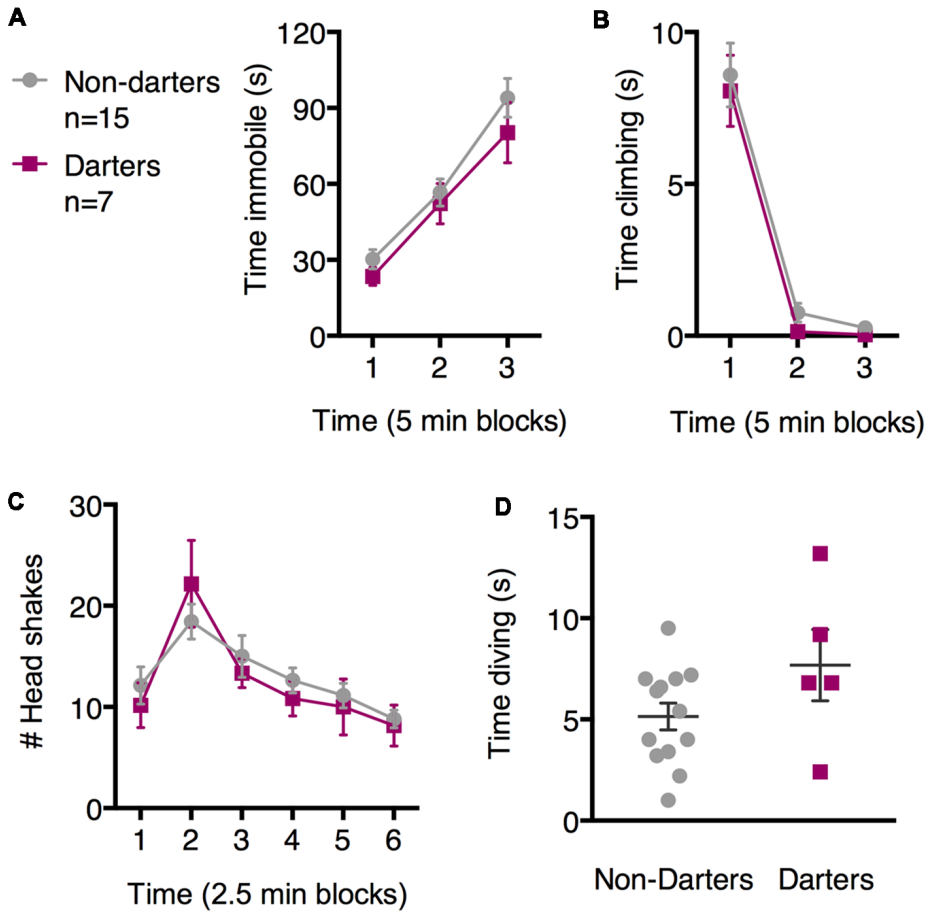

FIGURE 3 | Darter and Non-darter females did not differ in the SFS. No statistically significant differences were observed between Darters and Non-darters in measures of immobility **(A)**, climbing **(B)**, headshakes **(C)**, or time spent diving **(D)**.

males, engaging in a broader repertoire of coping behaviors. We emphasize the strain here because we and others have found that sex differences in the SFS can vary by strain (Kokras et al., 2015; Colom-Lapetina et al., 2017), and we have not yet assessed darting in other commonly used strains. The lack of differences between Darters and Non-darters in the SFS suggests that behavioral diversity itself is not necessarily an individual trait that transfers from one model to the next. In addition, this finding argues against the putative interpretation that Darters are simply hyperactive—a conclusion also supported by our findings here and previous that Darters do not exhibit greater BL locomotor activity (Gruene et al., 2015). Instead, we believe that Darters represent a true subpopulation of animals that switch strategies from passive (freezing) to active (darting) in conditioned fear paradigms. We look forward to dissecting the neural circuits and mechanisms that distinguish Darters from Non-Darters, as well as investigating the biological basis for darting's prevalence in females.

One common pattern between our current findings and those of Kent et al. (2017) is that female behavior in the SFS could not be predicted by previous responding in a stress test. In contrast, similar approaches were successful in identifying consistent subpopulations in males, as described above. This discrepancy points to potential sex differences in the interpretive value of standard behavioral measures. As we have argued previously (Shansky and Woolley, 2016; Shansky, 2018), many common behavioral tests were developed and validated in male animals, and therefore the readouts we use to assess the same states in females may need to be adjusted. For example, a Principal Component Analysis of the elevated plus maze, which is traditionally used to measure anxiety, found that time spent in open vs. closed arms was most directly related to anxiety in males, but locomotor activity in females (Fernandes et al., 1999). Therefore, in models of stress coping and depressive-like behavior such as the FST, it is critical to determine which behaviors and metrics most accurately capture the motivational state of the subjects. Identifying sex differences in these methodologies could improve the translational value of these tests (Kokras and Dalla, 2017).

It is also important to recognize that different ethological demands in males and females may render active vs. passive responding differentially advantageous for each sex. For example,

females may be more likely to survive a threat if they are able to escape it, while males may be better served by conserving energy and adopting more passive strategies. As we observed previously (Colom-Lapetina et al., 2017), a 2-day FST produces a sex-specific "learned helplessness" effect, such that immobility increased from day 1 to day 2 in males, but not females. One might interpret that to mean that females did not learn that escape from the FST is impossible, but an alternate interpretation could be that it is generally advantageous for females to try to escape stressful situations, and therefore they will maintain active coping responses longer than males will. This interpretation is supported by classic studies in the stress literature, showing that inescapable shock exposure impairs shuttlebox escape and active behaviors in the holeboard and elevated plus maze tests in males, but not females (Steenbergen et al., 1989, 1991). Given these early findings, it is perhaps unsurprising that we also observe a female-leaning propensity towards escape-like behaviors in standard fear conditioning and forced swim paradigms. This trend therefore warrants caution in interpreting effects such as those observed in the current study to mean that females are more adaptive or resilient than males simply because they exhibit greater behavioral diversity. In clinical populations, women are more susceptible to stress-related pathologies like PTSD (Breslau, 2009). If the ultimate goal of preclinical research is to improve disease treatment and prevention, we must accept the challenge of modifying our behavioral models and metrics to more precisely capture sex-specific vulnerabilities.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JC-L and RS designed the experiments. JC-L and TP-P conducted all behavior testing. AL wrote the Python code for darting analysis. JC-L and RS analyzed the data and wrote the manuscript.

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frontiers in Behavioral Neuroscience

REVIEW
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A Rodent Model of Exposure Therapy: The Use of Fear Extinction as a Therapeutic Intervention for PTSD

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The symptoms of post-traumatic stress disorder (PTSD) include cognitive impairment related to medial prefrontal cortical dysfunction. Indeed, a deficit of cognitive flexibility, i.e., an inability to modify previously learned thoughts and behaviors based on changes in the environment, may underlie many of the other symptoms of PTSD, such as changes in mood, hyper-arousal, intrusive thoughts, exaggerated and over-generalized fear, and avoidance behavior. Cognitive-behavioral therapies target the cognitive dysfunction observed in PTSD patients, training them to recalibrate stress-related perceptions, interpretations and responses. Preclinically, the extinction of conditioned fear bears resemblance to one form of cognitive therapy, exposure therapy, whereby an individual learns, through repeated exposure to a fear-provoking stimulus in a safe environment, that the stimulus no longer signals imminent threat, and their fear response is suppressed. In this review article, we highlight recent findings from our lab using fear extinction as a preclinical model of exposure therapy in rodents exposed to chronic unpredictable stress (CUS). We specifically focus on the therapeutic effects of extinction on stress-compromised set-shifting as a measure of cognitive flexibility, and active vs. passive coping behavior as a measure of avoidance. Finally, we discuss mechanisms involving activity and plasticity in the medial prefrontal cortex (mPFC) necessary for the therapeutic effects of extinction on cognitive flexibility and active coping.

Keywords: cognitive flexibility, coping, chronic unpredictable stress, infralimbic cortex, set shifting

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating illness that affects up to 8% of the general population in the United States (Kilpatrick et al., 2013), and as many as 20%–30% of combat veterans (Breslau, 2001). Development of PTSD symptoms is associated with experiencing or witnessing perceived life-threatening events, such as combat-related trauma, sexual abuse, and other uncontrollable and unpredictable events (Ozer et al., 2003). PTSD symptoms include fear generalization, intrusive re-experiencing of trauma, avoidance behaviors, cognitive impairments, and negative alterations in mood (DSM-V). PTSD has classically been conceptualized as a disorder of fear dysregulation, a traumatic event may subsequently cause an individual to generalize their fear of stimuli associated with the traumatic event to non-threatening stimuli, or to similar stimuli in non-threatening environments. Animal

models of PTSD have historically focused on mimicking the exaggerated fear responses observed in the patient population (Foa et al., 1992, 2006). Preclinical PTSD research studies often utilize Pavlovian fear conditioning and extinction as dependent measures to investigate the neurobiology underlying the exacerbation of fear in PTSD (Milad and Quirk, 2012). The neurobiological circuitry involved in fear acquisition and **fear extinction** has been extensively studied and delineated [see reviews by Johnson et al. (2012) and VanElzakker et al. (2014)]. Using this approach, investigations aimed at developing strategies to improve PTSD symptoms have identified substances that can accelerate the rate of extinction learning (Milad and Quirk, 2012). However, while excessively strong conditioned fear is a central component of the illness, PTSD is a complex and chronic disorder, encompassing other symptom domains that reflect, for example, avoidance behavior and withdrawal, and disruptions of executive function and cognition. These other symptom domains may not appear at first glance to be directly related to aberrant fear memory. However, chronic PTSD and the repeated process of re-experiencing fearful memories, i.e., the constant retrieval and reactivation of conditioned fear, may in itself induce a state of chronic stress. This state of chronic **stress** could then secondarily impair the function of brain regions such as the prefrontal cortex (PFC; see, for example Jett et al., 2017), contributing to the development and maintenance of a broader array of symptoms than present initially, including disruptions of executive function and **cognitive flexibility** that are characteristic of PTSD.

KEY CONCEPT 1 | Fear extinction
A decrease in conditioned fear responses (i.e., freezing) after repeated exposures of a non-reinforced conditioned stimulus (e.g., a tone).

KEY CONCEPT 2 | Stress
Any threat, either real or perceived, to one's health or well-being, that exceeds homeostatic regulatory capacity.

KEY CONCEPT 3 | Cognitive flexibility
The ability to modify previously learned thoughts, behaviors or associations based on new information from the environment.

Indeed, PTSD patients exhibit hypoactivity of the ventromedial and dorsomedial PFC (Etkin and Wager, 2007). The medial PFC (mPFC) exerts a top-down inhibitory influence on the fear and anxiety elicited by amygdala activation (Koenigs and Grafman, 2009; Likhtik et al., 2014), so dysregulation of mPFC function could contribute directly to inappropriate regulation and disinhibition of amygdala activation, and the resulting fear and anxiety in PTSD (Goossens et al., 2007). In addition, patients with PTSD also exhibit impairments of other higher order cognitive processes and executive functions mediated in the PFC, such as set shifting, spatial working memory, and response inhibition (Olff et al., 2014). They perform poorly in tests of cognitive flexibility, such as the Wisconsin Card Sorting Test (Kanagaratnam and Asbjørnsen, 2007). The PFC is involved in executive function (Girotti et al., 2018). Thus, prefrontal hypoactivity may contribute to the cognitive dysfunction observed in PTSD patients.

KEY CONCEPT 4 | Coping
Behavioral strategies mounted in response to a threatening stimulus or situation that serve to reduce or remove the threat, or to remove oneself from the threat.

In addition to deficits in cognitive flexibility, individuals with PTSD also often adopt passive **coping strategies**, associated with symptoms of avoidance and withdrawal (Olff et al., 2005). Passive coping strategies are associated with a greater neuroendocrine response to threat, and increase the likelihood that an individual will develop PTSD symptoms (Olff et al., 2005; Bronner et al., 2009). Continuous avoidance and ineffective, maladaptive coping can lead to persistence of intrusive thinking and negative emotions like fear, anxiety, and depression; thus, passive coping can contribute to both the onset and maintenance of stress-related psychiatric disease, as does cognitive inflexibility (Foa and Kozak, 1986; Wenzlaff et al., 1988; Creamer et al., 1992). Thus, although fear dysregulation is undoubtedly central to the symptomatology of PTSD, targeting the underlying cognitive dysfunction associated with mPFC dysregulation may improve treatment outcomes for PTSD patients.

Animal models of PTSD are limited in that they cannot recreate the uniquely human experience of the disorder in total. Animal models can, however, effectively model defined dimensional components of behavior that resemble specific symptom clusters, allowing researchers to pursue mechanistic questions addressing the neurobiological circuits underlying the dysregulation of those behavioral dimensions. This is supported by neuroimaging studies that characterize specific neural circuits that are dysregulated in PTSD patients (Bremner et al., 1995, 1997; Liberzon et al., 1999; Rauch et al., 2006; Bryant et al., 2008). Further, the advent of sophisticated preclinical chemogenetic and optogenetic tools to selectively manipulate fear-related circuitry and plasticity can advance efforts to elucidate the neurobiological mechanisms of behavioral therapy for the treatment of PTSD. This review will highlight preclinical findings from our lab using fear extinction, not as a dependent measure, but as a model of exposure therapy, with an emphasis on the effects of extinction in restoring cognitive flexibility and active coping behavior that has been compromised after chronic unpredictable stress (CUS).

MODELING PTSD IMPAIRMENTS IN RODENTS USING CHRONIC UNPREDICTABLE STRESS (CUS)

Cognitive flexibility is an executive function mediated by the mPFC that is impaired in patients with PTSD (Birrell and Brown, 2000; Walter et al., 2010; Olff et al., 2014). The attentional set shifting test (AST) measures cognitive flexibility performance in rodents, and the extradimensional (ED) set shifting stage of the AST relies specifically on the function of the mPFC (Birrell and Brown, 2000; Bissonette et al., 2008). Our lab has extensively used the CUS paradigm to model the hyperarousal and medial

prefrontal dysfunction observed in PTSD. The CUS procedure entails a series of several varied and robust acute psychogenic stressors applied once daily for a period of 2 weeks (Bondi et al., 2008, 2010). It is important to note that, in addition to preclinical models utilizing chronic stress to induce PTSD symptoms, models utilizing acute stress [such as the single prolonged stress (SPS) model; Lisieski et al., 2018] can also induce distinct PTSD-like phenotypes in rodents (see Goswami et al., 2013). Indeed, according to the DSM-V, both repeated and acute exposure to trauma can lead to a PTSD diagnosis. Therefore, the use of both chronic and acute stress models in the study of PTSD is informative, since the etiology of PTSD is complex (Scott and Stradling, 1994; Cloitre et al., 2009).

We have shown that this CUS treatment impairs performance on the ED set shifting stage of the AST (Bondi et al., 2008, 2010). Similar to the mPFC hypoactivity observed in PTSD patients, CUS decreases mPFC responsivity to afferent input in rodents (Jett et al., 2017). Other chronic stress paradigms also negatively alter the excitability of mPFC pyramidal neurons, and mPFC-mediated behaviors (Liston et al., 2006; Yuen et al., 2012). SPS is another rodent model of PTSD that induced impairments in executive function, including set shifting (George et al., 2015). We have also shown that set shifting impairment induced by CUS is reversed by several chronic and acute pharmacological interventions (Bondi et al., 2008, 2010; Naegeli et al., 2013; Jett et al., 2015).

In addition to cognitive impairment, uncontrollable and unpredictable stress in rodents can induce passive coping behaviors, consistent with avoidance symptoms observed in PTSD (Whitaker et al., 2016). Coping behavior is modulated by the mPFC in a top-down manner. In rodents, behavioral coping strategy in response to a threatening stimulus can be evaluated using the shock probe defensive burying test (SPDB; Lapiz-Bluhm et al., 2008). The SPDB involves placing a rat in a cage filled with bedding, with an electrified probe at one end of the cage (Fucich and Morilak, 2018). The rat approaches the probe and receives a shock, which evokes a rise in norepinephrine concentration in the lateral septum (LS) and a rise in plasma ACTH, an indicator of perceived stress (Bondi et al., 2007). Rats then engage in active coping behavior, defined by the amount of time they spend burying the probe with bedding, an ethologically-relevant defensive response, or passive coping behavior, defined by the amount of time spent immobile. Coping behaviors are assessed by analyzing both of these measures independently, and the relative amount of active vs. passive coping can then be expressed as a ratio (Fucich and Morilak, 2018). Following shock-probe exposure, we showed that rats allowed to bury the probe showed a return to baseline ACTH levels faster than rats that were unable to bury the probe because the bedding had been removed (Bondi et al., 2007). Thus, active burying in response to shock-probe exposure is an effective coping strategy that decreases stress. Further, the mPFC modulates activity in the LS, which promotes active coping in the SPDB test (Treit et al., 1993; Shah et al., 2004; Bondi et al., 2007). We have shown that CUS produces a shift from active to passive coping in the shock probe test, (Jett et al., 2015), modeling the avoidance behaviors seen in PTSD, and pharmacological interventions such as ketamine, vortioxetine, and desipramine prevent and/or reverse the stress-induced shift to passive coping (Bondi et al., 2007; Jett et al., 2015; Hatherall et al., 2017). These studies have highlighted the modulatory influence of monoaminergic neurotransmitters such as norepinephrine and serotonin, the targets of drugs such as traditional reuptake blocking antidepressants, and the essential role of glutamate as the primary excitatory neurotransmitter mediating the function and plasticity of prefrontal cortical circuits.

In sum, CUS produces functional and behavioral deficits similar to the mPFC-related cognitive impairment and avoidance-related symptoms seen in PTSD patients. Therefore, we used CUS to evaluate the therapeutic capacity of our rodent model of exposure therapy in reversing these effects.

FEAR EXTINCTION AS A PRECLINICAL MODEL OF EXPOSURE THERAPY

Cognitive behavioral therapy (CBT), developed by psychiatrist Aaron Beck, targets the underlying cognitive dysfunction observed in patients with psychiatric disorders, rather than treating only the individual symptoms that stem from those cognitive biases and cognitive dysfunction (Beck, 1976). Psychotherapeutic treatments for PTSD attempt to modify an individual's cognitive appraisal of their fear, and may also involve repeated exposure to fear-provoking stimuli (Foa et al., 1989). Cognitive behavioral therapies, of which exposure therapy is but one example, also aim to improve active adaptive coping (Brewin, 1996; Beck, 2005). Exposure-based therapies engage areas of the brain, such as the hippocampus, PFC and amygdala, that are affected by chronic stress and are associated with PTSD and related neuropsychiatric disorders (Mahan and Ressler, 2012). Indeed, individuals that responded to prolonged exposure treatment had greater baseline hippocampal volume than treatment non-responders (Rubin et al., 2016). Cognitive behavioral therapies, including exposure therapy, increase activation of the ventrolateral and dorsolateral PFC after treatment, and are effective in ameliorating PTSD symptoms (Helpman et al., 2016; Yang et al., 2018). Thus, effective cognitive behavioral therapies may restore compromised activity in the mPFC, a regulator of executive function and emotional modulation.

Fear extinction is a form of safety learning that consists of the formation of a new memory in the ventromedial PFC (vmPFC; Milad and Quirk, 2002). Cue-conditioned fear extinction consists of a decrease in fear response (i.e., freezing) that results from the repeated exposure to a conditioned fear stimulus (i.e., a tone), that is not reinforced or punished (Martinez et al., 2012; Milad and Quirk, 2012). Fear conditioning association occurs in the basolateral and central amygdala, which have reciprocal inhibitory connections to the infralimbic (IL) cortex in the vmPFC. This bears resemblance to the process of exposure therapy, whereby patients, by repeated exposure to fear-provoking stimuli learn that they are no longer threatening, and as a result suppress their fear behavior. Similar to the cognitive reappraisal during CBT, fear extinction

requires cognitive flexibility, i.e., modifying a previously learned association based on feedback from the environment.

EXTINCTION LEARNING REVERSES STRESS-INDUCED DEFICITS IN SET SHIFTING AND PROMOTES ACTIVE COPING

The effects of CUS are, at least partly, due to the attenuation of glutamatergic activity in the mPFC (Jett et al., 2017). Chronic stress induces reductions in apical dendritic spine numbers and dendritic length in the mPFC (Liston et al., 2006; Holmes and Wellman, 2009). Chronic stress also reduces AMPA receptor and NMDA receptor-mediated synaptic transmission, decreases the expression of glutamate receptors in the mPFC, and alters the expression and phosphorylation status of signaling molecules that mediate the transduction of neurotrophic signaling pathways that promote synaptic plasticity (Trentani et al., 2002).

Extinction is a learning process that promotes plasticity in the mPFC. Extinction learning activates the mPFC, much like exposure therapy in humans, and it enhances the excitability of glutamatergic pyramidal neurons in the vmPFC (Burgos-Robles et al., 2007). Therefore, we reasoned that restoring pyramidal cell function by engaging rats in a session of cognitive training by extinction learning would reverse the stress-induced deficits in set shifting. To test this, we first fear conditioned the rats by a standard procedure of four shock-tone pairings prior to stress, to avoid any effect of stress on the initial strength of fear learning. We then exposed them to 2 weeks of CUS or unstressed control treatment. Three days after the end of stress, we exposed them to a single session of 16 extinction trials with presentation of tones but no shock, and tested them on the set-shifting test 24 h after extinction. Another group of extinction controls were exposed to the same tone presentation, but without prior fear conditioning so that no learning took place during the session. We observed that extinction reversed the effects of CUS on set shifting, restoring performance back to non-stressed control levels (**Figures 1A–C**; Fucich et al., 2016). Extinction alone had no effect on set-shifting in unstressed rats, and exposure to tones alone without prior fear-conditioning did not improve set-shifting in stressed rats. Thus, training with a single session of cue-conditioned fear extinction had a therapeutic effect, reversing stress-induced deficits in cognitive set shifting.

We also investigated whether fear extinction could reverse the chronic stress-induced avoidance behavior modeled by a shift from active to passive coping on the SPDB test. We hypothesized that fear extinction, by engaging the mPFC and its modulatory influence on activity in its downstream target, the LS, would effectively restore active coping in stressed animals. The procedure and timing were as above. Active coping was measured by time spent burying the shock probe, and passive coping was measured by immobility. CUS induced a shift from active to passive coping on the SPDB test, and a single session of extinction 24 h before testing effectively restored active coping behavior back to unstressed control levels (**Figures 1D–F**; Fucich et al., 2016). Therefore, extinction as a model of exposure therapy ameliorated mPFC-dependent cognitive dysfunction and promoted active coping behavior that had been compromised by chronic stress.

MECHANISMS UNDERLYING THE THERAPEUTIC EFFECTS OF FEAR EXTINCTION AFTER STRESS: ACTIVITY OF PYRAMIDAL CELLS IN THE INFRALIMBIC CORTEX

Neuroimaging studies in clinical populations provide insight into the neural alterations that occur after effective psychotherapy. Studies show that activity of the vmPFC (corresponding to the IL mPFC in the rat brain) before CBT predicts symptom improvement (Ritchey et al., 2011). By contrast, hypoactivity in the mPFC is associated with increased symptom severity in major depressive disorder and PTSD (Shin et al., 2006). In addition, a recent study conducted in humans showed that stimulating the vmPFC with spatiotemporally focused transcranial magnetic stimulation (TMS) enhanced fear extinction learning, as measured by skin conductance responses (Raij et al., 2018). Fear extinction learning activates the mPFC, and its downstream targets in rodents (Sotres-Bayon et al., 2004). Further, retention of extinction memory requires the activity of pyramidal neurons in the vmPFC of rats, and stimulation of the vmPFC results in a decreased conditioned freezing response during fear extinction (Do-Monte et al., 2015). Thus, vmPFC activation may be necessary for the therapeutic effects of psychotherapy.

The IL and prelimbic (PL) sub-regions of the mPFC mediate opposing effects on fear expression behavior. Specifically, inactivating the PL impairs the expression of fear, but does not affect fear extinction memory. Conversely, inactivating the IL does not impair fear expression, but blocks fear extinction memory (Sierra-Mercado et al., 2011). Thus, we focused our attention in these studies on the IL cortex. We hypothesized that the activity specifically of glutamatergic pyramidal cells, the principle output neurons of the vmPFC, mediate the therapeutic effects of extinction learning on cognitive set-shifting and active coping behavior that have been compromised by CUS.

To test the necessity of pyramidal cell activity in the vmPFC for the beneficial effects of extinction therapy on cognitive set shifting after stress, we used AAV viral-mediated delivery of an inhibitory Gi-coupled Designer Receptor Exclusively Activated by Designer Drug (DREADD) into the IL cortex, under the control of a CaMKIIα promoter to induce expression specifically in glutamatergic neurons. Controls received a microinjection of virus expressing an inert GFP construct. Four-to-five weeks total time was allowed for expression of the DREADD protein before testing. Thus, approximately 2 weeks after injection, rats began the CUS or unstressed control procedures. Three days after the end of stress, 30 min prior to the extinction therapy session, rats received an injection of the DREADD ligand clozapine-N-oxide (CNO, 1 mg/kg in 2% dimethylsulfoxide, i.p.) to selectively inhibit pyramidal

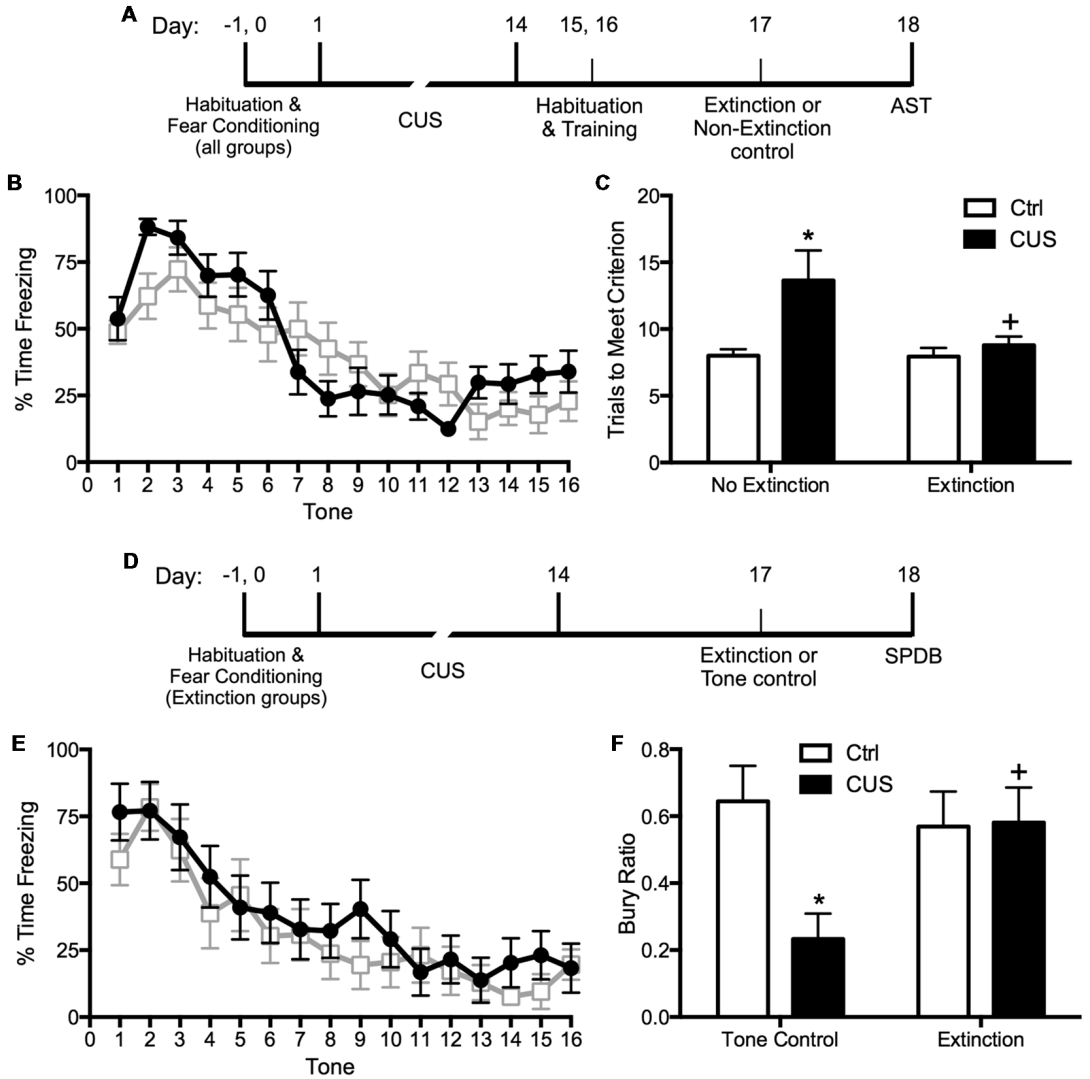

FIGURE 1 | Effects of chronic unpredictable stress (CUS) and extinction therapy on attentional set-shifting and coping behavior on the shock-probe defensive burying test (SPDB). **(A)** Time line for the experiment testing the effects of chronic stress and extinction therapy on cognitive set-shifting. **(B)** Extinction, administered 24 h before testing on the Attentional Set-shifting Test (AST), was comparable in the two extinction treatment groups (CUS and unstressed control; area under the curves, $p > 0.65$); $n = 14$ per group. **(C)** CUS induced a significant increase in the number of trials required to meet criterion (TTC) of six consecutive correct responses on the set-shifting task ($^{*}p < 0.05$, CUS compared to unstressed controls in the non-extinction groups). Extinction treatment reversed the effect of stress, restoring performance to unstressed control levels ($^{+}p < 0.05$, extinction compared to non-extinction in the CUS groups); $n = 14$–15 per group. **(D)** Time line for the experiment testing the effects of chronic stress and extinction therapy on coping behavior. **(E)** Extinction, administered 24 h before testing on the shock probe test, was comparable in the two extinction treatment groups (CUS and unstressed control; area under the curves, $p > 0.55$); $n = 12$ per group. Extinction control groups exposed to tone presentation but not fear conditioned ("Tone controls") showed low levels of freezing during tone presentation (not shown). **(F)** CUS induced a significant decrease in the Bury Ratio [calculated as bury time/(bury time + immobility time)]; $^{*}p < 0.05$, CUS tone controls compared to unstressed tone controls). Extinction treatment reversed the effect of stress, restoring the Bury Ratio to unstressed control levels ($^{+}p < 0.05$, CUS-extinction compared to CUS-tone controls); $n = 11$–12 per group. Data expressed as mean ± SEM. Reproduced and adapted with permission from Fucich et al. (2016).

cell activity in the IL cortex during extinction training. Rats were then tested for set-shifting on the AST 24 h after extinction. Our results showed that inhibiting pyramidal cell activity in the vmPFC, which had no effect on extinction itself, blocked the therapeutic effects of extinction on cognitive set shifting in stressed animals tested 24 h later (**Figure 2**; Fucich et al., 2018). Thus, activity of IL cortical pyramidal cells during extinction is necessary for its therapeutic effects on set shifting. We also tested whether activating these cells was sufficient to reverse the detrimental effects of stress on set-shifting. Rats received bilateral viral delivery of an excitatory Gq-coupled DREADD into the IL cortex. Three days after the end of CUS treatment, animals received an injection of CNO (1 mg/kg, i.p.) instead of extinction training, and were tested on AST 24 h post-injection. We found that transiently activating pyramidal cells in the IL cortex after

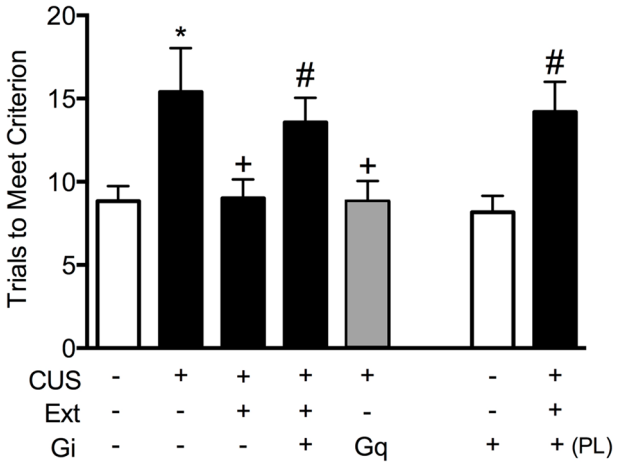

FIGURE 2 | Activity of glutamatergic pyramidal cells in the infralimbic (IL) cortex is necessary and sufficient for the therapeutic effects of extinction on cognitive set-shifting compromised by chronic stress. CUS compromised cognitive flexibility, significantly increasing trials required to meet criterion (TTC) of six consecutive correct responses on the extradimensional (ED) set-shifting task (*$p < 0.05$, GFP/CUS/tone controls compared with GFP/unstressed/tone controls). Both extinction treatment and hM3Dq activation with clozapine-N-oxide (CNO) reversed the effect of stress, restoring TTC to unstressed control levels ($^{+}p < 0.05$, GFP/CUS/extinction and hM3Dq/CUS compared with GFP/CUS/tone controls). hM4Di-mediated inhibition of IL projection neurons during extinction treatment prevented the therapeutic effect of extinction on set-shifting after CUS, as TTC were comparable to GFP/CUS/tone controls ($^{\#}p < 0.05$, hM4Di/CUS/extinction compared with GFP/CUS/extinction). Similarly, inhibiting glutamatergic neurons in prelimbic (PL) cortex during extinction also prevented the beneficial effect on set-shifting ($^{\#}p < 0.05$, hM4Di in PL/CUS/extinction compared with GFP/CUS/extinction). hM4Di-mediated inhibition of IL projection neurons alone in unstressed tone controls had no effect on set-shifting tested 24 h later; $n = 5$–9 per group. Data expressed as mean ± SEM. Reproduced and adapted with permission from Fucich et al. (2018).

CUS was sufficient to reverse the effects of stress on set shifting, mimicking the effects of extinction therapy (**Figure 2**; Fucich et al., 2018).

Using a similar DREADD strategy, we also investigated whether the activity of IL pyramidal neurons during extinction is necessary and sufficient for the therapeutic effects of extinction on active coping behavior on the SPDB in stressed animals, mediated by the LS (Treit et al., 1993; Bondi et al., 2007). The mPFC provides excitatory input to the LS, which in turn is composed of mainly inhibitory neurons that make reciprocal contacts with other sub-cortical regions associated with stress and fear, such as the amygdala, hypothalamus, and bed nucleus of the stria terminalis (Sheehan et al., 2004). Activity of LS neurons is increased during open arm exploration on the elevated plus maze (Thomas et al., 2013). By contrast, chronic stress blunts acute stress responsivity of the LS (Martinez et al., 1998). Thus, we reasoned that activity of IL pyramidal cells during extinction may induce plasticity downstream in the LS of stressed animals, promoting a shift back to active coping. We found that silencing pyramidal cells at the time of extinction prevented its beneficial effects on active coping behavior in stressed animals (**Figure 3A**), and that transiently activating pyramidal cells in the vmPFC after stress mimicked the beneficial effects of extinction therapy on coping behavior (**Figure 3B**; Fucich et al., 2018).

MECHANISMS UNDERLYING THE THERAPEUTIC EFFECTS OF FEAR EXTINCTION AFTER STRESS: ACTIVITY-DEPENDENT PROTEIN SYNTHESIS

In considering extinction as a learning process, it has been shown that extinction memory consolidation and retention require protein synthesis in the mPFC. Santini et al. (2004) further showed that extinction increased c-Fos expression in the dorsomedial PFC and vmPFC, but not in the insular cortex, suggesting that extinction learning initiates *de novo* protein synthesis in the mPFC. Based on what is known about the mechanisms underlying extinction learning and memory, together with our results discussed above showing the necessity of activity in the mPFC, we hypothesized that the therapeutic behavioral effects of extinction following chronic stress exposure may also be exerted through a process involving activity-dependent protein synthesis in the mPFC, similar to therapeutic mechanisms proposed for rapid-acting antidepressants, such as ketamine (Li et al., 2010; Autry et al., 2011; Monteggia et al., 2013; Duman et al., 2016).

We first observed that extinction increased phosphorylation at the S240/244 site of ribosomal protein S6 in the mPFC, but only in stressed rats (Fucich et al., 2016), indicating changes in protein synthesis (Roux et al., 2007; Knight et al., 2012). Changes in S6 phosphorylation are associated with activation of the mammalian target of rapamycin (mTOR) signaling cascade; mTOR activates the translational regulator ribosomal protein S6 kinase 1 (S6K1), which in turn activates ribosomal protein S6 by phosphorylation at serine S240/244 (Roux et al., 2007). The mTOR-p70S6K pathway has been linked to protein synthesis and structural changes in the mPFC that underlie the therapeutic effects of novel rapid-acting antidepressants (Li et al., 2010; Dwyer et al., 2015; Thomas and Duman, 2017).

We then tested whether *de novo* protein synthesis in the mPFC was necessary for the therapeutic behavioral effects of extinction on set shifting. Three days after the end of chronic stress treatment, animals received a local microinjection of the protein synthesis inhibitor, anisomycin into the mPFC 20 min prior to extinction. They were then tested on set-shifting 24 h later (Fucich et al., 2016). Blocking protein synthesis in the IL cortex during extinction had no effect on extinction learning itself. However, inhibiting protein synthesis in the IL during extinction completely blocked its subsequent therapeutic effects on set shifting (**Figure 4**). Importantly, anisomycin injection alone into the IL 24 h prior to testing did not affect set-shifting. Nor did blocking protein synthesis in the PrL cortex alter the therapeutic effects of extinction on set-shifting. Thus, these results support the hypothesis that activity-dependent protein synthesis specifically in the IL cortex is necessary for the therapeutic effects of extinction

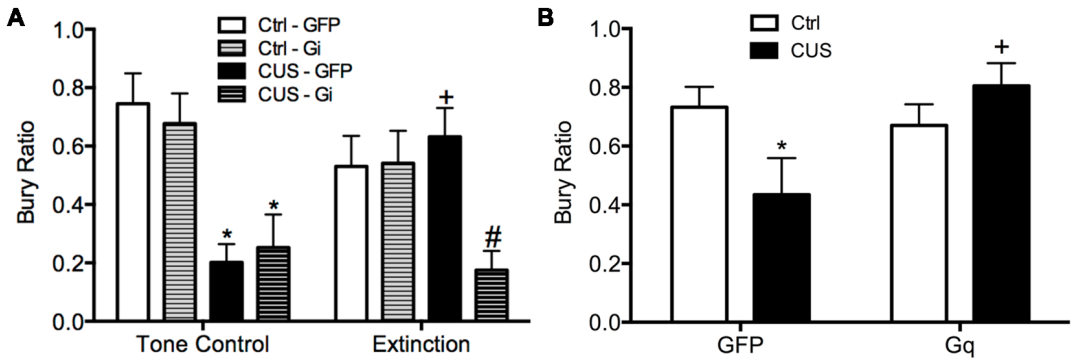

FIGURE 3 | Activity of glutamatergic pyramidal cells in the IL cortex is necessary and sufficient for the therapeutic effects of extinction on the shift from active to passive coping behavior induced by chronic stress on the SPDB test. **(A)** CUS induced a significant decrease in bury ratio ($^{*}p < 0.01$, for both GFP/CUS/tone controls and hM4Di/CUS/tone controls compared with GFP/unstressed/tone controls). Extinction treatment reversed the effect of stress, restoring the bury ratio to unstressed control levels ($^{+}p < 0.05$, GFP/CUS/extinction compared with GFP/CUS/tone controls). Inhibition of glutamatergic neurons in IL during extinction prevented the therapeutic rescue of CUS-compromised bury ratio ($^{\#}p < 0.02$, hM4Di/CUS/extinction compared with GFP/CUS/extinction); $n = 9$–14 per group. **(B)** CUS induced a significant decrease in the bury ratio ($^{*}p < 0.05$, GFP/CUS compared with GFP/unstressed controls). Activation of glutamatergic neurons in IL after transfection with the excitatory hM3Dq Designer Receptor Exclusively Activated by Designer Drug (DREADD) reversed the effect of stress, restoring the bury ratio to unstressed control levels 24 h after CNO administration ($^{+}p < 0.02$, GFP/CUS compared to hM3Dq/CUS); $n = 6$–10 per group. Data expressed as mean ± SEM. Reproduced and adapted with permission from Fucich et al. (2018).

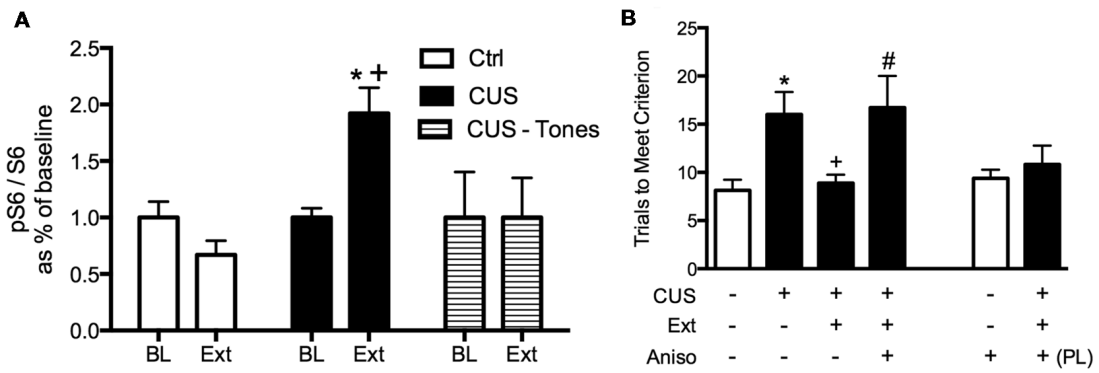

FIGURE 4 | Therapeutic effects of extinction after CUS require protein synthesis in the IL medial prefrontal cortex (mPFC). **(A)** Extinction induced a significant increase in phosphorylation of ribosomal protein S6, reflecting initiation of *de novo* protein synthesis in the mPFC of CUS-treated rats ($^{*}p < 0.05$, Extinction compared to Baseline), but not in unstressed controls, nor in CUS-tone control rats exposed to tone presentations but without prior fear conditioning ($^{+}p < 0.05$, CUS Extinction compared to unstressed Extinction and to CUS tone controls); $n = 4$–6 per group. **(B)** Inhibition of protein synthesis by microinjection of anisomycin (50 μg/0.5 μl) into the IL cortex prior to extinction prevented the rescue of cognitive set-shifting that had been compromised by CUS. Chronic stress induced a significant increase in trials to criterion (TTC) on the set-shifting task ($^{*}p < 0.05$, CUS-tone control-vehicle compared to unstressed-tone control-vehicle). Extinction treatment reversed the effect of stress, restoring TTC to unstressed control levels ($^{+}p < 0.05$, CUS-extinction-vehicle compared to CUS-tone control-vehicle). Microinjection of anisomycin into IL cortex before extinction treatment prevented the beneficial effect of extinction on set-shifting compromised by CUS, as TTC were comparable to CUS tone controls ($^{\#}p < 0.05$, CUS-extinction-anisomycin compared to CUS-extinction-vehicle); $n = 6$–8 per group. Administering anisomycin into the IL cortex of unstressed animals had no effect on set-shifting. Similarly, as a site-specificity control, administering anisomycin into the PL cortex of stressed animals prior to extinction did not prevent the therapeutic effect of extinction. Data expressed as mean ± SEM. Reproduced and adapted with permission from Fucich et al. (2016).

on cognitive set-shifting that has been compromised by chronic stress.

Our results show that protein synthesis in the IL is necessary for the therapeutic effects of extinction in stressed animals. We did not observe increased phosphorylation of ribosomal protein S6 after extinction in the mPFC of unstressed animals, consistent with previous reports suggesting that although protein synthesis is required for extinction, pS6 is not induced (Tedesco et al., 2014). However, we did observe phosphorylation of ribosomal protein S6 in the mPFC of stressed animals. Phosphorylation of S6 is not necessary for protein synthesis *per se*. However, induction of pS6 is associated with increased protein synthesis, and has been particularly associated with increased neural activity (Knight et al., 2012; Biever et al., 2015). Thus, the induction of pS6 in the mPFC of stressed animals but not in control

animals suggests that a specific set of proteins may be translated uniquely after extinction in stressed animals that are not translated in control animals. Identification of these proteins, and their potential role in the plasticity underlying therapeutic effects of extinction as perhaps distinct from the plasticity underlying fear extinction memory, will require further investigation.

Thus, our results suggest that extinction restores cognitive, behavioral, and functional properties of the mPFC that are compromised by CUS and that resemble pathological changes in PTSD. However, it is not yet clear whether extinction initiates processes that reverse the aberrant maladaptive changes caused by stress in the mPFC, or if extinction learning instead initiates adaptive processes that can compensate for, but are distinct from, the stress-induced pathology in the mPFC. For example, chronic stress results in dendritic atrophy and reduced excitability of pyramidal cells in the mPFC, as well as reduced population responsivity to afferent input from the medial dorsal thalamus (Liston et al., 2006; Yuen et al., 2012; Jett et al., 2017). Such morphological and electrophysiological alterations are associated with impaired cognitive performance on mPFC-dependent tasks. We performed electrophysiological recordings suggesting that extinction learning restored afferent-evoked responses in the mPFC that had been compromised by chronic stress (**Figure 5**; Fucich et al., 2018). However, chronic stress also has been reported to increase GABA-mediated inhibition of IL pyramidal neurons (McKlveen et al., 2016). Thus, it is possible that extinction can directly activate pyramidal cells without necessarily altering aberrant GABAergic inhibitory activity induced by chronic stress.

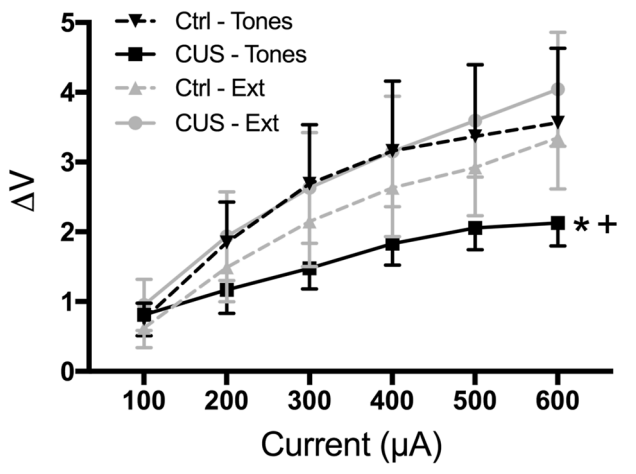

FIGURE 5 | Extinction therapy rescues chronic stress-induced attenuation of afferent-evoked electrical responses in IL medial prefrontal cortex. CUS compromised afferent-evoked field potentials recorded in the mPFC in response to stimulation of the medial dorsal thalamus (*$p < 0.01$, CUS/tone controls compared with unstressed/tone controls). Extinction treatment reversed the effect of stress, restoring evoked responses to unstressed control levels ($^{+}p < 0.001$, CUS/extinction compared with CUS/tone controls); n = 5–8 rats per group. Data expressed as mean ± SEM. Reproduced with permission from Fucich et al. (2018).

SUMMARY AND FUTURE DIRECTIONS

We and many others in both the basic and clinical literature have recognized that the fundamental process of exposure therapy is in fact a process of extinction (McNally, 2007; Hofmann, 2008; Craske et al., 2014). A question then is whether the mere extinction of a conditioned fear memory represents the entirety of the therapeutic effect of exposure therapy. We suggest that it does not. PTSD is more complex than just the memory of a stressful event, although that is an important and necessary component of a PTSD diagnosis. A related question might be whether cue-conditioned fear in and of itself represents a valid "model" of PTSD, which has been proposed (see Parsons and Ressler, 2013). We also suggest that it does not. First, it is unlikely that a few pairings of an innocuous tone with a 0.5 s, 0.7 mA foot shock is a traumatic stress. This was confirmed in our studies, in which fear conditioning alone had no effect on set-shifting (Fucich et al., 2016). Further, learning that the tone is to be feared in that context is not pathological. More practically, it would be circular logic to use cue-conditioned fear as a model of PTSD to test the extinction of cue-conditioned fear as a model of PTSD therapy. PTSD has other symptom domains, including a deficit of cognitive flexibility (Olff et al., 2014), which may contribute to the persistent and intrusive fear memory, but extends beyond fear memory alone. It is detected by neuropsychological tests that are not fear based, and from which the AST we use was back-translated (Birrell and Brown, 2000; Garner et al., 2006). PTSD also includes avoidance behavior and maladaptive coping, modeled by the shock probe test. Exposure therapy extinguishes the primary conditioned fear memory for the index event that initiated the pathology. But given the range of symptom domains in PTSD, we would argue that extinguishing the primary fear memory is not the sole therapeutic outcome of exposure therapy. Rather, we suggest that the process of learning involved in extinction induces plasticity in the mPFC that resolves or compensates for the pathology caused by the traumatic stress, which then accounts for the resolution of other symptom domains of PTSD. To test this in a preclinical model, and to avoid the circularity above, it is necessary to distinguish the target of the learning process *per se* (i.e., the cue-conditioned fear memory) from the dependent measures that characterize the stress-induced pathology and are used to assess therapeutic effect. A richer stress model than cue-conditioned fear alone is necessary to capture these other domains, hence our use of CUS to induce changes in cognition and coping behavior, measured by the set-shifting test and the shock probe test. To be clear, however, this is only necessary for a rigorous and valid preclinical test of the hypothesis that extinction learning, as a model of exposure therapy, induces plasticity in the mPFC that is therapeutic across symptom domains. It is not meant to imply that to treat PTSD it is necessary to induce a "second" fear memory that is then extinguished by exposure therapy to mitigate the pathology induced by the initial traumatic event. Extinction of the memory of the index event is the therapeutic learning process that accomplishes that. From a different perspective, this would also suggest that any process that induces similar

plasticity in the mPFC would be similarly beneficial across PTSD symptom domains. This may be one mechanism by which ketamine has been reported to be effective in PTSD (Girgenti et al., 2017). More interestingly, this may explain the efficacy of other forms of CBT, the goal of which is not necessarily to extinguish memory of the traumatic event, but to train patients to utilize more flexible thinking and to disengage from automatic, reflexive, habitual responding with a perseverative negative bias (Gallagher and Resick, 2012). Further, it is important to recognize that the extinction of conditioned fear responses can be malleable, and subject to spontaneous recovery of fear (Myers and Davis, 2007), which may reflect preclinical correlates of re-experiencing or relapse. Thus, future work is needed to investigate the relationship between the reappearance of conditioned fear responses and the duration and stability of the therapeutic effects of extinction on other measures after stress.

Another consideration for future studies is gender. PTSD affects both men and women, with women being twice as likely to develop the disorder after a trauma (Haskell et al., 2010). The studies described in this review have included only male rats to date. In future work, we will include both sexes in our studies and monitor the estrous cycle at the time of testing, since estrous cycle stage can influence extinction learning as well as responses to stress (Viau and Meaney, 1991; Milad et al., 2009).

In addition to the mPFC, both chronic stress and fear extinction involve other brain regions relevant to PTSD symptomatology, such as the hippocampus and amygdala (Shin et al., 2006; Milad et al., 2007; Garcia et al., 2008; Mahan and Ressler, 2012). To date, our studies have only addressed the necessity of activity-dependent plasticity in the mPFC for the therapeutic effects of extinction in stressed animals. However, we have not yet investigated the possibility that plasticity in the mPFC during extinction is driven by activity in other brain regions that are also engaged by extinction learning. Indeed, we have reported that inhibiting the activity of pyramidal cells in the PrL cortex during extinction also blocked the therapeutic effects of extinction on set shifting. By contrast, and unlike IL, inhibiting protein synthesis in the PrL was not sufficient to block the therapeutic effects of extinction on set shifting. These results suggest that activity-dependent plasticity induced by extinction in the vmPFC interacts with activity in other components of the fear learning circuit to reverse cognitive impairments caused by stress. In this manner, extinction-induced plasticity in the vmPFC may enhance the function of downstream target circuits, for example by facilitating the inhibitory influence of PrL on the amygdala, or by reversing maladaptive plasticity in the hippocampal-PFC pathway caused by stress (Cerqueira et al., 2007; Koenigs and Grafman, 2009). Future work will be needed to investigate the circuit-level plasticity that may be necessary for the therapeutic effects of extinction in specific components of these extended vmPFC networks.

The fact that extinction induced phosphorylation of ribosomal protein S6 only in stressed animals suggests that the molecular machinery underlying plasticity (e.g., S6 induction) may be specifically dysregulated in the stressed brain, and that extinction initiates unique molecular processes related to protein synthesis and plasticity in the stressed brain. Thus, the observation that extinction requires activity-dependent protein synthesis in the mPFC for its therapeutic effects in stressed animals prompts two important questions for future investigation. The first is to ask what proteins are synthesized in the vmPFC that lead specifically to plasticity mediating the therapeutic effects of extinction, and whether they are distinct from factors responsible for the consolidation and retention of extinction memory *per se*. The second is to identify the upstream molecular factors and signaling pathways that initiate the protein synthesis mechanisms responsible for the therapeutic benefits of extinction. Several molecular pathways have been shown to be necessary for extinction memory consolidation, such as MAPK/Erk, PI3K/Akt, and BDNF (Hugues et al., 2004; Kritman and Maroun, 2013; Rosas-Vidal et al., 2014). Because these pathways mediate long-lasting plastic changes associated with extinction memory, they may also be involved in the lasting therapeutic effects of extinction. Indeed, several of these same signaling pathways have been implicated in the mechanisms of action of both traditional and novel rapid-acting antidepressant drugs (Autry et al., 2011; Thomas and Duman, 2017). Identification of upstream factors and signaling pathways that initiate extinction-mediated protein synthesis, and downstream factors and pathways that mediate the resulting plasticity underlying its beneficial effects, may lead to the discovery of novel therapeutic targets and strategies to enhance the beneficial effects of extinction, and by translational extension, enhance the therapeutic efficacy of CBT for PTSD. More generally, identifying substrates and molecular mechanisms by which effective therapeutic interventions, whether behavioral or pharmacological, exert their beneficial effects will hopefully lead to the future development of more effective treatments, including rational evidence-based adjunct strategies combining complementary behavioral and pharmacotherapeutic approaches.

AUTHOR CONTRIBUTIONS

DP wrote and edited the manuscript. DM provided critical feedback and edited the manuscript.

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Sex Differences in Remote Contextual Fear Generalization in Mice

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The generalization of fear is adaptive in that it allows an animal to respond appropriately to novel threats that are not identical to previous experiences. In contrast, the overgeneralization of fear is maladaptive and is a hallmark of post-traumatic stress disorder (PTSD), a psychiatric illness that is characterized by chronic symptomatology and a higher incidence in women compared to men. Therefore, understanding the neural basis of fear generalization at remote time-points in female animals is of particular translational relevance. However, our understanding of the neurobiology of fear generalization is largely restricted to studies employing male mice and focusing on recent time-points (i.e., within 24–48 h following conditioning). To address these limitations, we examined how male and female mice generalize contextual fear at remote time intervals (i.e., 3 weeks after conditioning). In agreement with earlier studies of fear generalization at proximal time-points, we find that the test order of training and generalization contexts is a critical determinant of generalization and context discrimination, particularly for female mice. However, tactile elements that are present during fear conditioning are more salient for male mice. Our study highlights long-term sex differences in defensive behavior between male and female mice and may provide insight into sex differences in the processing and retrieval of remote fear memory observed in humans.

Keywords: fear generalization, remote generalization, contextual fear conditioning, fear memory, sex differences

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric illness that emerges following exposure to a life-threatening experience and is characterized by four symptom clusters: re-experiencing, avoidance, negative alterations in mood or cognition, and hyperarousal (APA, 2013). An important clinical manifestation of PTSD is the overgeneralization of fear or enhanced distress to environmental cues that resemble the life-threatening experience (APA, 2013). Patients who suffer from PTSD have greater difficulty in suppressing fear in a safe environment or in the presence of safety cues (Jovanovic et al., 2010). For example, in a laboratory setting, individuals with PTSD have greater difficulty relative to control subjects in discerning perceptually similar rings from those paired with a shock (i.e., fear-conditioned rings; Kaczkurkin et al., 2017). PTSD is therefore associated with broader generalization gradients (Grillon et al., 2009; Jovanovic et al., 2012; Homan et al., 2019; Starita et al., 2019).

The incidence of PTSD is significantly higher in women than in men (Kessler et al., 2005; Tolin and Foa, 2006). Given that PTSD involves alterations in fear learning and memory (Ross et al., 2017), understanding the environmental constraints that control fear generalization between sexes is an important area of research (Lissek et al., 2010; Dunsmoor and Paz, 2015; Lissek and van Meurs, 2015; Liberzon and Ressler, 2016; Lopresto et al., 2016; Jasnow et al., 2017). In this regard, rodent fear conditioning paradigms represent a powerful tool for examining how environmental parameters interact to influence fear generalization as a function of sex (Parsons and Ressler, 2013; Maeng and Milad, 2017; Asok et al., 2019a).

Like humans, when rodents are confronted with a potentially threatening stimulus or an environment, they must select an appropriate defensive response (Fanselow and Lester, 1988; Blanchard and Blanchard, 1989; Mobbs et al., 2015). Because current and past experiences are not identical, the selection of a response is based on the immediately available cues and contextual information that predict danger or safety. This process often entails the generalization of a defensive response (e.g., freezing in rodents) to an environment that was never explicitly learned to be dangerous (Dunsmoor and Paz, 2015; Dymond et al., 2015; Jasnow et al., 2017). In recent years, rodent behavioral studies have discovered a variety of molecular, cellular, and neural circuit mechanisms that influence fear generalization (for review, see Asok et al., 2019a). These studies have revealed how internal states may interact with environmental contingencies to modulate sex differences in fear generalization (Day et al., 2016; Keiser et al., 2017). For example, ovariectomized female rats given estradiol replacement exhibit enhanced fear generalization *via* activation of cytosolic estrogen receptors in the hippocampus (Lynch et al., 2013, 2016). However, the role of female hormonal fluctuations in fear generalization is not so clear in that other studies have shown that hormonal changes: (1) may have a greater influence on fear extinction (Milad et al., 2009); and (2) do not influence contextual fear generalization (Keiser et al., 2017).

Despite controversy on the role of hormones in fear acquisition, fear extinction, or fear generalization, these studies have been critical for probing the biological factors that influence aversive experiences in females. Yet, much of this work has focused on fear generalization at recent time points after conditioning (i.e., 24–48 h). Given that PTSD is associated with progressive and chronic symptomatology as well as a higher incidence in women (Kessler et al., 2005; Nemeroff et al., 2006; Tolin and Foa, 2006), it is therefore important to examine the environmental factors which modulate generalization between sexes over longer time intervals, as this may identify key environmental variables that modulate sex-dependent fear generalization in PTSD.

Environmental, or contextual, elements exert a powerful influence over fear generalization. This is especially true for rodent studies that examine fear generalization using a contextual fear conditioning (CFC) paradigm, where the elements of an environment (e.g., sounds, lighting, textures, space, etc.) are bound into a unitary contextual representation (O'Reilly and Rudy, 2001; Rudy et al., 2004; Rudy, 2009; Maren et al., 2013). In CFC, a neutral stimulus (i.e., a unique context) is paired with an unconditioned stimulus (US) such as a foot shock—which has been suggested to serve as a proxy for trauma (Liberzon and Ressler, 2016). The neutral stimulus is associatively transformed into a conditioned stimulus (CS) that can subsequently elicit freezing on future presentations (Maren, 2001). Following CFC, generalization occurs when a context that is perceptually related, but not identical, to the conditioning context elicits a similar conditioned response (Asok et al., 2019a). Moreover, the saliency of particular stimulus elements during conditioning can have a profound influence over whether fear becomes generalized. For example, tactile feedback from the electrified grid floors as well as odors present in the conditioning chamber are particularly salient features for mice and are capable of modulating fear memory, generalization, and context discrimination (Huckleberry et al., 2016).

In addition, fear generalization is subject to a number of temporal constraints. The order of context exposure prior to, or following conditioning, as well as the similarity between the conditioning and testing contexts, can produce differential effects on fear generalization (Tronel et al., 2005; Huckleberry et al., 2016; Keiser et al., 2017). However, fear generalization can naturally emerge with the passage of time in both rodents (Wiltgen and Silva, 2007) and humans (Leer et al., 2018), and may accompany the normal systems consolidation of a fear memory (Biedenkapp and Rudy, 2007; Wiltgen et al., 2010; Dudai et al., 2015; Poulos et al., 2016).

In light of the sex-dependent, contextual, and temporal factors that influence fear generalization, we examined how pre- and post-conditioning exposure to different contexts and elements of a context influence fear generalization and context discrimination at remote time intervals in both male and female mice. Our study highlights several key environmental parameters that may contribute to the stress-related, sex-dependent emergence of fear generalization.

MATERIALS AND METHODS

Animals

Wild-type male and female mice (C57BL/6J background) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) at 9–10 weeks of age. Animals were housed in the vivarium at the Zuckerman Institute at Columbia University, and maintained on a standard 12 h : 12 h light-dark cycle with *ad libitum* access to food and water. This study was carried out in accordance with the recommendations of the Animal Research Handbook made available by the Office of the Executive Vice President for Research at Columbia University. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Columbia University.

Behavioral Experiments

Estrous Cycle

Naturally cycling females were used in all experiments, given that the C57BL/6J background is relatively insulated from the effects of the estrous cycle with respect to fear conditioning (Meziane et al., 2007; Keiser et al., 2017). Indeed, the effects of estrous phase

on fear memory in rodents are more relevant to fear extinction (Milad et al., 2009; Blume et al., 2017). Nevertheless, in our study, we performed limited visual monitoring of estrous cycle phase, as described in Byers et al. (2012). Among five female cohorts evaluated in remote generalization experiments and for which estrous phase was assessed (n = 75 mice in total), there were no significant differences in phase distribution between experimental groups on the day of fear conditioning ($X^2_{(8)} = 8.73$, $p = 0.3656$). The percentage of mice in proestrus—when estradiol and progesterone levels are highest—on the day of fear conditioning ranged from 0 to 20% (0–3 mice per group, for a total of six animals in proestrus). Combining these five groups (n = 75 mice in total) and measuring post-shock freezing levels as a function of estrous phase, we detected no significant differences between groups by one-way analysis of variance (ANOVA; $F_{(2,72)} = 0.2827$, $p = 0.7546$). Although potential effects of proestrus on retrieval testing cannot be entirely ruled out due to the relatively small representation of proestrus mice during training, exclusion of these animals from statistical analyses of behavioral data has no impact on significance or interpretation (data not shown), and so these data points were retained. Our results are consistent with those of earlier reports (Meziane et al., 2007; Keiser et al., 2017).

Contextual Fear Conditioning and Generalization

All behavioral experiments were conducted on mice between 12–14 weeks of age. Fear conditioning experiments were conducted using a cubic chamber with the following dimensions: 30 cm (L) × 24 cm (W) × 21 cm (H). The fear conditioning chamber was housed in a sound-attenuating enclosure equipped with an infrared camera for automated measurement of freezing, which was quantitated using Video Freeze software (Med Associates, Inc., St Albans City, VT, USA). For standard CFC, mice were exposed to a 3 min session, with a 2 s shock (0.7 mA) presented at 2 min and again at 2.5 min. In the brief training protocol, two shocks of the same intensity and duration as in the standard protocol were administered over the course of an 8 s session, whereupon animals were immediately returned to their home cages. Control animals were exposed to the fear conditioning chamber for 3 min in the absence of shock. Three contexts were used as indicated: Context A (70% ethanol odorant, white light, metal floor grid, no roof insert); Context B (4% peppermint extract, no light, smooth flooring, triangular roof insert); and Context C (same as Context B, but with metal floor grid used in Context A). Animals were only exposed to foot shocks during initial fear conditioning in Context A. To evaluate retrieval, animals were exposed to the indicated test contexts for a duration of 3 min. For context pre-exposure experiments, test mice were placed in Context A for 10 min in the absence of foot shocks on one or two consecutive days as indicated, and then subjected to standard fear conditioning in Context A the following day. Control mice in the pre-exposure experiments were not pre-exposed to Context A, but were subjected to the same course of fear conditioning and retrieval as the other test groups. At the conclusion of the fear conditioning or retrieval sessions, mice were immediately returned to their home cages. Contextual memory retrieval or generalization were evaluated at 24 h, 48 h, or 21 days and 22 days later, as specified, in the absence of shocks. For quantitation of all behavioral data from fear conditioning and generalization experiments, % time freezing was used.

Data Analysis

All data were analyzed using the total percentage of time spent freezing and by computing a discrimination index [% time freezing in Context A/(% time freezing in Context A + Context B)]; (Wiltgen and Silva, 2007). All values are reported as the mean ± standard error of the mean (SEM). All behavioral data were analyzed by one-, two-, three-way ANOVA, or t-test as specified, using GraphPad Prism 7 software (GraphPad Software, Inc.). Cohort and sample sizes are specified in the text and figures. *Post hoc* comparisons performed after significant ANOVA results are specified when used. Statistical significance was set at $p < 0.05$ "*", $p < 0.01$ "**", $p < 0.001$ "***", and $p < 0.0001$ "#".

RESULTS

Experiment 1: Remote Contextual Fear Generalization in a Distinct Context Is Modulated by Sex and Test Order

Previous studies in mice have determined that contextual fear generalization at proximal time intervals (24–48 h after fear conditioning) is sensitive to both sex and the test order of the training and generalization contexts (Huckleberry et al., 2016; Keiser et al., 2017). Thus, we first examined the influence of sex and test order on the generalization of contextual fear at remote time-points (3 weeks after fear conditioning). In our initial experiments, we designed the training and generalization contexts to be as perceptually distinct as possible from one another to establish baseline levels of generalization (**Figure 1**). Male and female mice were conditioned in the training context (Context A) and then tested 21 days later to measure freezing in either Context A or the generalization context (Context B; **Figure 2A**). Both test orders (A→B and B→A) were evaluated in separate cohorts of mice, with a 24 h period between retrieval tests (**Figure 2A**).

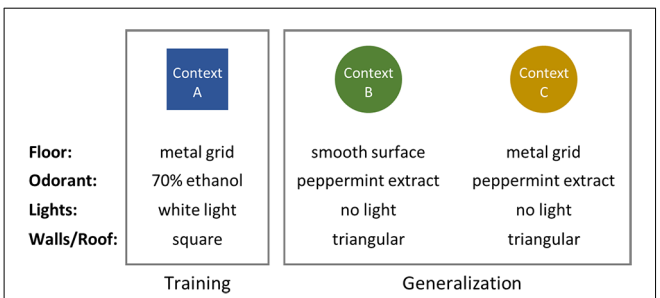
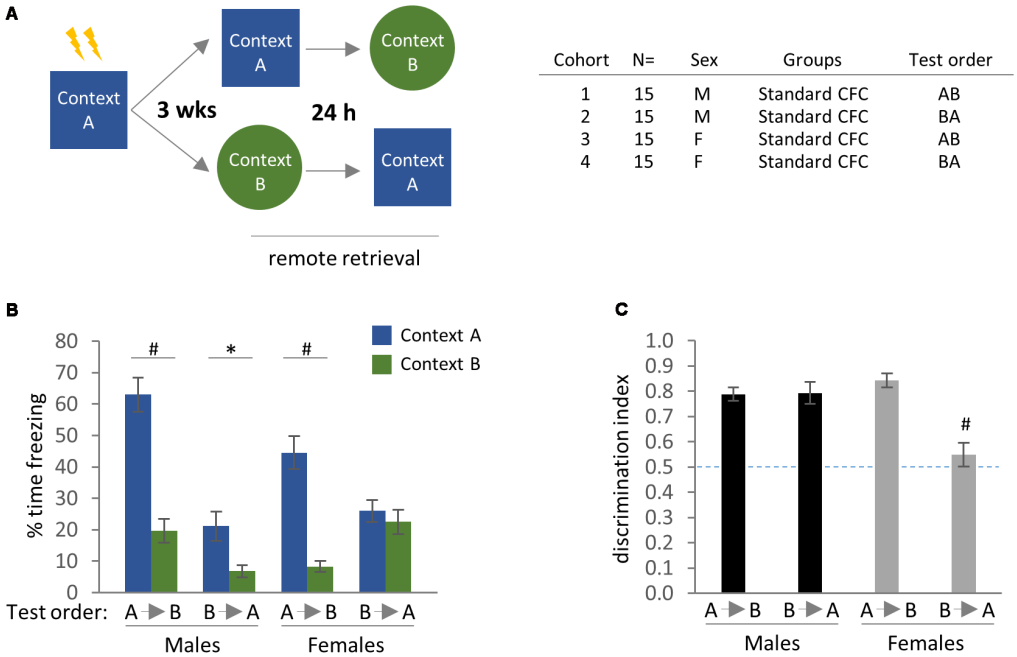


FIGURE 1 | Schematic of contexts used in fear conditioning and generalization experiments. Context B was designed to be as perceptually distinct as possible from the training context (Context A). Context C retains the metal floor grid used in Context A, but is otherwise identical to Context B.



Cohort	N=	Sex	Groups	Test order
1	15	M	Standard CFC	AB
2	15	M	Standard CFC	BA
3	15	F	Standard CFC	AB
4	15	F	Standard CFC	BA

FIGURE 2 | Remote contextual fear memory and generalization with perceptually distinct training and generalization contexts (Contexts A and B, respectively). **(A)** Experimental design and cohort information. **(B)** Effect of test order on freezing behavior in the training context (Context A) vs. a distinct novel context (Context B) at 3 weeks after standard contextual fear conditioning (CFC). Bonferroni *post hoc* comparisons following three-way analysis of variance (ANOVA) are indicated. **(C)** Discrimination index, calculated as % Freezing in Context A/(% Freezing in Context A + % Freezing in Context B). $^{\#}p < 0.0001$ for effect of test order in females, Bonferroni *post hoc* test following two-way ANOVA. $^{*}p < 0.05$, $^{\#}p < 0.0001$. Error bars are mean ± standard error of the mean (SEM).

We detected main effects of Test Context and Test Order on freezing behavior by three-way ANOVA, as well as a Test Context × Test Order interaction and Sex × Test Order interaction (**Figure 2B**; Test Context: $F_{(1,112)} = 74.97$, $p < 0.0001$; Test Order: $F_{(1,112)} = 27.61$, $p < 0.0001$; Test Context × Test Order: $F_{(1,112)} = 30.16$, $p < 0.0001$; Sex × Test Order: $F_{(1,112)} = 20.10$, $p < 0.0001$). Bonferroni *post hoc* comparisons indicated that male mice exhibited comparatively little freezing in the generalization context (Context B) vs. the training context (Context A) regardless of test order (**Figure 2B**; Males A→B: $p < 0.0001$ for freezing in Context A vs. Context B; Males B→A: $p = 0.0485$ for freezing in Context A vs. Context B), in agreement with earlier work examining contextual discrimination at 24–48 h (Keiser et al., 2017; but see Huckleberry et al., 2016). Female mice that were tested in Context A prior to Context B also exhibited relatively higher freezing in the training context, while those tested in the reverse test order (B→A) showed similar levels of freezing to both contexts (**Figure 2B**; Females A→B: $p < 0.0001$ for freezing in Context A vs. Context B). A more pronounced effect of test order in females than males was also observed for proximal time-points by Keiser et al. (2017).

To probe these effects further, we calculated a discrimination index based on the ratio of time spent freezing in each of the test contexts. This analysis confirmed our initial findings showing high levels of context discrimination [calculated as % time freezing in Context A/(% time freezing in Context A + Context B)] in the first three experimental groups (i.e., males A→B and B→A, and females A→B), while the female (B→A) cohort showed minimal departure from chance-level freezing. Analysis of the discrimination data by two-way ANOVA identified main effects of both Sex and Test Order, as well as a sex × test order interaction (**Figure 2C**; Sex: $F_{(1,56)} = 6.45$, $p = 0.0139$; Test Order: $F_{(1,56)} = 15.35$, $p = 0.0002$; Sex × Test Order: $F_{(1,56)} = 16.06$, $p = 0.0002$). Bonferroni *post hoc* comparisons revealed significant differences between the female (A→B) vs. (B→A) groups ($p < 0.0001$). Thus, in experimental paradigms employing completely distinct training and generalization contexts, only female mice tested in the (B→A) order were incapable of discriminating between contexts.

Finally, because there appeared to be additional meaningful comparisons in **Figure 2B** that were not detected by the original three-way ANOVA—in particular, freezing to Context B across experimental groups, and therefore generalization—we re-analyzed the effects of test order in males and females separately by two-way ANOVA to increase statistical power. For female mice, we again detected a main effect of Test Context as well as a Test Context × Test Order interaction by two-way ANOVA (**Figure 2B**; Test Context: $F_{(1,56)} = 27.68$, $p < 0.0001$; Test Context × Test Order: $F_{(1,56)} = 18.85$, $p < 0.0001$). Furthermore, Bonferroni *post hoc* comparisons revealed significant effects of test order on freezing in both Context A ($p < 0.01$) and Context B ($p < 0.05$). Analysis of male mice by two-way ANOVA identified main effects of Test Context and Test Order, as well as a Test Context × Test Order

interaction (**Figure 2B**; Test Context: $F_{(1,56)} = 47.83$, $p < 0.0001$; Test Order: $F_{(1,56)} = 43.09$, $p < 0.0001$; Test Context × Test Order: $F_{(1,56)} = 12.10$, $p = 0.0010$). While the effect of Test Order on freezing in Context A was determined to be significant by Bonferroni's *post hoc* test ($p < 0.0001$), freezing in Context B did not reach statistical significance ($p = 0.0666$). Thus, generalized freezing in Context B was more sensitive to test order for the female (B→A) group. In other words, within the parameters of this behavioral paradigm, female mice are predisposed to heightened freezing in a novel context that is presented before re-exposure to the training context, while males do not exhibit such a bias.

Experiment 2: Remote Fear Generalization in Female Mice Requires Associative Contextual Fear Memory

CFC and fear generalization are associative processes, whereby an animal requires a minimum time of exposure to a context in order to form a unitary representation from stimulus elements and subsequently associate that unitary representation with a foot shock (Fanselow, 1986, 1990; Rudy et al., 2004; Rudy, 2009; Sauerhofer et al., 2012; Maren et al., 2013). Given the female-specific influence of test order on discrimination between distinct contexts in Experiment 1, we compared the impact of brief training vs. standard CFC on remote memory and generalization to determine if the effects of test order are dependent on the formation of an associative contextual fear memory.

Mice were trained in either the standard CFC protocol or exposed to brief training (**Figure 3A**), which normally does not produce associative memory (Fanselow, 1990). A third group of mice was exposed to Context A for 3 min in the absence of foot shocks. Analysis of the effect of conditioning protocol on freezing to Context A or Context B by two-way ANOVA indicated a main effect of Training Protocol (**Figure 3B**; Training Protocol: $F_{(2,84)} = 43.25$, $p < 0.0001$). In particular, with the brief training protocol, we observed minimal levels of freezing to either context. Moreover, the levels of freezing produced by the brief training protocol were not statistically different from those observed in control mice that were exposed to the fear conditioning chambers in the absence of shock. However, Tukey's *post hoc* test revealed that freezing behavior produced by standard training was significantly greater than that produced by the brief training protocol (**Figure 3B**; $p < 0.0001$ for brief vs. standard training for either Context A or Context B). We conclude that, like contextual fear memory, the generalization of contextual fear at remote time points is an associative process for female mice in our paradigm.

Experiment 3: Female (B→A) Mice Exhibit Context Discrimination at Proximal Intervals

In Experiment 1, we observed that female mice tested in Context B prior to Context A exhibited similar levels of freezing in both contexts, indicating poor context discrimination

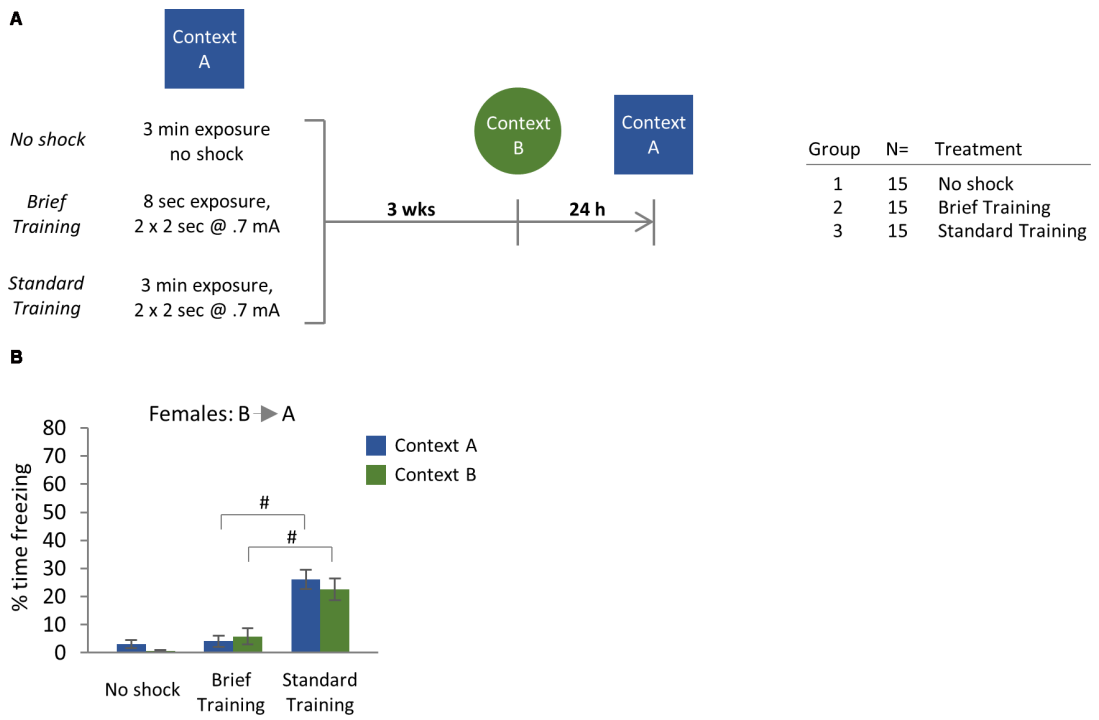


FIGURE 3 | (A) Experimental design and cohort information. **(B)** Effect of fear conditioning protocol (cage-exposed mice with no shock vs. brief training protocol vs. standard CFC) in female mice (B→A test order) at 3 weeks after initial training. Significant Tukey *post hoc* comparisons following two-way ANOVA are indicated. $^{\#}p < 0.0001$. Error bars are mean ± SEM.

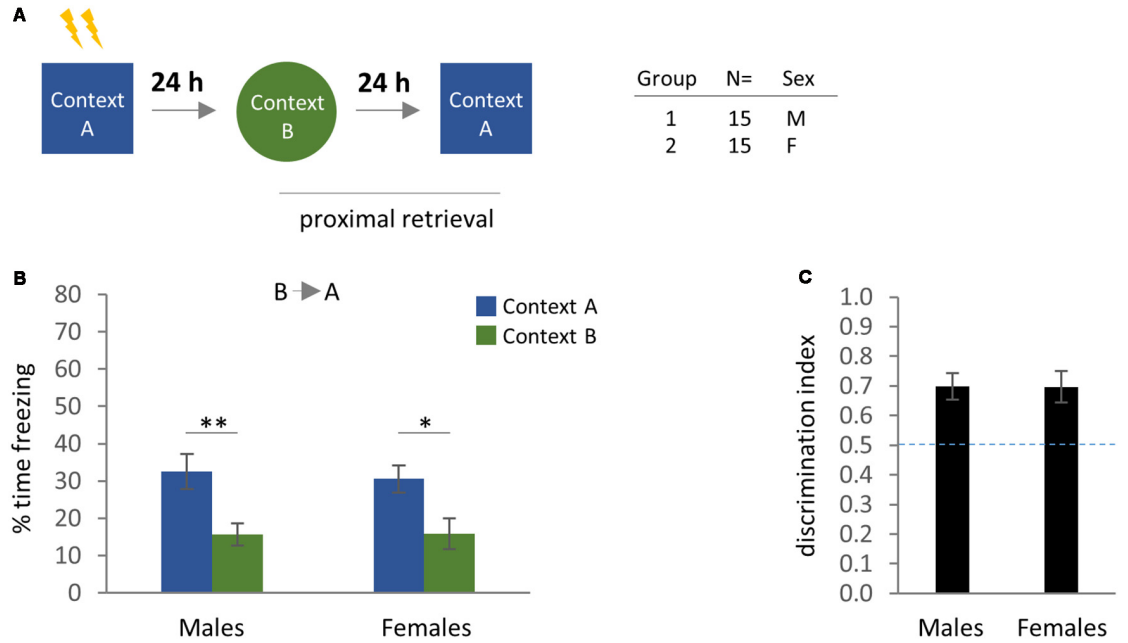


FIGURE 4 | Contextual fear memory and generalization at proximal time-points (BA test order). **(A)** Experimental design and cohort information. **(B)** Comparison of freezing behavior in male and female mice at 24–48 h after conditioning, using distinct training and generalization contexts. Bonferroni *post hoc* comparisons following two-way ANOVA are indicated. **(C)** Discrimination index for males and females at 24–48 h after conditioning. $^{*}p < 0.05$, $^{**}p < 0.01$. Error bars are mean ± SEM.

(**Figures 2B,C**). To determine whether the emergence of this phenotype was time-dependent, we performed the same experiment in female and male mice at proximal time intervals (**Figure 4A**). Here, a two-way ANOVA showed a main effect of Test Context (**Figure 4B**; Test Context: $F_{(1,56)} = 16.32$, $p = 0.0002$), but no significant effect of Sex. Bonferroni's *post hoc* test determined that freezing in Context A vs. Context B was significant for both males ($p = 0.0068$) and females ($p = 0.0208$). Additionally, an unpaired *t*-test did not identify a significant difference in the discrimination index between males and females (**Figure 4C**). Therefore, although test order was an important factor in females at remote time intervals, female mice were perfectly capable of discriminating between training and generalization contexts at proximal time-points, consistent with the idea that the generalization of fear increases over time (Wiltgen and Silva, 2007).

Experiment 4: Pre-exposure to the Training Context Enhances Context Discrimination in Females

Given that remote fear generalization in females is dependent on the formation of an associative memory, we hypothesized that pre-exposure to the training context (Context A) may enhance context discrimination by improving contextual learning and strengthening the representation of Context A. In theory, pre-exposure should ameliorate the effects of test order and reduce the generalized freezing in Context B that we observed in female mice if, in fact, generalization resulted from forming a weaker representation of Context A (Fanselow, 1990; Urcelay and Miller, 2014). To examine these possibilities, female mice were pre-exposed to Context A for either a single 10 min session or two 10 min sessions on consecutive days prior to conditioning (**Figure 5A**). Control mice were not pre-exposed to Context A, but were fear conditioned as usual in Context A, followed by the same type and order of retrieval tests as the other experimental groups. Like previous experiments, freezing in Context A and Context B was evaluated 3 weeks later.

In comparing the effects of pre-exposure to the female (B→A) data from Experiment 1, we detected a significant main effect of Test Context by two-way ANOVA, as well as a trend for a main effect of Pre-exposure Sessions (**Figure 5B**; Test Context: $F_{(1,84)} = 34.250$, $p < 0.0001$; Pre-exposure Sessions: $F_{(2,84)} = 2.837$, $p = 0.0642$). In addition, we observed a significant interaction between Test Context and Pre-exposure Sessions (**Figure 5B**; Test Context × Pre-exposure Sessions: $F_{(2,84)} = 6.106$, $p = 0.0033$). Bonferroni *post hoc* correction demonstrated that pre-exposure caused a significant increase in freezing to Context A vs. no pre-exposure, although there was no difference between one or two pre-exposure sessions (**Figure 5B**; Context A: 1 pre-exposure vs. no pre-exposure, $p = 0.0017$; two pre-exposure sessions vs. no pre-exposure, $p = 0.0076$). We observed a modest, dose-dependent reduction in freezing to Context B as a function of pre-exposure sessions, but the effect did not survive *post hoc* testing.

In comparing discrimination indices as a function of Pre-exposure using a one-way ANOVA, we found a significant main effect of Pre-exposure sessions (**Figure 5C**; Pre-exposure Sessions: $F_{(2,42)} = 11.370$, $p = 0.0001$). Tukey's multiple

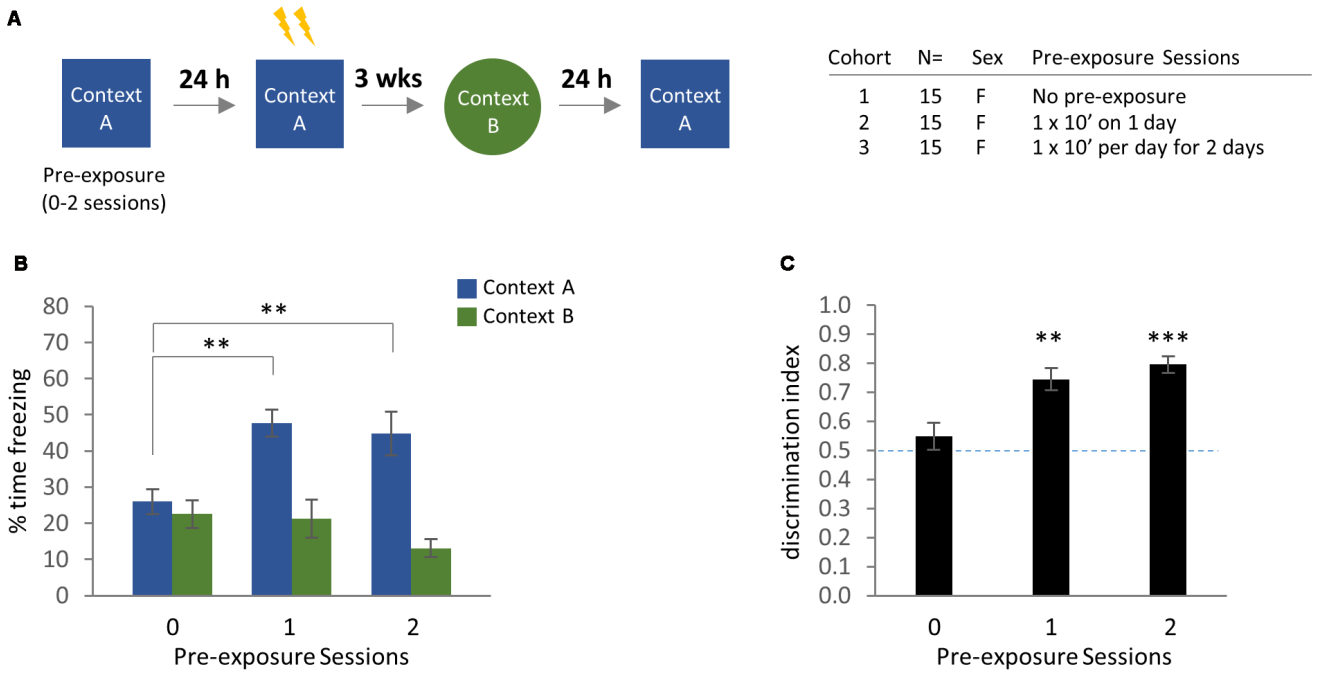


FIGURE 5 | Effect of context pre-exposure (0–2 sessions) on remote contextual fear generalization using distinct training and generalization contexts. **(A)** Experimental design. *N* = 30 mice per test order (AB or BA), with 15 mice for each pre-exposure condition. **(B)** Effect of test order on freezing behavior in the training context vs. novel context. Significant Bonferroni *post hoc* comparisons following two-way ANOVA are indicated. **(C)** Discrimination index calculations from previous data. Significant Bonferroni *post hoc* comparisons following one-way ANOVA are shown in graph. Dotted line indicates no-discrimination threshold. $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars are mean ± SEM.

comparisons test revealed a significant increase in discrimination index with Pre-exposure vs. without Pre-exposure (**Figure 5C**; No Pre-exposure vs. 1 Pre-exposure Session, $p = 0.0025$; no Pre-exposure vs. 2 Pre-exposure Sessions: $p = 0.0001$), but there was no difference between 1 or 2 Pre-exposure Sessions. Therefore, the ability of female mice to discriminate between contexts presented in the (B→A) test order was greatly enhanced by pre-exposure, and this effect was predominantly driven by improved contextual fear memory for the training context, rather than by a reduction in generalized freezing to Context B. These results suggest that pre-exposure enables female mice to form a more detailed contextual representation of the training context, which in turn supports greater memory precision.

Experiment 5: Tactile Contextual Elements Promote Generalization of Remote Contextual Fear and Reduce Discrimination in Males

As indicated earlier, the contexts used in the previous experiments were designed to be as distinct as possible to establish baseline levels of contextual fear generalization. We next asked if manipulating particular features between the training and generalization contexts could influence generalization aside from test order. A particularly salient feature in CFC is tactile information provided by the grid floor through which foot shocks are delivered (Huckleberry et al., 2016). Therefore, we examined whether inclusion of this contextual element in a novel test context C that was otherwise completely different from the training context would have an impact on a remote generalization (**Figure 6A**).

In this experiment, we detected main effects of Test Context, Test Order, and Sex by three-way ANOVA, as well as several two-way interactions between these factors (**Figure 6B**; Test Context: $F_{(1,96)} = 22.40$, $p < 0.0001$; Test Order: $F_{(1,96)} = 22.96$, $p < 0.0001$; Sex: $F_{(1,96)} = 11.13$, $p = 0.0012$; Test Context × Test Order: $F_{(1,96)} = 20.25$, $p < 0.0001$; Test Order × Sex: $F_{(1,96)} = 8.24$, $p = 0.0050$). A three-way interaction fell short of statistical significance ($p = 0.1153$), while Bonferroni *post hoc* comparisons identified significant effects of Test Context for males and females in the (A→C) groups (**Figure 6B**; Males: $p = 0.0006$; Females: $p < 0.0001$). Analysis of discrimination indices by two-way ANOVA revealed a main effect of Test Order as well as an interaction between Test Order and Sex (**Figure 6C**; Test Order: $F_{(1,48)} = 20.08$, $p < 0.0001$; Test Order × Sex: $F_{(1,96)} = 9.28$, $p = 0.0038$). In addition, Bonferroni *post hoc* comparisons identified a significant effect of Test Order for females only ($p < 0.0001$).

As in Experiment 1, we also examined the effects of test order on freezing to Context A and Context C within male and female groups using a two-way ANOVA to increase statistical power. Analysis of female mice revealed a main effect of Test Context as well as a Test Order × Test Context interaction (**Figure 6B**; Test Context: $F_{(1,48)} = 9.624$, $p = 0.0032$; Test

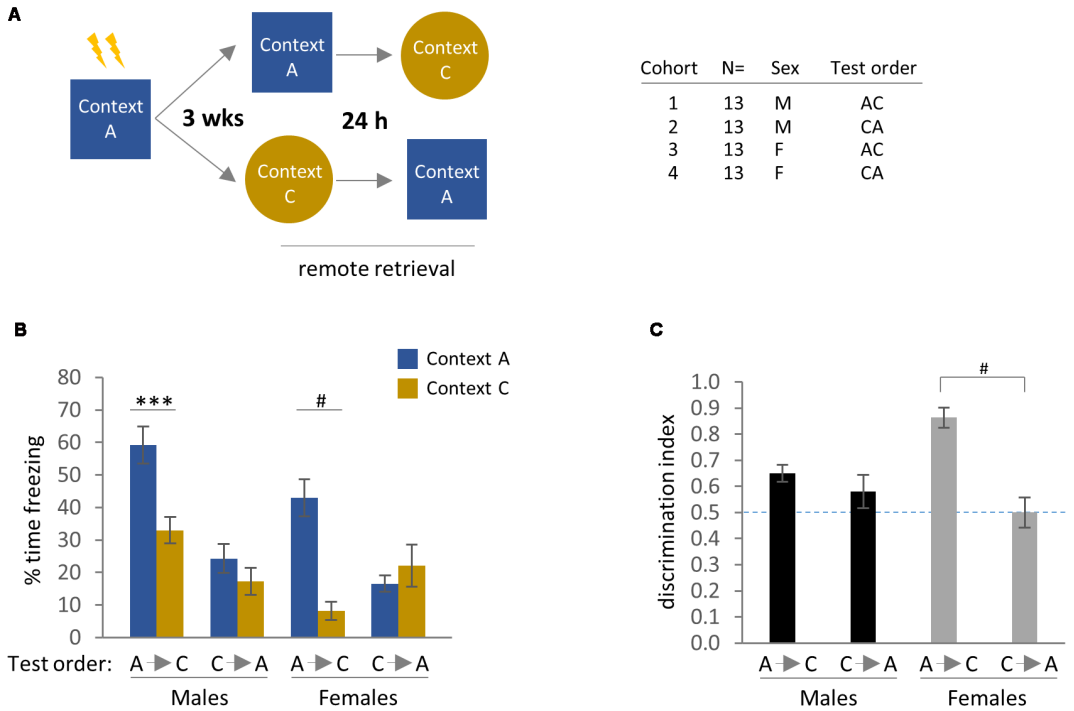


FIGURE 6 | Remote contextual fear generalization using context that retains metal grid floor used in the training context. **(A)** Experimental design. Context A and Context C are completely different in terms of odor, chamber shape, and lighting. However, the same metal grid floor through which shocks were delivered during training is present in both contexts. **(B)** Freezing behavior of male and female mice in remote contextual generalization, with both test orders (A→C and C→A). Significant Bonferroni *post hoc* effects following three-way ANOVA are indicated. **(C)** Discrimination index calculated from freezing data, with Bonferroni *post hoc* test following two-way ANOVA revealed a significant effect of test order in females. $^{***}p < 0.001$ and $^{\#}p < 0.0001$. Error bars are mean ± SEM.

Order × Test Context: $F_{(1,48)} = 18.13$, $p < 0.0001$), while Bonferroni's *post hoc* comparison indicated a significant effect of test order on freezing to Context A ($p = 0.0005$) and a trend for freezing to Context C ($p = 0.0895$). For males, two-way ANOVA detected main effects of Test Context and Test Order, as well as an interaction between these parameters (**Figure 6B**; Test Context: $F_{(1,48)} = 12.94$, $p = 0.0008$; Test Order: $F_{(1,48)} = 30.02$, $p < 0.0001$; Test Order × Test Context: $F_{(1,48)} = 4.33$, $p = 0.0427$). Bonferroni's *post hoc* test recovered significant effects of test order on freezing in both Context A ($p < 0.0001$) and Context C ($p = 0.0404$).

In summary, both male and female mice were able to discriminate between Context A and Context C when presented in the (A→C) test order, although the overall discrimination index for males was markedly lower than in the previous experiments utilizing completely distinct contexts (compare **Figures 2C**, **6C**). However, for both males and females, context discrimination was abolished by testing in the reverse order. This observation is consistent with earlier work demonstrating the importance of tactile elements in driving context discrimination at proximal time intervals in male mice (Huckleberry et al., 2016). On the other hand, female mice in the (A→C) group showed robust context discrimination, suggesting that tactile features are much less salient for females than for males at remote time intervals. Furthermore, test order became a significant variable for males only when the generalization context retained salient features of the training context (**Figure 6**), but not when contexts were sufficiently distinct (**Figure 2**).

Experiment 6: Tactile Contextual Elements Promote Generalization of Proximal Contextual Fear and Reduce Discrimination in Males and Females in the (C→A) Test Order

Given the generalized fear and absence of contextual discrimination observed in both males and females in the (C→A) group in **Figure 6**, we next evaluated whether such behavioral patterns are likewise present in the 24–48 h (**Figure 7A**) following initial CFC, or whether they develop over time. We observed that both males and females exhibited similar levels of freezing in Context A and Context C (**Figure 7B**), with no evidence of contextual discrimination (**Figure 7C**). Therefore, tactile information provided by the metal grid floor in an otherwise distinct context (Context C) is sufficient to promote levels of freezing similar to what we observed for the training context (Context A), at least for the (C→A) test order.

DISCUSSION

Contextual fear generalization and context discrimination at remote time-points are strongly influenced by several factors,

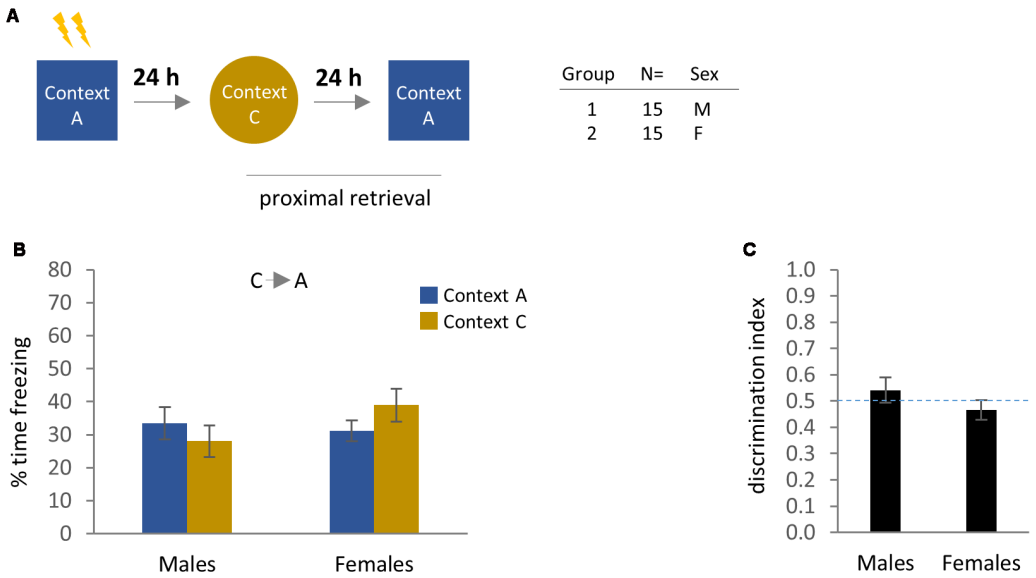


FIGURE 7 | Contextual fear memory and generalization at proximal time-points (C→A test order), in which the metal grid floor of the training context is retained in the otherwise contextually distinct generalization context. **(A)** Experimental design and cohort information. **(B)** Comparison of freezing behavior in male and female mice at 24–48 h after conditioning. **(C)** Discrimination index. Error bars are mean ± SEM.

including the saliency of specific contextual features, test order, and sex differences. Furthermore, these experimental variables interact to modulate behavior. The key findings of our studies over remote time intervals are as follows: (1) female mice are predisposed to exhibiting generalized fear in the first context that they encounter at remote time points after CFC, as well as poor context discrimination, even if the training and testing contexts are perceptually distinct; (2) the latter effects require the formation of an associative memory, and emerge over time; (3) for female mice, pre-exposure improves discrimination primarily by enhancing memory for the training context, rather than by reducing generalization; (4) both male and female mice exhibit greater freezing in the training context when presented before vs. after the generalization context, which may involve reconsolidation and interference rather than inter-trial extinction; and (5) tactile cues are more salient for male mice than for females.

Test Order Influences Remote Fear Generalization in Females

In our experiments, female mice exhibited generalized fear at remote time-points when first tested in a non-reinforced generalization context (Context B or C). This finding builds on previous observations showing an effect of test order at proximal time-points (Huckleberry et al., 2016; Keiser et al., 2017). However, in contrast to the latter studies, our observations were made using a generalization context (Context B) that was designed to be as perceptually distinct as possible from the training context (Context A). In fact, female mice showed robust differences in freezing in the training and generalization contexts as a function of test order, irrespective of whether the generalization context was distinct from, or shared at least one important contextual feature with, the training context. In addition, these effects were particularly apparent when evaluated in terms of discrimination indices, which permitted control over inter-individual variability in freezing levels. For male mice, despite the fact that overall differences in freezing levels varied as a function of a test order, remote discrimination between Context A and Context B was unaffected by test order and remained high. We conclude that male mice are, overall, less sensitive than females to the effects of a test order.

What can explain these observations? It is unlikely that the effect of test order is produced by sex differences in US processing because manual scoring of shock responsivity (e.g., running and jumping behavior) revealed no significant differences between males and females (data not shown). In addition, differences in inter-trial extinction are unlikely to be a contributing factor, because a single test session lasting only 3 min is not sufficient to support extinction (Lattal and Maughan, 2012), while the large difference in freezing levels in the training vs. generalization context in the (A→B or C) test order would entail a far greater rate of contextual fear extinction than one would typically observe in mice. Furthermore, the potential role of extinction is rendered all the more improbable by the substantial perceptual differences of the training and generalization contexts.

It is also unlikely that cues present during transport or from the experimenter strongly influenced our findings because female mice exhibited low levels of freezing in the generalization context in the (A→B or C) test order, even though both the experimenter and transport cues remained static. Moreover, in the brief training protocol, which emphasizes the salience of extra-contextual cues and features relative to the conditioning context, female mice did not show significant freezing to the

generalization context. While we cannot fully rule out the possibility that transport cues or the experimenter did not in some way act as "occasion setters" for heightened conditioned freezing exhibited by female mice upon placement in the first testing context (Holland, 1992), we would not expect to observe such low levels of freezing in the generalization context if extra-contextual information were important.

Although animals were not trained to asymptotic levels of freezing, another potential explanation for the bi-directional shift in freezing in the B→A or C test order is that the reinforced training context shared associative strength with the non-reinforced generalization context. However, this interpretation is also unlikely given that the environments (i.e., Context B vis-à-vis Context A) were as different as possible along tactile, olfactory, visual, and spatial dimensions. While earlier work demonstrates a time-dependent increase in generalization irrespective of test order and without a reduction in freezing to the training context (Wiltgen and Silva, 2007), it is important to recognize that differences in procedural variables such as fear conditioning parameters (e.g., shock number, intensity, and delivery schedule) and test design (e.g., similarity between training and generalization contexts, and timing of retrieval tests), as well as mouse genetic background, may preclude rigorous comparison of studies.

In our study, we speculate that the effect of test order in females may result from inadequate CS learning (see Spence, 1936) coupled with a mismatch between the expected and actual outcome, as captured by Pearce-Hall, Rescorla-Wagner, and Temporal-Difference theoretical models (Rescorla and Wagner, 1972; Pearce and Hall, 1980; Sutton, 1988). This hypothesis is supported by the fact that (1) our effects depended on associative learning; and (2) increasing pre-exposure to the training context, and thus increasing learning about the CS, ameliorated the test order effect, primarily by enhancing the strength of the context-specific fear memory. The reduced levels of freezing shown by females vs. males in Context A when tested prior to the generalization context are consistent with the idea of a weaker contextual representation. Although female mice can form a contextual representation of the training context, such a representation becomes more detailed as a consequence of pre-exposure (Rudy and O'Reilly, 1999; Keiser et al., 2017), which may be driven by learning-related structural changes in key hippocampal circuits that support memory precision (Ruediger et al., 2011). Finally, individuals with PTSD show greater reactivity to prediction errors, while females, in particular, show a greater difficulty with the encoding of prediction errors (Ross et al., 2018; Homan et al., 2019). Thus, it is plausible that fear generalization in female mice is a product of inadequate CS learning compounded by changes in prediction error.

Tactile Features Are More Salient for Males

Previous work has shown that tactile and olfactory elements exert the most powerful influence over the generalization of contextual fear at proximal time-points in males (Huckleberry et al., 2016). Thus, we also explored the consequences of retaining the metal grid floor in the generalization context (Context C) at a remote time-point. While this manipulation strongly inhibited remote contextual discrimination in male mice, females continued to exhibit strong discrimination between the training and generalization contexts as long as the training context was presented first. In other words, whereas female mice only exhibited heightened freezing in the generalization context when tested first—regardless of perceptual features—males showed pronounced generalization only when the training and generalization contexts shared at least one perceptual element (i.e., tactile cues provided by the metal grid). Thus, our observations support the notion that tactile features are, overall, more salient for males than for females. Finally, both males and females failed to exhibit context discrimination at proximal intervals when the generalization context (Context C) was presented first. Therefore, the absence of context discrimination at remote intervals with the latter test order is not a phenomenon that emerges over time, in contrast to what we observed in behavioral experiments using distinct training and generalization contexts.

Role of Interference and Reconsolidation on Test Order Effects

Both male and female mice exhibited heightened freezing to the training context at remote time intervals when tested prior to the generalization context, in comparison to the reverse order. Furthermore, we observed this effect for both generalization contexts (Contexts B and C). For reasons stated earlier, we would argue against inter-trial extinction as a contributing factor in the observed behavioral outputs. Instead, fear generalization is modulated by proactive and retroactive interference produced by exposure to novel contexts (Besnard and Sahay, 2016), and it is possible that initial exposure to the non-reinforced generalization context could drive a reassignment in cue value that produces a concomitant reduction of freezing to the training context. Initial exposure to the generalization context may support partial reactivation of the aversive training memory that is subsequently reconsolidated in an attenuated form. Conversely, initial testing in the training context serves as a reminder that promotes memory accuracy and therefore improves discrimination when animals are subsequently exposed to the generalization context (De Oliveira Alvares et al., 2013). However, the neurobiological mechanisms governing reconsolidation at remote time intervals are likely to be distinct from those operating at proximal time-points, when context discrimination remains high for female mice. Post-discrimination shifts in generalization gradients represent another potential mechanism by which freezing in the training context at remote time-points may be reduced by prior testing in the generalization context (ten Cate and Rowe, 2007). Furthermore, we speculate that memory storage processes such as pattern completion and reconsolidation may be differentially engaged among males and females (Rolls, 2013), as well as stress-induced alterations (Zoladz et al., 2011) on systems-level processes that operate during remote memory retrieval (Asok et al., 2019b). Additional experiments are needed to investigate these and other explanations.

Evolutionary Implications of Test Order Effects

The proclivity to exhibit a heightened fear response in the first context presented after an aversive event may represent an optimal evolutionary strategy for female mice (Kelley, 1988; Huckleberry et al., 2016; Bangasser and Wicks, 2017). For example, although an inappropriate or excessive defensive response may interfere with the acquisition of resources obtained through potentially risky behaviors such as foraging, such a strategy of erring on the side of safety is more likely to ensure reproductive success in the long run. In this regard, the increased generalization of contextual fear observed in female mice at remote time-points supports the idea that the selection of an optimal defensive strategy is sex-dependent (Gruene et al., 2015; Shansky, 2018). In the absence of sex differences in US processing, our findings are consistent with the idea that the CS or context representation is weaker in females. Importantly, in our study, conditioning parameters such as context placement-to-shock interval as well as the shock intensity and shock duration, produced levels of freezing comparable to studies that use a single-trial conditioning paradigm (Fanselow, 1986, 1990; Wiltgen et al., 2001). Finally, sex differences in generalization and context discrimination at proximal intervals are thought to reflect a differential recruitment of hippocampal and amygdalar circuitry (Keiser et al., 2017). This is likely true for remote memories and warrants further investigation.

PTSD

Animal models based on fear conditioning have generated a wealth of elementary knowledge into the molecular and neural circuit mechanisms that mediate the storage and retrieval of aversive memory (Schafe et al., 2001; Maren et al., 2013). Such studies have provided a useful framework with which to understand how the aberrant processing of fear memory may contribute to psychopathological changes observed in PTSD (Ross et al., 2017; Norrholm and Jovanovic, 2018; Zuj and Norrholm, 2019). Given that PTSD is a disorder of fear memory (McNally, 2006; Ross et al., 2017), and that fear is highly conserved throughout the animal kingdom (LeDoux, 2012; Adolphs, 2013), it is plausible that fear conditioning-based studies in rodents can reveal causative pathological mechanisms that govern the development of PTSD. However, we acknowledge that fear conditioning *per se* does not represent a complete model of PTSD. At best, animal studies can only model sub-components (i.e., intermediate phenotypes and endophenotypes) of these disorders, some of which are nonetheless highly amenable to experimentation in animals, such as fear generalization. Indeed, the generalization of contextual fear in humans is a relatively underexplored area of research (Andreatta et al., 2015), and our studies in mice should inform the design of experiments with human subjects.

Summary

Our findings reveal behavioral and parametric constraints of fear generalization and context discrimination in female mice at remote time-points. These findings also highlight how sex differences in the acquisition, consolidation, or retrieval of contextual representations may influence defensive behaviors to neutral environments long after the learning has occurred. Moreover, our findings point to the need for a better understanding of how contextual processing differs between sexes in hippocampal subfields to promote or prevent remote fear generalization, and how such differences might contribute to overgeneralization. Alterations in contextual processing have been proposed as a core feature in disorders including PTSD (Maren et al., 2013). Future studies that examine how hippocampal circuits interact with cortical networks to encode, store, and maintain contextual representations during systems consolidation will provide significant insights into the sex-specific neurobiological mechanisms of psychopathological disorders such as PTSD.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

JR and AA conceived and wrote the manuscript. JH and LH performed the experiments. JR, AA and JH analyzed the data. SK and EK helped prepare the manuscript and provided key conceptual insights.

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Role of the Bed Nucleus of the Stria Terminalis in PTSD: Insights From Preclinical Models

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Post-traumatic stress disorder (PTSD) afflicts approximately 8% of the United States population and represents a significant public health burden, but the underlying neural mechanisms of this and other anxiety- and stressor-related disorders are largely unknown. Within the last few decades, several preclinical models of PSTD have been developed to help elucidate the mechanisms underlying dysregulated fear states. One brain area that has emerged as a critical mediator of stress-related behavioral processing in both clinical and laboratory settings is the bed nucleus of the stria terminalis (BNST). The BNST is interconnected with essential emotional processing regions, including prefrontal cortex, hippocampus and amygdala. It is activated by stressor exposure and undergoes neurochemical and morphological alterations as a result of stressor exposure. Stress-related neuro-peptides including corticotropin-releasing factor (CRF) and pituitary adenylate cyclase activating peptide (PACAP) are also abundant in the BNST, further implicating an involvement of BNST in stress responses. Behaviorally, the BNST is critical for acquisition and expression of fear and is well positioned to regulate fear relapse after periods of extinction. Here, we consider the role of the BNST in stress and memory processes in the context of preclinical models of PTSD.

Keywords: PTSD, BNST, stress, fear conditioning, extinction, animal models

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INTRODUCTION

Stress-related disorders, such as post-traumatic stress disorder (PTSD), are among the most debilitating neuropsychopathologies in the world. PTSD is nonexclusive and can affect individuals either directly or indirectly exposed to actual or perceived life-threatening events (Herman, 1992; Nievergelt et al., 2018). According to the National Institute of Mental Health (NIMH), an estimated 31% of United States adults experience symptoms of an anxiety disorder at some point in their lives (Kessler et al., 1995, 2004, 2005, 2009; Merikangas et al., 2010), and approximately 8% of Americans have PTSD at any given time (PTSD Statistics, 2018). With increasing costs associated with diagnosis, treatment (and sometimes even misdiagnosis and under treatment), lost work productivity, and high comorbidity rates with addiction disorders, anxiety-related disorders cost the US as much as $42.3 billion annually (PTSD Statistics, 2018).

Despite the continued economic and social burden of stress-related disorders, the molecular mechanisms underlying PTSD and other stress- and anxiety-related disorders are just now being thoroughly investigated (Insel et al., 2010; Cuthbert and Insel, 2013; Cuthbert, 2014; Insel, 2014), although common symptoms of these debilitating conditions are well known and

include conditioned fear responses (Maren and Quirk, 2004; Todd et al., 2014; Giustino and Maren, 2015; Maren and Holmes, 2016; Maren, 2017; Trask et al., 2017; Ressler and Maren, 2019; Trask and Bouton, 2018). Once acquired, fear memories may span years or even decades in humans and contribute to the maintenance of fear and anxiety disorders like PTSD. Hence, there has been a considerable effort in the past several years to understand the neural mechanisms underlying acquisition of fear responses in animal models (Goode and Maren, 2017), particularly in regions associated with behavioral responses to stressor exposure. However, developing pre-clinical animal behavioral models to research the causes of anxiety disorders in the laboratory is challenging. Often, rodents are evaluated based on whether their behavioral changes following stressor exposure match a PTSD phenotype in humans after trauma (despite differences in motivation, emotion, and cognition between rodents and humans; for review, see Lezak et al., 2017), and whether these changes can be attenuated by medications used to treat stress-related disorders. Using this approach, early studies identified several brain regions, including the bed nucleus of the stria terminalis (BNST) and the amygdala, that are highly involved in mediating PTSD-like behaviors in rodents. Although these regions are in close proximity anatomically, they each make unique contributions to PTSD-associated behavioral phenotypes. Hence, we begin this review by discussing the differences between amygdala and BNST as they relate to PTSD. In subsequent sections, we address the role of the BNST in the stress response and identify neuropeptides that drive fear behavior. Finally, we consider current animal models of PTSD, critically review the specific involvement of BNST in each, and discuss the neural mechanisms driving behavioral manifestations of fear-related pathologies like PTSD. Based on these ideas, and a growing literature in BNST research, we suggest a critical role of BNST in PTSD-related behaviors and recommend continued research to explain how neural circuits involved in stress processing become dysregulated, and stay dysregulated, in response to trauma.

Differentiating the BNST and Amygdala

Anatomical Distinctions

The BNST is a limbic system structure widely implicated in mediating behavioral responses to anxiety and stressor exposure (Walker et al., 2003; Choi et al., 2007; Hammack et al., 2009; Kocho-Schellenberg et al., 2014; Lezak et al., 2014a,b; Miles et al., 2018). It has been linked to anxiety and stress in both clinical and laboratory settings (Walker et al., 2003; Avery et al., 2016), and is subject to long-term physiological alterations after stressor exposure (Dumont et al., 2008) that enhance BNST function and mediate stress-related behaviors. The rodent BNST lies just dorsal to the neighboring amygdala and shares similar morphology. Although often grouped together as the "extended amygdala," this term can be misleading, as studies have uncovered unique roles for each region in behavior that depend on their different projections, neuropeptides, and functional output. While a discussion of the multiple sub-regions and cell types associated with the BNST and amygdala are beyond the scope of this review, it is important to note the heterogeneity throughout each structure, as different regions and cell types may be activated in response to one stressor, for example, but not another (see Lebow and Chen, 2016). In short, BNST is often divided into anterior and posterior divisions that can be further categorized into distinct sub-regions including the well-recognized anterolateral, oval, and dorsomedial regions, among others. A heavy presence of androgen receptors, GABA receptors, adrenergic receptors, vesicular glutamate transporter 3 (VGLUT3) and PAC1 receptors are found in the anterior BNST, while posterior BNST also includes glutamate receptors and kainate receptors. Neuropeptide density also differs regionally: corticotropin releasing factor (CRF), dynorphin, enkephalin, pituitary adenylate cyclase activating peptide (PACAP), somatostatin, and neuropeptide Y (NPY) are all highly expressed in regions of the anterior BNST; glutamate, glutamate decarboxylase, and cholecystokinin, in contrast, have higher densities in the posterior BNST (see Lebow and Chen, 2016). Efferent projections both within the BNST and to surrounding regions [including amygdala, hypothalamus, ventral tegmental area (VTA), and lateral septum] help relay emotion-related information, whereas afferent projections [from frontal cortex, locus coeruleus (LC), ventral subiculum, VTA, amygdala, and even olfactory bulb] help integrate information about emotion.

The anatomy of the amygdala has also been reviewed elsewhere, but it includes the basolateral amygdala (which further subdivides into lateral and basal divisions, appropriately), the central amygdala (CeA; subdivided into lateral and medial components), and medial amygdala (Eleftheriou, 2013). Neuronal diversity in the amygdala is similar to the BNST: BLA contains glutamatergic neurons that serve to integrate and relay information between prefrontal cortex and hippocampus, among other regions, as well as GABAergic interneurons that gate information flow within the amygdala (Ramikie and Patel, 2012). The CeA, in turn, contains GABAergic neurons that integrate excitatory information from surrounding regions and project to the hypothalamus and BNST as well as downstream targets in the brainstem. Hence, the BLA serves as a sensory interface to the amygdala that directs the CeA to coordinate behavioral responses to biologically significant events.

BNST vs. Amygdala in Behavior Regulation

Although the BNST and amygdala are neuroanatomically distinct, they often communicate through CRF-containing projections: the BNST transmits information about stress-state through CRF projections to the amygdala, which then gets transmitted to hypothalamic-pituitary-adrenal (HPA) axis (Walker et al., 2003) neurons to stimulate the stress response system. Stress responses are generally protective short-term (Selye, 1955; Gold, 2015), but chronic stressor exposure results in many deleterious physiological and neuropsychological changes including increased risk for psychiatric disease (Vale, 2005; Pittenger and Duman, 2008; Gold, 2015). Upon stressor exposure, several stress response systems are activated, including the HPA axis and the sympathetic nervous system (SNS), as well as neural circuits important for emotional behavior such

as fear and anxiety. Brain areas involved in regulating these physiological and emotional processes, such as the BNST and amygdala, undergo neurochemical and morphological changes following stressor exposure; these changes may underlie several mental health disorders (including PTSD and depression, among others; Hammack et al., 2010, 2012; Maren and Holmes, 2016).

An effort to delineate the behavioral responses dependent on BNST activity has been ongoing since Davis and colleagues initially argued that the BNST mediates a delayed response system following stressor exposure (in which the stress response system becomes activated sometime after stressor exposure rather than in direct response to a footshock, for example—akin, perhaps, to human states of anxiety; Walker et al., 2003; Davis et al., 2010). Notably, these early studies showed that lesions to CeA attenuated conditioned fear responses, but did not affect anxiety-like responses; in contrast, BNST lesions lessened anxiety-like responses, but not fear responses (Walker and Davis, 1997; Walker et al., 2003), illustrating an important distinction between the BNST and amygdala. Since then, several additional studies have suggested a purely temporal role of the BNST in fear learning (Davis et al., 2010; Hammack et al., 2015; Goode et al., 2018) although the BNST also acts in response to contextual cue reminders of perceived (i.e., sustained; *not* immediate) threat. Amygdala activation, however, seems critical to the immediate threat. Human and animal studies corroborate this idea (Hammack et al., 2010; Alvarez et al., 2015; Fox et al., 2015). A functional magnetic resonance imaging (fMRI) study of humans with phobias found increased BNST (and *not* amygdala) activation in response to an image of phobia-related objects (Straube et al., 2007), but dual activation of BNST and amygdala in response to current phobia-related challenges. In rodents, local infusion of AMPA-receptor antagonist NBQX into BNST decreased fear-potentiated startle but had no effect when infused into amygdala, indicating a selective mediation of sustained fear responses by the former (for review, see Davis et al., 2010). Hence, BNST appears to mediate trepidation while amygdala comes on board only in the threat of imminent danger (Goode and Maren, 2017).

BNST vs. Amygdala in Pavlovian Fear Conditioning

The general distinction between BNST and amygdala has been carried over to the fear-conditioning realm. Both structures have been widely implicated in the dysregulated stress response observed in psychiatric patients, but the BNST, in particular, has provided insight on fear memory neural circuitry that may underlie PTSD. Indeed, Pavlovian fear conditioning, where laboratory animals associate an aversive event (the unconditioned stimulus, or US; i.e., a footshock) with a cue that predicts the event (the conditioned stimulus, or CS; i.e., a tone), is believed to mediate fear learning associated with PTSD. The acquisition, expression, extinction and relapse of fear memories is perhaps the most common symptom of PTSD and often manifests as behavioral responses to trauma-related cues. Studying the learning mechanisms associated with fear memories is critical to understanding the neural circuitry behind the development, maintenance, and subsequent treatment of PTSD. However, BNST circuits become activated in response to limited stimulus modalities. The status of a CS as a contextual stimulus does not appear to rely on BNST activity (although Zimmerman and Maren demonstrated that BNST inactivation blocks freezing to a conditioning context, but not a CS, suggesting that BNST activation plays a role in the expression of contextual fear; Zimmerman and Maren, 2011; Hammack et al., 2015). In contrast, long-duration stimuli (and not short) does involve BNST (Waddell et al., 2006; Hammack et al., 2015). Furthermore, BNST activation does not appear to become more or less prevalent depending on how long a stressor may be presented (Walker et al., 2009; Hammack et al., 2015). BNST circuits may also process ambiguous threat signals: BNST inactivation reversed freezing to a backward CS (ambiguous signal for shock onset that occurs after shock) but did not affect freezing in response to CS's that preceded shock exposure (Goode et al., 2018). Research on stress-network contributions has further informed this debate: ultra-high-field magnetic resonance imaging studies observed CeA and BNST responses to sustained threat and demonstrated decreases in intrinsic functional connectivity to regions including ventral medial PFC (Torrisi et al., 2018). Indeed, the role of vmPFC in human emotion regulation has been well studied (Dickie et al., 2008; Rougemont-Bücking et al., 2011; Bisson et al., 2013; Garfinkel et al., 2014), and if vmPFC drives BNST activity, activation of behavioral and physiological components of PTSD-like symptoms may occur. Further research should explore the direct connections between BNST, amygdala, and surrounding regions to identify involvement in the multiple stages of Pavlovian fear conditioning. Understanding these connections and how dysregulated neural circuitry drives trauma-related behaviors will become critical in developing new therapies.

BNST NEUROPEPTIDES INVOLVED IN PTSD

The heterogeneity of the BNST, and the neuropeptides present within its nuclei suggests that the regulation of BNST activity is complex. The BNST is thought to aid communication between limbic system structures and emotional processing systems, insofar as it projects to and receives projections from areas responsible for both emotional and physiological responses to a stressor. As such, the BNST may have a prominent role in mediating stress disorders (i.e., PTSD; Hammack et al., 2010; Roman et al., 2014; Hammack and May, 2015). Furthermore, the BNST may not only be activated by stressor exposure but may undergo physical alterations as a result of stressor exposure (Lezak et al., 2014a); BNST neurochemistry and morphology is altered with repeated exposure to stress. In a study of unpredictable stress in rats, several researchers observed an increase in BNST volume and behavioral manifestations of stress after 28 days of chronic stressor exposure (McEwen and Chattarji, 2007). Hence, the BNST appears well suited to mediate stress-related behavior.

Corticotropin Releasing Factor

CRF is widely regarded as a key regulator of the stress responses system (Bale and Vale, 2004; Slominski et al., 2013) as CRF

and its cognate receptors, CRFR1 and CRFR2, are abundant in stress-related brain regions including BNST. CRF also initiates HPA axis activation by binding to CRFR1 receptors in the anterior pituitary after stressor exposure (Tsigos and Chrousos, 2002; Bale and Vale, 2004), which suggests CRF activation may mediate behavioral and physiological stress responses related to PTSD. The BNST oval nucleus, in addition to containing its own CRF, also receives CRF inputs from the CeA, BLA, parabrachial nucleus (PBn), hippocampus, and medial prefrontal cortex (mPFC), and contains CRF afferents to regions of the hypothalamus (i.e., paraventricular nucleus), limbic system structures and brainstem nuclei that mediate emotional behavior (Crestani et al., 2013).

Perhaps not surprisingly, CRF receptor expression is correlated with stress- and PTSD-related behavior: CRFR1 expression increases after stressor exposure in BNST, while CRFR2 expression decreases in certain sub-regions of BNST (Elharrar et al., 2013). As CRFR1 antagonists can block the effects of stressor exposure, it is thought that stress effects are mediated by CRFR1 (Binder and Nemeroff, 2010). In support of this, CRFR1 knockout mice exhibit decreased anxiety-like behavior (Timpl et al., 1998). However, CRFR2 receptors appear to mediate sustained fear and actually attenuate stress responses after the threat has passed (Bale and Vale, 2004). Mice with PTSD-like symptoms also show increased levels of BNST CRFR2 mRNA levels, and lentiviral knockdown of CRFR2 attenuates anxiety-like behavior (Lebow et al., 2012). Indeed, CRFR2 activation may recruit "coping genes" and reduce PTSD symptoms (Lebow and Chen, 2016). Hence, an important role of CRF receptors has been implicated in stress- and anxiety-related behaviors. In the clinical realm, it is interesting to note that CRF antagonists have been developed to treat stress-related illnesses, but have thus far been unsuccessful in treating behavioral disparities related to the disorder (Zorrilla and Koob, 2010).

Research on behavioral stress responses, such as contextual fear conditioning, has also been shown to increase CRF mRNA in dorsal regions of the BNST (Shalev et al., 2001; Davis et al., 2010). Recently, Pomrenze et al. (2019) showed a distinct CRF-mediated CeA to BNST circuit related to the generation of anxiety-like behaviors in rodents. Stress has also been shown to increase Fos expression (indicative of neuronal activity; Lin et al., 2018) that can be reduced by CNO activation of Gi-coupled DREADDs in PBN regulating BNST CRF neurons (Fetterly et al., 2019), further implicating BNST CRF neurons in response to stressor exposure. Optogenetic activation of CRFR2 neurons has also been shown to decrease anxiety and reduce PTSD-like symptoms in rodents (Henckens et al., 2017), suggesting the importance of CRF receptors in modulating behavioral outputs associated with stressor exposure. Furthermore, lateral hypothalamus-BNST circuits mediating emotional states, such as PTSD-related anxiety, depends on CRF activation (Giardino et al., 2018). Several researchers have observed increases in CRF following chronic variable stress paradigms in the dorsal lateral aspect of BNST—changes that are linked to an increase in anxiety-like behavior (Lee and Davis, 1997; Schulkin et al., 1998) and subsequent PTSD-like behaviors. Additionally, the amygdala is a major source of CRF afferents to the LC that modulate noradrenergic (NE) activity, and NE afferents directly innervate CRF-containing amygdala neurons (Kravets et al., 2015). Indeed, hyperarousal is mediated by NE BNST projections (Forray and Gysling, 2004), and is a common symptom of PTSD in humans. Hence, if CRF is downstream of stress-activated norepinephrine, integration may occur in the BNST. In support of these findings, patients exhibiting symptoms of PTSD in a clinical setting (Arató et al., 1989; Nemeroff et al., 1991; Baker et al., 1999; Ressler et al., 2011) have higher levels of CRF in their system compared to healthy controls. Depressed individuals also have heightened levels of CRF mRNA in BNST and amygdala (Merali et al., 2006) which, given the high levels of comorbidity between PTSD and depression (Kessler et al., 1995; Flory and Yehuda, 2015), further implicates CRF as a critical component of PTSD symptomology. Notably, using animal models, Dunn and Berridge (1987) showed that central CRF administration produces the same physiological effects as those associated with stressor exposure: increased heart rate, increased anhedonia and anorexic-like behaviors (Dunn and File, 1987), low sex drive (Dunn and Berridge, 1990), and reduced social interaction (Dunn and File, 1987). Hence, CRF activity regulates stress- and PTSD-related behavior.

Pituitary Adenylate Cyclase Activating Peptide

PACAP has recently been implicated as a key regulator of peptide signaling in stress-related brain regions (Stroth et al., 2011; Hammack and May, 2015), and patients with PTSD show dysregulated PACAP activity that correlates with the severity of the disorder (Hashimoto et al., 2011, 2016; Ressler et al., 2011). A neurotrophic factor, PACAP promotes cell survival of multiple neuron types including progenitor cells, dorsal root ganglion cells, cerebellar granule cells, and peripheral sympathetic neurons (Stroth et al., 2011). Importantly, PACAP has been shown to increase cell survival in response to stressor exposure (Stroth et al., 2013), and regulates CRF *via* upstream activation in several stress-related regions to modulate CRF release and subsequent HPA axis activation (Gray and Cline, 2019). PACAP and its cognate G-protein coupled receptor, PAC1, are highly expressed in areas that project to the HPA axis, including the PVN where PACAP is heavily co-localized with CRF neurons (Hannibal et al., 1995). Furthermore, CRF transcription is increased in response to PACAP in hypothalamic cells (Stroth et al., 2011). Intracerebroventricular administration of PACAP has also increased CRF mRNA in PVN (Hashimoto et al., 2010), and PACAP-immunoreactive fibers form synapses in close proximity to CRF-expressing neurons in the PVN and BNST (Sherwood et al., 2000; Missig et al., 2014, 2017; Roman et al., 2014) suggesting PACAP may regulate neuroendocrine and behavioral responses to stressor exposure *via* CRF-dependent mechanisms. Indeed, PACAP null mice exhibit long-term HPA axis activation in response to chronic stressors, a finding that supports PACAP's role in regulating the HPA axis during stressor exposure (Vaudry et al., 2005; Hammack et al., 2010). PACAP-deficient

mice also exhibit lower CRF peptide content in PVN and subsequently demonstrate attenuated stress-hormone release after stressor exposure (Stroth and Eiden, 2010), indicating upstream activation of CRF activity by PACAP neurons. Hence, PACAP is involved in the regulation of PVN CRF neurons that activate HPA axis activity and subsequent stress response systems.

Recently, researchers observed that intra-BNST infusion of PACAP mimics behavioral and physiological responses to stressor exposure (Hammack et al., 2009; Miles et al., 2018). For example, when exposed to a 7-day chronic variable stress paradigm, rats exhibit anxiety- and stress-like behaviors that correspond with a significant increase in PACAP and PAC1 receptors in the BNST. Bilateral intra-BNST infusion of PACAP on its own is also sufficient to cause these same stress-related behaviors (Kocho-Schellenberg et al., 2014; Lezak et al., 2014a,b; Roman et al., 2014). Indeed, PACAP infusions mimic chronic stress-related responses by increasing startle and anxiety-like behavior on the elevated plus maze (i.e., rodents spend more time in the closed arm, indicative of anxiety), and elevating circulating corticosterone levels (Hammack et al., 2009; Stroth et al., 2011; Kocho-Schellenberg et al., 2014; Lezak et al., 2014a,b; Roman et al., 2014; King et al., 2017b). Additionally, the bilateral intra-BNST infusion of a PAC1 receptor antagonist reduced stress-induced consequences of repeated variable stress, suggesting that BNST PACAP is necessary for the stress responses observed (Roman et al., 2014). Combined, these data implicate PACAP as an important regulator of stress-related pathologies (Hammack et al., 2012) including PTSD.

Neuropeptide Y

Translational research also points to the NPY system as a critical mediator of stress responses. Although NPY is expressed widely throughout the brain, mRNA and peptide content have been observed specifically in the BNST. Interestingly, the heightened expression in this region may be due to axons from NPY interneurons synapsing in BNST rather than local expression among BNST neurons themselves (Kash et al., 2015), although NPY receptors also densely populate the BNST, which lends support to its label as an "anti-stress" peptide. Indeed, NPY exhibits anxiolytic properties (Sajdyk et al., 2004; Reichmann and Holzer, 2016), perhaps due to its heightened presence between the hypothalamic arcuate nucleus (a major source of NPY), the PVN (the major source of CRF; Reichmann and Holzer, 2016), and the BNST. NPY also innervates the BNST and facilitates afferent cellular and circuit-based activity within limbic system structures including the amygdala.

Few studies have observed the direct effects of BNST NPY manipulations and behavioral output as it relates to PTSD, although chronic restraint stress (discussed in detail below) has been shown to increase NPY expression in BNST in mice susceptible to stress-effects (DBA/2J mice; Pleil et al., 2012, 2015), suggesting an involvement in NPY activity after stressor exposure. Appropriately, rodents exposed to chronic variable stress also show reduced NPY levels in BNST and amygdala (Kautz et al., 2017). Intranasal administration of NPY prior to Single Prolonged Stress (SPS) attenuated PTSD-like behavior in rats (Serova et al., 2013) and reversed PTSD-like behavior when administered after stressor exposure (Serova et al., 2014). Human patients with PTSD exhibit consistent alterations in peripheral levels of NPY (Rasmusson et al., 2000) and combat veterans with a diagnosis of PTSD had significantly lower levels of cerebrospinal fluid NPY than combat-experienced controls who did not develop PTSD-like symptoms (Sah et al., 2014). Hence, NPY involvement has been implicated in stress- and PTSD-related behaviors (Pleil et al., 2015), but more research is needed to determine BNSTs role in this activity.

Cortisol/Corticosterone

A steroid hormone produced by the adrenal glands, cortisol (in humans; corticosterone in rodents) is known to be heavily involved in stress response systems. Although cortisol travels through the blood stream, receptors for cortisol are present on almost every bodily cell, facilitating vastly different effects depending on the location of peptide-receptor binding. The secretion of cortisol is controlled mainly by activation of the HPA axis through PVN CRF action after stressor exposure. Notably, BNST is anatomically situated to appropriately integrate synaptic activity from limbic system structures monitoring negative valance and PVN, and lesions to BNST attenuate corticosterone response to stress-related contextual stimuli (Sullivan et al., 2004) indicating an important role of BNST in stress- and PTSD-related behaviors. Furthermore, corticosterone injections in rodents facilitate an increase in anxiety-like behavior coupled with a decrease in dorsolateral BNST activity (Conrad et al., 2011), suggesting a distinct role of BNST/corticosterone interactions in stress responding.

The development of PTSD and other stress-related disorders facilitates a change in cortisol levels in humans. Recent data indicates lower levels of cortisol could be used as a predictor of risk to develop PTSD (Steudte-Schmiedgen et al., 2015). In rodent models, BNST inactivation leads to increased systemic corticosterone following restraint stress (see below; Myers et al., 2014), although sub-nuclei of the BNST may have different roles in mediating corticosterone release (Lebow and Chen, 2016). Intra-BNST (but not intraventricular) PACAP infusion increased plasma corticosterone levels in males and females, suggesting that BNST PACAP plays a key role in regulating stress responses (Lezak et al., 2014a). However, stress-induced elevations in corticosterone may not drive BNST peptide expression, as corticosterone treatment does not increase BNST PACAP transcript levels (Lezak et al., 2014b). Interestingly, PACAP knockout animals show reduced corticosterone levels after emotional stress (Ressler et al., 2011), and BNST PAC1 receptor antagonism blocks corticosterone release in a sensitized stress model (Roman et al., 2014). Recently, researchers showed that emotional stressors (i.e., open-field exposure or restraint stress), but not physical stressors, attenuate corticosterone release in PACAP knockout mice (Tsukiyama et al., 2011). Furthermore, blunted basal corticosterone levels appear to serve as a risk factor for PTSD-like behaviors in rats (Danan et al., 2018) and intraperitoneal injection of corticosterone following fear memory reactivation reduces

retrieval of strong contextual memories (Abrari et al., 2008). Finally, exposure to high levels of corticosterone leads to impaired fear extinction to contextual freezing (Gourley et al., 2009), which may be BNST dependent. Hence, hypocortisolism and subsequent HPA axis alterations may serve as a risk factor for PTSD development, although additional research is needed to thoroughly examine the relationship between cortisol release and BNST activity.

ANIMAL MODELS OF PTSD

Several animal models of PTSD have been developed (recently reviewed in detail by Flandreau and Toth, 2018) to model different aspects of PTSD symptomology widely used to study neural and behavioral manifestations of trauma. The remainder of this review discusses the role of the BNST in each of these models.

Restraint Stress

Restraint stress, in which a rodent is placed in an enclosed chamber and allowed minimal movement, is typically used to model PTSD-like anxiety symptoms in rodents and has been shown to induce structural remodeling throughout stress-related regions of the brain (Pham et al., 2003). In rats, restraint stress disrupts fear extinction compared to non-stressed animals (Izquierdo et al., 2006) consistent with human PTSD literature. Adami et al. (2017) showed that, after restraint, BNST glutamatergic neurotransmission in rodents influences changes in heart rate and tail skin temperature *via* co-activation of N-Methyl-D-aspartate (NMDA) and non-N-Methyl-D-aspartate (NMDA) receptors. Several studies have also demonstrated an involvement of CRF1 and CRF2 receptors (Oliveira et al., 2015), α1-adrenoceptors (Barretto-de-Souza et al., 2018), and endocannabinoid CB1 receptors (Gomes-de-Souza et al., 2016) in the BNST in cardiovascular adjustments during restraint stress (Oliveira et al., 2015). However, PVN CRF mRNA is not upregulated following restraint stress (Stroth et al., 2011) indicating upstream activation of CRF function (*via* PACAP, for example; Hammack et al., 2010; King et al., 2017b). Acute restraint also causes an increase in corticosterone release in mice, but this change is significantly attenuated in PACAP- and PAC1-deficient mice, suggesting that PACAP-PAC1 receptor binding in BNST may mediate central short-term effects of restraint stress (Mustafa et al., 2015). Relatedly, acute stress (i.e., a short, potent stressor) has been shown to elevate norepinephrine in the BNST: Schmidt et al. (2018) showed, using optogenetic-assisted fast-scan cyclic voltammetry, elevated norepinephrine release across several stimulation parameters and reduced sensitivity to norepinephrine auto-receptors in mice exposed to 5 days restraint stress. In contrast to reports of acute restraint, chronic immobilization increases BNST (but not amygdala) dendritic branching (Vyas et al., 2003), suggesting a role of stress in remodeling neurons in stress-related brain regions after the traumatic event that underlies PTSD. Chronic variable stress paradigms that include restraint stress (as well as pedestal stress, forced swim, footshock, and oscillation; but not single stress exposure) are also associated with increased levels of histone H2A-X phosphorylated at serine 139 (γH2AX), a marker of DNA damage associated with cell death in the BNST (Hare et al., 2018). Hence, chemical and morphological changes that occur in BNST as a result of restraint stress generate behavioral anxiety-like phenotypes that match human PTSD characteristics.

Footshock Stress

One of the most widely accepted methods of stressor exposure, footshock stress has been used to study effects of a single traumatic stressor (modeling, appropriately, a single traumatic experience that leads to the development of PTSD) and fear conditioning (observing the mechanisms underlying stressor exposure; for review, see Goode and Maren, 2017; Flandreau and Toth, 2018; Goode et al., 2018). Fear conditioning using footshock stress generally includes tests for fear extinction, which is impaired in PTSD patients, and dependent on BNST activity. Interestingly, the presentation of footshock stress not only induces reliable freezing in response to previously neutral cues associated with the shock, but suppresses instrumental responding for food (conditioned suppression; Bouton, 1986; Waddell et al., 2006; Allcoat et al., 2015) and increases startle responses to non-related aversive stimuli (fear potentiated startle; Walker and Davis, 1997), both of which are mediated by BNST activity. Chronically stressed rats exposed to a single footshock stressor also show enhanced neuronal activation in regions associated with stress responses, including the BNST and BLA. Indeed, exposure to an aversive event, in general, has been shown to activate or modify BNST signaling (Daniel and Rainnie, 2016; Marcinkiewcz et al., 2016; Rainnie et al., 2017) but with very little uniformity.

GABAergic neurons are widespread within the rodent BNST and active during fear conditioning. Photoinhibition of BNST-VTA projections during footshock, for example, reduces freezing behavior in contexts previously associated with stressor exposure (Ch'ng et al., 2018) and decreases closed arm entries on an elevated plus maze (akin to anxiety-like behavior in PTSD patients; Jennings et al., 2013), indicating a potentially anxiolytic role of GABAergic pathways projecting to and from the BNST. Additionally, chronic exposure to unpredictable footshock stress *increases* serotonin release in the BNST and modifies cell type-specific distribution of serotonin receptors within the BNST and amygdala (Hazra et al., 2012). Appropriately, dysregulation of the GABAergic and serotonin systems increases anxiety-states and has been linked to the pathophysiology of PTSD (Krystal and Neumeister, 2009; Kelmendi et al., 2016).

Learned helplessness (LH) has also recently been classified as a potential model of PTSD (although was initially developed to model depression; Seligman, 1974; Maier and Seligman, 2016). Here, animals exposed to controllable or uncontrollable shock stress ultimately learn appropriate avoidance responses that may be BNST-dependent (Hammack et al., 2012). Notably, behavioral changes associated with controllable or uncontrollable shock presentation appear mediated by the controllability of the stressor rather than the shock stress

itself (Hammack et al., 2012) and mimic anxiety-like states seen in PTSD and depression phenotypes. LH-associated shocks activate BNST neurons (Greenwood et al., 2005) that may drive CRF- or PACAP-induced behavioral responses. Given the high rates of comorbidity between depression and PTSD, continued research on the BNST mechanisms underlying LH may aid our understanding of the shared neural activity.

Forced Swim

The forced swimming model of depression has long been used as a measure of antidepressant drug efficacy (although the translational implications of this model are arguably weak), and requires that rodents swim for 15 min in a deep water-filled tank. Typically, rodents demonstrate escape behaviors but eventually adopt an immobile posture when escape fails. Subsequent tests show the rodents becoming immobile earlier (generally accepted as a sign of depression), but are eventually re-mobilized with the administration of antidepressants (Pezuk et al., 2008). Because the immobility, or signs of "giving up," mimics symptoms of mood disorders in humans, the forced swim test has been adopted as a model of PTSD. High levels of comorbidity between PTSD and depression suggest the two phenotypes share common neural circuitry underlying their respective behavioral manifestations.

BNST activity appears critical for both PTSD- and depression-like symptoms in rodents, and modulation of this region alters behavioral phenotypes associated with the disorders. BNST lesions (Pezuk et al., 2008) and temporary inactivation of synaptic transmission within the BNST (Crestani et al., 2010) result in increased immobility after multiple swim trials in both male and female rats compared to controls. Intraperitoneal injections of CRF antagonists (known to demonstrate antidepressant-like effects) decreased immobility (Jutkiewicz et al., 2005), as did the subcutaneous administration of δ opioid agonists (Broom et al., 2002), both of which may act on stress-related brain regions like BNST. CRF may also have a differential role at BNST and amygdala in forced swim: lentivirus overexpression of CRF in CeA attenuated swim-induced anxiety-like behaviors, but overexpression in BNST promoted depressive-like behaviors (Regev et al., 2011). Local BNST CRF administration also reduces activity in LC (an established brain region mediating arousal) following forced swim (Curtis et al., 1999), suggesting a role of stressors in mediating threshold activation of arousal. Furthermore, mice deficient in PACAP (upstream of CRF in BNST) show immediately increased immobility during forced swim (Hashimoto et al., 2009), indicating an importance of BNST CRF and PACAP in PTSD and depression phenotypes. Underwater holding has also been used to target PTSD phenotypes in rodents (Richter-Levin, 1998; Flandreau and Toth, 2018). Notably, 20–30 s of forced water submersion results in increased startle reactivity (Richter-Levin, 1998), which may be BNST-PACAP dependent (for review, see King et al., 2017a). The forced swim test of "behavioral despair" (Flandreau and Toth, 2018) has predictive validity for antidepressant medications (which have shown some efficacy in PTSD treatment; see Cryan and Kaupmann, 2005), but more research is needed to determine the relationship between models of depression, PTSD, and associated neural circuitry.

Predator Based Psychosocial Stress

The predator-based psychological stress (PPS) model of PTSD is an ethologically relevant stressor based on a threat to survival and a lack of social support (both factors of PTSD in humans). Rodents exposed to 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a synthetic derivative of fox feces, exhibit intense stress responses mediated (in part) by the BSNT (Janitzky et al., 2015). Notably, the BNST receives afferent projections from the olfactory bulbs (Kang et al., 2009; Janitzky et al., 2015) and is well suited to integrate neural information, endocrine responses, and behavioral manifestations of stress. Indeed, temporary inactivation of BNST (but not amygdala) with muscimol (a GABAa receptor agonist) blocked TMT-induced freezing in rats (Fendt et al., 2003), supporting a role of BNST in unlearned fear. This activity may be regulated by interactions between CRF and GABAergic transmission within the BNST, as higher stress sensitivity in mice (measured by working memory tasks) could result from high CRF expression and low GABAergic signaling (Janitzky et al., 2014). Exposure to a cat reduces pCREB expression in the BNST (Blundell and Adamec, 2007), but increases pCREB in CeA (Adamec et al., 2006) of rats. Importantly, some models of predator exposure cause long-term (i.e., months-long) behavioral changes that mimic the lasting clinical symptoms of PTSD in humans.

Social Defeat Stress

A particularly effective social stress model in rodents is the resident-intruder social defeat model in which mice are repeatedly exposed to an unfamiliar, dominant aggressor (Newman et al., 2018) and either socialize with this intruder (where the mice are then dubbed "resilient") or become anti-social (and are considered more "susceptible" to trauma). This distinction is then used to study biological markers of trauma in the susceptible population. Social defeat stress mimics PTSD symptoms including anxiety- and depressive-like behavior in rodents that persist long after the initial stressor exposure; it is, therefore, high in translational validity and commonly used as a model of PTSD.

In mice, social defeat tests demonstrate increases in brain-derived neurotrophic factor (BDNF; critical for the growth, maturation, and survival of neurons) protein in the BNST of females (and not males; Greenberg et al., 2014) and exaggerated social withdrawal. Intraspecific confrontation between male and female rats, in which an aggressor socially defeats a subordinate, increased c-fos expression in BNST, showing a cellular change in stress-related regions specific to stressor exposure. Furthermore, selective antagonism of CRFR2 (but not CRFR1) receptors reduces defensive behavior following social defeat in Syrian hamsters when administered intracerebroventricularly or directly into BNST. Previous studies, however, demonstrate that CRF receptors modulate social defeat at the BNST (and not amygdala; Jasnow et al., 2004), suggesting that even ICV infusion may act specifically on

BNST receptors (Cooper and Huhman, 2005). Despite altered activity of the BNST following social defeat, pCREB and pERK expression were unchanged (although perhaps these markers are not activated by social stimuli; Trainor et al., 2011). Finally, intermittent episodes of social stress escalate alcohol and cocaine intake in rats, but continuous exposure attenuates cocaine intake and increases alcohol intake (Newman et al., 2018), a behavioral change that may (like other models of stress-induced reinstatement to drug-seeking; see Miles et al., 2018) be BNST-dependent. Because BNST activity may drive PTSD-like symptomology, neurochemical and anatomical alterations after social defeat stress suggest a critical involvement of BNST in the maintenance and development of PTSD.

Single Prolonged Stress (SPS)

This animal model is based on the finding that PTSD may be induced after a person experiences a single traumatic incident. While SPS causes a number of behavioral changes similar to those described in PTSD patients, little work has been done to determine the role of BNST activity in this model. However, exposure to 2-h restraint followed by forced swim and ether anesthesia does successfully reproduce neuroendocrine and behavioral characteristics of PTSD including HPA axis activation (Liberzon et al., 1997) and increased acoustic startle response (Khan and Liberzon, 2004). These responses, in turn, may be BNST dependent, as intra-BNST administration of AMPA antagonists block light-potentiated acoustic startle response (Walker and Davis, 1997). Furthermore, rats exposed to an SPS procedure showed reduced fear extinction learning (likely due to disrupted retention) that led to enhanced renewal (Knox et al., 2012). SPS also has been shown to decrease open arm exploration on an elevated plus maze (indicating an anxiogenic state; Qiu et al., 2016), which could be attributed to BNST activation (Butler et al., 2016). Given the overlap between SPS-induced behaviors and PTSD-related characteristics in humans, the BNST may be a key area of interest for future investigations in this model.

Prenatal Stress

While not a model of PTSD *per se*, early life events often have long-lasting impacts on cardiovascular, neuroendocrine, and cognitive development (Harris and Seckl, 2011). Rodents that undergo restraint stress during pregnancy deliver offspring that demonstrate increased vulnerability to PTSD, anxiety disorders, and learning difficulties (Ward et al., 2000; Harris and Seckl, 2011). Early environmental challenges increase anxiety and depressive-like behavior as they age, corroborating human data (Meaney and Szyf, 2005). However, is it unclear whether HPA axis dysregulation and subsequent BNST activity, in these animals (and humans) is a cause or symptom of PTSD. Data from human women who were pregnant on September 11, 2001 and in/near the World Trade Center who developed PTSD show lower cortisol (Lupien et al., 2000) levels than women who did not develop PTSD (Yehuda et al., 2005). This reduction may be facilitated by BNST activation, as animal models of PTSD show lower levels of corticosterone following stressor exposure (see above). Offspring of these women also have significantly lower levels of cortisol compared to children from mothers without PTSD (Yehuda, 2002; Yehuda et al., 2005). Furthermore, extended amygdala CRF expression and receptor-bound activation were increased in rodent adult prenatally stressed offspring (Cratty et al., 1995; Ward et al., 2000). Elevated plasma corticosterone and adrenal hypertrophy were also observed in prenatally stressed rats (Ward et al., 2000), and prenatal stress leads to long-lasting increases in plasma corticosterone after restraint stress in adulthood (Louvart et al., 2009). CRF receptor antagonists can also attenuate the defensive withdrawal behavior of prenatally stressed offspring. Hence, prenatal stress may contribute to stress vulnerability later in life that manifests as susceptibility to stress-related disorders including PTSD.

BNST-Related Sex Differences in PTSD

Adult women exposed to trauma demonstrate a two-fold higher lifetime prevalence of PTSD than their male counterparts (Kessler et al., 1995; Breslau et al., 1997; Olff, 2017), and although it is clear that psychosocial and biological factors may contribute, the underlying neural mechanisms of this discrepancy are not well understood. To date, an animal model of PTSD does not exist that reliably shows females developing and maintaining PTSD-like characteristics at a two-fold higher rate than males that would match clinical manifestations of PTSD (Shansky, 2015; but also see Pooley et al., 2018). While a thorough discussion of sex differences in trauma (including PTSD) is beyond the scope of this review, a few points regarding BNST sex differences relating to PTSD are worth noting.

In several learning conditioning paradigms thought to mimic PTSD development and maintenance, BNST activity enhances the effect of stressor exposure on males and not females (Bangasser et al., 2005, 2016; Bangasser and Wicks, 2017), suggesting that different circuits mediate stress-responses depending on sex. Indeed, the BNST itself is sexually dimorphic: the human BNST region is approximately 2.5 times greater in males than females, a sexual dimorphism that also appears in rodents (Allen and Gorski, 1990). This size difference corresponds appropriately with differences in neurochemical composition and connections to other sexually dimorphic nuclei (Simerly and Swanson, 1986) that mediate behavioral outputs associated with stressor exposure, including PTSD-like behaviors. The regulation of sex-related peptides may also be estrogen-dependent (Ramikie and Ressler, 2016). PACAP transcripts, for example, are increased in BNST in ovariectomized female rats after continuous exposure to estrogen (Ressler et al., 2011), and the PACAP system itself appears sexually dimorphic in stress-related regions (King et al., 2017a). CRF activation of the HPA axis is also stronger in females than males (Bangasser and Valentino, 2012, 2014), and CRF receptor activation in stress-related regions appears lower in females after acute stress (Bangasser et al., 2010). In a study of contextual fear conditioning, researchers found that intra-BNST infusion of allopregnanolone, a metabolite of progesterone, in males suppressed freezing behavior, but inhibiting allopregnanolone in females enhanced fear conditioning (Nagaya et al., 2015), suggesting a role of BNST in anxiety-related behaviors that depend on steroidal regulation. Sex-dependent differences

in HPA axis activity (Stephens et al., 2016) also supports the divergence in stress-related behaviors observed in males compared to females. As mentioned, HPA axis dysregulation underlies stress-related disorders including PTSD and is linked to BNST activity. Continued research is needed to determine why females tend to show different neurochemical and behavioral responses to trauma than males and the underlying neural circuitry responsible for these differences.

CONCLUSION

The BNST has long been implicated in stress- and anxiety-related behaviors, but has only recently been considered a potential therapeutic target for PTSD symptoms. A review of animal behavior in preclinical models of PTSD suggests that the BNST may underlie symptoms of stress-related disorders in humans. Indeed, the BNST appears critical for the acquisition and expression of fear and is well positioned to regulate fear relapse after periods of extinction that mimics fear relapse in PTSD patients. Stress-related peptides are also prevalent in this region, suggesting interactions between BNST and HPA axis activation mediates behavioral outputs. It is important to note, however, that BNST activity and subsequent peptide release may differ depending on the preclinical model under investigation. Furthermore, the BNST itself comprises multiple sub-regions, cell types, and peptide expression, all of which may not contribute equally to stress-related behavioral outputs. A more thorough understanding of how these different sub-regions contribute to PTSD-related symptomology will be important moving forward. Indeed, continued research in this area may aid the development of novel pharmacotherapeutic treatments for PTSD in humans.

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OM and SM wrote and edited the manuscript.

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frontiers in Behavioral Neuroscience

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Single-Prolonged Stress Impairs Prefrontal Cortex Control of Amygdala and Striatum in Rats

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Medial prefrontal cortex (mPFC), amygdala, and striatum neurocircuitry has been shown to play an important role in post-traumatic stress disorder (PTSD) pathology in humans. Clinical studies show hypoactivity in the mPFC and hyperactivity in the amygdala and striatum of PTSD patients, which has been associated with decreased mPFC glutamate levels. The ability to refine neurobiological characteristics of PTSD in an animal model is critical in furthering our mechanistic understanding of the disease. To this end, we exposed male rats to single-prolonged stress (SPS), a validated model of PTSD, and hypothesized that traumatic stress would differentially activate mPFC subregions [prelimbic (PL) and infralimbic (IL) cortices] and increase striatal and amygdalar activity, which would be associated with decreased mPFC glutamate levels. *in vivo*, neural activity in the subregions of the mPFC, amygdala, and striatum was measured using manganese-enhanced magnetic resonance imaging (MEMRI), and glutamate and N-acetylaspartate (NAA) levels in the mPFC and the dorsal striatum (dSTR) were measured using proton magnetic resonance spectroscopy (^{1}H-MRS) longitudinally, in rats exposed to SPS or control conditions. As hypothesized, SPS decreased MEMRI-based neural activity in the IL, but not PL, cortex concomitantly increasing activity within the basolateral amygdala (BLA) and dorsomedial striatum (dmSTR). ^{1}H-MRS studies in a separate cohort revealed SPS decreased glutamate levels in the mPFC and increased NAA levels in the dSTR. These results confirm previous findings that suggest SPS causes mPFC hypoactivation as well as identifies concurrent hyperactivation in dmSTR and BLA, effects which parallel the clinical neuropathology of PTSD.

Keywords: post-traumatic stress disorder, single-prolonged stress, ^{1}H-MRS, MEMRI, striatum, amygdala, prelimbic cortex, infralimbic cortex

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Abbreviations: ^{1}H-MRS, proton magnetic resonance spectroscopy; ACC, anterior cingulate cortex; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; dl STR, dorsolateral striatum; dmSTR, dorsomedial striatum; dSTR, dorsal striatum; IL, infralimbic; MEMRI, manganese-enhanced magnetic resonance imaging; mPFC, medial prefrontal cortex; MPRAGE, rapid acquisition gradient echo; NAA, N-acetylaspartate; PDGE, proton density-weighted; PFC, prefrontal cortex; PL, prelimbic; PTSD, post-traumatic stress disorder; ROI, region of interest; SPS, single prolonged stress; STR, striatum.

INTRODUCTION

Dysfunction of "top-down" prefrontal cortex (PFC) control in post-traumatic stress disorder (PTSD) likely contributes to amygdala hyperactivity, which is thought to mediate disease characteristics, such as the inability to inhibit fear-related behaviors related to traumatic events (Shin and Liberzon, 2010). Specifically, the dorsal anterior cingulate cortex (ACC) and dorsolateral PFC [analogous to the rodent prelimbic (PL) cortex] are implicated in regulating acquisition and expression of conditioned fear behaviors by activating the basolateral amygdala (BLA). In contrast, the ventromedial PFC [analogous to the rodent infralimbic (IL) cortex] is known to inhibit fear response by regulating intercalated cells of the amygdala to attenuate central nucleus of the amygdala (CeA) output (Sierra-Mercado et al., 2011; Do-Monte et al., 2015). Functional neuroimaging studies in humans with PTSD consistently show an increase activity in dorsal ACC and a decrease activity in ventromedial PFC, which is concomitant with an increase in amygdala activity (Hayes et al., 2012). Fear conditioning studies in animals have helped define this circuitry (Milad et al., 2006); however, recapitulation of these neuronal (or neurochemical) changes in animal models of PTSD has not been reported. Using proton magnetic resonance spectroscopy (^{1}H-MRS), we and others have shown that glutamate (Glu) levels are decreased in the medial PFC (mPFC) of rodents exposed to the single-prolonged stress (SPS) model of PTSD (Knox et al., 2010; Perrine et al., 2016; Lim et al., 2017), consistent with human ^{1}H-MRS studies in the ACC (Yang et al., 2015). Considering these parallels (Pitman et al., 2012), the present study aimed to confirm and extend our previous ^{1}H-MRS findings by using manganese-enhanced magnetic resonance imaging (MEMRI) to quantify calcium-dependent neural activity in mPFC and amygdala, longitudinally, before and after SPS.

The PFC also influences striatal (STR) neural activity to regulate goal-directed, motivated, and habit behaviors (Peters et al., 2009; Everitt and Robbins, 2016). Importantly, a lack of inhibitory control in individuals with PTSD is associated with increased activation in the STR during a response inhibition test (Falconer et al., 2008) and with reduced striatal activation reward processing and responsivity (Sailer et al., 2008; Elman et al., 2009). PFC-STR projections have been identified in animals and, similar to the amygdala, the STR receives distinct glutamatergic mPFC input. The PL projects to the ventral STR, including the nucleus accumbens core and dorsomedial STR (dmSTR) to promote goal-directed behaviors, whereas the IL projects to the nucleus accumbens shell to inhibit goal-directed behaviors and to the dorsolateral STR (dlSTR) where its input stimulates the transition of goal-directed to habit-based behaviors (Peters et al., 2009; Everitt and Robbins, 2016). This neurocircuitry has been well-defined in the context of drug addiction and feeding behaviors, but the study of its role in traumatic stress responses is limited. Our findings using the SPS model show neurochemical changes in the STR, implicating it in anhedonia and cross-sensitization with drugs of abuse that relate to trauma exposure (Eagle et al., 2013, 2015; Enman et al., 2015; Matchynski-Franks et al., 2016). Therefore, a second goal of this study is to use longitudinal ^{1}H-MRS and MEMRI to assess glutamatergic tone and neural activity, respectively, in subregions of the STR before and after SPS.

Here, we used SPS to evaluate the effects of traumatic stress exposure on neurochemistry and neural activity within the mPFC, amygdala, and STR. SPS has been shown to be a valid rodent model of PTSD displaying many of the expected characteristics observed in individuals with PTSD, including hyperarousal (Khan and Liberzon, 2004; Ganon-Elazar and Akirav, 2012), avoidance of aversive cues (Brand et al., 2008), emotional and cognitive deficits (Wang et al., 2008, 2010; Li et al., 2010; Eagle et al., 2013), and increased alcohol drinking (Blanco et al., 2013; Matchynski-Franks et al., 2016). Particularly important in relation to the present study, animals exposed to SPS show a deficit in the retention of extinction learning in conditioned fear paradigms (Milad et al., 2006; Knox et al., 2012a), which may indicate impairment in the IL-amygdala pathway that regulates extinction learning. We hypothesized that, at a post-trauma interval during which behavioral deficits are typically apparent in the SPS model, SPS-exposed animals would show: (1) decreased glutamate levels in the mPFC; (2) augmented neural activity in the PL and attenuated activity in the IL; (3) increased neural activity in the BLA; and (4) increased neural activity in the medial STR. To test these hypotheses, we collected neuroimaging data using ^{1}H-MRS and MEMRI before and after SPS or control procedures, which allowed us to quantify Glu levels (Knox et al., 2012a) and neural activity (Perrine et al., 2015; Bosse et al., 2018), respectively, in a longitudinal *in vivo* design.

MATERIALS AND METHODS

Experimental Design

Two experiments were conducted using separate cohorts of rats to determine the region-specific effects of SPS on brain neurochemistry and neural activity using ^{1}H-MRS or MEMRI *in vivo*. In the first experiment, single-voxel ^{1}H-MRS data at 7T were acquired in two regions of interest (ROIs): the mPFC and dorsal STR (dSTR) to measure Glu as well as N-acetylaspartate (NAA), a marker of neuronal integrity. Spectra were collected before and after SPS or control treatment; prescan measurements occurred between 2 and 96 h before exposure and postscan measurements occurred on day 8 or 9 after exposure to SPS. In the second experiment, MEMRI was conducted 7 days prior to SPS to obtain baseline measurements and again 20 days following SPS. MEMRI data were used to assess neural activity in the mPFC (PL and IL), amygdala (BLA and CeA), and dSTR (dmSTR and dlSTR) before and after SPS or control treatment. Also in the second experiment, an object-location memory task was performed 7 days after SPS or control conditions but data are not included as the behavioral task is not regulated by the neurocircuitry being studied herein, furthermore the results showed that no significant differences between groups.

Animals

Thirty-six male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing >240 g at the beginning of the study were pair-housed in standard microisolator cages upon arrival. Animals were acclimated to the climate-controlled vivarium under a 12-h light/dark cycle (lights on at 7 AM) for 5 days and handled once daily for 3 days prior to initiation of procedures. Stress exposure and neuroimaging experiments were conducted during the light phase of the light/dark cycle. Rats were allowed *ad libitum* access to standard rat chow and water except during experiments. Each day, animals were transported from the vivarium to the laboratory and habituated for at least 1 h before beginning experiments. All procedures were approved by the Wayne State University Institutional Animal Care and Use Committee and abided by guidelines in the Guide for the Care and Use of Laboratory Animals.

Single-Prolonged Stress (SPS)

Rats were exposed to SPS as previously described (Knox et al., 2010; Eagle et al., 2015); for a review of the SPS model see (Lisieski et al., 2018). Rats were first restrained for 2-h in cylindrical clear plastic restraints. Immediately following this restraint, they were put into a large tub (48 cm top diameter) that was filled to a depth of 30 cm of room temperature water for a 20-min forced group swim (6–10 rats at a time). Following the swim, rats were towel-dried and given a 15-min rest period in a clean cage with fresh bedding. Finally, rats were exposed to diethyl ether vapor as a group (6–10 rats at a time) until loss of consciousness, as confirmed by absence of righting reflex and lack of response to toe and tail pinch (<5 min). Control animals were held in a separate room for an equivalent period of time, during which they were weighed and handled for ∼2 min. Following SPS or control exposure, rats were returned to the vivarium and left undisturbed (except for routine animal care) for 7 days. This 7-day "incubation period" has been shown to be necessary for the development of PTSD-like effects on behavior (Knox et al., 2012b) and neuroendocrine markers (Liberzon et al., 1997, 1999) that model characteristics observed in PTSD.

Proton-Magnetic Resonance Spectroscopy (^{1}H-MRS)

^{1}H-MRS spectra were acquired on a 7T Bruker ClinScan system with a Siemens console using a transmit-only whole-body coil and receive-only surface coil. Prior to each scan, animals were anesthetized with isoflurane (1%–5%, 0.4 L/min O_2) and maintained on the same percent of isoflurane through each scan. Animals were placed in a prone position and secured using blunted ear-bars and a tooth-bar before being placed into the magnet's horizontal bore, and rats were warmed throughout scanning using a heated water-circulation system. Voxels (3 mm × 2 mm × 3 mm) were placed in mPFC and dSTR ROI. For mPFC, the voxels were placed at the center approximately +2.0 mm from Bregma, on the midline, with the bottom edge just superior to corpus callosum (**Figure 1A**, left). For dSTR, the voxels were placed at the center, approximately at Bregma, 3 mm from the midline, with the top corners just inferior to corpus callosum (**Figure 1B**, left). These placements were based on the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007). Prior to each measurement, magnetic field homogeneity (a.k.a. shimming) over the voxel was adjusted to yield a water spectrum line width of 30–50 Hz using FASTESTMAP (Fast, Automatic Shim Technique using Echo-planar Signal readouT for Mapping Along Projections; Gruetter and Tkác, 2000). A PRESS sequence (repetition time = 4,000 ms, echo time = 3 ms, spectral width = 4 kHz; 2,048 data points; and at least 256 averages) was used to obtain spectra. Additionally, unsuppressed water spectra were acquired per animal for absolute metabolite quantification. The spectral data were analyzed using LCModel and with a basis set derived from simulated data. Only Cramér–Rao measurements <10 for both Glu and NAA were accepted.

Manganese-Enhanced Magnetic Resonance Imaging (MEMRI)

MEMRI images were acquired on the same 7T Bruker ClinScan system with a Siemens console using a transmit-only whole-body coil and receive-only surface coil with established parameters (Bissig and Berkowitz, 2009, 2011; Perrine et al., 2015; Ouyang et al., 2017; Bosse et al., 2018). Twenty-four hours prior to each scan, rats received intraperitoneal (i.p.) injection of manganese (Mn^{2+}; 66 mg/kg $MnCl_2{\cdot}4H_2O$). Before each scan, animals were anesthetized with isoflurane (1%–5%, 0.4 L/min O_2), and then placed in a prone position and secured as for ^{1}H-MRS before being placed into the magnet's horizontal bore. Rats were maintained on the same percent of isoflurane throughout the scan. Vital signs and cardiac gating were monitored, and body temperature was maintained at 37°C using a heated re-circulating water system located beneath the rat. Rapid acquisition gradient echo (MPRAGE) and proton density-weighted (PDGE) images were acquired sequentially using a dual coil mode per animal with principally mutual parameters (echo time = 3.03 ms, turbo factor = 9, echo spacing = 7.77 ms, field of view 2.50 × 2.50 × 2.91 cm^3, matrix size 192 × 192 × 112, resulting a resolution of 130 μm × 130 μm × 260 μm, slice thickness 260 μm).

T_1-weighted images were generated by dividing the signal intensity of MPRAGE images with the corresponding PDGE images on a voxel-by-voxel basis (Bissig and Berkowitz, 2009, 2011; Perrine et al., 2015; Bosse et al., 2018). MPRAGE, PDGE, and T_1-weighted ratio images were uploaded in ImageJ (Schneider et al., 2012) for ROI analysis. Construction of 2D ROI templates, representing PL/IL (**Figure 2A**, left), BLA/CeA (**Figure 2B**, left), and dmSTR/dlSTR (**Figure 2C**, left), were guided by neuroanatomical landmarks with careful comparison of MR images with a rat brain atlas (Paxinos and Watson, 2007). Prominent landmarks included white matter tracts (e.g., the genu of the corpus callosum, anterior and posterior commissures) as well as the overall brain and ventricle profile. The atlas-based, user-defined ROI templates were used to maintain uniform quantification of signal intensities from T_1-weighted ratio images across subjects. Average signal intensities were measured using ImageJ for each ROI and normalized to the mean signal intensity

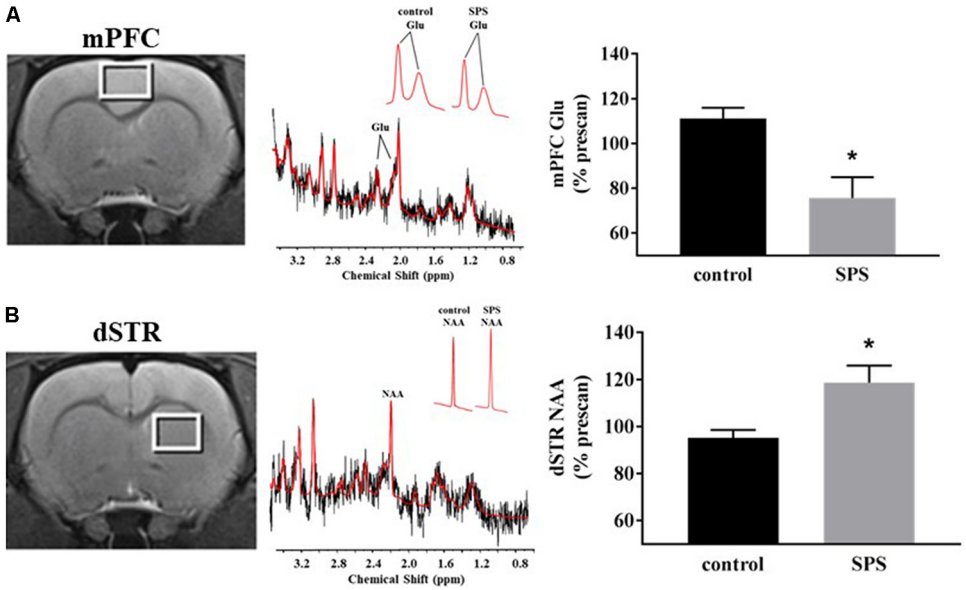

FIGURE 1 | Effects of single prolonged stress (SPS) on glutamate (Glu) and N-acetylaspartate (NAA) levels in the medial prefrontal cortex (mPFC) and dorsal striatum (dSTR). **(A)** Left, voxel placement in the mPFC (white box, voxel size 3 mm × 2 mm × 3 mm). Middle, representative spectrum from the mPFC [Inset: Glu spectrum (peaks at 2.10 ppm and 2.35 ppm) from (Left) a control rat and (Right) an SPS-exposed rat]. Right, SPS decreased Glu levels relative to prescan levels in the mPFC (control $n = 5$, SPS $n = 4$) compared to controls. **(B)** Left, voxel placement in the dSTR (white box, voxel size 3 mm × 2 mm × 3 mm). Middle, representative spectrum from the dSTR [Inset: NAA spectrum from (Left) a control rat and (Right) an SPS-exposed rat]. Right, SPS increased NAA levels relative to prescan levels in the dSTR (control $n = 4$, SPS $n = 5$) compared to controls. Data are plotted as % prescan and expressed as mean ± SEM. $^*p < 0.05$ compared to controls.

recorded for the temporalis muscle tissue (located adjacent to the skull); mPFC and amygdala were also normalized within three consecutive brain slices. Prior to muscle normalization, signal intensities from lateral ROIs were averaged from the left and right hemisphere for each subject.

Data and Statistical Analyses

The percent change from prescan (baseline) to postscan (post-SPS exposure) were calculated within each subject to acquire % prescan values for the ^{1}H-MRS and MEMRI data, graphs and statistical comparisons. As the directionality of our data was predicted by previous studies (Knox et al., 2010; Perrine et al., 2016; Lim et al., 2017), ^{1}H-MRS Glu and NAA data were analyzed by one-tailed Student's *t*-tests and MEMRI signal intensities for each ROI were independently analyzed by one-tailed Student's *t*-tests. Secondary analysis was also performed on the raw data using a 2 × 2 factorial design with repeated measures followed by multiple comparisons using Fischer's LSD test. Data from both statistical designs are presented in the results text, and statistical results presented in the figures focus on the primary analysis. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc, La Jolla, CA, USA) and criterion for statistical significance was $p < 0.05$.

RESULTS

^{1}H-MRS was used to quantify Glu levels in the mPFC and dSTR (**Figure 1**). **Figures 1A,B** (left) shows voxel placements in the mPFC and dSTR, respectively. **Figure 1A** (middle) shows a representative spectrum from the mPFC with insets illustrating the Glu spectra from (middle-left) a control rat and (middle-right) an SPS-exposed rat. **Figure 1B** (middle) shows a representative spectrum from the STR with insets illustrating the NAA spectra from a control rat (middle-left) and an SPS-exposed rat (middle-right). As hypothesized and previously shown (Knox et al., 2010; Perrine et al., 2016; Lim et al., 2017), SPS-exposed rats had significantly decreased Glu levels (as % prescan) in the mPFC compared to control rats [**Figure 1A** (right); control $n = 5$, SPS $n = 4$; ($t_{(7)} = 3.59$, $p = 0.004$)], and secondary analysis revealed a main effect interaction between stress exposure and scan-time $F_{(1,7)} = 14.13$ ($p = 0.007$) and *post hoc* effect showing SPS significantly decreased Glu in mPFC from prescan to postscan ($p = 0.007$) and a trend for a decrease in mPFC Glu postscan values in SPS-exposed rats compared to controls ($p = 0.060$). In the dSTR, SPS-exposed rats displayed an increase in NAA levels compared to controls [**Figure 1B** (right); control $n = 4$, SPS $n = 5$; ($t_{(7)} = 2.655$, $p = 0.016$)]. Secondary analysis did not reveal a main effect, but *post hoc* analysis showed a significant increase in striatal NAA between SPS and control postscan values ($p = 0.023$) and a trend for an increase in striatal NAA in SPS-exposed rats from prescan to postscan ($p = 0.056$). **Tables 1**, **2** summarize the prescan (baseline, before-exposure) and postscan (after SPS or control exposure) values of these ^{1}H-MRS metabolites of interest in the mPFC and dSTR, respectively.

Neural activity was assessed in SPS-exposed and control rats using MEMRI (**Figure 2**). Representative images depicting ROIs

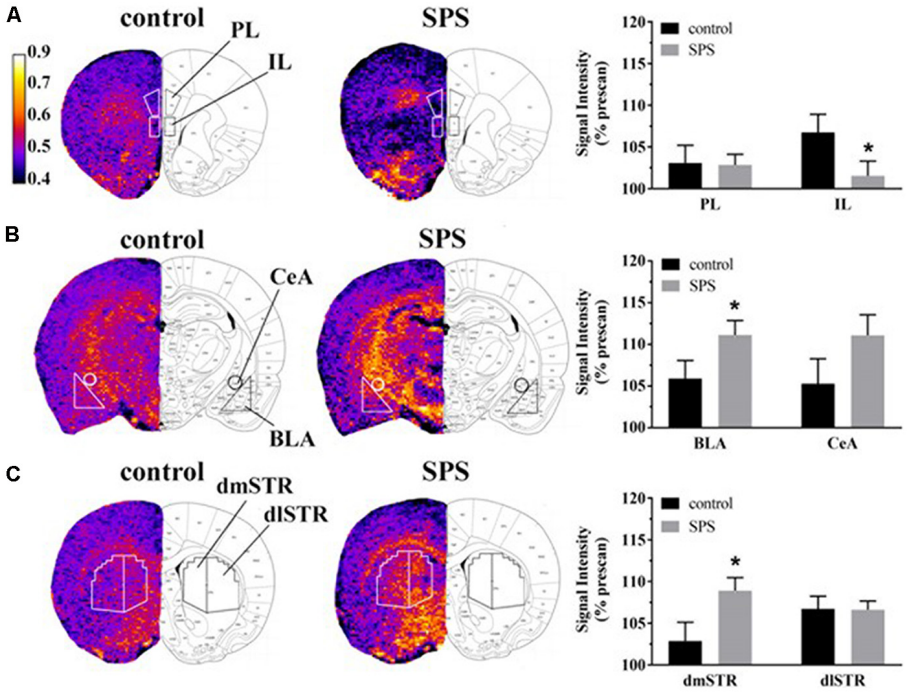

FIGURE 2 | Effects of SPS on neural activity in multiple brain regions assessed with manganese (Mn^{2+})-enhanced MRI (MEMRI). Magnetization prepared rapid acquisition gradient echo/proton density weighted (MPRAGE/PDGE) images of coronal sections containing **(A)** the prelimbic (PL) and infralimbic (IL) cortices, **(B)** the basolateral (BLA) and central extended (CeA) amygdala, and **(C)** the dorsomedial (dmSTR) and dorsolateral (dlSTR) striatum. Each panel **(A–C)** from left to right shows: region of interest (ROI) placements on a representative MPRAGE/PDGE ratio image (pseudocolor indicates signal intensity, scale bar: lighter color indicates higher activity with arbitrary units and Mn^{2+} uptake) adjacent to the corresponding rat brain atlas image (adapted from Paxinos and Watson, 2007), and average normalized signal intensities plotted as % prescan (mean ± SEM) for each ROI (control $n = 8$, SPS $n = 9$). $^*p < 0.05$ compared to controls.

TABLE 1 | Summary of metabolites in the medial prefrontal cortex (mPFC) as measured by proton magnetic resonance spectroscopy (^{1}H-MRS).

	Control		SPS	
Metabolite	**Prescan**	**Postscan**	**Prescan**	**Postscan**
Glutamate	7.56 ± 0.53	8.31 ± 0.29	8.92 ± 0.52	6.75 ± 0.84
N-acetylaspartate	6.15 ± 0.59	5.89 ± 0.11	6.03 ± 0.31	5.29 ± 0.32

Prescan and postscan values reported as mean ± SEM (arbitrary units) for control (n = 5) and single-prolonged stress (SPS)-exposed rats (n = 4).

TABLE 2 | Summary of metabolites in the dorsal striatum (dSTR) as measured by ^{1}H-MRS.

	Control		SPS	
Metabolite	**Prescan**	**Postscan**	**Prescan**	**Postscan**
Glutamate	6.18 ± 0.47	6.64 ± 0.30	6.70 ± 0.53	6.54 ± 0.58
N-acetylaspartate	4.71 ± 0.07	4.49 ± 0.22	4.77 ± 0.31	5.60 ± 0.30

Prescan and postscan values reported as mean ± SEM (arbitrary units) for control (n = 4) and SPS-exposed rats (n = 5).

used to quantify Mn^{2+} uptake are shown in **Figures 2A–C** from control (left, $n = 8$) and SPS exposed rats (middle, $n = 9$) alongside corresponding data (right) reported as % prescan. Within the mPFC subregions, MEMRI results indicate that neural activity, normalized to prescan values, was unchanged in SPS-exposed rats in the PL ($t_{(15)} = 0.09$, $p = 0.46$), but decreased in the IL ($t_{(15)} = 1.89$, $p = 0.039$), compared to control rats (**Figure 2A**). Secondary analysis of the IL neural activity data showed a main effect of scan-time $F_{(1,15)} = 8.161$ ($p = 0.012$) with *post hoc* analysis showing a trend for a decrease in IL neural activity in SPS-exposed rats compared to controls for postscan values ($p = 0.058$). Conversely, in amygdala subregions, SPS-exposed rats showed increased neural activity as % prescan in the BLA ($t_{(15)} = 1.89$, $p = 0.039$), but not in the CeA ($t_{(15)} = 1.51$, $p = 0.075$), compared to control rats (**Figure 2B**). Secondary analysis of the BLA neural activity data showed a main effect of scan-time $F_{(1,15)} = 37.45$ ($p < 0.0001$) with *post hoc* analysis revealing SPS significantly increased BLA neural activity from prescan to postscan ($p < 0.0001$). SPS-exposed rats also displayed increased neural activity, expressed relative to prescan levels, in the dmSTR ($t_{(15)} = 2.25$, $p = 0.020$), but not the dlSTR ($t_{(15)} = 0.05$, $p = 0.47$), compared to control rats (**Figure 2C**). Secondary analysis of the dSTR neural activity data showed a main effect of scan-time $F_{(1,15)} = 37.45$ ($p = 0.001$) with *post hoc* analysis revealing SPS significantly increased dSTR neural activity from prescan to postscan ($p = 0.0003$).

DISCUSSION

In this study, our first goal was to assess longitudinal Glu levels and NAA *in vivo* in the mPFC and the dSTR after SPS using ^{1}H-MRS. The second goal was to determine if SPS changed

longitudinal neural activity *in vivo* in subregions of the mPFC, amygdala, and STR using MEMRI. The *a priori* hypothesis of the current study was that SPS-exposed rats would show decreased Glu levels in the mPFC with increased neural activity in the PL, decreased neural activity in the IL, and increased neural activity in the BLA and dmSTR. As expected, and replicating our previous findings, we observed that animals exposed to SPS had decreased Glu levels in the mPFC (Knox et al., 2010; Perrine et al., 2016). We extend this finding by showing decreased neural activity in the IL, but not in the PL, cortices. Collectively, we parsimoniously synthesize these findings to suggest that SPS decreases Glu-based activity in the IL subregion of the mPFC. We also found increased neural activity in the amygdala after SPS, as anticipated based on human PTSD neuroimaging studies, and specifically, that this activity was significantly increased in the BLA, but not in the CeA. Our most novel findings were observed in the STR, where we showed that SPS increased NAA levels in the dSTR and increased neural activity in the dmSTR, both of which suggest increased neural hyperactivity in animals exposed to traumatic stress.

The PFC plays an important role in executive control and exerts "top-down" influence on the amygdala. The PFC-amygdala network mediates associative fear learning, (Marek et al., 2013; Arruda-Carvalho and Clem, 2015), which is critical to both overgeneralization (Kaczkurkin et al., 2017) and extinction deficits seen in humans with PTSD (Rabinak et al., 2017). Clinical studies using functional MRI and positron-emission tomography consistently show hypoactivity in the ventromedial PFC and hyperactivity in the dorsolateral PFC and amygdala complex during fear conditioning in humans with PTSD (Bremner et al., 2005; Etkin and Wager, 2007; Milad et al., 2009; Rougemont-Bücking et al., 2011; Pitman et al., 2012). In animal studies, SPS-exposed rodents show extinction retention deficits after fear conditioning, decreased c-fos immunoreactivity in the IL following fear extinction and increased BLA neural activity during fear extinction training (Knox et al., 2012a, 2016; Perrine et al., 2016). Our results demonstrate that SPS decreases activity in the IL, but not the PL, would indicate a lack of IL-mediated activation of the CeA, which would be important to inhibit fear response after SPS and suppress amygdala output through the BLA as presently observed. Hyperactivity in BLA, as shown herein, would alter fear extinction, without an effect on fear acquisition (Sierra-Mercado et al., 2011). A previous study from our lab has demonstrated that SPS reduces Glu and glutamine in the mPFC 7 days after exposure as measured by ^{1}H-MRS at 11.7T *ex vivo* (Knox et al., 2012a). Similar results have been reported in rats by another group (Lim et al., 2017) and in mice by our group (Perrine et al., 2016). These preclinical findings parallel human reports, where PTSD is associated with decreased Glu (Yang et al., 2015) in the ACC, which may predict hyperarousal symptoms (Meyerhoff et al., 2014). These effects appear to be brain-region-specific, because in temporal, parietal, occipital, and insular cortices, an increase in Glu is generally observed in individuals with PTSD (Averill et al., 2017). We show no change in PFC GABA levels (data not shown), which is consistent with a meta-analysis of ^{1}H-MRS studies in humans with PTSD (Rosso et al., 2014; Schür et al., 2016); however, ^{1}H-MRS studies in humans with PTSD have shown increased GABA levels in the ACC, but decreased GABA levels in other cortical areas (Michels et al., 2014; Averill et al., 2017). Finally, we observe no change in PFC NAA levels after SPS; whereas, albeit inconsistently reported, a reduction in NAA/creatine ratio in ACC has been observed in individuals with PTSD (Mahmutyazicioğlu et al., 2005; Ham et al., 2007; Schuff et al., 2008). This dysregulation in mPFC-amygdala neurocircuitry, leading to impaired prefrontal control of fear and emotion, is thought to underlie pathological fear responses in PTSD (Liberzon and Sripada, 2008). As indicated, our results match with previous preclinical and clinical PTSD studies that show decreased Glu levels in the mPFC. Our data further suggest that SPS affects Glu-based mPFC and amygdala subregions, which is reflected as IL hypoactivity and proposed to cause BLA hyperactivity.

Increasing evidence suggests that PTSD results in reward circuitry dysfunction. In those with PTSD, functional MRI studies show decreased striatal activation during a monetary reward task, indicating anhedonic behavior (Elman et al., 2009). In parallel rodent studies, Perrine and colleagues have shown that rats exposed to SPS demonstrate anhedonic behavior during a sucrose preference task and in cocaine self-administration studies, which was associated with decreased dopamine and dopamine 2 receptor levels in the STR (Enman et al., 2015). Similarly, studies from our group indicate that SPS-exposed mice show impaired behavioral sensitization to ethanol that is accompanied by decreased striatal dopamine 2 receptor levels and increased striatal postsynaptic density protein 95, a marker of increase neuronal plasticity (Matchynski-Franks et al., 2016). PTSD has also been associated with deficits in inhibitory control, suggesting that the dSTR is activated to support high-demand inhibitory processing (Falconer et al., 2008). These data support the hypothesis that changes in dSTR activation may be related to impaired inhibitory control observed following SPS. Similar to the amygdala, PFC also exerts "top-down" control of STR function. The dmSTR receives projections from the PL and is responsible for action-outcome association, whereas the dlSTR receives projections primarily from the IL and is responsible for habit-like behaviors (Moussa et al., 2011; Burton et al., 2015; Kalivas and Kalivas, 2016; Kaczkurkin et al., 2017; Ma et al., 2017). Our results show that SPS decreases activity in the IL and increases activity in the dmSTR, but not dlSTR, which may suggest that SPS affects action-outcome association, but not habit-like behaviors. In the current study, dSTR Glu levels did not change, however NAA levels increased in the dSTR after SPS exposure. No studies have yet measured NAA levels in dSTR in humans with PTSD, adding novelty to the present findings. A clinical study demonstrating a reduction in NAA within the ACC and hippocampus of patients with PTSD interpreted this change as indicating a disruption of neuronal integrity (Ham et al., 2007), suggesting in our study that SPS influenced dSTR neuronal integrity without affecting its excitatory tone.

In conclusion, results from the present study corroborate results from clinical and pre-clinical studies. We show that

SPS-exposed rats have decreased glutamate in the mPFC with decreased neural activity in the IL and increased neural activity in the BLA. Our novel findings in this study show that traumatic stress also affects reward neurocircuitry in the dSTR with increased neural activity and neuronal integrity in the dmSTR, confirming that coordinated function across the neurocircuitry of mPFC, STR, and amygdala is important in the pathology of PTSD. Our data indicating that the presently used animal model of traumatic stress recapitulates several neurochemical features of clinical PTSD. These findings support its utility to explore therapeutic interventions aimed at mitigating the aberrant functionality of these regions and to gain further mechanistic insight into brain-behavior abnormalities of PTSD.

AUTHOR CONTRIBUTIONS

SP designed the experiments. SP, FG, and ML performed the experiments with technical input from JSta. VP, ML, JStr and KB analyzed the data. SP and AC reviewed the data and interpreted findings. VP and KB drafted the manuscript. All other authors provided critical input and revisions.

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The Divergent Effects of CDPPB and Cannabidiol on Fear Extinction and Anxiety in a Predator Scent Stress Model of PTSD in Rats

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Post-traumatic stress disorder (PTSD) currently has no FDA-approved treatments that reduce symptoms in the majority of patients. The ability to extinguish fear memory associations is impaired in PTSD individuals. As such, the development of extinction-enhancing pharmacological agents to be used in combination with exposure therapies may benefit the treatment of PTSD. Both mGlu5 and CB1 receptors have been implicated in contextual fear extinction. Thus, here we tested the ability of the mGlu5 positive allosteric modulator 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) and cannabidiol (CBD) to reduce both conditioned and unconditioned fear. We used a predator-threat animal model of PTSD which we and others have previously shown to capture the heterogeneity of anxiety responses observed in humans exposed to trauma. Here, 1 week following a 10-min exposure to predator scent stress, rats were classified into stress-Susceptible and stress-Resilient phenotypes using behavioral criteria for elevated plus maze and acoustic startle response performance. Two weeks after classification, rats underwent 3 days of contextual fear extinction and were treated with vehicle, CDPPB or CBD prior to each session. Finally, the light-dark box test was employed to assess phenotypic differences and the effects of CDPPB and CBD on unconditioned anxiety. CDPBB but not CBD, reduced freezing in Susceptible rats relative to vehicle. In the light-dark box test for unconditioned anxiety, CBD, but not CDPPB, reduced anxiety in Susceptible rats. Resilient rats displayed reduced anxiety in the light-dark box relative to Susceptible rats. Taken together, the present data indicate that enhancement of mGlu5 receptor signaling in populations vulnerable to stress may serve to offset a resistance to fear memory extinction without producing anxiogenic effects. Furthermore, in a susceptible population, CBD attenuates unconditioned but not conditioned fear. Taken together, these findings support the use of predator-threat stress exposure in combination with stress-susceptibility phenotype classification as a model for examining the unique drug response profiles and altered neuronal function that emerge as a consequence of the heterogeneity of psychophysiological response to stress.

Keywords: fear extinction, TMT, resilient, mGlu5, mPFC, BLA, Fos

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INTRODUCTION

Post-traumatic stress disorder (PTSD) develops in a subset of individuals following a traumatic event (Perkonigg et al., 2000). A characteristic feature of PTSD is impaired fear memory extinction (Orr et al., 2000; Guthrie and Bryant, 2006), which contributes to the persistent anxiety and hyperarousal experienced by affected individuals (Herman, 1992; Norrholm et al., 2011). Fear extinction is an active learning process where stimuli that previously elicited fear are repeatedly presented in the absence of threat to produce a gradual reduction in fear response (Bouton et al., 2006). While extinction-based exposure therapies are frequently used as a strategy for treating anxiety-like disorders, PTSD-associated extinction deficits reduce the efficacy of these treatments (Schottenbauer et al., 2008). Consequently, there is a need to improve currently available therapies for PTSD. One approach that directly addresses extinction deficits in PTSD would involve the co-administration of extinction-enhancing pharmacological agents with exposure therapy to improve treatment outcomes (e.g., Rothbaum et al., 2014).

Animal models are essential to interrogate the neurobiology underlying fear extinction and for the development of novel extinction-enhancing therapeutics. The most commonly used models are grounded in Pavlovian fear conditioning principles (Pavlov, 1927; Rescorla, 1988). Fear conditioning involves pairing an unconditioned aversive stimulus (US; e.g., mild electric shock) with neutral conditioned stimuli (CS; e.g., a discrete cue or context) until a conditioned fear response (CR; e.g., freezing, changes in heart rate) is produced following delivery of the CS alone. Like exposure therapy, fear extinction training involves prolonged, or repeated presentations of the CS alone, and ideally results in the gradual elimination of the CR (Rothbaum and Schwartz, 2002; Barad, 2005).

Footshock stress is commonly used to study fear learning, and although these models have contributed substantially to our understanding of neural circuits involved in conditioning and extinction of fear, several alternatives to footshock have been established, each offering unique and complementary contributions to the field. Notably, inescapable exposure to species-relevant predator odors (also termed as predator scent stress, PSS) can evoke persistent alterations in behavioral and physiological response in rats that mirror the symptom profile of fear and anxiety related disorders such as PTSD. Exposure of rodents to 2, 3, 5-Trimethyl-3-thiazoline (TMT), a synthetically derived component of fox feces (Vernet-Maury et al., 1984) induces hyperarousal (Hebb et al., 2003), anxiety (Rosen et al., 2015), social dysfunction (Stockman and McCarthy, 2017), vulnerability to substance use (Schwendt et al., 2018), and contextually cued defensive behaviors (Fendt and Endres, 2008; Homiack et al., 2017), indicating the incidence of both sensitized and conditioned fear and anxiety like behaviors.

A key advantage of using PSS models is the ability to examine physiological features associated with the individual differences in vulnerability to such stress. As previously established for PSS using cat odor (Cohen et al., 2003, 2014; Nalloor et al., 2011), rats can be separated into Susceptible, Resilient and Intermediate phenotypes based on scores in both the elevated plus maze (EPM) and habituation in the acoustic startle response (ASR) 7 days after PSS exposure. Control rats are placed into the PSS context without predator odor and are later assessed in the EPM and ASR. Most humans exposed to trauma initially display symptoms of distress and anxiety which dissipate within 1–4 weeks following the trauma (Foa et al., 2006). A similar pattern is observed in the PSS model: 1 day following PSS exposure, approximately 90% of PSS exposed rats are classified as Susceptible; by the 7th day post-exposure, this rate drops to 25%, nicely paralleling the human condition (Cohen et al., 2003). We and others have demonstrated that in unstressed Control rats, the percent of rats classified as Susceptible is much lower, at 1.33–4% (Cohen et al., 2003; Schwendt et al., 2018). Thus, the anxiety phenotype in Susceptible rats is induced by PSS exposure, and is not present in the absent of such exposure.

Likewise, we have recently reported that a single 10-min exposure to TMT gives rise to distinct stress-Susceptible and Resilient phenotypes in Sprague-Dawley rats, with each group presenting distinct behavioral, hormonal, and molecular signatures (Schwendt et al., 2018). Notably, we found that while all TMT-exposed rats and Control rats displayed similar freezing during the PSS exposure, only Susceptible rats displayed increased freezing upon re-exposure to the PSS context whereas Resilient and Control rats did not. Furthermore, Susceptible rats do not decrease freezing over the course of 5 days of extinction exposures to the PSS context (Schwendt et al., 2018). Taken together, these findings indicate phenotypic heterogeneity among populations of stressed animals which may have an unseen influence on the conclusions gained measuring fear extinction within the entire population of stressed animals. Thus, studies addressing differential vulnerabilities may reveal novel fear-associated adaptations.

In healthy humans, neuroimaging studies have revealed an important role for neural activity in the circuitry encompassing medial prefrontal cortex (mPFC) and amygdala during fear extinction. Increased activity is observed in the ventral medial prefrontal cortex (vmPFC) and decreased activity observed in the dorsal lateral prefrontal cortex (dlPFC; Milad et al., 2007) and amygdala (LaBar et al., 1998). Opposite patterns are demonstrated in humans with PTSD, with low vmPFC activity and high activity in both dlPFC and amygdala (Milad et al., 2009). As noted above, the neural correlates of fear extinction in rodents have been extensively studied using footshock models, and suggest a conserved mechanism also involving functional interactions between the mPFC and amygdala. In the rodent mPFC, the prelimbic (PL) and infralimbic (IL) cortices (analogous to the dlPFC and vmPFC in humans, respectively) are strongly interconnected with the basolateral amygdala (BLA; Hoover and Vertes, 2007). The BLA is required for extinction of conditioned footshock (Falls et al., 1992), and serves to regulate fear response through output to the central amygdala (CeA) and brainstem regions (Royer et al., 1999; Haubensak et al., 2010). Chemogenetic, or electrical stimulation of IL or PL pathways targeting the BLA reveal opposing influences (Herry et al., 2008; Senn et al., 2014), with IL enhancing, and PL impairing extinction (Sierra-Mercado et al., 2011). Additionally, inhibitory and excitatory IL and PL projections (respectively)

regulate BLA excitability, stabilizing fear response inhibition (Cho et al., 2013). This evidence suggests that extinction of footshock conditioned fear requires a switch from PL- to IL-mediated reciprocal signaling through the BLA. Indeed, the assessment of neuronal activity using c-Fos immunoreactivity reveals high Fos expression in the IL, but not PL following extinction, and PL and BLA Fos expression correlating with extinction resistance (Knapska and Maren, 2009). While TMT exposure is also associated with changes in amygdala, IL, and PL activity (Sevelinges et al., 2004; Hwa et al., 2019), and recent studies implicate these regions in the extinction of conditioned fear with alternative predator odors, how the coordinated activity across these regions may contribute to the suppression of TMT conditioned fear remains undetermined.

Glutamate receptor signaling has been the focus of many efforts in the development of extinction-enhancing agents. Metabotropic glutamate receptor 5 (mGlu5) subtype regulates bidirectional synaptic plasticity in fear-associated brain regions including the mPFC and BLA (Niswender and Conn, 2010). Pharmacological and genetic inhibition of mGlu5 impairs extinction of both cues and contexts paired with footshock (Xu et al., 2009; Fontanez-Nuin et al., 2011; Sepulveda-Orengo et al., 2013; Sethna and Wang, 2016), and administration of mGlu5 positive allosteric modulators (PAMs) enhances extinction of a footshock-paired context (e.g., Sethna and Wang, 2014). Although antagonism of mGlu5 receptors impairs consolidation of extinction memory, these drugs have also been found to produce anxiolytic effects (Porter et al., 2005; Rahman et al., 2017). The consequences of glutamate receptor modulation on the extinction of predator odor conditioned fear has been assesses in only one study that demonstrated partial agonism of NMDA receptors with D-cycloserine enhanced extinction of a cat odor-paired context (Sarıdoğan et al., 2015). We have previously found increased mGlu5 gene expression in the amygdala and mPFC of Resilient rats following re-exposure to the TMT-associated context (Schwendt et al., 2018). In the same study, daily systemic treatment with the mGlu5 PAM CDPPB during extinction of the TMT-paired context increased freezing in a cohort of Susceptible rats that previously underwent cocaine self-administration (Schwendt et al., 2018). However, as chronic cocaine can alter both function and mGlu5 receptors numbers in brain regions associated with fear signaling (Hao et al., 2010; Ghasemzadeh et al., 2011; Schmidt et al., 2011), a primary goal here was to examine the effects of CDPPB on fear extinction in cocaine-naïve rats using this model.

Like mGlu5, CB1 receptors are abundantly expressed in the BLA and mPFC and are important modulators of fear and anxiety signaling (Chhatwal and Ressler, 2007). Previous studies have revealed dysregulated expression of CB1 receptors and abnormal levels endocannabinoids in subjects with PTSD (Neumeister et al., 2015), as well as in rodent PTSD models (Schwendt et al., 2018). In rodents, genetic or pharmacological inhibition of CB1 receptors impairs extinction (Marsicano et al., 2002), while CB1 agonists have extinction-enhancing effects (Chhatwal et al., 2005; Campolongo et al., 2009). However, CB1 agonists can also produce biphasic anxiogenic and anxiolytic effects (Haller et al., 2004; Sink et al., 2010), which may compromise their clinical usefulness. Several recent studies have demonstrated that cannabidiol (CBD), a component of cannabis which lacks THC-like psychoactive effects (Campos et al., 2012b), may serve to mitigate symptoms of PTSD by increasing extinction and reducing post-trauma anxiety in both humans and rodents (Bitencourt et al., 2008; Das et al., 2013).

Here we evaluated the effects of CDPPB and CBD on the extinction of contextual fear in a PSS model. We focused our investigation on rats with stress-Susceptible phenotype, as Resilient and Control rats do not demonstrate freezing upon re-exposure to the conditioning context (Schwendt et al., 2018). Further, this study explored possible changes in neuronal activity (via Fos expression) produced by fear extinction training within the PL, IL, and BLA regions. Given the involvement of PL, IL, and BLA neuronal activity in extinction to conditioned footshock (Cho et al., 2013), and evidence indicating an important role for mGlu5 receptor function (Sethna and Wang, 2016), we predicted that treatment with CDPPB would (a) enhance extinction of contextual fear, and (b) increase Fos expression in all three regions. Finally, this study also considered the effects of CDPPB and CBD treatment on unconditioned anxiety, as anxiogenic effects may compromise the utility of these drugs for fear-extinction therapies.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Charles River; N = 307) were individually housed in ventilated cages in a vivarium maintained on a 12:12 light-dark cycle (lights off at 7:00 am). Prior to the beginning of the study, rats were acclimated to the vivarium for 7 days with *ad libitum* access to food and water. Beginning 72 h after arrival, rats were carefully handled to become familiar with experimenters (always one male and one female experimenter) prior to stress induction. Food access was restricted to 20 g/day from the beginning of testing to be consistent with our previous publication with this model (Schwendt et al., 2018). All procedures were performed within 4 h of the beginning of the dark cycle. Rats arrived in 3 cohorts of 80–110 rats over the course of 1 year. Procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Drugs

3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB, 30 mg/kg; Abcam Biochemical) was suspended in 10% Tween 80 (Sigma-Aldrich) in phosphate-buffered saline (PBS) to a final concentration of 30 mg/ml and injected subcutaneously (s.c.). The dose of CDPPB was based on previous studies indicating an effect on fear extinction (Sethna and Wang, 2014). Cannabidiol (CBD, 5 mg/kg) was provided by the NIDA controlled substances program (RTI, Research Triangle, NC) and dissolved in a mixture of 100% ethanol, Cremophor, and 0.9% NaCl to 5 mg/ml and injected intraperitoneally (i.p.). The dose of CBD was based on previous studies demonstrating an effect on enhancing footshock conditioned contextual fear conditioning (Jurkus et al., 2016),

and is within a range of doses found to produce anxiolytic effects (Guimarães et al., 1990). Locomotor testing was not performed as given doses of CDPPB and CBD do not affect locomotion in rats (Gass and Olive, 2009; Ren et al., 2009). 2, 3, 5-Trimethyl-3-thiazoline (TMT, 5 μl; BioSRQ) was presented undiluted (97% purity). The amount of TMT used for predator odor exposures was based on previous studies by our laboratory and others (Tanapat et al., 2001; Day et al., 2004; Schwendt et al., 2018).

Experimental Procedures

Predator-Scent Stress Exposure

The timeline for the predator-scent stress exposure and assessment of anxiety is shown in **Figure 1A**. Six to 10 days after arriving in the vivarium, rats received a single exposure to TMT in a covered, clear cylindrical Plexiglas chamber (BioBubble Pets; 40 cm diameter × 35 cm height) with steel mesh flooring above a clear plastic dish. Prior to each session, TMT (5 μl) was placed on a square of filter paper positioned in the center of the dish. Rats were individually placed in the test chamber for a single 10 min exposure. Test chambers were cleaned with 70% ethanol between sessions. Exposures were videotaped.

Power analyses (G power) indicated the number of animals needed was 7–8/treatment group with a significance level of 0.05. We have previously shown that the incidence of the Susceptible phenotype amongst TMT-exposed rats ranges from 14 to 21.8% (Schwendt et al., 2018). Thus, with a target of 8 rats/group for a total of 16 Susceptible rats needed for Experiments 1 and 32 needed for Experiment 2, we initially exposed 307 rats to TMT.

Elevated Plus Maze (EPM)

Seven days after TMT exposure, all rats were tested on the EPM according to previously described procedures (Pellow et al., 1985). The EPM apparatus (Med Associates) was made from black acrylic and consists of four arms (50 cm length × 10 cm width) raised 50 cm from the floor. Two open arms (2.5 cm high walls) and two closed arms (50 cm high walls) are joined by a center square platform (10 cm × 10 cm) illuminated at 50 lux. Rats were individually placed on the center platform facing a closed arm and allowed to move freely for 5 min. Sessions were filmed by a camera secured above the maze. The EPM was cleaned with 70% ethanol between tests. Total time spent in the open arms excluding time in the center area (OA time) was recorded with EthoVision XT 14 software (Noldus Information Technology) and served as a measure for anxiety.

Acoustic Startle Response

Immediately after EPM testing, habituation of acoustic startle response (ASR) was assessed according to Valsamis and Schmid (2011). Four ventilated soundproof chambers (San Diego Instruments) each contained a transparent plexiglass cylinder that rested on a pressure-sensitive platform. An accelerometer fitted to the platform measured changes in pressure created from movement of the rat's body, and the maximum response amplitude was registered during presentation of acoustic stimuli. Accelerometer calibration and acoustic sound levels were routinely checked, and chambers were cleaned with CaviCide disinfectant (Metrex) and 70% ethanol between sessions. Rats (four at a time) were secured in the plexiglass cylinders and acclimated to the chamber for 5 min. Next, 30 pulses of 110 db white noise were delivered for 40 ms followed by a variable (30–45 s) intertrial interval. Startle habituation was calculated as the percent change in startle amplitude from the first six trials to the last six trials.

Experiment 1 – The Effects of CDPPB of Contextual Fear Extinction and Context-Induced Fos Protein Expression

Fear extinction

The timeline for this experiment is shown in **Figure 2A**. Rats first underwent predator stress induction and EPM/ASR assessment (see above and **Figure 1A**). Rats classified as Susceptible were randomly subdivided into two groups: Sus-Veh and Sus-CDPP (n = 7/group). Two weeks after anxiety assessment, rats underwent three contextual fear extinction sessions on three separate days. We previously found that in Susceptible rats with a history of cocaine self-administration, treatment with CDPPB immediately prior to placement into the TMT context increased freezing on Days 2–4 of extinction (Schwendt et al., 2018). Here we sacrificed rats immediately after Day 3 of extinction in order to examine neuronal activity during the last extinction session. Twenty minutes before sessions, rats were injected with either vehicle or CDPPB and returned to their home cage. Rats were then placed into the exposure chamber in the absence of TMT for 10-min sessions (one session/day). To avoid potential residual scent, we used plexiglass chambers which never had contact with TMT. Sessions were filmed, and freezing was quantified offline using the mobility detection function in Ethovision XT 14 software according to Pham et al. (2009). Freezing in rats is a species-specific threat-related defensive strategy that is defined by the absence of movement except for respiration (Fanselow, 1980).

Combined Fos immunohistochemistry and fluorescent in situ hybridization

Next, we performed fluorescent immunolabeling for Fos protein to measure neuronal activity during the third extinction session. Fos is widely used relative marker for neuronal activation (Lanahan and Worley, 1998; Kovács, 2008). As previous studies have demonstrated a role for mGlu5 in the regulation of cFos, and stimulation of mGlu5 with CDPPB can enhance cFos expression (Mao and Wang, 2003; Mao et al., 2005; Uslaner et al., 2009), we also performed dual-labeling for mGlu5 mRNA. Two hours after the third extinction session, rats were administered an overdose of sodium pentobarbital (Euthasol, 1 ml, i.p.) and transcardially perfused in nuclease free 0.9% NaCl followed by cold 4% paraformaldehyde (PFA) in PBS. Brains were removed, post-fixed for 12 h at 4°C in 4% PFA in PBS, cryopreserved in 30% sucrose in PBS, frozen and kept at −80°C until sectioning. Twelve-μm-thick tissue sections corresponding to the rat PL/IL (+3.00 mm relative to Bregma) and BLA (−2.52 mm) according to rat brain atlas (Paxinos and Watson, 2005), were collected using a freezing cryostat (Leica CM1950). Tissue sections were direct-mounted onto Superfrost Plus Gold slides (Fisher Scientific), dried, and stored at −80°C. Fluorescent *in situ* hybridization of *GRM5* (mGlu5) mRNA was

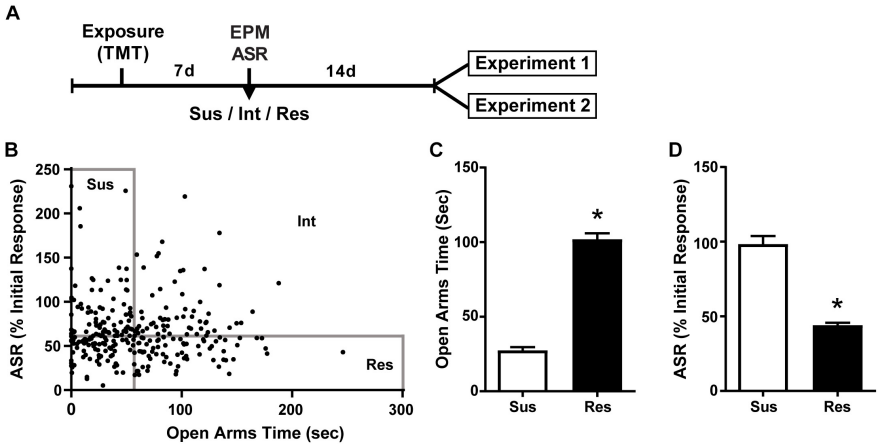

FIGURE 1 | Susceptible and Resilient rats display distinct behavioral phenotypes. **(A)** Timeline for behavioral classification. **(B)** Time spent in the open arms of the EPM plotted against % habituation of acoustic startle response (ASR) for all TMT exposed rats (n = 299). Median splits performed on EPM open arm time (median = 56.2 s) and % ASR habituation (median = 61.2%) were used to classify rats into Susceptible (Sus, n = 74), Resilient (Res, n = 74), or intermediate (Int, n = 151) phenotypes. **(C)** Sus rats spent less time in the open arms of the EPM and **(D)** exhibited attenuated habituation to ASR relative to Res rats. *$p < 0.0001$.

performed using the RNAscope Multiplex Fluorescent Reagent Kit (ACDBio) according to the manufacturer's instructions with a few modifications (Wang and Zhuo, 2012). Slides were first equilibrated to room temperature (RT) before heating to 60°C for 45 min. Next, sections were fixed in cold 4% PFA in PBS and dehydrated using ethanol gradient of 50, 70, 100, and 100% in consecutive 5 min incubations. Slides were boiled in target retrieval reagent (ACDbio), washed in nuclease-free H_2O, and again dehydrated in 100% ethanol. Proteinase digestion of sections was conducted using pretreatment #3 (ACDbio) at 40°C for 30 min under humidity-controlled conditions (HybEZ hybridization oven; ACDbio). The RNAscope probe for *GRM5* (ACDBio: 471241-C2, lot 17335A) was diluted with C1 diluent probe (1:50) and applied to sections. Slides were then incubated 2 h at 40°C for hybridization of probe to target mRNAs. Signal amplification was performed with preamplifier and amplifier probes at 40°C (AMP 1, 30 min; AMP 2, 15 min; AMP 3, 30 min; AMP 4, 15 min). For AMP4 (15 min), Alt-A was selected so that the target probe could be detected with ATTO 550 (Cy3) fluorescent label. Immediately following *in situ* hybridization, slides were rinsed three times in Tris-buffered saline (TBS) and blocked (0.3% Triton x-100 and 5% NGS in TBS) for 1 h at RT. TBS was used for all rinses and antibody dilutions. Sections were incubated in rabbit anti-Fos antibody (1:1000; Synaptic Systems) overnight at 4°C. The next day slides were rinsed and then incubated in goat anti-rabbit Alexa 594 secondary antibody (1:1000; Invitrogen) for 2 h at RT. Finally, sections were rinsed again before counterstaining with DAPI (Invitrogen) and coverslipped using ProLong Gold antifade mounting reagent (Invitrogen).

Imaging

Fluorescent images were captured using Zeiss LSM70 inverted Axio-Observer 3-channel spectral confocal microscope and Zen 2012 software. Multitrack sequential imaging settings were applied to avoid inter-channel crosstalk effects. The 405, 488, and 561 nm laser lines were used for excitation of DAPI, Fos (Cy3), and mGlu5 (FITC). Two regions of interest were selected for each brain area (PL, IL, and BLA; **Figure 2D**) and Z-stacks were acquired at 1 μm intervals using a 63× oil immersion objective. Image stacks were imported into NIH Image J software (Schneider et al., 2012) and analyzed offline. Only the middle five focal planes from each Z-stack were used for analysis. To measure co-expression, Z-stacks were first converted to composite images for separation of individual color channels. The red channel (mGlu5) was then turned off, and cells containing green (Fos) staining were marked using the NIH Image J multi-point tool on each focal plane. Then, the green channel was then turned off, and the red channel was used to mark mGlu5 puncta in the same manner. Individual cells were distinguished based on DAPI nuclei staining. Fos expression was established if staining within a cell was detectable on three consecutive focal planes, and mGlu5 expression was established if a cell contained mRNA puncta on three consecutive focal planes. Under these conditions, cells expressing both Fos and mGlu5 were considered positive for co-expression. The total number of Fos expressing and Fos/mGlu5 co-expressing cells were counted for each selected area. The average number of cells for each parameter across the two selected areas was calculated for the three brain regions for each rat.

Experiment 2 – The Effects of CDPPB (or CBD) on Unconditioned Anxiety and Contextual Fear Extinction

The timeline for this experiment is shown in **Figures 3A**, **4A**. Rats first underwent predator stress induction and anxiety assessment as described above. Susceptible rats were randomly selected from the sample to create vehicle (Sus-Veh; n = 8), CDPPB treated (Sus-CDPPB; n = 8), and CBD treated (Sus-CBD; n = 8) groups. Resilient rats were treated with vehicle (Res-Veh; n = 8).

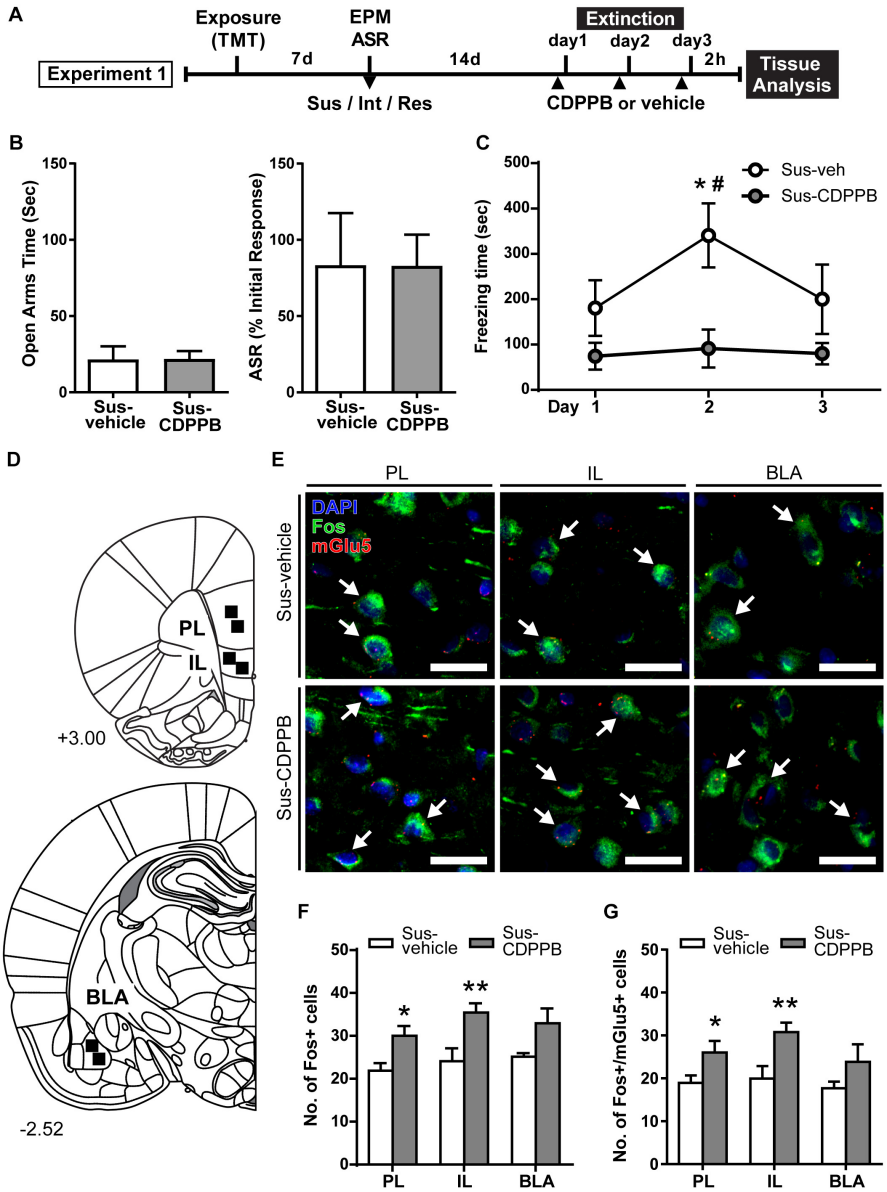

FIGURE 2 | CDPPB reduces context dependent freezing in Sus rats and increases Fos expression in the medial prefrontal cortex. **(A)** Timeline for Experiment 1. **(B)** Treatment groups did not differ in EPM open arm time or % ASR habituation. **(C)** Mean ± SEM of freezing in vehicle (n = 7) or CDPPB (n = 7) treated Susc rats during 10 min extinction sessions in the TMT context initiated 14 days after phenotype classification; CDPPB reduced freezing on day 2 of extinction. **(D)** Schematic indicating the regions of interest (ROI) used for characterization of extinction dependent neuronal activity in the prefrontal cortex (top) and basal lateral amygdala (BLA; bottom). **(E)** Representative confocal images from the selected ROIs in the prelimbic cortex (PL; left), infralimbic cortex (IL; middle) and BLA (right) showing fluorescent immunolabeling of Fos (green) protein, labeling of mRNA for mGlu5 using fluorescent *in situ* hybridization, and DAPI (blue) nuclear staining, in vehicle (top panel) and CDPPB (bottom panel) treated susceptible rats; scale bar: 20 μm, 63× magnification. Fos+mGlu5 co-expression is indicated with white arrows. **(F)** CDPPB increased the number of Fos labeled cells in the PL and IL regions on day 3 of extinction. **(G)** The effects of CDPPB on the number of Fos+/mGlu5+ double-labeled cells in the PL, IL, and BLA on day 3 of extinction. $^{*}p < 0.05$, $^{**}p < 0.01$ relative to vehicle, $^{\#}p < 0.05$ relative to Days 1 and 3.

Light-dark box

Two weeks after anxiety assessment and phenotype classification, rats underwent light-dark box testing based on previously described procedures (Crawley, 1981). Light-dark box apparatus consisted of a plexiglass box with two separate compartments of equal dimensions (40 cm × 44 cm × 37 cm); a light compartment (light box) with translucent walls illuminated at 300 lux, and a dark compartment (dark box) with blackened opaque walls. An opening in the dividing wall allowed free movement of rats between the compartments. Prior to testing, rats were treated with vehicle, CDPPB, or CBD in their home cage. Following pretreatment (CDPPB: 20 min; CBD: 30 min), each animal was individually placed in the center of the light box and allowed to roam freely for 10 min. Sessions were

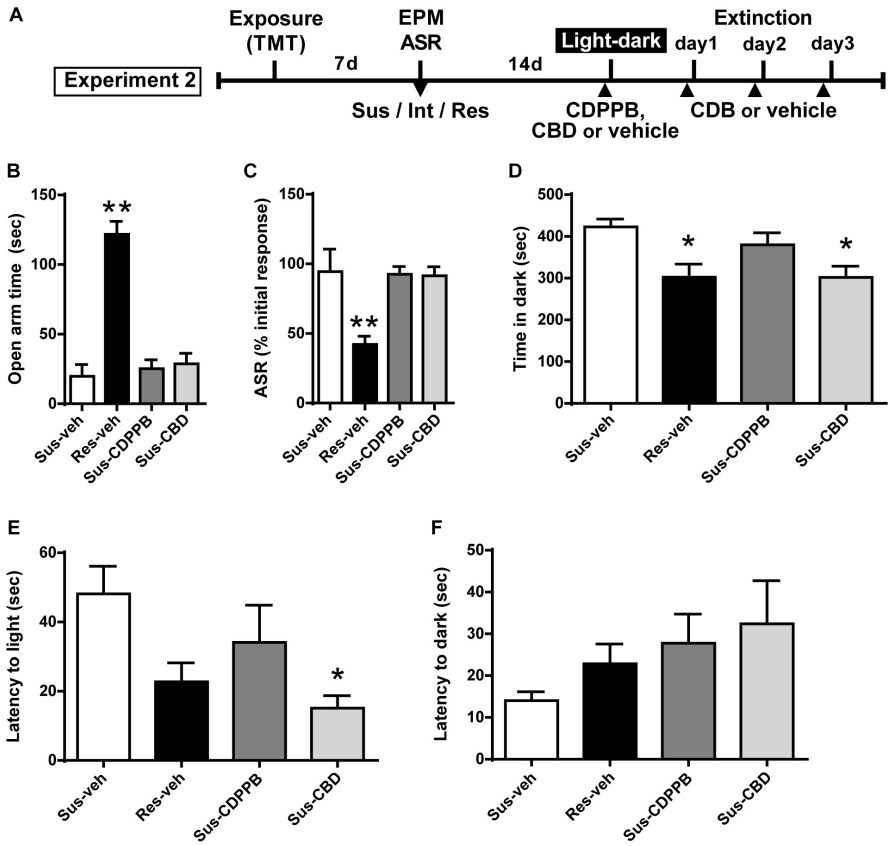

FIGURE 3 | Cannabidiol attenuates increased anxiety-like behavior in the light-dark box test in Susceptible rats. **(A)** Timeline for Experiment 2. **(B)** Vehicle (Sus-veh, $n = 8$), CDPPB (Sus-CDPPB), and CBD (Sus-CBD, $n = 8$) treated Sus rats spent less time in the open arms of the EPM and **(C)** exhibited reduced habituation to ASR relative to vehicle treated Res rats (Res-veh, $n = 8$). **(D)** CBD treated Sus rats and Res rats spent less time in the dark-box relative to vehicle treated Sus rats. **(E)** CBD treatment reduced the latency to enter the light-box in Sus rats. **(F)** No differences were observed in latency to enter the dark-box. $^{**}p < 0.05$ relative to Res-veh; $^{*}p < 0.05$ relative to Sus-veh.

filmed, and the following behaviors were hand scored by an experimenter blind to the conditions: (1) latency to enter the dark box from the light box and vice versa; (2) the number of transitions between compartments; and (3) the duration of individual compartment visits. The apparatus was cleaned with 70% ethanol between trials.

Fear extinction

Next, rats were used to assess differences between Resilient and Susceptible rats in fear extinction and the ability of CBD to enhance the extinction of TMT conditioned fear. The day after light-dark box testing, rats were administered vehicle or CBD 20 min prior to testing (**Figure 4A**). Rats received the identical treatment that they had received on the day prior for light-dark box testing. Procedures for fear extinction were identical to Experiment 1.

Data Analysis

GraphPad Prism (version 6.0) was used for statistical analysis with the alpha level set at $p \leq 0.05$. Unpaired *t*-tests were used to compare phenotypic differences in EPM and ASR behavior for rats used in Experiments 1–2. Unpaired *t*-tests were also used to test for within region differences in Fos expression between treatment groups. One-way analyses of variance (ANOVAs) were used to assess differences in EPM and ASR behavior for rats later treated with vehicle, CBD or CDPPB to ensure no pre-existing differences prior to initiation of testing and pharmacological treatment. Freezing during fear extinction was analyzed by two-way mixed factorial repeated-measures (RM) ANOVAs with Treatment as the between-subjects factor and Day as a within-subjects factor. Significant interactions were followed by Tukey's *post hoc* analyses with corrections for multiple comparisons.

RESULTS

Predator Odor Stress and Susceptibility Classification

A total of 307 rats were exposed to TMT. ASR data files for 8 rats were corrupted and thus the following calculations are based on the data from 299 rats. The median time spent in the OA of the EPM was 56.2 s and median habituation of the ASR was 61.2%. Rats that fell below the median for time spent

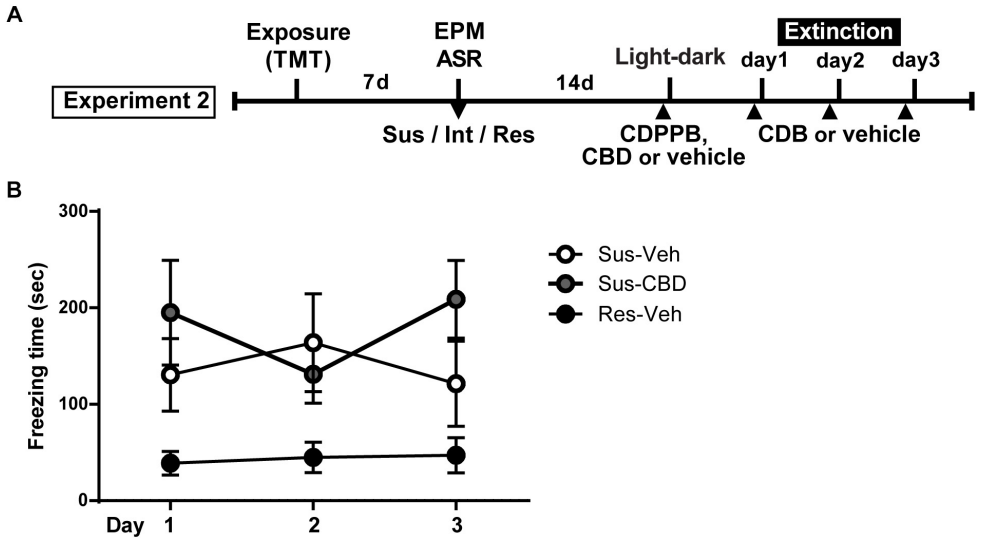

FIGURE 4 | Cannabidiol does not reduce increased context dependent freezing in Susceptible rats. **(A)** Timeline for Experiment 2. **(B)** Mean ± SEM of freezing in vehicle (Sus-veh; $n = 8$) and CBD (Sus-CBD; $n = 8$) treated Sus rats and vehicle treated Res rats (Res-veh; $n = 7$) during 10 min extinction sessions. A main effect of Group was detected with Sus-veh and Sus-CBD displaying increased freezing relative to Res-veh rats.

in the OA and above the median for ASR habituation were classified as Susceptible as in our previous report (Schwendt et al., 2018). Rats were classified as Resilient if they fell above the median for time spent in the OA and below the median for ASR habituation. This resulted in 74 rats (25%) meeting criteria for the Susceptible phenotype and an equal number meeting criteria for the Resilient phenotype (**Figure 1B**). A total of 46 Susceptible rats and 8 Resilient rats were used for the present set of experiments. More Susceptible and Resilient rats were generated than were needed for the present set of experiments and were used for other experiments to be published at a later date. Rats not classified as either Susceptible or Resilient (Intermediate; $n = 151$) were eliminated from the experiment. Susceptible (Sus) rats spent less time in the open arms of the EPM [$t(26) = 4.482$, $p < 0.0001$, **Figure 1C**], and exhibited less reductions in ASR magnitude [$t(26) = 4.515$, $p < 0.0001$, **Figure 1D**] compared to Resilient rats. Freezing was assessed on the day of TMT exposure in a subset of Susceptible ($n = 20$) and Resilient ($n = 20$) rats; no phenotypic differences in freezing were found (mean ± SEM: Susceptible 10.2 ± 2.438 s; Resilient 10.8 ± 2.624 s).

Experiment 1

Rats were assigned to receive vehicle (Sus-vehicle, $n = 7$) or CDPPB (30 mg/kg, s.c.; Sus-CDPPB, $n = 7$) prior to extinction sessions such that there were no differences in prior EPM and ASR scores between treatment groups (**Figure 2B**). To examine the effects of CDPPB on fear extinction, a 2-way RM ANOVA conducted on freezing behavior (**Figure 2C**) revealed a main effect of Treatment [$F(1,11) = 6.803$, $p = 0.024$] and Day [$F(2,22) = 3.905$, $p = 0.035$], and a significant Treatment × Day interaction [$F(2,22) = 5.134$, $p = 0.015$]. *Post hoc* tests revealed that on Day 2, Sus-CDPPB rats froze significantly less than Sus-veh rats, and no between-group differences in behavior were observed on Days 1 or 3. Within the Sus-veh group, Day 2 freezing was greater than Days 1 and 3 (**Figure 2C**). Analysis of Fos immunoreactive cells revealed increased Fos expression in the PL [$t(10) = 2.80$, $p = 0.02$], IL [$t(10) = 3.03$, $p = 0.01$] of Sus-CDPPB rats compared to Sus-veh (**Figure 2F**). In the BLA, a trend toward increased Fos protein expression was detected in Sus-CDPPB group ($p = 0.054$). Co-expression of Fos and mGlu5 was abundant across analyzed regions of both treatment groups, however, the level of Fos/mGlu5 expression overlap varied between regions (Sus-veh, [$F(2,15) = 2.756$, $p = 0.0125$]; Sus-CDPPB [$F(2,15) = 0.830$, $p < 0.0001$]), with lower co-expression in the BLA (∼70%) compared to both IL and PL regions (∼90%; data not shown). Perhaps due to this high degree of co-localization, CDPPB treatment increased Fos expression in mGlu5-positive cells in a manner that was similar to overall Fos expression: expression was increased in the PL [$t(10) = 2.92$, $p = 0.04$] and IL [$t(10) = 3.30$, $p = 0.01$], but not in the BLA (**Figure 2G**). Representative images of dual Fos/mGlu5 labeling from the regions depicted in **Figure 2D** are shown in **Figure 2E**.

Experiment 2

Subsets of Susceptible (Sus) and Resilient (Res) rats were used to generate four treatment groups: Sus-veh, Sus-CDPPB, Sus-CBD, and Res-vehicle. Examining only the rats utilized for Experiment 2, a one-way ANOVA revealed phenotypic differences in EPM open arm time [$F(3,27) = 37.61$, $p < 0.0001$, **Figure 3B**] and ASR habituation [$F(3,27) = 8.028$, $p = 0.0006$]. All three groups of Sus rats spent less time compared to Res rats in the open arms of the EPM (Sus-veh, $p < 0.0001$; Sus-CDPPB, $p < 0.0001$; Sus-CBD, $p < 0.0001$, **Figure 3B**), and exhibited less habituation of the ASR relative to Res rats (Sus-veh, $p = 0.0023$; Sus-CDPPB, $p = 0.0023$; Sus-CBD, $p = 0.0029$, **Figure 3C**). In the light-dark box test, Group differences in time spent in the dark side were

found [$F(3,27) = 4.686$, $p = 0.0092$, **Figure 3D**]. *Post hoc* analysis revealed Sus-veh rats spent more time in the dark side compared to Res-veh, indicating the preservation of anxiety phenotypes 2 weeks after classification with EPM and ASR. In Susceptible rats, CDPPB did not alter time spent in the dark ($p = 0.692$, Sus-CDPPB vs. Sus-veh), however, CBD had an anxiolytic effect, demonstrated by a reduction in dark box time ($p = 0.023$, Sus-CDB vs. Sus-veh). Time spent in the dark in Sus-CBD rats did not differ from Res-veh rats. One-way ANOVA also revealed differences in latency to enter the light box [$F(3,25) = 3.315$, $p = 0.036$, **Figure 3E**], with CDPPB again having no effect, and CBD reducing the time to enter relative to the Sus-veh treated group. No phenotypic or treatment differences were observed in the latency to enter the dark side (**Figure 3F**).

On the day following the light-dark box test, rats began fear extinction trials that continued for 3 days. Prior to each trial animals received CBD (5 mg/kg, i.p.) or vehicle. A two-way repeated measures ANOVA on time spent freezing revealed a significant main effect of group [$F(2,20) = 4.106$, $p = 0.032$] and a trend toward a significant group × time interaction [$F(4,40) = 2.197$, $p = 0.08$]. There was no main effect of time [$F(2,40) = 0.2389$, n.s.] (**Figure 4B**).

DISCUSSION

Here we found that 7 days after a single exposure to the predator odor TMT, populations of stress Susceptible and Resilient rats emerged following phenotype classification using EPM and ASR scores. Similar to previous studies (e.g., Cohen and Zohar, 2004), including our own (Schwendt et al., 2018), 25% of rats met criteria for the Susceptible phenotype and an equal number met criteria for the Resilient phenotype. Moreover, we found that a majority of rats displayed EPM and ASR behavior intermediate between Susceptible and Resilient rats, suggesting that the two phenotypes were indeed representative of extremes of the susceptibility spectrum. In the current study, light-dark box testing 2 weeks after the initial classification revealed that un-conditioned anxiety also differs between Susceptible and Resilient phenotypes.

Consistent with our previous report (Schwendt et al., 2018), the results of Experiment 2 indicate that 3 weeks following the initial TMT context pairing, conditioned fear in Susceptible rats was greater than in Resilient rats. While some previous studies failed to produce a conditioned fear response with TMT (McGregor et al., 2002; Blanchard et al., 2003; Rosen, 2004), these studies only tested for such a response 24 h after TMT exposure. We hypothesize that conditioned freezing in the TMT-paired context here and in our previous report (Schwendt et al., 2018) may be due to the inclusion of a post-stress incubation period. A lengthier exposure time in a medium size chamber may also have contributed to an accumulation of contextual CS information sufficient to generate a defensive response upon re-entry to the TMT-paired context; exposures in smaller chambers do not have this effect (Takahashi et al., 2008). Furthermore, past studies examining TMT fear conditioning have not distinguished stress-Susceptible rodents from Resilient (Wallace and Rosen, 2000; McGregor et al., 2002; Blanchard et al., 2003), hence any conditioning effects may have been obscured.

Consistent with our previous report, we observed an increase in freezing in vehicle-treated Susceptible rats from Day 1 of extinction to Day 2 (Schwendt et al., 2018). CDPPB treated rats demonstrated less freezing on the 2nd day of re-exposure compared to vehicle rats. One potential interpretation of these data is that vehicle-treated rats experienced a potentiation of fear memory reconsolidation on Day 1 that resulted in greater freezing on Day 2; CDPPB prevented this fear memory reconsolidation, yielding reduced freezing. Alternatively, CDPPB treatment may have aided in extinction memory consolidation on Day 1 by enhancing mGlu5 receptor activity within extinction associated neuronal pathways, manifesting as a decrease in freezing on Day 2. As freezing decreased in vehicle-treated rats from Days 2 to 3, these rats may have engaged in extinction acquisition and consolidation during day 2 which was exhibited on Day 3. Extinction of conditioned fear cannot be said to have occurred in the CDPPB-treated group, as no differences in freezing were observed in this group across days, potentially due to a floor effect on freezing on Day 1. Assessment of freezing in CDPPB-treated rats on a subsequent "recall" test conducted in the absence of CDPPB would potentially have shed some light on whether CDPPB is acting acutely to enhance extinction or fear; however, we had designed the present experiment to assess Fos expression on Day 3. Thus, future work will aim to understand whether CDPPB is reducing freezing through the acquisition, consolidation, or expression of fear extinction behavior by administering CDPPB in different regimens, such as immediately after the re-exposure session or only on Day 1 of extinction.

A reduction in conditioned fear in CDPPB-treated rats is in contrast to our previous report that this dose of CDPPB increases freezing when administered prior to fear extinction in rats with a history of cocaine self-administration. It can be argued that chronic self-administration of cocaine altered the neurobiology underlying the extinction of conditioned fear. Alternatively, previous studies in rats and humans have demonstrated bi-phasic effects of the NMDA receptor partial agonist D-cycloserine, which can either produce a weakening or a strengthening of fear memory contingent upon an individual's experience during the extinction session (Orr et al., 2000; Bolkan and Lattal, 2014). Furthermore, in cocaine users, D-cycloserine *prevents* the reductions in craving and brain activation produced by extinction of cocaine-associated cues. Taken together, this suggests that cocaine alters the role of glutamate receptors in mediating extinction of both fear and cocaine-associated cues (Price et al., 2013; Prisciandaro et al., 2013). This should be considered in the treatment of anxiety disorders in patients with cocaine use disorder.

A substantial literature devoted to understanding signaling dynamics involved in the extinction of footshock conditioned fear indicates implicates roles of the IL and PL. Indeed, many studies examining expression of neuronal activity markers in these regions have correlated activity in the PL with freezing, and IL activity with reductions in freezing during extinction (Herry et al., 2008; Cho et al., 2013; Senn et al., 2014). Here we found

increased Fos activation in CDPPB-treated rats in the absence of significant decreases in freezing on the day that the tissue was collected. It is possible that although extinction may have been occurring in both treatment groups on Day 3, the timing of tissue collection did not permit the assessments of the temporal patterns of PL and IL activity during the session. For instance, we cannot determine whether a brief fear recall event at the beginning of Day 3 extinction produced a depolarization of PL cells which may no longer have been firing near the end of the session. Analysis of Fos+mGlu5 co-expression revealed that the increased Fos expression was most likely a result of CDPPB binding, due to the fact that (a) very few Fos-positive cells did not contain mGlu5 mRNA, and (b) increased Fos expression in mGlu5-positive cells mirrored overall Fos protein increase. Thus, it is possible that the increase in Fos expression is an artifact of CDPPB that is not related to freezing behavior or extinction. It may also possible be the case that CDPPB treatment affected non-extinction associated mGlu5 containing cells. In order to understand the role of this circuitry in contextual fear extinction in Susceptible rats, future work will infuse mGlu5-targeting drugs directly into the mPFC and BLA prior to or after fear extinction sessions.

While mGlu5 receptors represent a promising target for reducing conditioned fear, mGlu5 agonists can produce anxiogenic effects under certain conditions (De Jesús-Burgos et al., 2016; Rahman et al., 2017). Therefore, we evaluated the ability of CDPBB to alter unconditioned fear in the light-dark box. We found that CDPPB neither attenuated nor enhanced anxiety in the light-dark box task. This is in contrast to a recent report that the same dose of CDPPB increased anxiety measures in the light-dark box in unstressed mice and in mice that had consumed alcohol (Lee et al., 2018). Thus, CDDPB or other mGlu5 PAMs may be beneficial in a PTSD population as they would reduce conditioned fear without inducing general anxiety, but caution should be exerted in alcohol users.

Although prior studies in heterogeneous rodent populations have observed extinction enhancing properties of CBD in a conditioned footshock model (Bitencourt et al., 2008; Stern et al., 2012; Do Monte et al., 2013), here there was no effect of CBD on conditioned fear in Susceptible rats. It is possible that TMT exposure was not sufficient to evoke the neuroadaptations necessary for CBD-mediated effects. Indeed, one recent study demonstrated that while CBD enhanced extinction in footshock conditioned rats, extinction was unaffected by CBD in rats administered a reduced number of shocks (Song et al., 2016). Although the dose of CBD used in the current study (5 mg/kg) has been previously demonstrated as effective in mitigating the response to contextually conditioned fear (e.g., Jurkus et al., 2016), this dose is at the lower end of an inverted U-shaped response curve for extinction-enhancing effects, which ranges from 3 to 30 mg/kg (Stern et al., 2012). Here, we utilized a lower dose because higher doses of CBD can also produce anxiogenic effects (Song et al., 2016; Lee et al., 2017). The mechanisms by which CBD exerts pro-extinction and anti-anxiety effects are multifaceted, however, several studies have demonstrated a capacity for indirect potentiation of CB1 receptors (Bisogno et al., 2001; Campos et al., 2013). Endocannabinoid (eCB) signaling is implicated in fear extinction through its involvement in synaptic plasticity (Heifets and Castillo, 2009), and dysregulated eCB signaling has been implicated in PTSD (Neumeister et al., 2013). In addition, CBD can function as a partial agonist of the 5-HT1a receptor (Russo et al., 2005; Campos et al., 2012a; Fogaça et al., 2014). Abnormal 5-HT signaling contributes to PTSD like symptoms (Zhao et al., 2017), and is a target for treatment of the disorder (Sullivan and Neria, 2009). Thus, further study is needed to more exhaustively characterize the potential of CBD in PTSD treatment. While CBD did not enhance extinction, it was effective in reducing anxiety in the light-dark box test. Our findings are consistent with previous work showing benefits of CBD in alleviating heightened anxiety in animals that have experienced prior footshock or restraint-stress (O'Brien et al., 2013; Fogaça et al., 2014; Song et al., 2016). While chronic CBD administration following exposure to a live predator was effective in mitigating predator-induced anxiety 1 week later (Campos et al., 2012a), we are the first to show an immediate effect of CBD in a population of Susceptible rats after only a single dose.

CONCLUSION

Here we found evidence that mGlu5 PAMs, such as CDPPB, represent a potential treatment strategy for the reduction of conditioned fear in a PTSD population, an effect possibly mediated by an activation of the PL/IL circuitry. However, some preclinical work (Lee et al., 2018; Schwendt et al., 2018) suggests that caution should be exerted when using this class of drugs in patients with a history of cocaine or alcohol use, as it may inhibit, rather than facilitate extinction. Finally, while CBD produced anxiolytic effects in response to unconditioned fear, it failed to enhance the extinction of conditioned fear in a Susceptible, PTSD-like population of rats. As our results indicate distinct roles for these drugs in unconditioned vs. conditioned anxiety, future consideration should be given to polytherapy with both CBD and mGlu5 PAMs for the enhancement of extinction and relief of anxiety that accompany PTSD. For example, the combination of CBD and an mGlu5 PAM such as CDPPB should be administered for the assessment of unconditioned and conditioned fear.

ETHICS STATEMENT

Procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

AUTHOR CONTRIBUTIONS

JS, MS, and LK involved in the conception and design of the study. JS, PH, MR, and AB involved in the acquisition of data. JS, AB, LK, and MS involved in analysis and interpretation of the data. JS drafted the manuscript. LK and MS made critical revisions. JS, PH, AB, MR, LK, and MS approved the final version of the manuscript to be published.

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frontiers in Behavioral Neuroscience

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Autonomic and Redox Imbalance Correlates With T-Lymphocyte Inflammation in a Model of Chronic Social Defeat Stress

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Patients diagnosed with post-traumatic stress disorder (PTSD) are at a significantly elevated risk of developing comorbid inflammatory conditions, but the mechanisms underlying this predilection remain unclear. Our previous work has shown that T-lymphocytes exposed to elevated levels of norepinephrine (NE) displayed a pro-inflammatory signature reminiscent of an autoreactive phenotype. With this, we hypothesized that the increased sympathetic tone observed during psychological trauma may be promoting pro-inflammatory T-lymphocytes, which causes a predisposition to comorbid inflammatory conditions. Here, we examined the consequences of psychological trauma on splenic T-lymphocytes using a mouse model of repeated social defeat stress. Social defeat led to anxiety-like and depression-like behavior as has been previously described. The spleens of socially-defeated mice showed significant elevations of NE, tyrosine hydroxylase (TH), and acetylcholinesterase (ACHE) levels, which appeared to be due in part to increased expression within T-lymphocytes. Additionally, T-lymphocytes from stressed animals showed higher levels of pro-inflammatory cytokines and mitochondrial superoxide. Interestingly, in this model system, close associations exist within splenic T-lymphocytes amid the autonomic, inflammatory, and redox environments, but these only weakly correlate with individual behavioral differences among animals suggesting the psychological and physiological manifestations of trauma may not be tightly coupled. Last, we describe, for the first time, elevations in calprotectin levels within T-lymphocytes and in circulation of psychologically stressed animals. Calprotectin correlated with both behavioral and physiological changes after social defeat, suggesting the potential for a new biological marker and/or therapeutic target for psychological trauma and its inflammatory comorbidities.

Keywords: post-traumatic stress disorder, PTSD, behavior, norepinephrine, immune, IL-6, IL-17, calprotectin

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INTRODUCTION

Approximately 70% of adults in the United States have experienced some form of traumatic event, and development of post-traumatic stress disorder (PTSD) in this population is estimated at over 20% or 45 million Americans (Kessler et al., 1995, 2005, 2012). PTSD is classified as a trauma and stressor-related disorder, and the disease manifests itself in several behavioral changes including intrusion symptoms, avoidance, and negative alterations in cognitions and mood (American Psychiatric Association, 2013). PTSD patients also demonstrate significantly

elevated risks for the development of comorbid somatic illnesses such as cardiovascular, metabolic, and autoimmune diseases (Boscarino, 2004; Edmondson et al., 2013; Mikuls et al., 2013; Britvic et al., 2015; Lee et al., 2016; Edmondson and von Känel, 2017). While PTSD patients frequently partake in activities that independently increase the chances of developing these disorders (e.g., smoking, drug use, poor diet, lack of exercise, etc.), statistical analyses have explicitly shown a consistent and significantly elevated comorbid disease risk even after controlling for these precarious activities. Moreover, it is unclear if treatment of the behavioral manifestations of PTSD impact the development of these comorbid somatic conditions, suggesting the control mechanisms remain elusive.

One characteristic physiological change of PTSD that may partially explain the development of comorbid somatic diseases is elevated sympathetic nervous system activity and norepinephrine (NE) outflow (Park et al., 2017). Compared to other psychological conditions such as chronic depression, bipolar, or schizophrenia disorders, PTSD patients show significantly elevated NE levels in both urine and cerebrospinal fluid compared to matched controls (Mason et al., 1988; Geracioti et al., 2001, 2008; Strawn et al., 2004). Moreover, targeting of NE *via* pharmacological means using prazosin or clonidine (α1 adrenergic antagonist and α2 adrenergic agonist, respectively), physical manipulation by denervation of the sympathetic chain, as well as anesthetic ganglion blockade have all demonstrated benefits in attenuating the psychological manifestations of the disease (Sutherland and Davidson, 1994; Brady et al., 2000; Telaranta, 2003; Raskind et al., 2007; Lipov et al., 2008, 2012; Lipov and Kelzenberg, 2012). These treatment modalities are highly suggestive of a sympathetic component contributing to PTSD, however, it remains unclear if this dysregulation of autonomic tone is causal to the development of comorbid somatic diseases.

Inflammation is also a theme of all the comorbid diseases described in PTSD to date, and the immune system, particularly T-lymphocytes, appear to be highly sensitive to the psychobiological and sympathetic changes after trauma. For example, PTSD patients have decreased numbers of naïve and regulatory (anti-inflammatory) T-lymphocytes with concurrent increases in memory T-lymphocytes (Sommershof et al., 2009; Wilson et al., 2012). Additionally, circulating levels of various pro-inflammatory cytokines such as interleukin 6 (IL-6) and interleukin 17A (IL-17A) have been shown to be elevated in the PTSD population (von Känel et al., 2007; Zhou et al., 2014; Imai et al., 2018; Maloley et al., 2019). Animal models have corroborated these results showing alterations in both T-lymphocyte populations and cytokine production with various modalities of traumatic stress induction (Avitsur et al., 2002; Hodes et al., 2014). We and others have previously demonstrated that exposure to simply elevated levels of NE can have profound effects on T-lymphocyte activation and cytokine production (Padro and Sanders, 2014; Case and Zimmerman, 2015; Case et al., 2016), and our recent report has elucidated a novel role for the mitochondrial redox environment in NE-mediated T-lymphocyte regulation (Case et al., 2016). Taken together along with the observation that glucocorticoid levels are often not elevated in patients with PTSD (Mason et al., 1988), we hypothesized that the increased sympathoexcitation observed in PTSD is leading to an increased pro-inflammatory T-lymphocyte phenotype *via* redox mechanisms, and it is this inflammation that predisposes these patients to increased incidences of comorbid somatic diseases.

To address this hypothesis, herein, we utilized an established and accepted mouse model of psychological trauma known as repeated social defeat (Golden et al., 2011; Deslauriers et al., 2018). We show that these animals demonstrated altered behavior, dysregulated autonomic balance with elevated sympathetic tone, and increased T-lymphocyte pro-inflammatory cytokine production concurrent with a disrupted mitochondrial redox environment, which confirms and extends our previous observations using *in vitro* systems (Case et al., 2016). However, examination of individual animal differences identified that only a few physiological parameters associated significantly with specific behavioral phenotypes, but are highly related to other respective physiological elements. Last, T-lymphocyte RNA sequencing identified expression of a novel and unexpected inflammatory protein (i.e., calprotectin) within these cells from stressed animals that correlates with both behavioral phenotypes and physiological readouts, suggesting the potential for a new biomarker and/or regulatory player of psychological trauma.

MATERIALS AND METHODS

Mice

All control and experimental stress animals were 8-12 week-old male wild-type mice of a C57BL/6J background (Jackson Laboratory #000664, Bar Harbor, ME, USA). The social defeat stress paradigm precludes the use of female mice, thus, sex differences were not examined and not within the scope of the study described herein. All aggressive mice were 4-6 month-old retired breeder male mice of a CD-1 background (Charles River #022, Wilmington, MA, USA). Experimental mice were bred in-house to eliminate shipping stress and environmental changes. Littermates were group housed ($\leq$5 mice per cage) prior to the stress induction protocol to eliminate social isolation stress. Mice were housed with standard corncob bedding, paper nesting material, and given access to standard chow (Teklad Laboratory Diet #7012, Harlan Laboratories, Madison, WI, USA) and water *ad libitum*. Mice were euthanized by pentobarbital overdose (150 mg/kg, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) administered intraperitoneally. All mice were sacrificed between 07:00 and 09:00 Central Standard Time to eliminate circadian rhythm effects on T-lymphocyte function. Mice were randomized prior to the start of all experiments, and when possible, experimenters were blinded to the control and stress groups of mice until the completion of the study. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Social Defeat Stress Paradigm

An adapted version of the social defeat stress paradigm described by Golden et al. (2011) was utilized for all studies, and is

summarized in **Figure 1A**. First, retired male breeder CD-1 mice (pre-screened thrice for aggressive behavior) were allowed to inhabit standard cages outfitted with two sets of food, water, and bedding 3 days before the start of an experiment to allow territory establishment by these mice. On day 1, all elements of the cage (except corncob bedding) were temporarily removed, and an experimental mouse was introduced into the cage for 5 min to allow for a physical confrontation. After the 5 min interaction period, the mice were separated within the same cage by a transparent perforated barrier, and all housing elements were placed back into the cage. The mice were then co-housed with physical separation for the remainder of the 24 h period, and the process was repeated again by rotating the experimental mouse to a different CD-1 cage for 10 days. Mice were excluded from the study if they showed signs of wounding or lameness after social defeat sessions. Control mice were pair housed using identical separation and barrier housing techniques, but not allowing for any physical confrontation between mice during the 24 h periods. At the end of the 10 day period (day 11), all mice were assessed for behavioral changes using both social interaction and elevated zero tests. After testing, control and experimental mice remained in their former co-housed barrier cage until the following day (day 12) when they were sacrificed for biological analysis.

Elevated Zero Maze

The elevated zero maze test was utilized to assess anxiety-like behavior (Walf and Frye, 2007). An elevated circular maze consisting of 50% open and 50% enclosed quadrants was applied for these tests (50 cm diameter, 5 cm track width, 20 cm wall height, 61 cm stand height; Noldus Information Technology, Leesburg, VA, USA). Control and stress mice were introduced into a closed arm of the maze and allowed to explore the novel environment for 5 min. Runs were performed with one mouse at a time, and the maze was thoroughly cleaned using water followed by 70% ethanol (allowing time for evaporation) to eliminate olfactory variables before the next mouse was tested. Sessions were recorded, tracked, and analyzed using Noldus Ethovision XT 13 software. Tests were performed within the housing room of mice during the light cycle using approximately 265 lux of ambient lighting at the testing arena.

Social Interaction Test

The social interaction test was utilized to assess depressive-like behavior (Golden et al., 2011). An open field chamber (40 cm wide, 40 cm long, 30 cm walls; Noldus Information Technology, Leesburg, VA, USA) was outfitted with a small wire mesh enclosure (6.5 cm wide, 10 cm long, 30 cm height; Noldus Information Technology, Leesburg, VA, USA) on one side. Control and stress mice were introduced into the open field and allowed to explore their environment with an empty mesh enclosure for 2.5 min. Runs were performed with one mouse at a time, and the chamber was thoroughly cleaned using water followed by 70% ethanol (allowing time for evaporation) to eliminate olfactory variables before the next mouse was tested. After all mice were assessed with an empty mesh enclosure, a different mesh enclosure was introduced into the open field containing a novel CD-1 aggressive mouse. Control and experimental mice were run in the aforementioned manner in the presence of an enclosed CD-1 mouse for 2.5 min. Sessions were recorded, tracked, and analyzed using Noldus Ethovision XT 13 software. Social interaction and corner zone ratios were calculated by the amount of time spent in the respective zones with a CD-1 present in the enclosure vs. absent. Tests were performed within the housing room of mice during the light cycle using approximately 265 lux of ambient lighting at the testing arena.

T-Lymphocyte Isolation

T-lymphocytes were isolated and cultured as previously described (Case et al., 2016). Briefly, splenic T-lymphocytes were negatively selected using the EasySep Mouse T-Cell Isolation Kit (StemCell Technologies #19851, Vancouver, BC, USA). The purity (assessed by flow cytometry) and viability (assessed by a Bio-Rad TC20 Automated Cell Counter using trypan blue exclusion) of the T-lymphocytes were randomly quality controlled and found to be >90%.

Catecholamine ELISA

Total catecholamines were assessed in plasma and splenic lysates using the 3-CAT research ELISA (Rocky Mountain Diagnostics #BAE-5600, Colorado Springs, CO, USA) as per manufacturer's instructions. Splenic catecholamine amounts were normalized to starting splenic weights, and then to controls within respective experiments.

Western Blot Analysis

Western blotting for the quantification of proteins was performed as previously described (Case et al., 2013). Briefly, whole-cell soluble lysate (30 μg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with antibodies directed against tyrosine hydroxylase (TH; 1:1,000 dilution, EMD Millipore #AB152, Burlington, MA, USA) and actin (1:1,000 dilution, Sigma Aldrich #A2066, St. Louis, MO, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000, Thermo Fisher #31460, Waltham, MA, USA). Densitometric analysis of band intensity was determined using ImageJ analysis software.

RNA Extraction, cDNA Production, and Quantitative Real-Time RT-PCR

Assessment of mRNA levels was performed as previously described (Case and Zimmerman, 2015). Briefly, total RNA was extracted from purified T-lymphocytes using the RNAeasy mini kit (Qiagen # 74104, Valencia, CA, USA) according to the manufacturer's protocol. Concentration of RNA was determined spectrophotometrically using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems #4374966, Grand Island, NY, USA) was used to obtain cDNA from total RNA. Generated cDNA was then subjected to SYBR green (Applied Biosystems #4385612, Grand Island, NY, USA) quantitative real-time PCR with primers specific to the coding sequence of the respective genes (**Supplementary Table S1**). PCR product

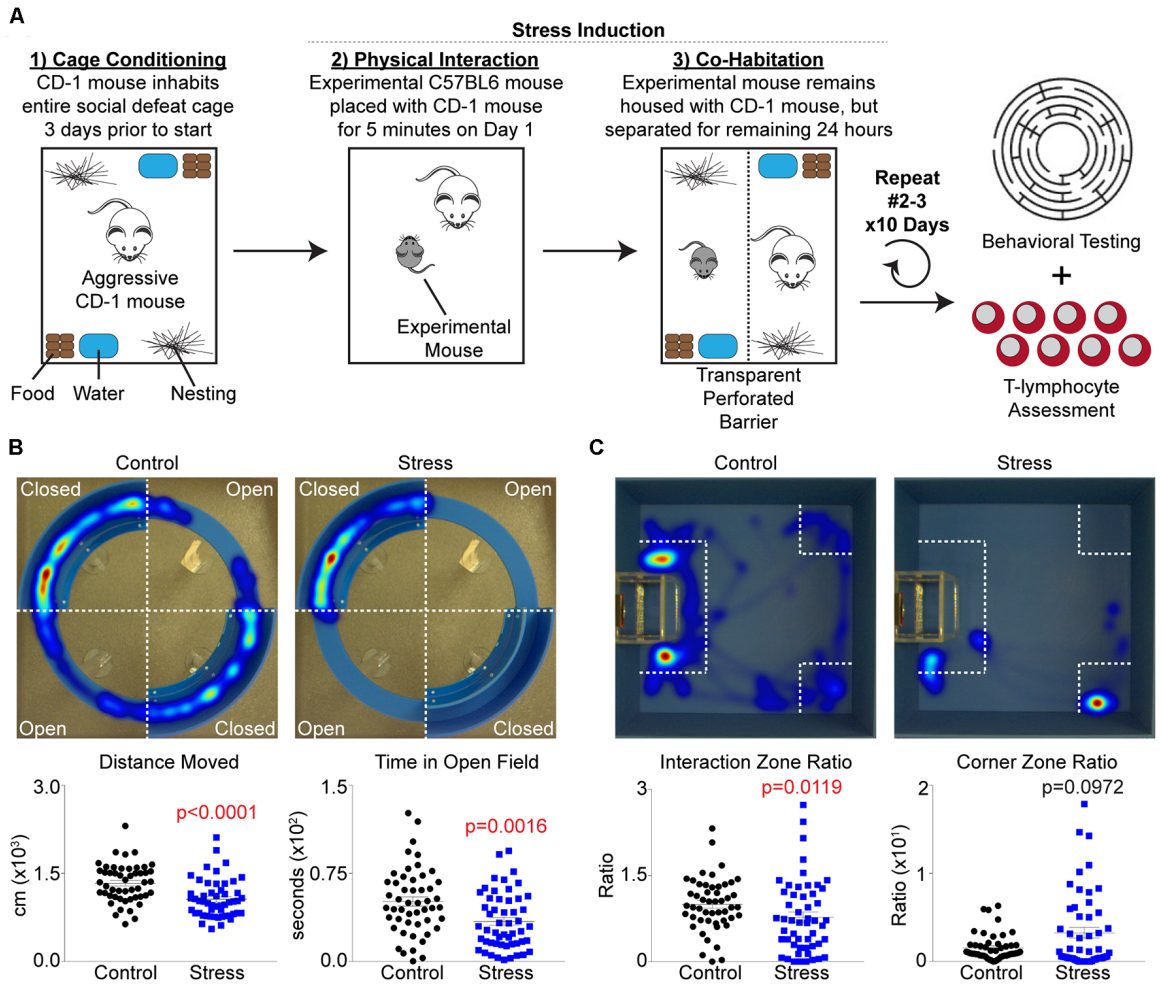

FIGURE 1 | Repeated social defeat stress increases anxiety-like and depression-like behavior. **(A)** Overview of experimental setup and timeline. **(B)** *Upper*, representative heat map tracings of the elevated zero maze analysis for control and socially-defeated (Stress) animals. Hot colors indicate more time spent in a specific area, while cool colors represent less time. *Lower*, quantification of elevated zero maze parameters. Distance Moved (N = 50 controls, 52 stress; 2-tailed; Mann-Whitney U = 655.5), Time in Open Field (N = 50 controls, 52 stress; 2-tailed; Mann-Whitney U = 832.5). **(C)** *Upper*, representative heat map tracings of the social interaction test. Color spectrum identical to elevated zero maze. *Lower*, quantification of social interaction test parameters. Interaction Zone Ratio (N = 50 controls, 52 stress; 2-tailed; Mann-Whitney U = 926.0), Corner Zone Ratio (N = 50 controls, 52 stress; 2-tailed; Mann-Whitney U = 1295.0).

specificity was determined by thermal dissociation. A threshold in the linear range of PCR amplification was selected and the cycle threshold (Ct) determined. Levels of transcripts were then normalized to the 18s rRNA loading control (ΔCT). For all analyses, 1/ΔCT was utilized to assess levels of transcripts in a directional manner relative to expression with only normalization to the 18s rRNA loading control.

Flow Cytometric Redox Assessment

Mitochondrial-specific assessment of specific redox species was performed as previously described (Case et al., 2016). Briefly, cells were stained with 1 μM MitoSOX Red ($O_2^{\bullet-}$-sensitive mitochondrial-localized probe, Thermo Fisher Scientific #M36008, Waltham, MA, USA) for 30 min at 37°C. Cells were analyzed on a LSRII flow cytometer at 488/610 nm ex/em, and data analyzed using FlowJo software.

Cytokine Analysis

Analysis of circulating levels of cytokines was performed using a Meso Scale Discovery 35 U-Plex Mouse Biomarker Group (#K15083K-1, Rockville, MD, USA) per manufacturer's instructions. Samples were assessed using a Meso Scale QuickPlex SQ 120, and analyzed using Meso Scale Discovery software.

Single-Cell RNA Sequencing

Single-cell RNA sequencing was performed on one control and one socially-defeated animal (verified by behavior testing) as a preliminary discovery method for changes in T-lymphocyte gene expression. Erythrocyte-depleted splenocyte cell suspensions were evaluated by light microscopy for debris and viability and were counted using a hemocytometer. Cell concentrations were 932 and 888 live cells/ul respectfully. Targeting approximately

2,400 single cells per sample, single cells were captured, lysed, and RNA was reverse transcribed and barcoded using a 10× Genomics Chromium instrument and Chromium Single Cell 3′ Reagent Kits v2 reagents (10× Genomics, Pleasonton, CA, USA). To construct Illumina compatible sequencing libraries the cDNA was fragmented, A-tail repaired and a double-sided bead cleanup was performed. Adapters were ligated to the cDNA fragments and the fragments were PCR amplified using unique sample index primers per manufacturer's recommendations. Libraries were quantified by qPCR using the KAPA Library Quant Kit (Illumina) from KAPA Biosystems (Roche, Pleasonton, CA, USA). Libraries were loaded on two Illumina MidOutput V2 150 cycle flowcells at a concentration of 1.3 pM. FASTQ files were delivered to the Bioinformatics and Systems Biology Core where raw sequencing data went through 10× Genomics software cell ranger pipeline in the following order: (1) demultiplexed the Illumina sequencer's base call files (BCLs) for each flowcell directory into FASTQ files; (2) generated single cell feature counts for each of the libraries and performed mapping/clustering; and (3) aggregated the analysis results from different libraries. A software loupe and cell ranger R kit were used for differential analyses of selected cell groups by marker gene identifications. Counts were then compared between control and stressed samples by Log2 fold changes applying two-tailed Fisher exact tests based on false discovery rate (FDR) cut-off of 0.05. The expression data was submitted to ArrayExpress repository.

Statistics

A total of 102 animals (50 control, 52 stress) were utilized in these studies. Not all physiological parameters were able to be run on a single animal, thus, each graph is individually labeled with *N* values and statistical information utilized for a specific set of experiments. Individual data are presented along with mean ± standard error of the mean (SEM). For two group or three group comparison, significance was assessed using the Mann-Whitney *U*-test or Kruskal-Wallis test due to the non-parametric distribution of the data. Correlations were performed using linear regression with Pearson correlation coefficient calculations. Differences were considered significant at $p < 0.05$, and exact *p*-values are displayed on individual graphs.

RESULTS

Repeated Social Defeat Stress Increases Anxiety-Like and Depression-Like Behavior

Several animal models exist that mimic the behavioral changes of human PTSD, and while repeated social defeat stress (**Figure 1A**) does not recapitulate all of these human PTSD phenotypic changes, it was chosen for this study due to its reproducible impact on inflammation (Deslauriers et al., 2018). Specific behavioral changes were first tested using an elevated zero maze to assess anxiety-like behavior and locomotor activity. Socially-defeated mice demonstrated decreased locomotor activity as evidenced by decreased total distance moved as well as increased anxiety-like behavior due to less time spent in the open arms of the elevated zero maze compared to control animals (**Figure 1B**). Additional parameters such as latency to first, body elongation, or head directed towards the open field only trended significance (data not shown). Depression-like and antisocial behavior was also confirmed by the use of a social interaction test, where stressed animals displayed less time spent in the interaction zone and more time in the corner zone relative to controls (**Figure 1C**). Together, these data support previous reports that repeated social defeat stress increases anxiety-like and depression-like behavior (Krishnan et al., 2007; Golden et al., 2011). However, these previous reports have suggested that stressed mice may be classified as "susceptible" or "resilient" based on a social interaction ratio threshold of 1.0 (Krishnan et al., 2007; Golden et al., 2011). When this analysis was performed on our mice, we observed that the "resilient" group only represented approximately 30% of the stressed mice, but moreover, was statistically different from control animals in regards to the average social interaction ratio (**Supplementary Figure S1A**). Furthermore, when examining locomotor activity and anxiety-like behavior on the elevated zero maze, no differences were observed between resilient and susceptible groups (**Supplementary Figure S1B**). Due to these discrepancies, data were processed using only major group categories (i.e., control vs. stress) as well as correlation analyses among all mice to identify dimensional individual differences among behavioral and physiological parameters.

Increased Sympathetic Signatures Are Observed in T-Lymphocytes From Stressed Mice, but Do Not Correlate With Behavior

Aforementioned, sympathoexcitation is a hallmark of PTSD. To assess this in our animal model, we first measured circulating levels of catecholamines at the completion of the stress induction paradigm. Unexpectedly, we did not observe any differences in circulating levels of catecholamines but identified significant increases within the spleen following social defeat (**Figure 2A**). This lack of increased circulating catecholamines suggested the potential for elevated sympathetic neuronal activity to the spleen. To assess this, we next evaluated the level of TH (the rate-limiting enzyme of catecholamine synthesis) protein in the spleen and observed significant increases in stressed animals (**Figure 2B**). To understand if the increased TH was due to potentiated neuronal expression of the protein, we excluded neurons by performing quantitative real-time RT-PCR analysis for TH mRNA in purified T-lymphocytes. To our surprise, we identified large and significant increases for TH message within T-lymphocytes (**Figure 2C**). We further evaluated other neurotransmitter synthetic and degradative enzyme mRNA levels within purified splenic T-lymphocytes and identified trending increases in monoamine oxidase A (MAO-A), trending decreases in choline acetyltransferase (CHAT), as well as significant increases in acetylcholinesterase (ACHE; **Figure 2C**). Together, these enzyme levels displayed a pro-sympathetic neurotransmission gene

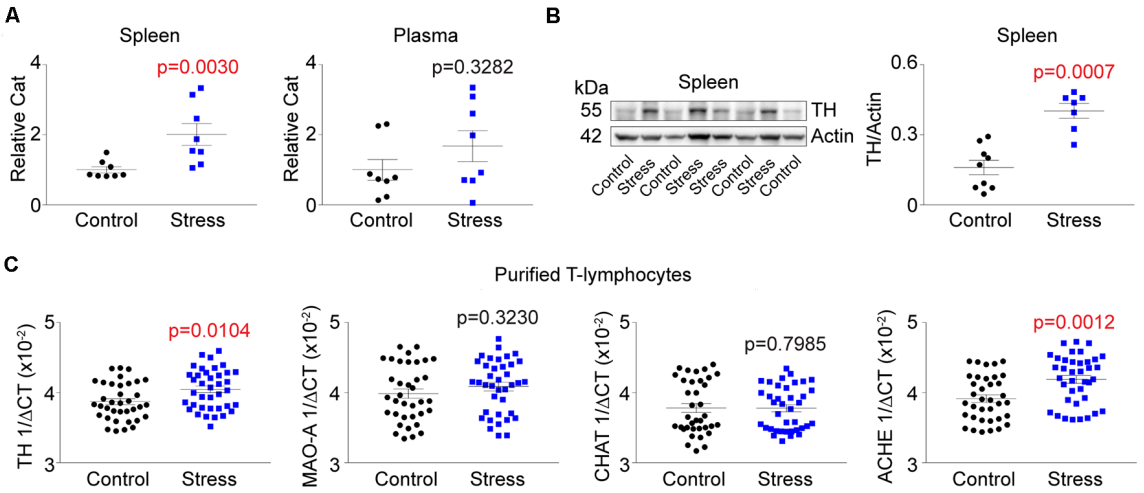

FIGURE 2 | T-lymphocyte sympathetic tone is increased with psychological trauma. Plasma, whole spleens, and purified splenic T-lymphocytes were isolated following the social defeat (Stress) paradigm. **(A)** Quantification of total catecholamines (Cat) in the spleens and plasma. Splenic values were first normalized to spleen weight. All values are displayed normalized to respective controls per experiment. Spleen (*N* = 8 controls, 8 stress; 2-tailed; Mann-Whitney *U* = 5), Plasma (*N* = 8 controls, 8 stress; 2-tailed; Mann-Whitney *U* = 22). **(B)** *Left*, representative western blot analysis for splenic tyrosine hydroxylase (TH) content. *Right*, quantification of TH content by western blot. (*N* = 9 controls, 7 stress; 2-tailed, Mann-Whitney *U* = 2). **(C)** Quantitative real-time RT-PCR analysis for various neurotransmission enzyme mRNA levels in purified T-lymphocytes. Data are shown as 1/ΔCT as normalized by 18s rRNA loading control. Monoamine oxidase A (MAO-A); choline acetyltransferase (CHAT); acetylcholinesterase (ACHE). TH (*N* = 36 controls, 38 stress; 2-tailed, Mann-Whitney *U* = 448.5), MAO-A (*N* = 36 controls, 38 stress; 2-tailed, Mann-Whitney *U* = 592.0), CHAT (*N* = 36 controls, 38 stress; 2-tailed, Mann-Whitney *U* = 660), ACHE (*N* = 36 controls, 38 stress; 2-tailed, Mann-Whitney *U* = 338.5).

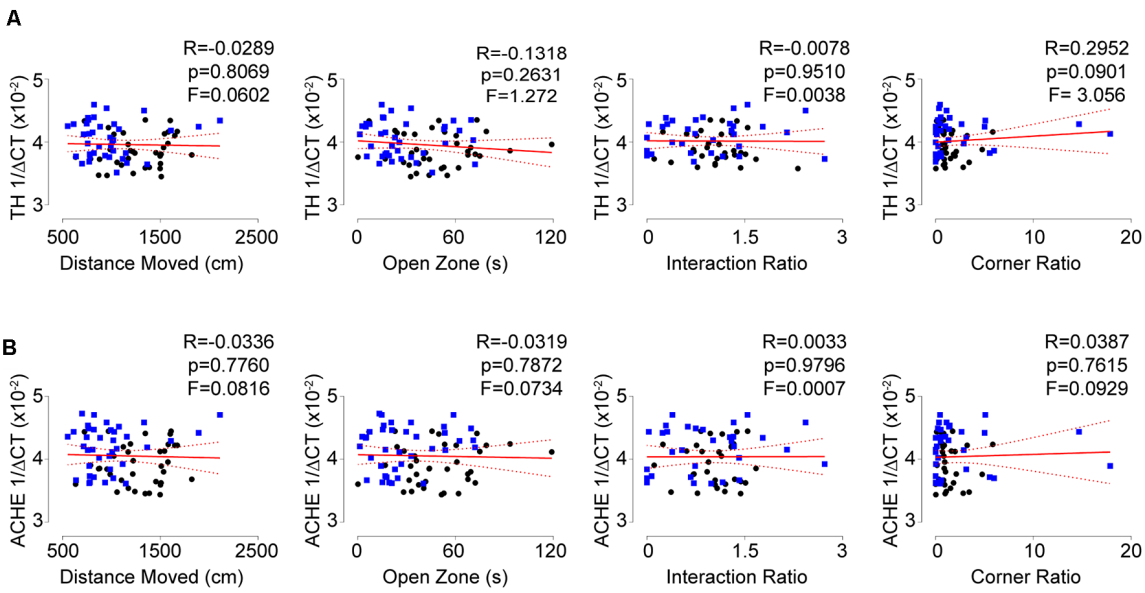

FIGURE 3 | Pro-sympathetic neurotransmission signatures in splenic T-lymphocytes do not correlate with behavior. **(A)** Correlation of TH mRNA levels within splenic T-lymphocytes with anxiety-like and depression-like behavior indices. (*N* = 36 controls, 38 stress. DFn, Dfd = 1,72 for all). **(B)** Correlation of acetylcholinesterase (ACHE) mRNA levels within splenic T-lymphocytes with anxiety-like and depression-like behavior indices. (*N* = 36 controls, 38 stress. DFn, Dfd = 1,72 for all). Black circles indicate control animals; blue squares indicate socially-defeated (Stress) animals. Statistics obtained using linear regression with Pearson correlation coefficient calculations (red line; 95% confidence interval indicated as dotted red line).

signature within purified T-lymphocytes after stress, which suggested the potential for lymphocyte-specific neurotransmitter production in response to stress. Interestingly, T-lymphocyte expression of genes driving a pro-sympathetic environment did not correlate with individual differences in anxiety-like or depression-like behavior (**Figure 3**).

Psychological Trauma Elicits Elevations in T-Lymphocyte Mitochondrial Superoxide and Pro-inflammatory Cytokine Expression

Our previous work demonstrated that T-lymphocytes exposed to NE expressed increased levels of IL-6 and IL-17A that was driven in part due to amplified mitochondrial superoxide production (Case et al., 2016). Understanding that social defeat elevated catecholamine levels in proximity to T-lymphocytes, we examined the effects of stress on T-lymphocyte redox and inflammatory environments. We first observed that social defeat caused a significant decrease in the percentage of splenic T-lymphocytes (**Figure 4A**), which has been previously reported in a similar trauma animal model (Avitsur et al., 2002). Assessment of these remaining splenic T-lymphocytes showed an approximate 2-fold induction of mitochondrial superoxide levels compared to controls (**Figure 4B**). Mitochondrial superoxide was not altered in circulating T-lymphocytes (data not shown), which further supported the importance of direct interaction with catecholamines. No change was observed in splenic T-lymphocyte nitric oxide levels from socially-defeated animals (**Supplementary Figure S2**), demonstrating not all redox signaling is perturbed with psychological stress. IL-6 and IL-17A levels were increased in circulation of socially-defeated animals (**Figures 4C,D**), and mRNA levels for these cytokines were also specifically and significantly elevated within splenic T-lymphocytes (**Figures 4E,F**). Overall, these data confirm and extend our previous *in vitro* findings in a relevant *in vivo* model of psychological trauma that catecholamines impact T-lymphocyte inflammation likely *via* redox mechanisms.

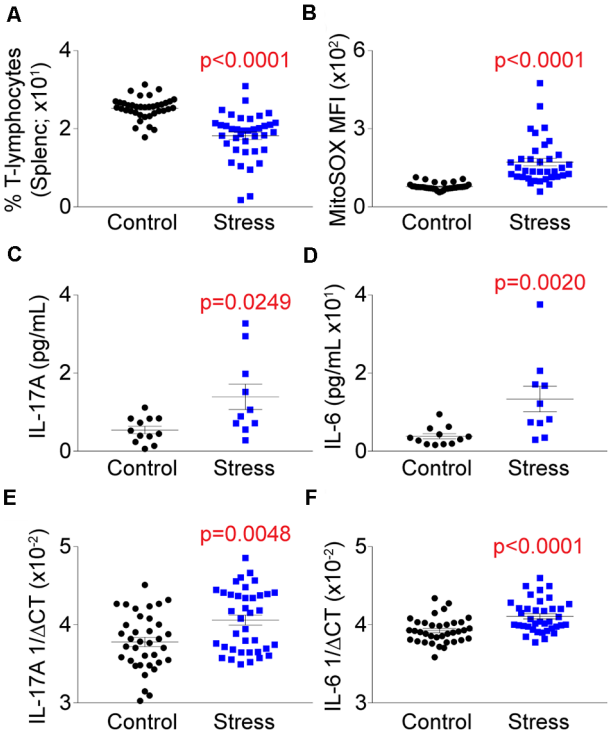

FIGURE 4 | Social defeat stress elevates the mitochondrial redox and pro-inflammatory environments of splenic T-lymphocytes. Plasma, whole spleens, and purified splenic T-lymphocytes were isolated following the social defeat paradigm. **(A)** T-lymphocyte counts from control and socially-defeated (Stress) animals represented as percent of total splenocyte count. (N = 38 controls, 38 stress; 2-tailed; Mann-Whitney U = 156.5). **(B)** Quantification of MitoSOX Red mean fluorescent intensity (MFI) of splenic T-lymphocytes assessed by flow cytometry. (N = 35 controls, 36 stress; 2-tailed; Mann-Whitney U = 56). **(C,D)** Circulating levels of interleukin 17A (IL-17A) and interleukin 6 (IL-6) assessed by mesoscale multiplex analysis. IL-17A (N = 12 controls, 10 stress, 2-tailed; Mann-Whitney U = 26.0), IL-6 (N = 12 controls, 10 stress, 2-tailed; Mann-Whitney U = 15.0). **(E,F)** Quantitative real-time RT-PCR analysis of IL-17A and IL-6 mRNA within splenic T-lymphocytes. Data are shown as 1/ΔCT as normalized by 18s rRNA loading control. (N = 36 controls, 38 stress; 2-tailed; Mann-Whitney U = 425.5), IL-6 (N = 36 controls, 38 stress; 2-tailed; Mann-Whitney U = 330.5).

T-Lymphocyte Mitochondrial Superoxide Correlates With Anxiety-Like Behavior, While IL-6 Expression Associates With Depression-Like Behavior

To assess if the T-lymphocyte mitochondrial redox and inflammatory environments had any impact on behavior, we performed correlation analyses on all animals comparing individual behavioral indices and these physiological readouts. Intriguingly, a positive correlation was observed between T-lymphocyte mitochondrial superoxide levels and anxiety-like behavior, but not depression-like behavior (**Figure 5A**). In contrast, splenic T-lymphocyte expression of IL-6 or IL-17A did not correlate with anxiety-like behavior indices but IL-6 positively correlated with depression-like behavior indices (**Figures 5B,C**), which has been previously reported (Hodes et al., 2014). Together, these data suggest that splenic T-lymphocyte mitochondrial superoxide levels may serve as an indicator of anxiety-like behavior, whereas specific inflammatory components may be more predictive of depressive-like symptoms in the social defeat stress model.

Significant Associations Exist Between the Autonomic, Redox, and Inflammatory Signatures of T-Lymphocytes

While the biological changes observed with repeated social defeat did not completely associate with individual behavioral phenotypes, we next set out to address if these physiological changes correlated with each other. Strikingly, we observed strong and significant positive correlations among all combinations of biological measures including T-lymphocyte mitochondrial superoxide and IL-6, IL-17A, TH, and ACHE transcript levels (**Figures 6A,B**, **Supplementary Figures S3A,B**). These data are highly suggestive of crosstalk between the autonomic, redox, and inflammatory pathways in T-lymphocytes during psychological trauma.

Identification of Calprotectin as a Novel Indicator of Behavioral and Physiological Changes During Psychological Trauma

Understanding that repeated social defeat stress significantly impacted the expression of several genes within T-lymphocytes,

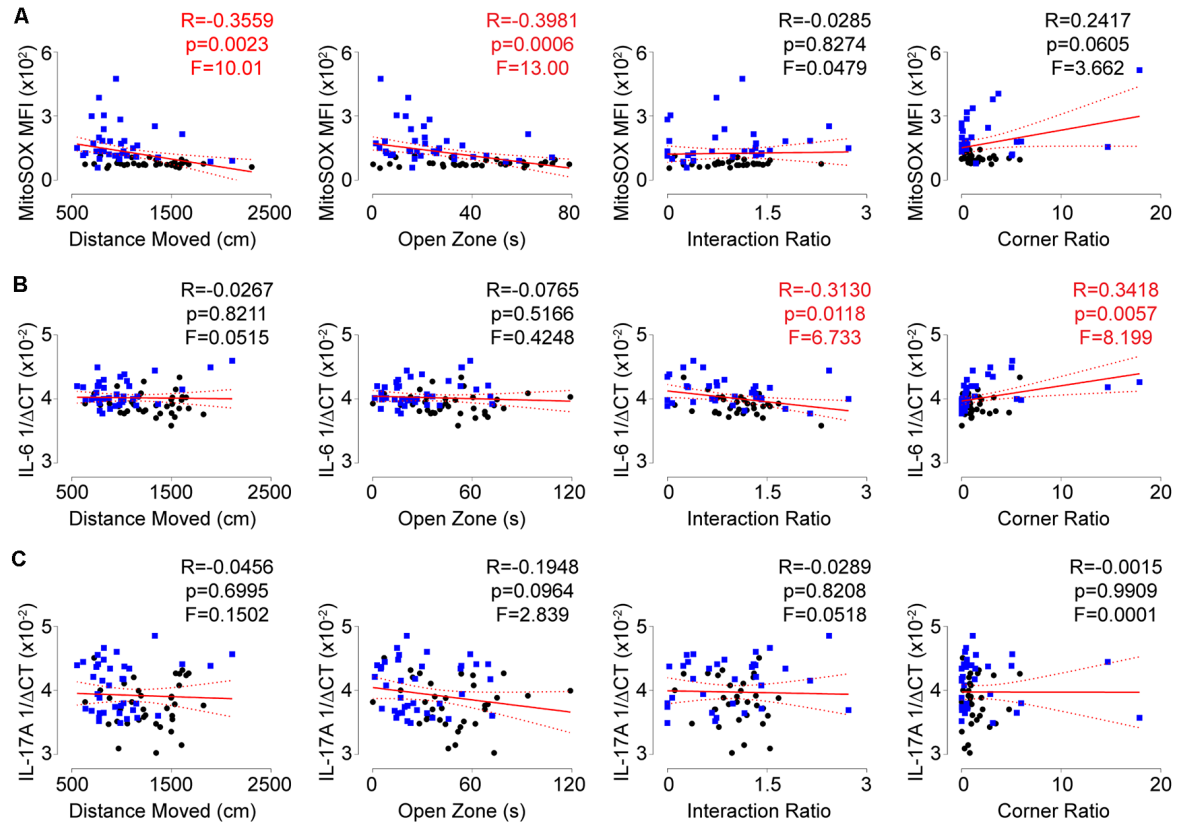

FIGURE 5 | Redox and inflammatory parameters show differential associations with behavior. **(A)** Correlation of splenic T-lymphocyte MitoSOX Red MFI with anxiety-like and depression-like behavior indices. (*N* = 35 controls, 36 stress. DFn, Dfd = 1,69 for all). **(B)** Correlation of splenic T-lymphocyte IL-6 mRNA levels with anxiety-like and depression-like behavior indices. (*N* = 36 controls, 38 stress. DFn, Dfd = 1,72 for all). **(C)** Correlation of splenic T-lymphocyte IL-17A mRNA levels with anxiety-like and depression-like behavior indices. (*N* = 36 controls, 38 stress. DFn, Dfd = 1,72 for all). Black circles indicate control animals; blue squares indicate socially-defeated (Stress) animals. Statistics obtained using linear regression with Pearson correlation coefficient calculations (red line; 95% confidence interval indicated as dotted red line). Values highlighted in red demonstrate statistical significance.

we next performed single-cell RNA sequencing analysis on splenocytes from socially-defeated and control animals (**Supplementary Table S2**). Interestingly, two of the most significantly upregulated genes in the T-lymphocyte population were calgranulin A (S100a8; Log2 fold increase +4.41, $p = 8.6 \times 10^{-19}$) and calgranulin B (S100a9; Log2 fold increase 4.28, $p = 1.3 \times 10^{-13}$), which together form the heterodimeric protein calprotectin. Calprotectin possesses both intracellular and extracellular properties but is thought to be primarily produced by neutrophils and monocytes making its detection in T-lymphocytes quite unexpected. We validated mRNA levels of the respective transcripts and observed large inductions within splenic T-lymphocytes from stressed animals that correlated strongly with one another (**Figures 7A,B**). Calprotectin protein was also increased approximately 3-fold in circulation of stressed animals and positively correlated only with anxiety-like behavior (**Figures 7C,D**). T-lymphocyte expression of S100a8 and S100a9 showed a similar positive correlation with anxiety-like behavior, but also positively correlated with the corner zone ratio of the social interaction test (**Supplementary Figures S4A,B**). Interestingly, S100a8 and S100a9 mRNA levels in splenic T-lymphocytes only correlated with mitochondrial superoxide levels, IL-6, and ACHE expression, suggesting a dissociative expression pattern compared to the other dysregulated genes (**Supplementary Figures S5A,B**). Together, we describe for the first time the observation of psychological trauma-induced calprotectin expression that associates with both behavioral and physiological alterations of repeated social defeat stress.

DISCUSSION

In the current study, we identify several previously undescribed links between the autonomic, redox, and inflammatory environments of splenic T-lymphocytes during psychological trauma. Together, these provide new insights into the regulatory control of the adaptive immune system during stress and suggest potential pathways which may lead to the increased incidence of inflammatory comorbidities in diseases like PTSD.

PTSD is a multifaceted disease that has proven difficult to replicate in animal models. A recent review by Deslauriers et al. (2018) has elegantly summarized accepted animal models that recapitulate PTSD behavioral and biological phenotypes.

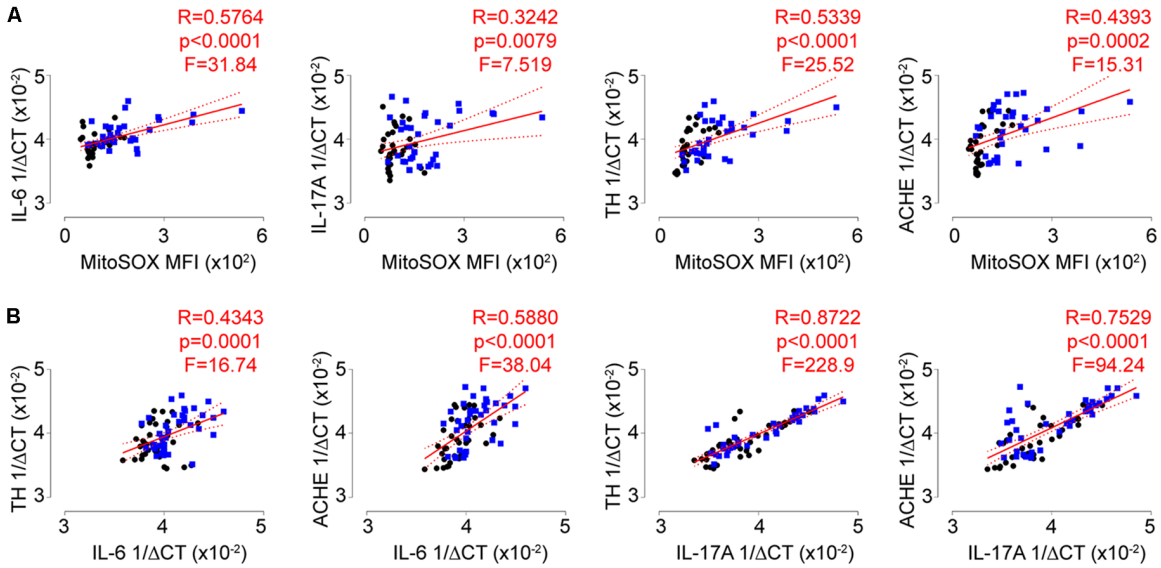

FIGURE 6 | Autonomic, redox, and inflammatory T-lymphocyte signatures are highly correlated. **(A)** Correlation of splenic T-lymphocyte MitoSOX Red MFI with splenic T-lymphocyte inflammatory (interleukin 6, IL-6; interleukin 17A, IL-17A) and autonomic (tyrosine hydroxylase, TH; acetylcholinesterase, ACHE) genes. **(***N* = 32 controls, 34 stress. DFn, Dfd = 1,64 for all). **(B)** Correlation of splenic T-lymphocyte inflammatory (IL-6; IL-17A) with autonomic (TH; ACHE) genes. (*N* = 36 controls, 38 stress. DFn, Dfd = 1,72 for all). Black circles indicate control animals; blue squares indicate socially-defeated (Stress) animals. Statistics obtained using linear regression with Pearson correlation coefficient calculations (red line; 95% confidence interval indicated as dotted red line). Values highlighted in red demonstrate statistical significance.

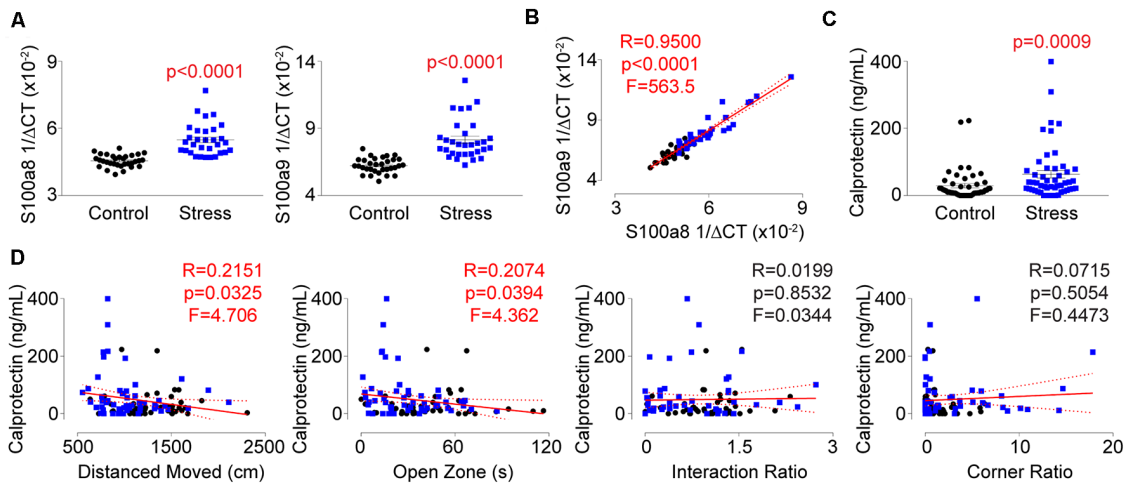

FIGURE 7 | Identification of calprotectin as a novel marker of psychological trauma. Plasma, whole spleens, and purified splenic T-lymphocytes were isolated following the social defeat paradigm. **(A)** Quantitative real-time RT-PCR analysis of S100a8 and S100a9 mRNA levels within splenic T-lymphocytes. Data are shown as 1/ΔCT as normalized by 18s rRNA loading control. S100a8 (*N* = 32 controls, 31 stress; 2-tailed; Mann-Whitney *U* = 58.0), S100a9 (*N* = 32 controls, 31 stress; 2-tailed; Mann-Whitney *U* = 47.5). **(B)** Correlation of splenic T-lymphocyte levels of S100a8 and S100a9 mRNA levels. (*N* = 32 controls, 31 stress. DFn, Dfd = 1,61). Statistics obtained using linear regression with Pearson correlation coefficient calculations (red line; 95% confidence interval indicated as dotted red line). **(C)** Circulating calprotectin levels assessed by ELISA. (*N* = 50 controls, 52 stress; 2-tailed, Mann-Whitney *U* = 756). **(D)** Correlation of circulating calprotectin levels with anxiety-like and depression-like behavior indices. (*N* = 50 controls, 52 stress. DFn, Dfd = 1,100 for all). Black circles indicate control animals; blue squares indicate socially-defeated (Stress) animals. Statistics obtained using linear regression with Pearson correlation coefficient calculations (red line; 95% confidence interval indicated as dotted red line). Values highlighted in red demonstrate statistical significance.

There are currently six animal models that are able to mimic the behavioral and biological phenotypes of PTSD, but each has demonstrated at least one phenotype of the human disease that is not able to be fully recapitulated. We chose the repeated social defeat model based on its ability to robustly and consistently produce increased anxiety-like behavior,

depression-like behavior, as well as peripheral inflammation (Deslauriers et al., 2018). As described in this review article, repeated social defeat is only one of two accepted animal models that produce a peripheral inflammatory response similar to human PTSD, which was the primary focus of this work. However, social defeat is limited in the fact that it does not demonstrate hallmark phenotypes of PTSD such as decreased fear extinction or increased HPA feedback. Additionally, the standard repeated social defeat does not allow for the use of female mice, which precludes examination of sex differences [An alternative version of social defeat using genetically modified CD-1 mice has been reported to be used with females (Takahashi et al., 2017)]. Therefore, while we have identified novel redox and inflammatory findings using a repeated social defeat model, due to the limitations of this model, further investigation is warranted to validate these observations in additional models of PTSD such as unpredictable variable stress, predator exposure, inescapable foot shocks, or single prolonged stress.

Repeated social defeat has also previously been reported to generate both "resilient" and "susceptible" phenotypes across stressed animals (Krishnan et al., 2007; Golden et al., 2011; Friedman et al., 2014). This grouping is based off of the social interaction behavior test, and the threshold cut-off is set at 1.0 (the value in which a mouse will enter the interaction zone at the same frequency with and without a CD-1 present). Using these categories, these two groups of mice have been shown to have different physiological phenotypes (Krishnan et al., 2007; Friedman et al., 2014; Hodes et al., 2014), but in contrast, also show no differences in many behavioral phenotypes (Krishnan et al., 2007). When performing this categorization on our animals, we found that approximately 30% of stressed animals were found to be "resilient," which is within the range (albeit the extreme low end) previously reported by Golden et al. (2011). However, unlike previous reports, the resilient group here was statistically different in regards to their social interaction ratio (and other parameters) compared to control animals. When examining the data closely, this difference is because animals demonstrating low social interaction ratios exist among control animals, but yet are all averaged as one composite group overlooking this natural variation. Therefore, we have pursued an alternative approach examining both group statistics (i.e., control vs. stress), and also individual statistics across dimensions of behavior and physiology. By using this type of analysis, we aim to mirror the National Institutes of Mental Health's Research Domain Criteria (RDoC) method that attempts to limit categorization diagnoses but instead examine individuals across various dimensions of behavior. In doing so, we find that correlations hold true across all animals dependent upon behavior phenotype, not trauma exposure, which we believe may be more reflective of the human condition.

One of the first intriguing findings of this work is that we show that T-lymphocytes express their own neurotransmission synthetic and degradative machinery that is dysregulated during psychological stress, suggesting the potential for T-lymphocyte-driven microenvironmental control of inflammation. Indeed, expression of these pro-sympathetic genes was tightly correlated with both mitochondrial superoxide and pro-inflammatory cytokine levels within T-lymphocytes, further supporting this pro-inflammatory hypothesis. However, T-lymphocyte expression of neurotransmission enzymes is a relatively new observation, and little is known regarding the contribution of these signaling pathways in these adaptive immune systems. TH expression and endogenous catecholamine production by T-lymphocytes was first observed in the mid-1990s and its function was shown to suppress lymphocyte proliferation and differentiation, which was suggested as a possible negative feedback mechanism to attenuate inflammation (Bergquist et al., 1994). Work from Yu-Ping Peng and Yi-Hua Qiu have also shown an overall suppressive phenotype of TH expression within T-lymphocytes. Several studies from this group have shown that TH expression and T-lymphocyte catecholamine production leads to a pro-T_H2 phenotype with suppression of T_H1 (Qiu et al., 2004, 2005; Liu et al., 2012). They have more recently demonstrated that TH expression correlates with the pro-inflammatory T_H17 subtype of T-lymphocytes, however, forced over-expression of TH in T-lymphocytes suppressed the polarization to the T_H17 phenotype (Wang et al., 2016). This data again suggests that T-lymphocytes may upregulate TH in pro-inflammatory subtypes as an auto-regulatory feedback mechanism to control inflammation. However, contradicting results exist showing both positive and negative effects of endogenous catecholamine production on anti-inflammatory regulatory T-lymphocyte function (Cosentino et al., 2007; Wang et al., 2016). Here, we demonstrate a significant elevation of TH expression within T-lymphocytes after repeated psychological trauma. Given the high correlation between TH and pro-inflammation gene expression levels among T-lymphocytes, these data support that either TH promotes inflammation within T-lymphocytes, or is upregulated in a compensatory manner to counteract the pro-inflammatory phenotype. Interestingly, T-lymphocyte TH levels did not correlate with behavioral changes after stress-induction, suggesting psychological manifestations after stress may not be directly coupled to autonomic changes.

The cholinergic system has also shown significant regulatory control over T-lymphocytes, yet, lymphoid organs such as the spleen are not cholinergically innervated (Dale and Dudley, 1929; Nance and Sanders, 2007). Acetylcholine, CHAT, and ACHE are all endogenously produced within T-lymphocytes (Szelényi et al., 1982; Rinner and Schauenstein, 1993; Rinner et al., 1998; Kawashima et al., 2007), and the regulation of acetylcholine on T-lymphocytes is complex, extensive, and has been previously reviewed (Fujii et al., 2017). Overall, due to the spleen being exclusively innervated by the sympathetic splenic nerve, it is believed the primary source of acetylcholine in this organ is CHAT-expressing T-lymphocytes (Pavlov and Tracey, 2005; Rosas-Ballina et al., 2011). Because of this, much work has focused on the role of CHAT-positive T-lymphocytes, while those expressing ACHE have been relatively understudied. Similar to CHAT, ACHE is significantly upregulated during T-lymphocyte activation suggesting a critical regulatory role in the modulation of cellular function (Szelényi et al., 1987). Additionally, pharmacological inhibition

of ACHE in T-lymphocytes reduced the production of IL-17A, suggesting the degradation of acetylcholine by ACHE may be important in the pro-inflammatory response (Nizri et al., 2008). Here, we also demonstrate a significant induction of ACHE mRNA in splenic T-lymphocytes after stress, which like TH, correlated significantly with pro-inflammatory cytokine expression and mitochondrial superoxide levels in these cells. Together, these data suggest the potential for a causal relationship between sympathetic autonomic balance and the inflammatory phenotype of T-lymphocytes, but further studies are needed to identify the mechanistic nature of these neurotransmission enzymes during psychological trauma.

While our work demonstrated the effects of psychological stress on T-lymphocytes, it is interesting to note that previous work has shown that peripheral T-lymphocytes may conversely impact behavior. Original studies utilized immunodeficient mice and could demonstrate that reconstitution with naïve T-lymphocytes restored enhanced cognitive function (Kipnis et al., 2004; Ziv et al., 2006). Anxiety-like and depression-like behavior have also been improved by the addition of naïve T-lymphocytes to immunodeficient animals (Cohen et al., 2006; Lewitus et al., 2008, 2009), whereas pro-inflammatory T-lymphocytes potentiate pathological behavior (Beurel et al., 2013). However, behavioral changes in response to T-lymphocytes do not appear to be universal and vary among stress-induction paradigms (Clark et al., 2014). Here, we demonstrate that repeated social defeat stress increases pro-inflammatory gene expression within T-lymphocytes, however, expression of these cytokines only associated with depression-like and not anxiety-like behavior. Correlations between circulating IL-6 levels and depression-like behavior have been previously observed when categorizing socially-defeated animals into susceptible and resilient groups (Hodes et al., 2014), but our data suggest individual differences display more as a spectrum as opposed to two separate entities. Additional studies are needed to identify if these correlations are specific to the social defeat paradigm, or may be more broadly applied.

Herein, we additionally identified two previously undescribed observations. First, we elucidate that repeated social defeat stress significantly increases mitochondrial superoxide levels within T-lymphocytes. This phenomenon appears specific to splenic T-lymphocytes, in that other cells of the spleen or even T-lymphocytes in circulation did not alter their mitochondrial redox environments after stress (data not shown). We previously observed that exogenous NE applied to T-lymphocytes *ex vivo* could increase mitochondrial superoxide levels, which appeared to regulate both IL-6 and IL-17A expression in these cells (Case et al., 2016). We posit that the elevated catecholamine levels in the spleens of social defeat animals may be eliciting a similar effect. Additionally, the highly significant positive correlations between mitochondrial superoxide and IL-6 or IL-17A levels with repeated social defeat is suggestive of similar mechanisms at play as well. The underlying cause of the increase in mitochondrial superoxide is currently unknown, but it is hypothesized that this induction may be occurring due to a potentiated metabolic state of the T-lymphocytes that occurs during activation (Pearce et al., 2013). However, our previous work would suggest that mitochondrial superoxide plays a critical regulatory role in T-lymphocyte activation and differentiation, and is not simply a by-product of another cellular process (Case et al., 2016). Another potential source of superoxide within T-lymphocytes could be from the direct oxidation of catecholamines. Utilizing a mouse model of NE infusion along with a combination of adrenergic receptor and catecholamine transport inhibitors, we previously identified that direct intracellular oxidation of catecholamines was not the primary source of superoxide within T-lymphocytes (Case and Zimmerman, 2015), but this possibility has not been exhaustively tested yet in our psychological trauma model. Last, we observed that T-lymphocyte-specific mitochondrial superoxide levels positively correlated with anxiety-like behavior after social defeat stress. Taken together with the tight positive correlations among mitochondrial superoxide levels, pro-inflammatory cytokine expression, and neurotransmission gene expression in T-lymphocytes, our findings suggest the mitochondrial redox environment of these cells may be causally involved in the pro-inflammatory nature of these cells, which could potentiate pathological anxiety-like behavior similar to what is observed in PTSD.

The additional novel observation described here was the identification of elevated calprotectin levels in socially-defeated animals. Aforementioned, calprotectin is a heterodimer of two proteins: calgranulin A and calgranulin B (encoded by S100a8 and S100a9, respectively). Calprotectin primarily serves as an extracellular protein where its known antimicrobial function is the sequestration of metals such as iron, manganese, and zinc, and is mainly produced by neutrophils and monocytes (Striz and Trebichavsky, 2004). Thus, our observation of stress-regulated S100a8 and S100a9 mRNA expression in T-lymphocytes was highly unexpected. In addition to its antimicrobial functions, calprotectin also serves as a damage-associated molecular pattern (DAMP) to activate other immune cells and promote inflammation (Vogl et al., 2007; Foell et al., 2008; Ehrchen et al., 2009). In fact, calprotectin binding to toll-like receptor 4 (TLR4) on the surface of T-lymphocytes enhances the IL-17A production from these cells, and this ability is attenuated in cells lacking TLR4 (Loser et al., 2010). While we did not observe a correlation with T-lymphocyte expressed calprotectin and IL-17A, we did observe a positive correlation between T-lymphocyte IL-17A and circulating calprotectin (data not shown). Interestingly, we observed correlations between calprotectin and IL-6 mRNA levels in T-lymphocytes, but not between IL-6 and circulating calprotectin. This is suggestive of different intracellular and extracellular functions of calprotectin that may be regulating T-lymphocyte inflammation. Additionally, calprotectin has previously been shown to be redox-regulated within its canonically-expressed cell types, and this protein plays a direct role in intracellular redox signaling (Jia et al., 2014). We observed that calprotectin expression correlated with mitochondrial superoxide levels in T-lymphocytes, which further suggests this protein may be a previously undescribed regulatory player in T-lymphocyte redox signaling. Last, calprotectin (in T-lymphocytes and in circulation) correlated with anxiety-like behavior. The downstream ramifications of this

observation are unclear but suggest that calprotectin may serve as a biological marker for this specific behavioral manifestation. Moreover, knowing that calprotectin has a functional role in promoting inflammation in autoimmune diseases, the presence of this protein may also suggest a predisposition to the development of comorbid inflammatory diseases after trauma. Taken together, this research opens a new avenue of investigation into the mechanistic roles of neurotransmission, inflammation, and redox into the long-term consequences of psychological traumatic diseases like PTSD.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of Nebraska Medical Center Institutional Animal Care and Use Committee. The protocol was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CM, SE, CC and AC designed research studies. CM, SE, CC, AK and AC conducted experiments and acquired, analyzed data. AC provided experimental oversight and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00103/full#supplementary-material

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Dissociation in Effective Treatment and Behavioral Phenotype Between Stress-Enhanced Fear Learning and Learned Helplessness

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Post-traumatic stress disorder (PTSD) is a debilitating disease with relatively high lifetime prevalence. It is marked by a high diversity of symptoms and comorbidity with other psychiatric disease. Furthermore, PTSD has a high level of origin and symptom heterogeneity within the population. These characteristics taken together make it one of the most challenging diseases to effectively model in animals. However, with relatively little headway made in developing effective disease interventions, PTSD remains as a high priority target for animal model study. Learned Helplessness (LH) is a procedure classically used to model depression, but has in recent years transitioned to use as a model of PTSD. Animals in this procedure receive 100 inescapable and unpredictable tailshocks or simple restraint without shock. The following day, the animals are tested in a shuttle box, where inescapably-shocked subjects exhibit exaggerated fear and profound deficit in escape performance. Stress-enhanced fear learning (SEFL) also uses an acute (single session) stressor for modeling PTSD in rodents. The SEFL procedure begins with exposure to 15 footshocks or simple context exposure without shock. Animals that initially received the 15 footshocks exhibit future enhanced fear learning. In this review, we will compare the behavior, physiology, and interventions of these two animal models of PTSD. Despite considerable similarity (a single session containing inescapable and uncontrollable shock) the two procedures produce a very divergent set of behavioral consequences.

Keywords: learned helplessness, stress-enhanced fear learning, PTSD, depression, fear, stress

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Up to 20% of the population that experiences a trauma will go on to develop Post-Traumatic Stress Disorder (Kilpatrick et al., 2013; PTSD). PTSD is a debilitating disease marked by symptoms such as dissociative amnesia, avoidance behaviors, hypervigilance, anhedonia, exaggerated fear startle, and insomnia (Association, 2013). Lifetime prevalence of PTSD in the United States is approximately 7%, with U.S. military incidence reaching as high as 15–20% (Gradus, 2007; Gates et al., 2012). A large ongoing research effort has focused on identifying the neurobiological consequences of stress that lead to the development of disorders such as PTSD. Despite great headway made in understanding the neurobiology of the disease, improvement in efficacious intervention has been bare. This point was highlighted in a recent public message from the Director

of the National Institute of Mental Health, Dr. Joshua Gordon. In some cases, this lack of progress has led to criticism of the animal models available to study PTSD.

In a recent review by Richter-Levin, Stork, and Schmidt, the authors weigh-in on the current stress research climate (Richter-Levin et al., 2018). The authors suggest that while animal model research has proved invaluable in the study of PTSD, modifications should be made to adequately capture the complexity and heterogeneity of the disease in order to increase translational relevance. Those authors suggest that animal models of PTSD should be modified to accurately represent exposure to risk factors and individual genetic and behavioral differences. They also suggest careful selection of stressor and behavioral phenotypes measured, suggesting that just as we see in humans, different stressors produce dissociable neurobiological and behavioral consequences in rodents.

In this review, we will examine this notion of stressor-induced heterogeneity. We will critically evaluate the reported effects of two animal stress procedures that have been claimed to model PTSD psychopathology. Learned helplessness (LH) is a half-century old procedure which commonly uses 100, 8 s inescapable and unpredictable 1 mA tailshocks over a 2-h session to produce a behavioral phenotype that parallels many of the symptoms of PTSD and Major Depressive Disorder (MDD). Stress-enhanced fear learning (SEFL) is a procedure that presents 15, 1 s inescapable and unpredictable 1 mA footshocks over a 90-min session to induce its PTSD-like phenotype. By critically examining two models that share several dimensional similarities, we can evaluate the specific consequences of stress volume on stress-induced psychopathology. In this case, we are defining volume as the product of shock number, shock duration, and shock intensity (current). It can also be thought of as the total number of coulombs received during stress. Previous work has shown that variation in coulombs qualitatively changes reactions to a stressor (Fanselow, 1984).

In this review we will focus on learned helplessness and SEFL stress procedures in rats only. SEFL in mice is still in its infancy, and we therefore do not feel comfortable discussing these findings at this time. Furthermore, there are several important changes made to the LH procedure when using mice and there is some controversy due to these changes (Landgraf et al., 2015).

LEARNED HELPLESSNESS

History

The learned helplessness procedure is a traditional method for analyzing the effects of acute, traumatic stress and modeling related symptoms of post-traumatic stress disorder and comorbid major depression in rats (Minor et al., 1991, 2010; Başoglu et al., 1997; Hammack et al., 2012; Minor and Plumb, 2012). Seligman and colleagues first discovered in 1967 that exposure to inescapable shock, but not escapable shock, results in failure to perform future escape responding in a novel apparatus (Overmier and Seligman, 1967; Seligman and Maier, 1967). The classic experiments utilized dogs and a triadic design. In this design there are three groups. One group is able to perform a response to escape the shock. Another group is able to perform the same response non-contingently, as their exposure to shock is yoked to that of the escapable group. A final group is exposed to the same apparatus, but no shock is administered. This design allows for dissociable assessment of the effects of escapable and inescapable shock. The term "learned helplessness" was originally coined as it was initially believed that the escape latency deficits were due to the animals learning that they had no control over the environment (Seligman and Maier, 1967; Maier and Seligman, 2016). However, others have provided subsequent evidence which has suggested that it instead may be the unpredictability of shock that is the root of the subsequent maladaptive behavior (Dess et al., 1990; Minor et al., 1991; Minor and Hunter, 2002). The model has since transitioned to rats and LH has been used extensively as an animal model of human disorders, such as PTSD and MDD (Maier, 1984; Foa et al., 1992). Though the learned helplessness model has been used extensively as a model of depression and PTSD, it does have a scientifically contentious history. The relatively short 24–72-h lifespan of many of the observed behavioral and cognitive deficits, which can be moderately extended using a reinstatement procedure (Maier, 2001), has been a point of which its opponents cite when discussing its inefficacy as a model of psychiatric disease (Anisman and Sklar, 1979; Jackson et al., 1979; Minor et al., 1988; Dess et al., 1989; Yehuda and Antelman, 1993). However, face, construct, and predictive validity maintain its place as one of the leading models of PTSD and MDD.

It should be noted that it is common to drop the escapable group in studies more concerned with modeling human stress disorders, and less concerned with questions on the effects of escapability. Both SEFL and the LH procedures induce behavioral changes by delivering inescapable shocks but the two procedures differ substantially in terms of the amount of shock delivered. Seeing as the effect of shock volume, and not escapability, is the focus of this review, we will be discussing the overall behavioral and physiological consequences of inescapable shock and not learned helplessness, *per se*. In other words, we will not disentangle if the effects discussed are specific to inescapable shock or if they also occur in rats that receive equivalent escapable shock (see Greenwood and Fleshner, 2008; Maier and Seligman, 2016 for review on the behavioral effects of escapability).

Induced Phenotype

Animals exposed to 100 inescapable and unpredictable shocks exhibit several behavioral characteristics similar to the symptoms of PTSD (see **Table 1**). Rats pre-exposed to inescapable shock enter the subsequent test situation in an anxious/agitated state and show exaggerated fear responding during initial escape testing. As testing progresses, inescapably shocked rats rapidly transition to an unresponsive, depression-like state, termed *conservation-withdrawal*. The transition to conservation-withdrawal is evident as a profound deficit in escape performance (Minor et al., 1994a,b; Plumb et al., 2013). Experience with inescapable shock also results in behavioral depression as defined by the forced swim task (Weiss et al., 1981) and sucrose preference (Christianson et al., 2008; but see Dess, 1992), disturbances in sleep

(Adrien et al., 1991), exaggerated startle (Servatius et al., 1995), anorexia (Weiss, 1968; Dess et al., 1989), anhedonia (Zacharko and Anisman, 1991), anxiety as measured by decreased social interaction (Short and Maier, 1993) and the elevated plus maze (Steenbergen et al., 1989), reinstatement of drug seeking (Figueroa-Guzman et al., 2011) and attentional/cognitive deficits in rats (Jackson et al., 1980; Minor et al., 1984; Shors, 2004). However, it should be noted that many of the behavioral deficits are short lived and fail to occur 72 or more hours following the traumatic stress session (Jackson et al., 1978; Grau et al., 1981; Weiss et al., 1981; Maier, 1990; Short and Maier, 1993; Will et al., 1998). Several of the neurochemical changes induced by inescapable shock also persist for only a few days (Weiss et al., 1981; Maier, 2001).

Interestingly, this severe stress procedure does not appear to enhance future fear learning as appreciably as the more moderate, SEFL stress procedure. One notable study provides evidence that inescapable shock may enhance, while escapable shock reduce, subsequent fear learning (Baratta et al., 2007). Furthermore, in a similar stress protocol (of considerably smaller total stress volume), inescapable tailshock has also been shown to enhance trace eyeblink conditioning (Beylin and Shors, 1998) However, it should be noted that the effects observed in both are relatively modest in comparison to the effect found using the moderate, SEFL stress procedure.

Behavioral Interventions

Several behavioral factors and interventions have profound effects on the phenotype produced by severe stress. For example, rats no longer exhibit post-stress escape latency deficits if they are given 6 weeks of free access to a running wheel prior to the trauma, and the protective effects of wheel running are dependent on the duration of activity (Greenwood et al., 2005). Perhaps more surprisingly, prior exposure to subthreshold stress has also exhibited beneficial effects following exposure to the traumatic stress session (Plumb et al., 2015).

Several design aspects are critical in the development of the phenotype produced by severe stress. For one, though the pretreatment and testing contexts differ on many dimensions, shuttle-escape deficits are contingent upon the stress and test contexts sharing the same olfactory cues (Minor and LoLordo, 1984). Traditionally, this is done by allowing feces and urine of the stressed animals to accumulate over the day. If one of the contexts is cleaned, the learned helplessness phenotype is abolished. Furthermore, if the contexts are cleaned and instead both scented with a common artificial odor, the behavioral phenotype persists. It should be noted that the effect of contextual odor generalization has not been tested for other behaviors induced by inescapable shock and does not likely play a similar role. Another essential dimension of the design is that the shocks remain variable and unsignaled. If the shocks are cued, the behavioral phenotype no longer persists (Dess et al., 1990).

Pharmacological Interventions/Defined Neurocircuitry

Research into the neural mechanisms of the behavioral consequences of severe stress was spearheaded early on by Steve Maier. Through decades of research, the Maier lab has characterized the importance of serotonin (5-HT) signaling in the dorsal raphe nucleus (DRN) in the development of the LH phenotype (for review, see Maier and Seligman, 2016). Within this model, he proposes that DRN activity is modulated by the controllability of the stressor via detection and activation in the ventromedial prefrontal cortex (vmPFC). For example, activation of the vmPFC using picrotoxin eliminates subsequent LH behavior in rats exposed to inescapable shock (Amat et al., 2008). Through Maier's body of work, he also implicates roles of the bed nucleus of the stria terminalis (Hammack et al., 2004, 2012), amygdala (Maier et al., 1993), and dorsal striatum (Strong et al., 2011). The habenula- DRN circuit has also been identified to play a role using a unique behavioral outcome in juvenile rats (Dolzani et al., 2016; see Metzger et al., 2017 for review). Additionally, research from several labs has suggested an integral role of norepinephrine signaling in the development on LH behaviors (Minor et al., 1988; Grahn et al., 2002).

Thomas Minor focused on the energetic demands of the stressor as a critical aspect that leads to future maladaptive behavior in the animal. Minor suggests both serotonin and corticosterone likely play only permissive roles in the development of the behavioral consequences induced by severe stress. This is based on their time course of release during stress exposure and testing (see Minor and Hunter, 2002 for review). Instead, he suggests that the state of fear invoked by the stress session is energetically costly and depletes the animal's energy reserves (Conoscenti et al., 2019). Thus, the animal enters the test session in a state of conservation withdrawal, a behavior deemed to conserve energy resources. This behavior limits the animal's motivation to escape, and is mediated by adenosine signaling in the nucleus accumbens core (Minor et al., 1994a,b, 2001, 2006, 2008, 2010; Plumb et al., 2013). Furthermore, consumption of glucose following the trauma, which has been shown to replete energy reserves (Conoscenti et al., 2019), eliminates the negative behavioral consequences of stress (Minor and Saade, 1997; Conoscenti et al., 2017, 2019). This theory accounts for the transient nature of the behavioral effects, as the effects disappear as the animal recovers from the energy deficit. However, it should be noted that it does not account for experiments showing that inescapably shocked rats with amygdalar lesions will still exhibit shuttle escape latencies despite lacking a fear response (Maier et al., 1993).

Another line of evidence implicates the role of the immune response in the development of LH behavior. Specifically, several studies have suggested that interleukin-1 (IL-1), an inflammatory cytokine, is critical for LH's characteristic shuttle escape latency deficits (Maier and Watkins, 1995; Minor et al., 2006; Goshen and Yirmiya, 2009; Hanff et al., 2010). Following inescapable shock, hippocampal, hypothalamic, and peripheral concentrations of IL-1 increase. This upregulation of IL-1 is necessary, but not sufficient, for the induction of stress-induced behavior, as it

TABLE 1 | Summary of LH and SEFL-induced change.

Phenotype	Present in LH?	Present in SEFL?	References
Future enhanced fear learning	Yes	Yes	(Rau et al., 2005; Baratta et al., 2007; Rau and Fanselow, 2009)
Anxiety; Elevated plus maze	Yes	Yes	(Steenbergen et al., 1989; Poulos et al., 2014)
Anxiety; Open field	Yes	Yes	(Fleshner and Greenwood, 2013; Perusini et al., 2016)
Anxiety; Exaggerated startle	Yes	Yes	(Servatius et al., 1995; Perusini et al., 2016)
Anxiety; Social interaction	Yes	Not reported	(Short and Maier, 1993)
Depression; Shuttle escape deficit	Yes	No	(Seligman and Maier, 1967; Minor et al., 1994a)
Depression; Forced swim	Yes	Maybe	(Weiss et al., 1981; Perusini et al., 2016; Tribble and Fanselow, 2019)
Depression; Sucrose preference	Yes	Not reported	(Dess, 1992; Christianson et al., 2008)
Anorexia	Yes	Not reported	(Weiss, 1968; Dess et al., 1989)
Reinstatement of drug seeking	Yes	Yes	(Figueroa-Guzman et al., 2011; Meyer et al., 2013)
NEUROBIOLOGY			
Amygdala	Yes	Yes	(Maier et al., 1993; Perusini et al., 2016)
Ventromedial prefrontal cortex	Yes	Yes	(Maier and Seligman, 2016; Pennington et al., 2017)
Dorsal raphe nuclei	Yes	Not reported	(Maier and Seligman, 2016)
Nucleus accumbens	Yes	Not reported	(Plumb et al., 2013)
Dorsal striatum	Yes	Not reported	(Strong et al., 2011)
BNST	Yes	Not reported	(Hammack et al., 2004, 2012)
Habenula	Yes	Not reported	(Dolzani et al., 2016)
Corticosterone	Yes	Yes	(Hanff et al., 2010; Poulos et al., 2014; Perusini et al., 2016)
Serotonin	Yes	Not reported	(Maier and Seligman, 2016)
Norepinephrine	Yes	Not reported	(Minor et al., 1988; Grahn et al., 2002)
Interleukin-1	Yes	Yes	(Goshen and Yirmiya, 2009; Jones et al., 2015)
Glucose	Yes	Not reported	(Minor and Saade, 1997; Conoscenti et al., 2017)
Adenosine	Yes	Not reported	(Minor et al., 1994a,b; Plumb et al., 2013)

This table displays a summary of the behavioral, neural, and pharmacological effects of LH and SEFL stressors.

has been shown that blocking IL-1 mitigates the behavioral consequences of shock. It has been posited that IL-1 exerts its stress mediating effects by inducing an increase in HPA-axis activation (see Goshen and Yirmiya, 2009 for review).

SEFL

History

Our first indication of enhanced fear learning following stress was suggested by two papers published in 1979 (Fanselow and Bolles, 1979a,b). In these experiments rats that received an identical single shock in the same novel context froze at very different rates depending on whether or not they received prior experience with a robust fear conditioning protocol in a completely different context (see **Figure 1**). Interestingly, while both 15 forward (tone-shock) and backward (shock-tone) trials enhanced subsequent contextual fear conditioning, predictive signaling of the shock reduced the magnitude of this enhancement. Importantly, the lack of freezing observed prior to the single shock indicated that this enhancement was not caused by generalization of fear from the 15 shock to the 1 shock contexts.

This ability of stress to enhance fear learning was then used as a tool to explore two deficits in contextual fear conditioning (Fanselow et al., 1993). One was the deficit seen when only a minimal period of exploration was allowed prior to delivery of a single shock. Prior stress facilitated conditioning with this procedure that typically supports little to no conditioning. Another deficit in contextual fear conditioning occurs when shocks are closely spaced rather than given in a more distributed manner. In this case, prior stress eliminated the difference between massed and spaced trials. These studies also revealed an important boundary conditioning to SEFL; when multiple conditioning shocks were well spaced prior stress caused no enhancement in fear learning. These findings indicate that stress enhances the rate but not the asymptote of the learning curve.

Induced Phenotype

As previously discussed, the quintessential phenotype measured using this model is the enhancement of future fear learning (Rau et al., 2005; Rau and Fanselow, 2009). However, animals exposed to 15 inescapable and unpredictable shocks also exhibit several similar symptoms to LH-stressed animals (see **Table 1**). Animals exposed to 15 footshocks exhibit reinstatement of drug seeking (Meyer et al., 2013) as well as several anxiety-like phenotypes. For example, animals that receive shock exhibit decreased time in the open arms of the elevated plus maze (Poulos et al., 2014), decreased exploration during an open field test (Perusini et al., 2016), and potentiated startle (Perusini et al., 2016). Unlike LH-stressed animals, there is no evidence suggesting that these animals exhibit depression-like behavior after to exposure to 15 shocks. With this stress volume, animals fail to exhibit escape

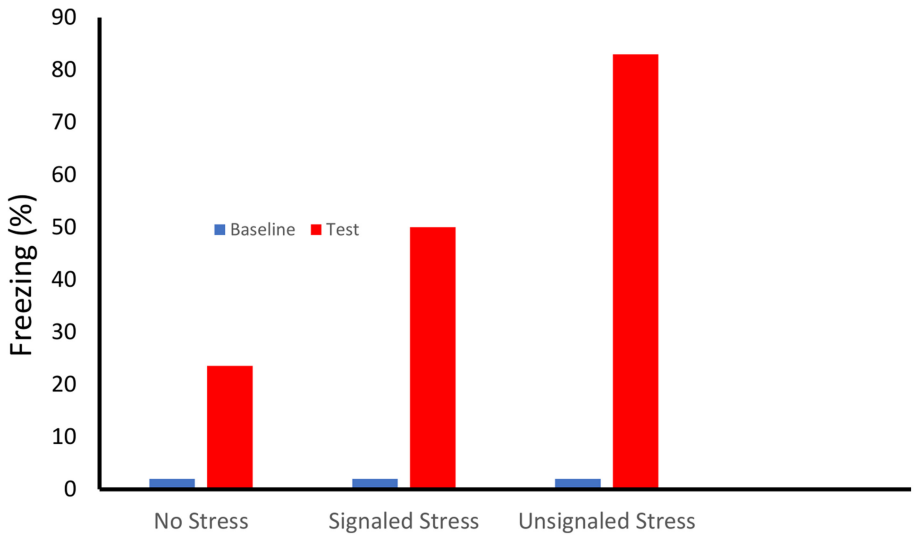

FIGURE 1 | On Day One female Long-Evans rats received either no treatment, or 15 shocks (0.71-mA, 0.75-s) that were either preceded by a 30 s tone (Signaled Stress) or followed by the tone (Unsignaled Stress) in a rectangular shuttle box. Subsequently the rats received a single conditioning shock (1.0-mA, 0.75-s) in a conditioning chamber that differed in term of shape, smell, location, dimensions and lighting. Prior to the conditioning shock the there was little freezing (<2%) in the conditioning chamber. Animals that received a prior signaled shock stressor showed more than twice the level of freezing of the unstressed controls. Fear learning showed an even greater enhancement in that rats whose stress was unsignaled [Based on Fanselow and Bolles (1979a,b)].

latency deficits (Minor et al., 1994c), though it should be noted that this study used tailshock, not footshock. While one study did show an effect of shock on float time in the forced swim test (Perusini et al., 2016), this effect has subsequently failed to replicate (Tribble and Fanselow, 2019). Interestingly, the behavioral effects of the SEFL stress have been shown to persist for several months (Rau and Fanselow, 2009). This symptom persistence is notable, seeing that the LH procedure has a much higher stress volume and yet several of the behavioral effects are much more transient in nature.

Unlike shuttle-escape performance deficits in LH, it appears that the SEFL behavior does not rely on associative processes such as context generalization (Rau et al., 2005; Poulos et al., 2014; Pennington et al., 2017). Stress during adolescence still results in SEFL even though this early life stress does not produce associative fear learning (Poulos et al., 2014). SEFL behavior is also resistant to extinction of the trauma context, further suggestion that there are non-associative processes at play (Rau et al., 2005; Long and Fanselow, 2012). However, it has been hypothesized that perhaps animals that undergo the SEFL procedure are learning a shock-shock association. That is, the animal is learning that one footshock predicts subsequent footshock, and the enhancement of fear to the 1-shock context is due to this learned association. To support the notion that the effects of SEFL are not due to a shock-shock association, we have found that stress pretreatment exposure will enhance subsequent fear learning when using a loud noise as the stressor (Pennington et al., 2017). Furthermore, the SEFL effects no longer appear if the 1-shock exposure precedes the 15-shock session (Rau et al., 2005). It should be noted that while this evidence does not eliminate the possibility of shock-shock associations from playing a role in SEFL, this explanation is less applicable to the behavioral changes produced by LH-stress due to their transituational nature.

Behavioral Interventions

The SEFL phenotype is relatively robust, and therefore has seen little success in terms of behavioral interventions. In the majority of stress models, animals are singly housed, as pair-housed animals often show decreased behavioral effects of stress (Liu et al., 2013). However, a series of studies aimed at probing the effects of single vs. pair-housing animals showed no significant effects in eliminating the SEFL phenotype (Tribble and Fanselow, 2019).

Several aspects of SEFL design are in direct contrast with the LH stress procedure. The most apparent is that the SEFL behavior is not dependent on shared cues between contexts. Indeed, great care is taken in the SEFL procedure to eliminate any similarity between the stress and conditioning contexts. Additionally, the effects of signaling shock show slightly different outcomes. As previously mentioned, evidence suggests that signaling shock during stress pre-exposure may act to reduce, but not eliminate, SEFL behavior (Fanselow and Bolles, 1979a,b).

Pharmacological Interventions/Defined Neurocircuitry

Compared to the decades of research dedicated to identifying the neural mechanisms of LH behavior, the neurocircuitry of SEFL behavior remains relatively scant (see **Table 1**). Similar to LH, it appears that corticosterone is necessary, but not sufficient, for the induction of SEFL behavior (Perusini et al., 2016). Furthermore, the SEFL stress procedure produces a similar dysregulation of the diurnal cycle of corticosterone

(Poulos et al., 2014). Finally, a series of studies suggest that glucocorticoids may be acting via activation glucocorticoid receptors in the basolateral amygdala, which in turn upregulate the GluA1 AMPA receptor subunit in this structure (Perusini et al., 2016).

It appears that the ventromedial prefrontal cortex also plays an important role in the SEFL phenotype. A study showed that when the vmPFC is lesioned, future enhanced fear learning is attenuated, while the trauma memory remains intact (Pennington et al., 2017). Interestingly, this means that the impacts of the vmPFC on LH and SEFL are opposite: activation of vmPFC may be necessary for SEFL, while inactivation of vmPFC during stress pretreatment appears to be necessary for the formation of LH behaviors (Amat et al., 2005, 2008).

Stress-induced immune reactivity also appears to play an essential role in SEFL. Donald Lysle has reported a series of studies which suggest that IL-1beta, specifically, is necessary for the induction of the SEFL phenotype (Jones et al., 2015, 2018). Similar to LH, IL-1 antagonists block the induction of SEFL and shock stress increases both central and peripheral concentrations of IL-1. Furthermore, repeated morphine injection into the dorsal hippocampus following stress pretreatment has been reported to eliminate the stress-induced increases in IL-1 and subsequent SEFL behavior (Szczytkowski-Thomson et al., 2013; Jones et al., 2015, 2018).

Summary and Conclusions

In this review, we discussed the behavioral and physiological consequences of two acute stress paradigms that vary on one major dimension: volume. Exposure to inescapable, unpredictable shock appears to incorporate some homogenous peripheral and central mechanisms and induce a series of consistent trans-situational behaviors, regardless of volume. It appears that stress-induced anxiety phenotypes are first to arise during exposure to a stressor, as anxiety-related behaviors are conserved across the two shock-stress models. The HPA axis appears to play a critical, permissive role in the development of both LH and SEFL-induced behavior. It also appears that the immune response, specifically IL-1, plays a critical role in the development of stress-induced psychopathology. Regarding neurocircuitry, converging evidence suggests that the amygdalar complex is involved in the neurocircuitry of shock stress regardless of volume. The vmPFC has also been implicated in both behavioral models, though it appears to have opposing effects.

Several dissociable behavioral and neurobiological aspects of the two procedures stand out. The most obvious division is the induction of a depression-like phenotype in LH-stressed animals that appears absent in SEFL-stressed animals. Another interesting difference is the apparent generalization necessary for LH's characteristic deficits in shuttle-escape performance, which does not appear necessary for the SEFL phenotype. Perhaps the most perplexing difference is that of symptom persistence. The LH-stressor produces many behavioral changes that appear to persist for only a few days. Meanwhile, SEFL produces a set of behaviors which persist for at least several months. Given that there is a much greater volume of stress in the LH procedure it is surprising that many of its effects do not persevere. However, it should be noted that several of these short-lived changes are in behaviors that do not overlap with the behavioral effects of SEFL. Therefore, it may be a product of the behavioral phenotype assayed, and not an effect directly related to stress volume. It is important to note that there are several outstanding questions that have been left unanswered. For example, the role of 5-HT neurons in the DRN has been well characterized in LH, but has yet to be investigated in SEFL.

Use of the same stressor can produce dissociable behavioral and neural consequences by simply modulating stress volume. Notably, the degree of stress does not necessarily make the effects quantitatively greater, but rather there seems to be qualitative changes in the consequent behavioral reactions. Based on the literature reviewed, it appears that the SEFL procedure may produce several phenotypes specific to model PTSD without depression comorbidity, while LH may model a PTSD comorbid with depression. This notion sits perfectly in-line with the heterogeneity of PTSD described in the review by Richter-Levin et al. (2018). Within that review, the authors describe an outstanding fundamental question about PTSD: is PTSD with depression a unique subtype, or do the diseases merely show a high comorbidity. Approximately half of patients diagnosed with PTSD also concurrently meet criteria for Major Depressive Disorder (Kessler et al., 1995; Breslau et al., 1997; Rytwinski et al., 2013; Caramanica et al., 2014; Flory and Yehuda, 2015). Perhaps even more staggering is the statistic that 95% of those with PTSD will be diagnosed with MDD within their lifetime (Hammack et al., 2012). Patients with MDD exhibit symptoms such as chronic depressed mood, anhedonia, anorexia or hyperphagia, insomnia or hypersomnia, fatigue, and cognitive deficits (Association, 2013). These symptoms are consistent with several of the symptoms observed following LH, but not SEFL, stress exposure. It is possible that human PTSD development is influenced by similar factors. For example, stress volume may influence both the quality and quantity of symptoms. It is also possible, that disease persistence does not positively correlate with stress volume, but may be predicted by another variable of stress exposure. Only through careful, focused study examining the neurobiological effects of modulating stress volume may we begin to unravel the dissociable aspects of PTSD and PTSD with comorbid depression.

Further precise exploration to assess the behavioral and neurobiological dissociation between the two procedures is necessary. By further understanding the mechanisms of each stressor we may be able to more accurately target investigation into neural mechanisms and effective treatment of specific disease phenotypes. This goal can best be reached by minimizing the lab-specific stress procedure permutations that are presently under use and focusing on stressors that can be parametrically titrated and objectively compared.

ARTICLE DEDICATION

We dedicate this article to the memory of Dr. Thomas R. Minor, a major contributor to the reviewed work on learned helplessness. Tom was an exceptional mentor, a supportive colleague, and a caring friend whose ideas both challenged and sparked our scientific endeavors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Heritable Differences in Catecholamine Signaling Modulate Susceptibility to Trauma and Response to Methylphenidate Treatment: Relevance for PTSD

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Alterations in cortical catecholamine signaling pathways can modulate acute and enduring responses to trauma. Heritable variation in catecholamine signaling is produced by a common functional polymorphism in the catechol-O-methyltransferase (COMT), with Val carriers exhibiting greater degradation of catecholamines than Met carriers. Furthermore, it has recently been suggested that drugs enhancing cortical catecholamine signaling may be a new therapeutic approach for posttraumatic stress disorder (PTSD) patients. We hypothesized that heritable differences in catecholamine signaling regulate the behavioral response to trauma, and that methylphenidate (MPD), a drug that preferentially blocks catecholamine reuptake in the prefrontal cortex (PFC), exerts COMT-dependent effects on trauma-induced behaviors. We first examined the contribution of the functional mutation COMTval158met to modulate enduring behavioral responses to predator stress in a unique "humanized" COMTval158met mouse line. Animals were exposed to a predator (cat) for 10 min and enduring avoidance behaviors were examined in the open field, light-dark box, and "trauma-reminder" tests 1–2 weeks later. Second, we examined the efficacy of chronic methylphenidate to reverse predator stress effects and if these effects were modulated by COMTval158met genotype. Mice were exposed to predator stress and began treatment with either saline or methylphenidate (3 mg/kg/day) 1 week after stress until the end of the testing [avoidance behaviors, working memory, and social preference (SP)]. In males, predator stress and COMTval158met had an additive effect on enduring anxiety-like behavior, with Val stressed mice showing the strongest avoidance behavior after stress compared to Met carriers. No effect of COMT genotype was observed in females. Therefore methylphenidate effects were investigated only in males. Chronic methylphenidate treatment reversed the stress-induced avoidance behavior and increased social investigation independently of genotype. Methylphenidate effects on working memory, however, were genotype-dependent, decreasing working memory in non-stressed Met carriers, and improving stress-induced working memory deficit in Val carriers. These results suggest that heritable

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variance in catecholamine signaling modulates the avoidance response to an acute trauma. This work supports recent human findings that methylphenidate might be a therapeutic alternative for PTSD patients and suggests that methylphenidate effects on anxiety (generalized avoidance, social withdrawal) vs. cognitive (working memory) symptoms may be modulated through COMT-independent and dependent mechanisms, respectively.

Keywords: COMT, catecholamine, PTSD, predator stress, methylphenidate

INTRODUCTION

Posttraumatic stress disorder (PTSD) affects 7%–8% of the American population while rates are as high as 22% in veterans (Kessler et al., 2010). In the US, 50%–90% of the population endorse at least one trauma in their lifetime, but only 10%–40% of individuals who endorsed sufficiently high numbers of traumatic events develop PTSD symptoms (Kessler et al., 2010; Kilpatrick et al., 2013), characterized by intrusive re-experiencing of a trauma event, avoidance of trauma-related cues and hyperarousal (Neuner et al., 2004; Kolassa et al., 2010). Since only a fraction of individuals who experience trauma develop PTSD, a better understanding for the biological risk factors that contribute to susceptibility to stress and PTSD will be crucial for the development of novel preventative or early intervention therapeutics.

Cortical catecholamine signaling is critical for acute and enduring responses to trauma (Arnsten, 2009). In humans, heritable variation in peripheral and central catecholamine signaling is produced by the common carried val158met functional single nucleotide polymorphism (SNP; rs4680) in the catechol-O-methyltransferase (COMT) gene, localized on chromosome 22q11.2. The COMTval158met SNP is a coding variant with known functional effects, with COMT being an enzyme that degrades catecholamines: epinephrine, norepinephrine, and dopamine. In the coding sequence for COMT, a valine (Val) is substituted by a methionine (Met) at amino acid residue 158, resulting in a 40% reduction in COMT enzymatic activity in the prefrontal cortex (PFC) of human Met/Met carriers. Therefore, Val/Val carriers have increased catecholamine clearance and reduced catecholamine tone in the cortex (Chen et al., 2004). Preclinical studies of this SNP confirms that it results in reduced enzymatic activity as well as more rapid degradation of the COMT enzyme (Tunbridge, 2010; Risbrough et al., 2014). COMTval158met SNP, which has been associated with a greater risk of PTSD in some, but not all candidate gene studies (Boscarino et al., 2011; Clark et al., 2013; Almli et al., 2014; Goenjian et al., 2015) is yet to be confirmed in larger genome-wide association studies (GWAS; Nievergelt et al., 2015; Li et al., 2016; Duncan et al., 2018). These discrepancies may be due in part to relatively small effect sizes associated with common SNPs, differences in PTSD outcome measures and differences in the ancestral background across studies (Kolassa et al., 2010; Boscarino et al., 2011; Valente et al., 2011; Arnsten et al., 2015). Contributions of genetic variance in catecholamine signaling to PTSD risk are also likely *via* gene × environment interactions (i.e., timing and intensity of trauma exposure). Animal models of the COMTval158met mutation will be critical in evaluating any causal contributions and mechanisms of heritable differences in catecholamine signaling to enduring stress response because only animal models can enable isolation of genetic effects against a fixed genetic background and "trauma-type" of fixed intensity and time-course.

Only 60% of PTSD patients adequately respond to the existing pharmacological treatments, antidepressants, and approximately 20%–30% of them achieve full remission (Ravindran and Stein, 2010; Steckler and Risbrough, 2012). Thus, there is a major need for novel treatment strategies (Krystal et al., 2017). Evidence suggests that cortical dopamine-enhancing treatments may be a novel therapeutic alternative for PTSD. The psychostimulant methylphenidate (MPD; Ritalin), a dopamine and norepinephrine transporter inhibitor, is a first-line therapeutic for attention deficit hyperactivity disorder (ADHD). Methylphenidate preferentially increases dopamine signaling in the frontal cortex over striatal release when used at low-doses (Koda et al., 2010; Swanson et al., 2011). Interestingly, methylphenidate reverses the enduring anxiety-like traits in animal models of PTSD (Aga-Mizrachi et al., 2014; Ritov and Richter-Levin, 2017), and a small randomized placebo-controlled trial has also reported decreased PTSD symptoms following methylphenidate treatment (McAllister et al., 2016). These effects are yet to be confirmed in larger randomized controlled trials, and the effects of cortical dopamine-enhancing drugs remain largely unexplored in PTSD patients. More importantly, it is unknown how heritable variance in COMT function modulates treatment response to methylphenidate.

To address the role of the heritable variation in COMT function in response to trauma and methylphenidate treatment, we used a "humanized" *COMT*val158met mouse line, in which either the human Val or Met version of the COMT gene is knocked into the mouse locus (mouse COMT has 70% homology with the human gene). In agreement with the known 40% decrease of COMT enzymatic activity in PFC (Chen et al., 2004) and cognitive traits in human Met/Met carriers (Diaz-Asper et al., 2008; Quednow et al., 2009), Met/Met mice display a 30% reduction in enzymatic activity, and exhibit similar cognitive phenotypes (i.e., higher working memory and fear conditioning compared to Val/Val carriers; Risbrough et al., 2014). These behavioral and neurobiological similarities suggest this "humanized" mouse line as a predictive model of heritable

differences in COMT function found in humans and thus offer rigorous causal testing of the role of this variation in susceptibility to trauma events and in treatment response. We hypothesized that heritable variance in COMT function regulates the response to a severe trauma by increasing susceptibility to the enduring effects of trauma. We also hypothesized that COMTval158met may modulate the response to methylphenidate treatment. To test these hypotheses we used a predator stress model of PTSD to examine the sensitivity of mice "humanized" for the *COMT*val158met polymorphism to a traumatic event in adulthood (Adamec et al., 2009; Bakshi et al., 2012; Deslauriers et al., 2018). We utilized two different stress protocols, full and protected predator exposure, to examine how COMTval158met modulates response to both high and low "trauma" conditions. We also examined the efficacy of chronic low-dose treatment with methylphenidate on trauma-induced effects on several PTSD-relevant domains, mainly generalized avoidance, social withdrawal and deficits in working memory. Finally, we tested the hypothesis that COMTval158met would modulate methylphenidate efficacy across anxiety (generalized avoidance, social withdrawal) and cognitive (working memory) domains.

MATERIALS AND METHODS

Animals

The humanized *COMT* mouse line was generated as previously described (Risbrough et al., 2014). Heterozygous *COMT* Met/Val breeders were used to produce Met/Met and Val/Val littermates with $N > 9$ backcross to a C57Bl6J background. All subjects were group housed (3–4 per cage) after weaning (PND28) in a temperature controlled (21–22°C) room under a reverse 12 h light/dark cycle (lights off at 7:00 AM). One week before stress exposure, mice were single housed, as it has been shown that predator stress had stronger effects in isolated mice due to lower baseline levels of avoidance behaviors (Adamec et al., 2009). Mice were tested after reaching adulthood (90–95 days old at start of testing). The behavioral tests were performed from 9:00 AM to 12:30 PM, except the social preference (SP) test that was run from 9:00 AM to 6:00 PM and conducted in accordance with the *Principles of Laboratory Animal Care*, National Institutes of Health guidelines, as approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC, Protocol S09179). To reduce the stress related to transport and new environment, mice were habituated to the testing room for 60 min under red light (15 lux) conditions, with *ad libitum* access to food and water, before each behavioral assessment.

Exploratory Behavior and Locomotor Activity

Differences in exploratory and locomotor activities are a potential confound for the interpretation of the behavioral outcomes described in the experimental timeline (**Figure 1**). To counterbalance all the groups for baseline locomotor activity/exploratory behavior, these were assessed in behavioral pattern monitor (BPM) chambers (San Diego Instruments, San Diego, CA, USA; Risbrough et al., 2006) 1 week before stress exposure for all experiments. Each BPM chamber is a clear Plexiglas box containing a 30 × 60 cm holeboard floor. A grid of 12 × 24 photobeams 1 cm above the holeboard floor providing a resolution of 1.25 cm (+16 beams detecting rears) allows us to locate the mouse. Mice were placed in the

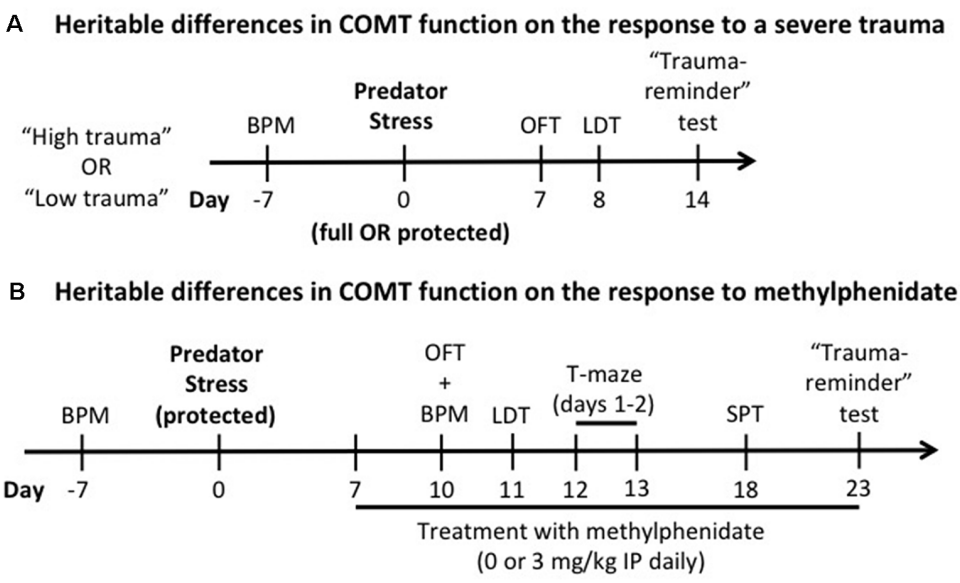

FIGURE 1 | Experimental design. The effects of heritable differences in catechol-O-methyltransferase (COMT) function on the response to a trauma **(A)** and to chronic administration of methylphenidate **(B)** were determined. In both experiments, a week before predator stress exposure, Met/Met and Val/Val carriers were tested in the behavioral pattern monitor (BPM) a week before predator stress exposure to counterbalance all the groups for baseline locomotor activity/exploratory behavior. OFT, open field test; LDT, light-dark box test; IP, intraperitoneal; SPT, social preference test.

BPM chambers (under light) and total distance traveled and exploratory behavior (transitions) were recorded for 60 min (**Figure 1**). In all experiments, there was no effect of genotype and no pre-manipulation group effects of stress or methylphenidate on locomotor activity (counts; **Supplementary Figure S1**).

Experiment 1: Effects of Heritable Differences in COMT Function on the Response to Low and High Intensity Trauma

Experimental Design and Predator Stress Exposure

The predator stress paradigm is known to induce several behavioral and biological phenotypes relevant to PTSD (for review, see Deslauriers et al., 2018). The predator stress exposure was performed from 9:00 AM to 6:00 PM as previously described (Toth et al., 2016) using two alternate protocols to manipulate trauma severity. In the full exposure condition ("high trauma") the mouse was individually placed with a cat (Liberty Research, Waverly, NY, USA) in a well-lit room (2.3 × 1.8 m; 150–200 lux) for 10 min in which both were allowed to freely explore and interact. Predator engagement was measured as described previously (Adamec et al., 2006b; Toth et al., 2016; Deslauriers et al., 2017): sniffing (bringing its nose near the mouse to explore the mouse's scent), pawing (playing gently with the mouse using its paws), and mouthing (touches the mouse with its mouth without biting). In the protected exposure condition ("low trauma"), the mouse was individually placed in a transparent cage with a wire lid, allowing the cat to freely move around and interact with the cage without being able to touch the mouse directly. This modified protocol aimed to avoid ceiling effects of predator stress as previously described (Adamec et al., 2006a). We based the protected vs. unprotected comparison on (Adamec et al., 2006a,b), which showed that the "low intensity" protected exposure enabled detection of mechanisms of "risk" using 5-HT transporter mutant mice. Both protocols show significant effects of stress (indicating predator exposure was adequate to induce long-term effects), however, the shift in avoidance was stronger in the unprotected version (0.3 vs. 0.6 standard deviation shift between non-stressed and stressed groups, see **Figure 2**). Thus, the two protocols differ in strength of trauma exposure to induce PTSD-like phenotype as operationalized in this model with avoidance behavior from 7 to 14 days after exposure. Only one cat is used per exposure, and the cat is exposed to 3–6 mice per day and there is an 1-h break between exposures to prevent habituation. COMT Met/Met and Val/Val mice were assigned to non-stressed or stressed groups (n = 85, 8–14 per group per sex for full exposure; n = 99, 10–18 per group per sex for protected exposure). Non-stressed mice were handled for 1 min. Generalized avoidance behaviors were assessed as described in **Figure 1A**.

Open Field Test

Open field test (PFT) was performed in an open arena (40 × 40 × 40 cm; 800 lux). Mice were placed in a corner and their activity was assessed for 10 min. Total distance traveled, entries into and time exploring the center area (25 × 25 cm), and latency of the first entry in the center area were analyzed using Ethovision Tracking Software (Noldus, Leesburg, VA, USA).

Light-Dark Box Test

The open field arena was divided in two 20 × 40 × 20 cm chambers (one well-lit; 950 lux and one covered; <5 lux) joined by a 6 × 6 cm door. Mice were placed in the dark chamber with door closed for 30 s and the test was started by opening the door. For 10 min, the number of entries into and time spent in the light chamber, and the latency of the first entry were assessed using Ethovision Tracking Software.

"Trauma-Reminder" Test

To measure avoidance of trauma-associated cues (Toth et al., 2016; Deslauriers et al., 2017), two perforated conical tubes (50 mL) containing either clean mouse bedding (no odor tube) or dirty cat litter (odor tube; from the cat used for stress exposure; containing urine and fur) were affixed to the floor of the open field arena in a cross-over design. In mice not exposed to the predator stress, the ratio of time spent around the odor tube vs. total time spent in either no odor or odor tube is 47%–60% while it is 75%–80% in stressed mice (Toth et al., 2016; Deslauriers et al., 2017), suggesting that stress exposure increases avoidance of predator odor in these mice. Mice were placed in an empty corner and their activity was assessed for 10 min. The number of approaches, the latency of first approach and time spent within a 3-cm radius zone around the tubes were measured using Ethovision Tracking Software.

Statistical Analysis

Since the three behavioral paradigms measure generalized avoidance behaviors, we calculated a composite avoidance score (average z-score of the number of entries/approaches into, time spent in, and latency of the first entry/approach in the aversive area) as previously described (Toth et al., 2016; Deslauriers et al., 2017). The aversive areas correspond to the center area, the light chamber and the tube containing dirty cat litter in the open field, light-dark box and "trauma-reminder" tests, respectively. This approach allows a more accurate and robust evaluation of the overall long-term effect of the stressor across multiple measures and time points and reduces family-wise error due to multiple testing. This procedure essentially reduces nine avoidance-related variables to 1 composite score. By creating this composite, variables that have relatively little differential meaning [time in open area of OFT vs. time in lit area of light-dark box test (LDT)] cannot be arbitrarily chosen for effects. This composite also prevents the problem of multiple sampling and the need for statistical power to overcome statistical corrections. A Pearson correlation between the nine dependent variables across the three behavioral tests was performed to describe the relationship between the components of the composite avoidance score (**Supplementary Table S1**). All variables contribute equally to the score, and by creating a composite of assessments over a 1-week period the consistency of the behavioral phenotype is emphasized. In other words, if on one test an animal is highly avoidant, but on other tests is not, it's overall "PTSD-like phenotype" score will be relatively modest. For each experiment (full and protected exposure), the

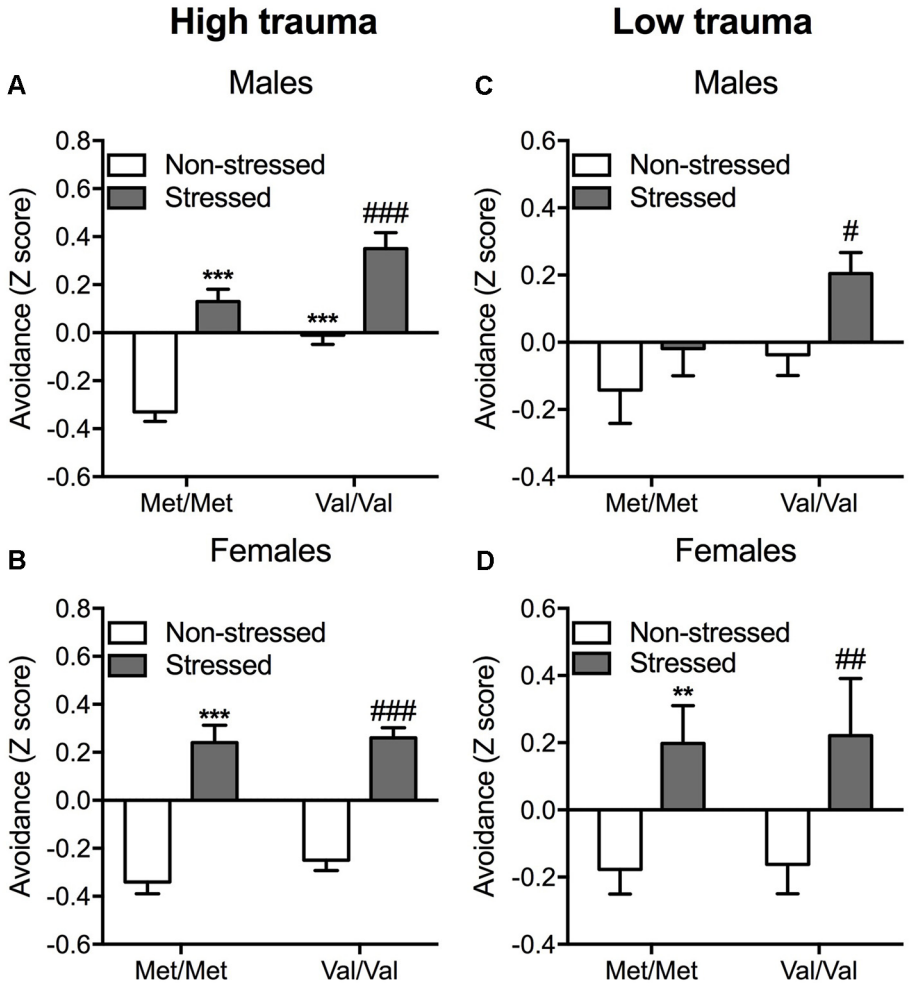

FIGURE 2 | Enhanced avoidance behavior induced by COMT Val/Val genotype and exposure to a predator in mice. A composite *z*-score combining all the parameters across the open field, the light-dark box and the "trauma-reminder" (7, 8, and 14 days post stress respectively) testing paradigms was calculated. Data are presented as mean ± standard error of the mean (SEM) for full (high) trauma **(A,B)** and protected (low) trauma **(C,D)** in both male and female mice. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. non-stressed Met/Met mice; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. non-stressed Val/Val group (n = 8–14 per group).

composite *z*-score was then analyzed using multifactorial analysis of variance (ANOVA) with *COMT* genotype and predator stress as between-subject factors, followed by Sidak *post hoc* comparisons (IBM SPSS Statistics). Since we and others have found that sex modulates COMTval158met SNP associations and COMT functional effects are sex-dependent in both humans and animals (Tunbridge, 2010; Risbrough et al., 2014), we performed the analysis for each sex separately.

Experiment 2: Effects of Heritable Differences in COMT Function on the Response to a Methylphenidate

Experimental Design and Predator Stress Exposure

Male COMT Met/Met and Val/Val mice were assigned to non-stressed or stressed groups (n = 92, 9–14 per group). Female mice were dropped from the experiment because they did not show genotype effects on predator stress. The predator stress exposure was performed as described above using the alternate mild "low trauma" protocol (see "Experimental Design and Predator Stress Exposure" section) to avoid ceiling effects of predator stress (Adamec et al., 2006a). Non-stressed mice were handled for 1 min. Starting 7 days after predator stress (or handling for the control group), methylphenidate (3 mg/kg intraperitoneal; Sigma-Aldrich, Saint-Louis, MO, USA) or vehicle (saline) was administered daily until the end of the study. Since differences in COMT enzymatic activity and dopamine tone are observed in the PFC of human and mouse Met/Met and Val/Val carriers (Chen et al., 2004; Tunbridge, 2010; Risbrough et al., 2014), the dose was chosen based on known increases in catecholamines in cortex but not striatum (Koda et al., 2010) with a peak effects 30 min post-injection. Treatment with methylphenidate was performed in the morning or 30 min before behavioral testing. Starting 10 days after predator exposure, several behavioral tests were performed to assess generalized avoidance (OFT, light-dark box, and "trauma-

reminder" test, as described above). We also extended the behavioral test battery to include additional measures relevant to PTSD and potential efficacy of methylphenidate, such as working memory (T-maze) and social withdrawal (social preference test, SPT; see experimental design in **Figure 1B**). The order of testing was established according to previous studies, and we conducted the least disruptive tests first (Contet et al., 2001; Deslauriers et al., 2017).

Behavioral Pattern Monitor

To verify that the chronic low-dose treatment did not affect locomotor and exploratory activities, these parameters were assessed in BPM chambers (San Diego Instruments, San Diego, CA, USA; Risbrough et al., 2006) as described above (see "Exploratory Behavior and Locomotor Activity" section). Mice were placed in the BPM chambers (under dark) and transitions (ambulations) were recorded for 60 min.

Working Memory

Patients with PTSD often exhibit deficits in working memory, which can also be related to disruption in inhibited processing speed, behavioral inflexibility, attention, and response inhibition (Vasterling et al., 2009). To examine working memory after predator stress, we used the spontaneous alternation task, a common test of spatial working memory in rodents which is sensitive to both cortical catecholamine function and COMT gene function (Lalonde, 2002; Risbrough et al., 2014). For each trial, the mouse begins in the stem of the maze and is allowed to choose one of two arms to explore. Typically rodents choose to explore arms that were not visited in the previous trial, and alternate between arms. This test takes advantage of a rodent's natural motivation to explore novel vs. familiar environments, and requires the mouse to remember what arm was just visited, while disregarding all other previous trials. The spontaneous alternation test was conducted as previously described (Risbrough et al., 2014) using a black plastic T-maze. The T-maze (81 cm length × 10 cm width × 25 cm height) includes two arms (35 cm length × 10 cm width × 25 cm height) and a start box (8 cm length × 10 cm width) that is separated from the main stem by a horizontal sliding door. Horizontal sliding doors (20 cm high) are also placed at the entrance of each arm. The 1st day, mice were habituated to the maze for 5 min, during which they could freely explore all three arms. The 2nd day, mice were placed in the start box for 30 s before the start of each trial. After 30 s the door was slid open and mice had a free choice into either the left or the right arm. After the mouse made a choice (all four paws in the chosen arm), the arm door was closed and the mice were allowed to explore the arm for 30 s before being removed and replaced in the start box for the next trial. A total of eight trials were completed (seven possible alternations). Percentage of spontaneous alternation was calculated as 100 × (number of alternations/7).

Social Approach Test

Social withdrawal is commonly observed in PTSD patients (Gold et al., 2000). Therefore, the preference of the mice for a social stimulus over an inanimate object was assessed. Social approach was tested using a three-chambered box similar to what we have been previously described (Naviaux et al., 2014). Briefly, a Plexiglas box (60 cm length × 60 cm width × 30 cm height) was divided into three equal compartments by Plexiglas partitions containing an opening through which the mice could freely enter the three chambers. The test was conducted in two phases. During the first phase, the test mouse was first allowed to explore the chambers for 10 min. Each of the two outer chambers contained an empty, inverted stainless steel wire cup (Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH, USA). During the second phase, the test mouse was briefly removed. An unfamiliar mouse, age- and sex-matched, was placed under one of the wire cups and Lego blocks were placed under the other wire cup. The test mouse was then gently placed back in the arena and given 5 min to explore. The amount of time spent in each chamber was recorded using Ethovision Tracking Software and hand-scored by an experimenter blinded to the condition/treatment. The location (left or right) of the novel object and novel mouse alternated across subjects. SP in percent was calculated as 100 multiplied by the hand-scored time spent exploring (sniffing; bringing its nose in a 2-cm radius zone around the cage) the stranger mouse (t_M) divided by the sum of the time with stranger plus time with object ($t_M + t_L$): $SP = 100 \times [t_M/ (t_M + t_L)]$.

Statistical Analysis

For the three paradigms measuring generalized avoidance behaviors (OFT, LDT, and "trauma-reminder" test), we calculated a composite avoidance score as described above (see "Statistical Analysis" section; Toth et al., 2016; Deslauriers et al., 2017). The composite *z*-score, spontaneous alternation and SP were then analyzed using multifactorial ANOVA with *COMT* genotype, predator stress, and methylphenidate treatment as between-subject factors. For locomotor and exploratory activities assessed in the BPM, repeated measures ANOVAs were conducted with the same between-subject factors and with time (10-min block) as within-subject factor. All ANOVAs were followed by Tukey's *post hoc* comparisons (IBM SPSS Statistics).

RESULTS

Experiment 1: Effects of Heritable Differences in COMT Function on the Response to Low and High Intensity Trauma

High Trauma Condition

In males, unprotected predator exposure increased the avoidance composite score ($F_{(1,38)} = 64.16$; $p < 0.001$) independently of genotype as confirmed with *post hoc* comparisons ($p < 0.001$ vs. the corresponding non-stressed group; **Figure 2A**). *COMT* Val/Val carriers also showed increased avoidance compared to Met/Met carriers ($F_{(1,38)} = 27.83$; $p < 0.001$), as confirmed by *post hoc* test ($p < 0.001$; **Figure 2A**; **Supplementary Table S2**). No interaction between *COMT*val158met and stress were detected suggesting additive effects of stress and COMT genotype on avoidance behavior. In females, predator stress resulted in

significant increases in avoidance behavior independently of genotype (main effect of stress: $F_{(1,39)} = 109.90$; $p < 0.001$; Sidak *post hoc* comparisons: $p < 0.001$ vs. the corresponding non-stressed group; **Figure 2B**; **Supplementary Table S3**).

Low Trauma Condition

As in the high trauma condition, *COMT* Val/Val genotype and stress exposure increased avoidance in males ($F_{(1,51)} = 4.79$; $p < 0.05$ and $F_{(1,51)} = 5.87$; $p < 0.05$, respectively; **Figure 2C**). Sidak *post hoc* analysis showed that predator exposure exerted a greater effect in Val/Val mice, as demonstrated by increased avoidance in response to stress ($p < 0.05$ vs. non-stressed Val/Val mice; **Figure 2C**; **Supplementary Table S4**). In this lower stress condition, female Val/Val and stressed mice show increased amount of avoidance ($F_{(1,38)} = 10.85$; $p < 0.01$) independently of the genotype (**Figure 2D**). *Post hoc* comparisons confirmed the increased avoidance in both stressed Met/Met and Val/Val carriers ($p < 0.01$ compared to the corresponding non-stressed group; **Figure 2D**; **Supplementary Table S5**).

Experiment 2: COMTval158met Modulation of Response to Chronic Treatment With Methylphenidate

Avoidance

To examine the overall effects of chronic treatment with methylphenidate on stress-induced PTSD-relevant behaviors, the mild "low trauma" protocol was used for predator stress since it allowed us to prevent ceiling effects of stress that might blur COMTval158met effects (Adamec et al., 2006a). Since the main effect of genotype was found only in males (see above), methylphenidate effects were tested only in male mice. Predator stress increased avoidance behavior which was reversed with methylphenidate treatment (stress × methylphenidate: $F_{(1,84)} = 4.50$; $p < 0.05$). Methylphenidate effects were independent of COMT genotype (**Figure 3**). *Post hoc* tests confirmed that stressed Met/Met and Val/Val mice treated with methylphenidate exhibited lower avoidance behaviors as compared to the same stressed carriers treated with vehicle ($p < 0.001$; **Figure 3**). To confirm a genotype effect a two-way ANOVA in vehicle-treated animals alone (genotype × stress) revealed a main effect of genotype ($F_{(1,42)} = 2.95$; $p < 0.05$) and stress ($F_{(1,42)} = 8.40$; $p < 0.01$). *Post hoc* comparisons confirmed that Val/Val carriers showed greater avoidance behavior in response to stress exposure than Met/Met mice ($p < 0.05$ compared to non-stressed Met/Met carriers; **Figure 3**; **Supplementary Table S6**).

Locomotor Activity

Repeated measures ANOVAs revealed no effect of or interaction with methylphenidate treatment on locomotor activity. A time × genotype interaction was observed ($F_{(1,84)} = 3.69$; $p < 0.01$; **Supplementary Figure S2**) however no significant differences were detected in *post hoc* analyses at each time point, although Val/Val carriers had slightly higher transitions compared to Met/Met mice at later time points. Univariate ANOVA within each 10-min block did not reveal any main effect or interaction of genotype, stress, or methylphenidate.

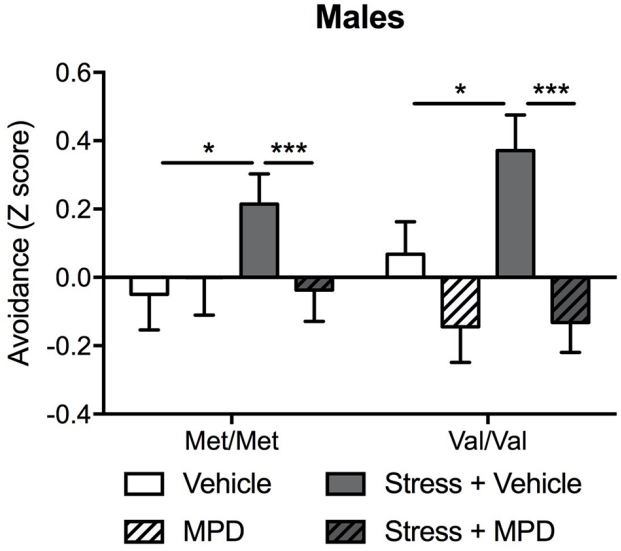

FIGURE 3 | Chronic treatment with methylphenidate (MPD) decreased avoidance behaviors in stressed male mice regardless of their genotype. A composite *z*-score combining all the parameters across the open field, the light-dark box and the "trauma-reminder" (10, 11, and 23 days post stress respectively) testing paradigms was calculated. Data are presented as mean ± SEM. $^*p < 0.05$; $^{***}p < 0.001$ (n = 9–14 per group).

Working Memory

To determine the effects of chronic administration of methylphenidate on working memory, spontaneous alternation in the T-maze was assessed. Methylphenidate treatment effects were modulated by genotype (methylphenidate × genotype: $F_{(1,83)} = 7.61$; $p < 0.01$) and stress (methylphenidate × stress: $F_{(1,83)} = 6.94$; $p = 0.01$). *Post hoc* comparisons revealed that methylphenidate decreased spontaneous alternation in non-stressed Met/Met carriers compared to the same non-stressed carriers treated with vehicle ($p < 0.05$; **Figure 4**). The same treatment had the opposite effect in stressed Val/Val mice by increasing spontaneous alternation ($p < 0.05$ as compared to the same stressed mice treated with vehicle; **Figure 4**). To replicate our previous observation of COMTval158met modulation of working memory (Risbrough et al., 2014) a two-way ANOVA (genotype × stress) in vehicle-treated mice confirmed that Val/Val carriers exhibited lower spontaneous alternation compared to Met/Met mice ($F_{(1,40)} = 6.10$; $p < 0.05$, *post hoc* comparisons, $p < 0.05$).

Social Approach Behavior

We then evaluated the effects of chronic treatment of methylphenidate on social approach. Methylphenidate effects on social approach were dependent upon stress exposure ($F_{(1,83)} = 5.49$; $p < 0.05$; **Figure 5**). Indeed, stressed mice showing decreased social approach, regardless of genotype. Methylphenidate treatment prevented the development of social approach deficits in both stressed Met/Met and Val/Val carriers (Tukey's *post hoc* comparisons $p < 0.05$ compared to the corresponding non-stressed vehicle-treated group; **Figure 5**).

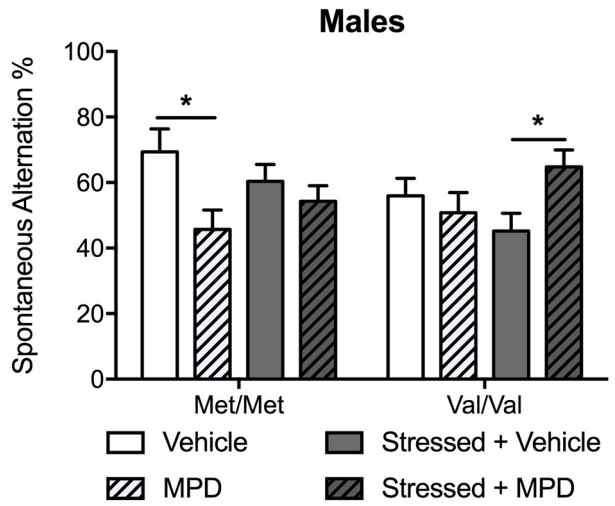

FIGURE 4 | Chronic treatment with MPD had opposite effects on working memory in non-stressed Met/Met carriers and in stressed Val/Val mice. Working memory was determined with the spontaneous alternation assessed in the T-maze 13 days after stress. Data are presented as mean ± SEM in male mice. $^*p < 0.05$ (n = 9–14 per group).

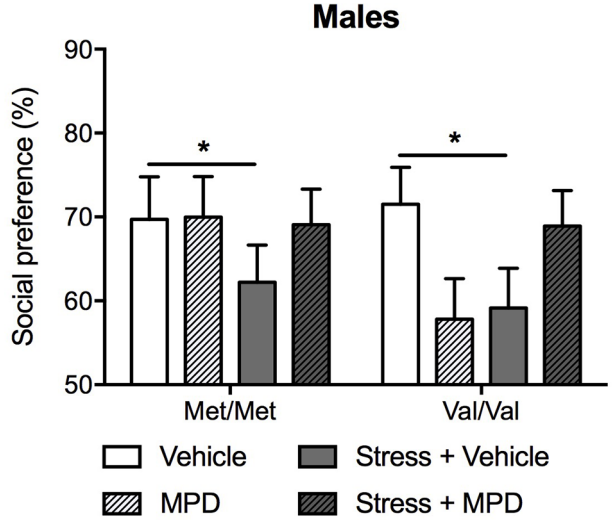

FIGURE 5 | Chronic treatment with MPD prevented stress-induced decrease in social approach in male mice, regardless of their genotype. Social behaviors were assessed in the social approach test 18 days after stress. Data are presented as mean ± SEM. $^*p < 0.05$ (n = 9–14 per group).

DISCUSSION

The present study aimed to test whether the heritable variance in COMT function produced by the *COMT*val158met SNP modulates response to trauma and to chronic treatment with methylphenidate. In a "humanized" *COMT*val158met mouse line (Risbrough et al., 2014), male Val/Val carriers exhibited higher avoidance behaviors at baseline and in response to a predator stress model of PTSD compared to Met/Met carriers. This phenotype in Val/Val mice was observed across both low and high "trauma" protocols, and in the treatment protocol, underscoring the robustness of the phenotype. In female mice, the stress effects were COMT-independent across both "trauma" protocols, indicating that effects of COMTval158met on avoidance-like traits are sex-dependent. Male Val/Val carriers had lower working memory performance as measured by spontaneous alternation compared to Met/Met carriers. However, no differences were found in SP at baseline or after stress exposure between male Met/Met and Val/Val carriers. Hence in males, COMTval158met modulates avoidance, working memory as well as trauma response, but not social behaviors. Methylphenidate treatment reduced avoidance and increased SP in stressed mice independently of their genotype. However, COMTval158met genotype did modulate methylphenidate effects on spontaneous alternation, decreasing alternation in non-stressed Met/Met mice while increasing alternation in stressed Val/Val mice. These findings suggest that the effects of methylphenidate on avoidance and social behaviors involve COMT-independent mechanisms, and COMT-dependent pathways may modulate the effects of the same treatment on working memory in male mice.

The current study shows that higher COMT function by carrying the Val allele of the *COMT*val158met SNP induced higher avoidance behaviors in male mice. These findings are in line with a meta-analysis (n = 2,913) confirming an association between the Val allele and panic disorder in studies with participants of European ancestry (Howe et al., 2016). These results also concur with a previous study linking the Val allele with higher PTSD incidence following mild traumatic brain injury (mTBI) in a predominantly Caucasian sample (Winkler et al., 2017). Together with our preclinical findings, these data support the double-hit hypothesis (gene × environment interaction) of trauma-related disorders (Daskalakis et al., 2013; Smoller, 2016). In female mice, COMT genotype was not associated with different baseline or post-trauma avoidance behavior across either "trauma" protocols. Sex-dependent effects of COMTval158met are in line with previous studies reported a strong sexually dimorphic effect of COMT on brain and behavior (Tunbridge and Harrison, 2010). However, stronger effects of COMT inhibition were found with tolcapone in female rats (Laatikainen et al., 2013) or in COMT-deficient female mice (Gogos et al., 1998) compared to male rodents. These discrepancies with our findings may be due to complete inhibition of COMT function, whereas our COMT Met/Met mice exhibit a 50% inhibition of COMT function compared to Val/Val carriers (Risbrough et al., 2014). Animal studies have also shown that estrogen enhances dopamine activity in the PFC (Jacobs and D'Esposito, 2011), suggesting that estrogen may mitigate COMTval158met effects. Nevertheless, further work characterizing the effects of estrogen on catecholamine signaling in our model needs to be conducted to understand the mechanisms underlying the sex-dependent effects of the COMTval158met. In the spontaneous alternation test, we replicated past observations of low alternation in Val/Val mice compared to Met/Met mice (Risbrough et al., 2014), which is in line with some human studies reporting poorer working memory performance in of Val/Val carriers (Giakoumaki et al.,

2008; Costa Dde et al., 2016; Geller et al., 2017; Miskowiak et al., 2017). We observed no effects of COMTval158met genotype on SP, which concurs with a previous clinical study showing no association between COMTval158met and social functioning in patients with schizophrenia or bipolar disorder (Goghari and Sponheim, 2008).

COMTval158met effects on response to a trauma could be *via* modulation of cortical catecholamine signaling both during and after the trauma. COMT has a significant contribution to catecholamine signaling in the cortex because of relatively low dopamine transporter expression (Harrison and Tunbridge, 2008). The PFC modulates fear responses through reciprocal inputs to the amygdala and is involved in multiple forms of fear inhibition including fear extinction, which can be impaired in PTSD patients (Bremner, 2005; Gamo and Arnsten, 2011). The *COMT*val158met SNP alters catecholamine signaling pathways in the PFC, with Val/Val carriers exhibiting higher COMT activity in this circuit compared to Met/Met carriers. Subsequently, mouse and human Val/Val carriers have lower dopamine tone than Met/Met carriers (Tunbridge and Harrison, 2010; Risbrough et al., 2014). There is some evidence that lower cortical dopamine tone can promote anxiety responses. Chronic stress reduces dopamine levels in the mesocortical region in rodents (Burke and Miczek, 2014), and cortical dopamine depletion is anxiogenic in rodents (Sullivan et al., 2014). Most pertinent to the current study, exposure to predator odor elevates dopamine turnover in the medial PFC (mPFC), which is correlated with increased avoidance (Morrow et al., 2000). Hence it is possible that the combination of stress-induced reductions in cortical dopamine signaling along with higher dopamine catabolism in Val/Val carriers underlies the modulation of *COMT*val158met on enduring stress response (Arnsten et al., 2015). It should also be noted that a potential role of noradrenergic signaling in *COMT*val158met effects on stress responses should not be excluded (Arnsten et al., 2015). Indeed, since the COMTval158met affects noradrenergic pathways (Tunbridge and Harrison, 2010), it would be interesting to investigate the effects of COMTval158met and predator stress on hyperarousal and corticosterone levels. Further studies using this "humanized" *COMT* mouse line exposed to predator stress will be necessary to confirm the underlying mechanisms of *COMT*val158met modulation of enduring response to trauma.

We also investigated the effects of chronic treatment with methylphenidate in the predator stress model of PTSD in males and asked if COMTval158met modulated treatment response. Acute low doses of methylphenidate (<5 mg/kg) reduce anxiety-like behaviors (Koike et al., 2009; Mioranzza et al., 2010), whereas high doses (>7 mg/kg) have anxiogenic effects (Ihne et al., 2012). We chose a dose of MPD that selectively increases catecholamine release in cortex while having little effect in striatum in mice, to specifically target circuits that are most sensitive to COMT variation (Chen et al., 2004; Tunbridge, 2010; Risbrough et al., 2014). Higher doses of MPD induce increases in striatal dopamine and concomitant stimulant activity, which could also confound activity-based measures used in this study (Koda et al., 2010). Here, we confirmed that this dosing regimen did not induce changes in locomotor and exploratory behavior. This dose is also very similar to effective doses of MPD in rat models of PTSD (Avital et al., 2011; Zubedat et al., 2015).

Here, the treatment was tested only in males since females did not show significant *COMT* variant effects on predator stress. Methylphenidate treatment ameliorated both stress-induced avoidance and reduction in SP. These data are in line with other reports of methylphenidate efficacy in animal models of PTSD (Aga-Mizrachi et al., 2014; Ritov and Richter-Levin, 2017) and social approach deficits (Aga-Mizrachi et al., 2014; Gill et al., 2014). It is also in line with reports of efficacy in PTSD patients (McAllister et al., 2016) and for social functioning in ADHD (Abikoff et al., 2004). COMTval158met did not modify treatment effects in these tests, suggesting these treatment effects are independent of heritable differences in COMT function. There are a number of potential mechanisms underlying the effects of methylphenidate to treat enduring avoidance and other stress-related symptoms after trauma. In rodents, chronic administration of low-dose methylphenidate increases attention in several cognitive tasks *via* activation of α_2 adrenergic and D1 dopaminergic receptors in the rat PFC (Spencer et al., 2015). Chronic low-dose methylphenidate also restores stress-induced alterations in PFC dendritic spine densities and impairments in NMDAR function (Zehle et al., 2007; Cheng et al., 2014). It also amplifies long-term potentiation (LTP) in rat hippocampal CA1 area *via* the activation of β-adrenergic and D1/D5 dopaminergic receptors (Rozas et al., 2015). As stated above, PFC and hippocampal dysfunctions are consistent "biophenotypes" in PTSD, and there is also mounting evidence for glutamatergic disruption in PTSD (Averill et al., 2017; Deslauriers et al., 2018). Nevertheless, further work needs to be conducted to investigate the exact therapeutic mechanisms of chronic low-dose methylphenidate, as well as its sex-dependent effects in the context of PTSD.

In line with the COMT-dependent effects of methylphenidate on working memory reported here, treatment with COMT inhibitor tolcapone improves working memory in human Val carriers but reduces the high working memory performance in Met carriers (Giakoumaki et al., 2008). These data support the hypothesis that COMTval158met modulates the "inverted U" effects of dopamine signaling on PFC functions such as working memory. No significant association was found between COMTval158met and response to methylphenidate treatment in adolescent and adult patients with ADHD (Contini et al., 2012; Unal et al., 2016). However, a meta-analysis (n = 889) reported increased response to methylphenidate in ADHD children carrying the Val/Val genotype (Myer et al., 2018), suggesting that the COMT-dependent effects of methylphenidate may be age-dependent. Here, methylphenidate exerted COMT-dependent effects on working memory, but not avoidance and social behaviors in mice. Together with the clinical findings reported above, these findings suggest that the COMT-dependent effects of methylphenidate may be specific to attention and memory.

The present study showed an increased response to predator-induced trauma in male Val/Val mice. Furthermore, methylphenidate reversed the predator stress-induced avoidance behaviors and social withdrawal in males regardless of genotype while methylphenidate effects on cognition were dependent

upon COMTval158met genotype. These results suggest that individual differences in catecholamine signaling may modulate response to a trauma and support the double-hit gene × environment hypothesis of PTSD. These findings also support recent human findings that methylphenidate might be a therapeutic alternative for PTSD patients (McAllister et al., 2016) and suggest that methylphenidate effects anxiety vs. cognitive symptoms in males may be modulated through COMT-independent and dependent mechanisms respectively. Nevertheless, further work needs to be conducted to investigate the COMT variant-specific response to methylphenidate in females.

ETHICS STATEMENT

The experiments were conducted in accordance with the Principles of Laboratory Animal Care, National Institutes of Health guidelines, as approved by the University of California, San Diego, San Diego, CA, USA.

AUTHOR CONTRIBUTIONS

VR designed the experiment, and MT conducted the experiments using the full exposure to predator stress. JD performed all the other experiments, analyzed and interpreted the data. XZ developed the "humanized" COMT mouse line. JD and VR wrote the manuscript, and MT and XZ provided critical feedback.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00111/full#supplementary-material

FIGURE S1 | No difference on baseline locomotor activity was found across all groups in all experiments. Locomotor activity (counts) was assessed **(A–D)** for Experiment 1 (COMTval158met modulation of response to low and high trauma; **A–D**) and Experiment 2 (COMTval158met modulation of response to methylphenidate; **E**) were assessed in the behavioral pattern monitor 1 week before stress. Data are presented as mean ± SEM (n = 8–14 per group).

FIGURE S2 | Heritable differences in COMT function, stress, and methylphenidate did not affect locomotor activity in male mice. Locomotor activity (transitions) was assessed in the behavioral pattern monitor 10 days after stress. Data are presented as mean ± SEM for non-stressed and stressed Met/Met carriers **(A,B)**, as well as for non-stressed and stressed Val/Val mice **(C,D)** (n = 9–14 per group).

TABLE S1 | Pearson correlation matrix of all variables included in the composite avoidance score across all three experiments (unprotected and protected predator exposures, and chronic methylphenidate experiment). Since the three behavioral paradigms measure generalized avoidance behaviors, we calculated a composite avoidance score (average z-score of the number of entries/approaches into, time spent in, and latency of the first entry/approach in the aversive area) as previously described (Toth et al., 2016; Deslauriers et al., 2017). The aversive areas correspond to the center area, the light chamber and the tube containing dirty cat litter in the open field, light-dark box and "trauma-reminder" tests, respectively (n = 187).

TABLE S2 | Avoidance behavior in the open field, light-dark box and trauma-reminder tests following full predator exposure (high trauma) in male mice. Data are presented as mean ± SEM for the number of entries/approaches, time spent and latency of first entry/approach in the aversive area across all the behavioral tests. The aversive areas are the center area, the lit chamber and the odor tube in the open field, light-dark box and trauma-reminder tests, respectively. Two-way ANOVAs (genotype × stress) revealed a main effect of stress in the open field test ($F_{(1,38)} = 13.76$; $p < 0.01$), with decreased time spent in the center area in predator-exposed mice. Stress also increased the latency of first approach to the cat odor tube in the trauma-reminder test ($F_{(1,38)} = 4.49$; $p < 0.05$). $^{**}p < 0.01$ vs. non-stressed Met/Met mice following Sidak *post hoc* test.

TABLE S3 | Avoidance behavior in the open field, light-dark box and trauma-reminder tests following full predator exposure (high trauma) in female mice. Data are presented as mean ± SEM for the number of entries/approaches, time spent and latency of first entry/approach in the aversive area across all the behavioral tests. The aversive areas are the center area, the lit chamber and the odor tube in the open field, light-dark box and trauma-reminder tests, respectively. Two-way ANOVAs (genotype × stress) revealed a main effect of stress in the open field test, with decreased number of entries time spent in the center area ($F_{(1,39)} = 10.57$; $p < 0.01$ and $F_{(1,39)} = 8.23$; $p < 0.01$, respectively), and increased latency of first entry in the center arena ($F_{(1,39)} = 7.70$; $p < 0.01$) in predator-exposed mice. In the light-dark box, stressed mice exhibited reduced number of entries in the lit chamber ($F_{(1,39)} = 4.76$; $p < 0.05$). $^{*}p < 0.05$ vs. non-stressed Met/Met mice; $^{\#}p < 0.05$ vs. non-stressed Val/Val mice following Sidak *post hoc* test.

TABLE S4 | Avoidance behavior in the open field, light-dark box and trauma-reminder tests following protected predator exposure (low trauma) in male mice. Data are presented as mean × SEM for the number of entries/approaches, time spent and latency of first entry/approach in the aversive area across all the behavioral tests. The aversive areas are the center area, the lit chamber and the odor tube in the open field, light-dark box and trauma-reminder tests, respectively. Two-way ANOVAs (genotype × stress) revealed a main effect of stress in the open field test ($F_{(1,51)} = 2.66$; $p < 0.001$), with increased latency to first entry to center area in predator-exposed mice. Also, the genotype decreased the time spent in the lit chamber of the light-dark box ($F_{(1,51)} = 10.79$; $p < 0.01$) and around to cat odor tube in the trauma-reminder test ($F_{(1,51)} = 11.04$; $p < 0.01$). The COMT genotype also increased the latency to the first approach to the odor tube in the trauma-reminder test ($F_{(1,51)} = 8.73$; $p < 0.01$). $^{*}p < 0.05$ vs. non-stressed Met/Met mice; $^{\#\#\#}p < 0.001$ vs. non-stressed Val/Val group following Sidak *post hoc* test.

TABLE S5 | Avoidance behavior in the open field, light-dark box and trauma-reminder tests following protected predator exposure (low trauma) in female mice. Data are presented as mean ± SEM for the number of entries/approaches, time spent and latency of first entry/approach in the aversive area across all the behavioral tests. The aversive areas are the center area, the lit chamber and the odor tube in the open field, light-dark box and trauma-reminder

tests, respectively. Two-way ANOVAs (genotype × stress) revealed a main effect of stress in the open field and the trauma-reminder tests, mainly on latency to first entry to center area ($F_{(1,51)} = 9.72$; $p < 0.01$) and the latency to first approach to the odor tube ($F_{(1,51)} = 4.41$; $p < 0.05$), respectively. Also, the genotype interacted with stress (main effect of genotype: $F_{(1,51)} = 4.32$, $p < 0.05$; genotype stress: $F_{(1,51)} = 5.67$, $p < 0.05$) in the trauma-reminder stress, with Val/Val carriers exposed to a predator exhibiting lower time spent around the cat odor tube. $^{*}p < 0.05$ vs. non-stressed Met/Met mice; $^{\#}p < 0.05$ vs. non-stressed Val/Val group following Sidak *post hoc* test.

TABLE S6 | Avoidance behavior in the open field, light-dark box and trauma-reminder tests following protected predator exposure and chronic treatment with methylphenidate in male mice. Data are presented as mean ± SEM for the number of entries/approaches, time spent and latency of first entry/approach in the aversive area across all the behavioral tests. The aversive areas are the center area, the lit chamber and the odor tube in the open field, light-dark box and trauma-reminder tests, respectively. In the open field test, a three-way ANOVAs (genotype × stress × methylphenidate) revealed a main effect of methylphenidate ($F_{(1,84)} = 12.87$; $p < 0.001$; genotype × methylphenidate: $F_{(1,84)} = 4.41$; $p < 0.05$) on the number of entries in center area, with increased number of entries in the center area in Val/Val mice treated with methylphenidate vs. corresponding group treated with vehicle. Methylphenidate also decreased the time spent in the center area ($F_{(1,84)} = 5.48$; $p < 0.05$), and a genotype × stress interaction ($F_{(1,84)} = 4.53$; $p < 0.05$) was found on the latency of first entry in the center area. In the trauma-reminder test, methylphenidate increased the number of approaches to cat odor tube ($F_{(1,84)} = 26.19$; $p < 0.001$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. corresponding group treated with vehicle following Tukey's *post hoc* test.

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REVIEW
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Animal Models of PTSD: The Socially Isolated Mouse and the Biomarker Role of Allopregnanolone

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Post-traumatic stress disorder (PTSD) is a debilitating undertreated condition that affects 8%–13% of the general population and 20%–30% of military personnel. Currently, there are no specific medications that reduce PTSD symptoms or biomarkers that facilitate diagnosis, inform treatment selection or allow monitoring drug efficacy. PTSD animal models rely on stress-induced behavioral deficits that only partially reproduce PTSD neurobiology. PTSD heterogeneity, including comorbidity and symptoms overlap with other mental disorders, makes this attempt even more complicated. Allopregnanolone, a neurosteroid that positively, potently and allosterically modulates $GABA_A$ receptors and, by this mechanism, regulates emotional behaviors, is mainly synthesized in brain corticolimbic glutamatergic neurons. In PTSD patients, allopregnanolone down-regulation correlates with increased PTSD re-experiencing and comorbid depressive symptoms, CAPS-IV scores and Simms dysphoria cluster scores. In PTSD rodent models, including the socially isolated mouse, decrease in corticolimbic allopregnanolone biosynthesis is associated with enhanced contextual fear memory and impaired fear extinction. Allopregnanolone, its analogs or agents that stimulate its synthesis offer treatment approaches for facilitating fear extinction and, in general, for neuropsychopathologies characterized by a neurosteroid biosynthesis downregulation. The socially isolated mouse model reproduces several other deficits previously observed in PTSD patients, including altered $GABA_A$ receptor subunit subtypes and lack of benzodiazepines pharmacological efficacy. Transdiagnostic behavioral features, including expression of anxiety-like behavior, increased aggression, a behavioral component to reproduce behavioral traits of suicidal behavior in humans, as well as alcohol consumption are heightened in socially isolated rodents. Potentials for assessing novel biomarkers to predict, diagnose, and treat PTSD more efficiently are discussed in view of developing a precision medicine for improved PTSD pharmacological treatments.

Keywords: post-traumatic stress disorder, translational neuroscience, social isolation, biomarker axis, neurosteroids, endocannabinoids, PTSD rodent models, PTSD treatments

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a multifaceted psychiatric disorder characterized by a high worldwide prevalence in the general population and a consistent global burden and disability. In the U.S., about 50%–85% of individuals during their lifetime experience traumatic events, of these, about 6.8% develop PTSD (Kessler et al., 2005). However, its prevalence is even higher, reaching 25%–50%, in individuals exposed to warzones or in victims of domestic violence and abuse, including children and battered women, respectively (Goldstein et al., 2016). Importantly, women are particularly susceptible to develop PTSD as compared with men (Shansky, 2015; Yehuda et al., 2015). Other predictors for developing PTSD, include characteristics of the traumatic event for a given exposed individual (Bichescu et al., 2005). Comorbidity with other psychiatric disorders, such as major depressive disorder, anxiety spectrum disorders, and alcohol use disorder (AUD), or with suicide, as well as, overlapping of symptoms with these disorders are very common in individuals affected by PTSD (Shalev, 2001; Lassemo et al., 2017; Gagne et al., 2018). Together, these complications result in a general difficulty in diagnosing PTSD and make treatment selection difficult (Greene et al., 2016). Current pharmaco-treatment for PTSD relies in the administration of the selective serotonin reuptake inhibitors (SSRIs), such as paroxetine and sertraline, the only FDA-approved drugs for PTSD (Friedman and Bernardy, 2017). These drugs are associated with poor response rate in a consistent number of treatment-seeking patients, with active military members and veterans who are relatively non-responsive to SSRIs (Bernardy and Friedman, 2015; Starke and Stein, 2017). Developing suitable animal models for PTSD and discovering reliable biomarkers that allow a more accurate diagnosis, based on objective measures, may improve quality of healthcare. Biomarker discovery will indeed permit developing targeted drugs and may generally offer more treatment options, which is highly desirable and needed (discussed in Aspesi and Pinna, 2018).

While a number of animal models in mice and rats were developed in the past decades that, at least, partially recapitulate several neurochemical and behavioral deficits encountered in the wide ranging PTSD symptoms clusters, none of them is currently recognized as an optimal match with the human neuropathology (reviewed in Aspesi and Pinna, 2019). However, some of them reproduce core aspects of PTSD, including deficits in fear extinction and fear extinction retention and even transdiagnostic aspects relevant for comorbidity with depression, suicide and AUD. Notwithstanding sex matters with PTSD, sex as a biological variable in research including females has only recently being intensified and the sex-effect or the effect of the menstrual cycle or pregnancy in women with PTSD only recently has been taken into examination (Onoye et al., 2013; Pineles et al., 2017, 2018). In rodent PTSD models, these sex-related effects were scantily studied with very few studies that have attempted to reproduce endophenotipic expression of female PTSD neurobiology into female rodents (Cohen and Yehuda, 2011; reviewed in Keller et al., 2015; Aspesi and Pinna, 2019). Hence, sex-related studies in PTSD neurobiology are urgent and a priority in both clinical and preclinical research.

Furthermore, to add to the general complexity and heterogeneity of PTSD, it is conceivable that factors, including the type and the duration in time of a traumatic event, as well as, the condition of individuals in a given time when they are exposed to trauma, altogether, may dictate the development of subtypes of PTSD (Stein et al., 2016). Collectively, all these factors are important aspects that may drive establishing successful PTSD animal models. Often, the question arises as to whether an experimental model of PTSD should exclusively recapitulate core traits of PTSD, such as extinction deficits and avoidance or rather should take into account what is often encountered in the diagnosis of PTSD patients, for example, comorbidities with other mental disorders (discussed in Aspesi and Pinna, 2019).

The recent progress that has been made in understanding PTSD neurobiology has facilitated the development of experimental stress-induced animal models (Torok et al., 2018). However, PTSD remains a neuropathology with no specific pharmacological treatments, no established and reliable biomarkers, and PTSD animal models only reproduce PTSD neurobiology to a limited degree. While previous recent articles examined a number of animal models of PTSD and the validity of several biomarker candidates that have been proposed for PTSD (Aspesi and Pinna, 2018, 2019), this review will focus on the socially isolated mouse model of stress-induced fear extinction deficits. Other abnormal behavioral deficits will be discussed as well as commonalities with PTSD neurobiology in humans, such as reproducing endophenotipic features observed in PTSD patients. Transdiagnostic aspects shared with depression, anxiety, suicide and AUD are also discussed. This review article also analyses running findings suggesting the neurosteroid, allopregnanolone biosynthesis and its targets may prove valuable for establishing a *biomarker axis* suitable for PTSD. It is conceivable that allopregnanolone may play a key role to predict, diagnose and suggest an optimal treatment selection for PTSD in the near future.

ALLOPREGNANOLONE FROM ITS DISCOVERY IN ADRENAL GLANDS TO A ROLE IN MOOD DISORDERS

Following its discovery in 1938 by Beall and Reichstein in the adrenal glands (**Figure 1**), allopregnanolone was recognized as a 5α-reduced metabolite of progesterone (Beall and Reichstein, 1938). It was named a *neurosteroid* in 1981 by Baulieu's team who discovered that the brain "acting like a peripheral gland," expresses the enzymatic machinery required to synthetize allopregnanolone *de novo* starting from pregnenolone, the precursor of all neurosteroids (Corpéchot et al., 1981). Allopregnanolone's anti-convulsant, anxiolytic and anti-depressant pharmacological effects after its administration in animal models and humans were soon recognized to be mediated by a mechanism of action that includes the fast allosteric modulation of the action of GABA

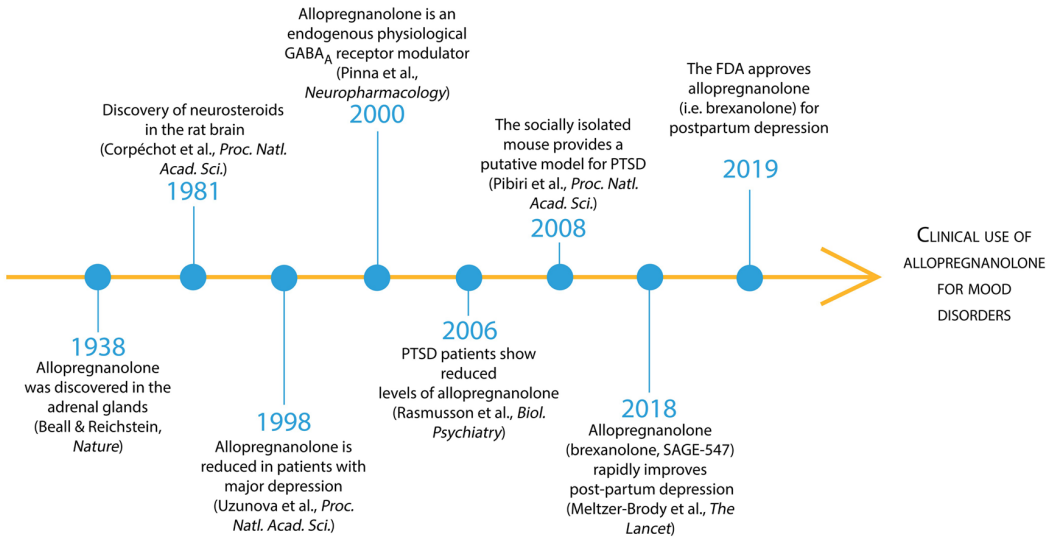

FIGURE 1 | Timeline of allopregnanolone from its discovery to FDA preapproval for the treatment of mood disorders. Beall and Reichstein discovered allopregnanolone in 1938 in the adrenal glands where 5α-reductase metabolizes progesterone into 5α-dihydroprogesterone and then the enzyme 3α-hydroxysteroid dehydrogenase produces allopregnanolone (Beall and Reichstein, 1938). In 1981, Baulieu's team discovered that the brain "acting like a peripheral gland" synthetize allopregnanolone *de novo* starting from pregnenolone, the precursor of all neurosteroids (Corpéchot et al., 1981). Allopregnanolone's pharmacological effects following its administration in animal models and humans are mediated by the fast allosteric modulation of the action of GABA at GABA$_A$ receptors (Majewska et al., 1986; reviewed in Belelli et al., 2009). The neurophysiological role of allopregnanolone in fine-tuning GABA$_A$ receptors to agonists, positive allosteric modulators, and GABAmimetic agents, was unveiled thereafter (Pinna et al., 2000). Allopregnanolone levels were found decreased in mood disorders, including major unipolar depression and PTSD (Romeo et al., 1998; Uzunova et al., 1998; Rasmusson et al., 2006, 2019). An animal model of stress-induced behavioral dysfunction, including fear extinction deficits and aggressive behavior associated with a corticolimbic allopregnanolone biosynthesis downregulation was proposed therein after (Pinna et al., 2008; Pibiri et al., 2008). More recently, phase 3 clinical trials have established the clinical relevance of allopregnanolone in mood disorders (Kanes S. J. et al., 2017; Meltzer-Brody et al., 2018). Intravenous allopregnanolone (brexanolone or SAGE-547) or an orally-active, allopregnanolone's analog (SAGE-217), showed a rapid and long-lasting remission of post-partum depression and major depressive disorder symptoms, respectively. These successful clinical trials led to the FDA approval of brexanolone for the treatment of post-partum depression in March 2019 and encouraged the possible future clinical use of brexanolone or SAGE-217 for the treatment of mood disorders, including PTSD.

at GABA$_A$ receptors (Majewska et al., 1986; reviewed in Belelli and Lambert, 2005; Belelli et al., 2009, 2018). In the year 2000, the neurophysiological role of allopregnanolone in permitting the fine-tuning and regulating the strength of GABA$_A$ receptors to agonists, positive allosteric modulators, and GABAmimetic agents, was unveiled (Pinna et al., 2000). By acting at GABA$_A$ receptors, allopregnanolone also regulates emotional behavior in rodent stress models of behavioral abnormalities and humans with PTSD and major unipolar depression (Uzunova et al., 1998; Pinna et al., 2003, 2008; Rasmusson et al., 2006, 2019; Pineles et al., 2018). More recently, several phase 3 clinical trials have established the clinical relevance of allopregnanolone in mood disorders. Intravenous allopregnanolone (brexanolone or SAGE-547) or an orally-active, allopregnanolone's analog, named SAGE-217, showed a rapid and long-lasting remission of post-partum depression and major depressive disorder symptoms, respectively (Kanes S. J. et al., 2017; Kanes S. et al., 2017; Meltzer-Brody et al., 2018[1]). These studies, in March 2019, led to the FDA approval of allopregnanolone (i.e., brexanolone) as the first specific treatment for post-partum depression that will allow this "endogenous tranquillizer" to be prescribed as a novel treatment for mood disorders starting in Summer 2019. On the other hand, if successfully developed, SAGE-217 will be the first durable, rapid-acting, oral, short-course treatment for mood disorders and potentially may be applied to test whether administered during prolonged exposure therapy for PTSD, it facilitates recovery in patients. The new generation of *rapid-acting antidepressants* has just emerged and may likely dominate the field of neuropsychopharmacology for the next decades to come.

The finding that the traditional gold-standard treatment option for PTSD, the selective serotonin reuptake inhibitors (SSRIs), is efficient in about half of the treated patients (reviewed in Golden et al., 2002; Rush et al., 2006; Kemp et al., 2008; Bernardy and Friedman, 2015), suggests that mood disorders emerge from complex neurobiological backgrounds and only one molecular deficit may not reflect a valid biomarker for the disorder under examination. Likewise, only one treatment cannot be the answer to improve symptoms in all patients, following the one-fit-all treatment expectation (Brewin, 2001; Aspesi and Pinna, 2018). Overall, discovering biomarkers that may lead to precision medicine for PTSD is in high demand. Novel advances in the field have been possible by employing state-of-the-art technologies and more reliable animal models (reviewed in Ngounou Wetie et al., 2013;

[1]http://investor.sagerx.com/news-releases/news-release-details/sage-announces-pivotal-phase-3-trial-status-sage-217-major

Aspesi and Pinna, 2018, 2019). However, more research is needed to establish a reliable biosignature for PTSD and other mood disorders.

ALLOPREGNANOLONE: A BIOMARKER CANDIDATE AND A TREATMENT ENDPOINT FOR MOOD DISORDERS

Research for effective biomarkers in psychiatric disorders still remains backward when compared to most fields of medicine that heavily rely on biomarkers for their prediction, prevention, diagnosis and assessment of the most effective treatments (discussed in Fernandes et al., 2017; Aspesi and Pinna, 2018). Diagnosis of PTSD and mood disorders still rely on subjective measures, including questionnaires and description of symptoms by the patients to the psychiatrist or psychologist and are based on the Diagnostic and Statistical Manual of Mental Disorders version 5 (DSM-V) criteria. Unfortunately, a number of factors complicate the nature of these diagnostic assessments. These include the poor general understanding of the neurobiological underpinnings of psychiatric disorders, such as PTSD and major depressive disorder (Pinna, 2018). These conditions are multifaceted and heterogenic for symptoms and for the way they manifest in different patients. The finding that symptoms overlap and comorbidity among various psychiatric disorders, including depression, anxiety, substance abuse and suicide, further complicates diagnosis (Locci and Pinna, 2017; Franklin et al., 2018). Objective neurobiological parameters are not yet in the clinical practice unlike in the diagnosis of most of the medical conditions. In recent years, several biomarker candidates have been suggested for PTSD, however, their diagnostic value remains to be yet established (reviewed in Aspesi and Pinna, 2018). As in the symptoms and comorbidity of mood disorders, these biomarkers for PTSD are often common to other neuropsychopathologies, such as major depressive disorder. For example, downregulation of neurosteroid biosynthesis, including the concentrations of the GABAergic endogenous modulator allopregnanolone and of its equipotent stereoisomer, pregnanolone was found in cerebrospinal fluid (CSF), plasma, serum of major depression and PTSD patients (Romeo et al., 1998; Uzunova et al., 1998; Rasmusson et al., 2006, 2019; Pineles et al., 2018). In PTSD patients, CSF allopregnanolone levels inversely correlated with levels of dehydroepiandrosterone (DHEA), likely generating an imbalance between inhibitory and excitatory neurotransmission underlying PTSD symptoms (Rasmusson et al., 2006). Importantly, sleep disturbance in the context of PTSD was previously associated with DHEA responses following adrenal activation as well as with decreased allopregnanolone levels (reviewed in Pitman et al., 2012). The significance of allopregnanolone biosynthesis downregulation as a biomarker of psychiatric disorders has been highlighted in numerous reports (Uzunova et al., 1998; Nemeroff, 2008; Agis-Balboa et al., 2014; Dichtel et al., 2018; reviewed in Schüle et al., 2011; Zorumski and Mennerick, 2013; Schüle et al., 2014; and Locci and Pinna, 2017). Neurosteroid biosynthesis deficit observed in PTSD patients has been successfully modeled in rodents subjected to chronic stress, such as in mice exposed to prolonged (3–4 weeks) social isolation stress.

Clinical and preclinical observations suggest that allopregnanolone may serve as a *biomarker* for symptoms overlapping in neuropsychopathologies encompassing from PTSD and depression (Pibiri et al., 2008; Pinna et al., 2008; Pinna and Rasmusson, 2012; Locci and Pinna, 2019b). In this respect, the synergic interplay of multiple neurochemical alterations that have been newly proposed within neurosteroid levels, their receptors and biosynthetic enzymes, as possible biomarkers, which is, establishing a *biomarker axis* may be the most accurate path to predict, diagnose, prevent or treat mood disorders (discussed in Aspesi and Pinna, 2018).

These summaries also suggest that by counteracting the downregulation of allopregnanolone biosynthesis, novel treatment may ameliorate symptoms in PTSD and depression (Rupprecht, 2003; Rupprecht et al., 2009, 2010; reviewed in Locci and Pinna, 2017). Indeed, allopregnanolone biosynthesis promises to be instrumental for a much-needed precision medicine for mood disorders (Aspesi and Pinna, 2018).

ANIMAL MODELS OF PTSD

Establishing reliable biomarkers and specific treatments for PTSD has been hampered not only by the relative difficulty in establishing PTSD animal models but also because of the limited knowledge on PTSD neurobiology (Borghans and Homberg, 2015; Pinna and Izumi, 2018; Aspesi and Pinna, 2019). However, establishing correlative analyses among altered neuroactive chemicals in patients' plasma, serum, and CSF is key to translate findings to animal models. Animal models are essential investigative tools to understand the etiopathology of a disease/disorder, how this develops over time and what targets can be affected by new pharmacological treatments. While it is beyond impossible to precisely model complex behavioral expressions of human symptoms that recapitulate to PTSD, basic behavioral endophenotypes can be reproduced in animals (reviewed in Siegmund and Wotjak, 2006, 2007). At this regard, animal models must satisfy criteria including *face, construct* and *predictive validity* Geyer and Markou, 2002). *Face validity* is the collection of phenotypes (behavioral and neurochemical) that relate finding in PTSD patients to rodent stress or genetic models. *Construct validity* is the process involved in the onset and the manifestation of the disorder and this, ultimately, is recapitulated in the animal model. Finally, p*redictive validity* reflects the capability of animals to inform by means of predictors on the human disorder.

Probably the most commonly used stressful experimental condition to elicit stress-induced behavioral deficits that recapitulate to PTSD symptoms includes *the restraint stress*. Rodents are generally restraint under one single exposure that may last up to 2 h (Whitaker et al., 2014) or during repeated sessions that vary from few days to several weeks (Gameiro et al., 2006).

Pairing the restraint stress with forced swimming and other stressors is part of *the unpredictable variable stress*,

which reproduces PTSD behavioral deficits that are ameliorated by administration with SSRIs or ketamine (Garcia et al., 2009; Yin et al., 2016). This procedure is believed to model the unpredictable stress that soldiers often experience in warzones (Wakizono et al., 2007; Goswami et al., 2013; Shepard et al., 2016). In addition to a PTSD-like phenotype, the unpredictable protocol is associated with depressive-like deficits typically observed in PTSD patients with comorbidity with depression.

The inescapable shocks is another unpredictable stressor-based model, which relies on an unexpected single stress-exposure, an electric foot or tail shock and is generally used to model fear responses and fear extinction learning (Pryce et al., 2011; Desmedt et al., 2015). The inescapable shock model can be combined with restraint (Nagata et al., 2009).

The predator-stress model protocol includes the exposure of rodents to a predator or to its scent (Adamec et al., 2004; Wilson et al., 2014a). This stressor induces hyperarousal, avoidance, fear, and reduces fear extinction (Cohen et al., 2010; Zoladz et al., 2015; Seetharaman et al., 2016). Exposure to predators also increases anxiety-like behavior (Adamec et al., 2005). Behavioral deficits are heightened when rodents are directly exposed to a predator rather than the predator scent. These animals also respond to sertraline, which reduces anxiety-like behavior and cue avoidance (Zoladz et al., 2013; Wilson et al., 2014b).

The single prolonged stress consists in three stressors that are administered in succession: restraint stress (2 h), forced swimming (20 min) and exposure to diethyl ether (Liberzon et al., 1997, 1999). Cue-conditioned fear and its extinction are unaffected; however, this procedure induces consistent impairment in extinction retention (George et al., 2015). This model also induces hyperarousal and enhanced contextual freezing (Imanaka et al., 2006; Yamamoto et al., 2009). Cue-induced fear can be attenuated by paroxetine (Perrine et al., 2016).

The social defeat stress model is mostly performed in male rodents by a resident-intruder test, which results in aggressive behavior and social stress for the intruder (Björkqvist, 2001; Hammels et al., 2015). This increases social avoidance and other behavioral traits of PTSD, including hyperarousal and anhedonia (Warren et al., 2013; Der-Avakian et al., 2014).

The 129S1/SvlmJ genetic mouse model of PTSD (Camp et al., 2009) is characterized by impaired fear extinction (Hefner et al., 2008). Importantly this model allows investigating the molecular and genetic mechanisms underlying fear extinction from a genetic perspective allowing studies on individual vulnerability, as well as, their predisposition to PTSD. Similarly to most of PTSD rodent models reviewed above, the 129S1/SvlmJ mouse also responds to SSRIs, such as fluoxetine that improves the fear responses (Camp et al., 2012).

Finally, serotonin 2C receptors (5-HT2CR) are well characterized in anxiety, and a new model in mice having the fully VGV edited isoform of 5-HT2CR, which overexpresses brain 5-HT2CR, was recently established to study PTSD predisposition (Règue et al., 2019). VGV mice expressed greater fear responses, fear extinction deficits, and fear generalization. These dysfunctions were normalized by paroxetine in VGV mice given acutely and decreased when administered chronically. This treatment also improved deficits in brain derived neurotropic factor (BDNF) expression in the amygdala and the hippocampus. VGV-transgenic mice express neurobiological features relevant to PTSD and its treatment (Règue et al., 2019).

By far, "PTSD model" has often been an overused terminology to depict basic research studies that include a number of stressors to induced behavioral deficits (Siegmund and Wotjak, 2006). The human condition should probably be modelled by applying an uninterrupted chronic stress in combination with an acute traumatic event. Generally, the first serves an essential substrate for "trauma/fear incubation" and the second is a trigger that challenges the individual susceptibility to develop resilience or PTSD symptoms. However, reproducing chronic stress in animal models is a hard task in that most paradigms administer repeated acute stressors, which results in an intermittent stress model. Protracted social isolation stress may offer an alternative to this methodological problem and provide the advantage of administering the chronic stressor continuously and for as long as desired (often weeks; reviewed in Zelikowsky et al., 2018). This phase of neurochemical changes, such as social isolation stress-induced neurosteroid biosynthesis downregulation, may provide the required conditions that precipitate PTSD-like behavior following the administration of acute stressors (i.e., foot shocks that are part of the fear conditioning paradigm; Torok et al., 2018).

THE SOCIALLY ISOLATED MOUSE

The protracted social isolation stress, in humans, called perceived social isolation (PSI) or loneliness, elicits a number of physical, neurological and psychological deficits that range from Alzheimer's disease to major depression, anxiety disorders and suicidality (Cacioppo and Cacioppo, 2016). Social and community support is fundamental for emotional regulation following traumatic stress, their absence puts at risk for PTSD and other mental disorders (Nemeroff et al., 2006; Charuvastra and Cloitre, 2008; Mehnert et al., 2010). An individual inability to manage emotional memories often results in avoidance, re-experiencing symptoms and hypervigilance (Cahill et al., 2003; Rothbaum and Davis, 2003; Pitman et al., 2006; Rauch et al., 2006).

Rodents that have been exposed to a prolonged *social isolation* in individual cages for 3–4 weeks, express time-dependent behavioral deficits, including increased anxiety-like behavior and aggression (Guidotti et al., 2001; Pinna et al., 2003; Rau et al., 2005; Pibiri et al., 2008; discussed in Locci and Pinna, 2019b). Individual housing is likewise a powerful stressful condition that may increase the susceptibility to develop behavioral dysfunctions when rodents are additionally exposed to an acute traumatic stressor, for example, the electric shocks that constitute the fundamental of the Pavlonian fear conditioning test (Charuvastra and Cloitre, 2008; Pinna, 2010).

Behavioral deficits following protracted social isolation are associated with a number of physical and neuronal dysfunctions, including impairment of the HPA axis, neurotransmitter systems, neuropeptides, neurohormones, and neurotropic

factors (reviewed in Nin et al., 2011a). Importantly, studies have investigated the potential role for tachykinins in regulating social isolation-induced aggression in mice. Studies focusing on the neuropeptide tachykinin 2 (Tac2)/neurokinin B (NkB) showed that in the central amygdala the peptide plays a role in fear memory consolidation. A more recent study showed that Tac2/NkB is dramatically upregulated throughout the brain following protracted social isolation, which resulted in aggression and impairment of other behaviors by acting on multiple brain regions (Zelikowsky et al., 2018).

Studies from this lab have mainly focused on the effects of social isolation on the GABAergic neurotransmission dysfunction caused by impaired neurosteroid biosynthesis, and changes in the expression of several $GABA_A$ receptor subunit subtypes. The role of neurosteroids in regulating the expression of neurotropic factors (i.e., BDNF) during social isolation has also been one important research interest.

Behavioral Deficits in Socially Isolated Mice

Mice that are socially isolated for 3–4 weeks post-weaning (PN21) express a number of behavioral deficits relevant to model aspects of human mood disorders (reviewed in Pinna and Rasmusson, 2012; Zelikowsky et al., 2018; Aspesi and Pinna, 2019; Locci and Pinna, 2019b). Specifically, male mice when exposed to a fear conditioning test with administration of a conditioned (CS, acoustic tone) and an unconditioned stimuli (US, footshock, please see **Figure 2**), in a novel context, comprising a contextual chamber (Pibiri et al., 2008; Pinna et al., 2008), show elevated freezing, which is an index of elevated fear responses, 1 day post-training session. Time-course experiments have unveiled that freezing increases time-dependently during 4 weeks of isolation and similarly to the expression of aggressive behavior, reaches a plateau between week 4 and 6 of isolation (Pibiri et al., 2008; Pinna et al., 2008). In this mouse model of enhanced fear responses, socially isolated mice exhibit an impaired fear extinction memory as compared with group-housed control male mice and a re-emergence of fear after the passage of time or, in other words, they show impaired fear extinction retention (Pibiri et al., 2008). On a translational standpoint, social isolation can be seen as a prolonged stress that is often associated with a precipitating traumatic event, which leads to maladaptive post-stress adaptations and emergence of PTSD in patients. Thus, social isolation offers a suitable model to study vulnerability to PTSD (discussed in Aspesi and Pinna, 2019).

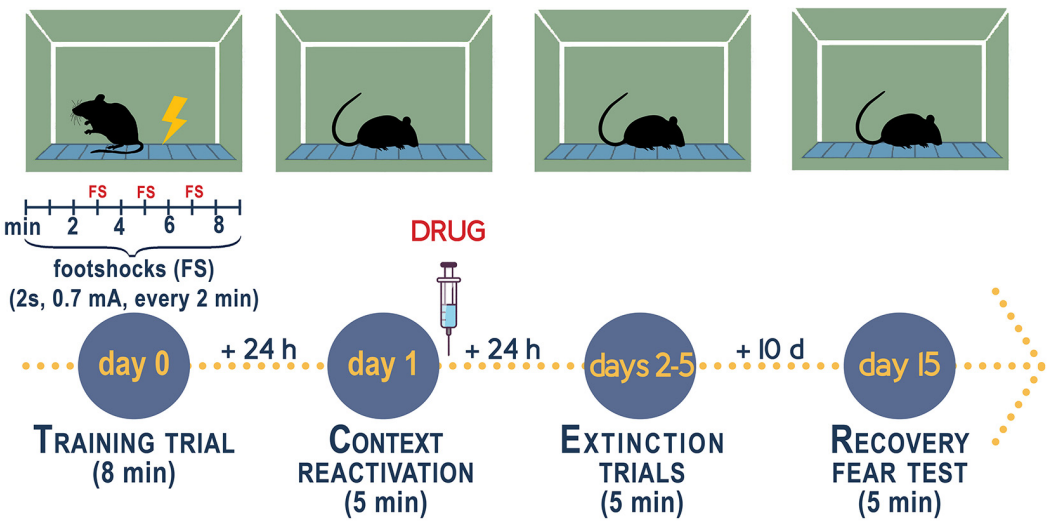

FIGURE 2 | Experimental procedure to measure fear conditioning responses, fear extinction, and fear extinction retention in socially isolated mice. Contextual fear conditioning responses in socially isolated is studied after 4 weeks of isolation when the decline of allopregnanolone is maximal (Pibiri et al., 2008; Pinna et al., 2008). Group-housed mice of the same age as the socially isolated mice serve as control. Socially isolated mice express a decrease of corticolimbic allopregnanolone levels that is associated with an enhancement of contextual fear responses and impaired fear extinction (Pibiri et al., 2008). The fear-conditioning apparatus, which schematized in the figure, consists of a transparent acrylic chamber measuring 25 cm wide, 18 cm high, and 21 cm deep (San Diego Instruments). The cage floor is composed of stainless-steel rods connected to an electric shock generator. A small fan is located on the top wall of the enclosure. The chamber is surrounded by a frame with 16 infrared photo beams. A computer controls the delivery of electric foot shocks and auditory stimuli and records beam interruptions and latencies to beam interruptions (freezing time). *Training Test*. During the training, mice are placed into the training chamber and allowed to explore it for 2 min. After this time, they receive an unconditioned stimulus (US, electric footshock, 2 s, 0.5 mA). The footshock is repeated three times every 2 min. After the last tone plus shock delivery, mice are allowed to explore the context for an additional minute before removal from the training chamber (total of 8 min). *Contextual Test*. Twenty-four hours after training, the mice are placed in the contextual cage, and freezing behavior is measured for 5 min (Freeze Monitor System, San Diego Instruments) without footshock presentation. *Extinction Test*. For contextual extinction experiments, mice are placed in the contextual cage for 5 consecutive days starting 24 h after the training session. *Fear extinction retention*. Retention of fear extinction is measured by placing the mice to the context for 5 min following an interval of 10 days. Freezing behavior is measured for 5 min without tone or footshock presentation. Freezing is defined by the absence of any movement except for those related to respiration while the animal is in a stereotypical crouching posture (Pibiri et al., 2008). To disrupt aversive memories through a reconsolidation blockade (Stern et al., 2012), drugs are given immediately after a contextual fear conditioning reactivation session (Pinna and Rasmusson, 2014; Locci and Pinna, 2019b).

Other behavioral deficits expressed by socially isolated mice include increased aggression to a same-sex intruder, as well as, anxiety-like and depressive-like phenotypes. These behavioral traits are consistent with behavioral aspects that are reminiscent of PTSD symptoms often observed in PTSD patients following re-exposure to trauma reminders (Grillon and Morgan, 1999; Rauch et al., 2006). Limitations of this animal model include studies that were conducted mostly in male mice. Socially isolated female mice investigation was mostly limited to the study of depressive-like behavior (Weiss et al., 2004; Grippo et al., 2007).

A number of pharmacological agents, including SSRIs administered at low doses that act like *selective brain steroidogenic stimulants* (SBSSs) and increase corticolimbic allopregnanolone levels (Pinna, 2015), or allopregnanolone analogs, including ganaxolone, by a contextual fear reconsolidation blockade, normalize fear response and facilitate fear extinction (Pibiri et al., 2008; Pinna and Rasmusson, 2014; Rasmusson et al., 2017). Most importantly, these agents prevent the reemergence of fear after the passage of time, during recall (Pinna and Rasmusson, 2014; reviewed in Aspesi and Pinna, 2019; Locci and Pinna, 2019a; Raber et al., 2019). Furthermore, the novel allopregnanolone's analogs BR297 and BR351 showed strong anti-aggressive effects in isolated mice (Locci et al., 2017). Another strategy to increase allopregnanolone levels and enhance activation of emotion regulation neurocircuits includes administration with the allopregnanolone precursor pregnenolone (Sripada et al., 2013). Recently, neurosteroidogenic agents, including the endocannabinoid-like, PEA by a similar mechanism, which include upregulation of allopregnanolone biosynthesis, showed to improve fear extinction and its retention in socially isolated mice compared to non-stressed mice (Locci and Pinna, 2019b). PEA also decreased anxiety-like and depressive-like behavior and aggression in socially isolated mice (Locci et al., 2017; Locci and Pinna, 2019b). Recently, by directly manipulating the endocannabinoid system by administering the endocannabinoid reuptake inhibitor AM404 facilitated safety learning in a CB1-dependent manner and attenuated the relapse of avoidance (Micale et al., 2017). Although a direct evidence that endocannabinoids stimulate brain neurosteroid biosynthesis has not been provided, recent studies show THC increases allopregnanolone's precursor, pregnenolone by activating CB1 (Vallée et al., 2014; Vallée, 2016). The detailed description on endocannabinoid and neurosteroidogenic neuronal targets and novel molecules that are currently investigated for the development of new treatments for PTSD has been the focus of recent reviews (Pinna, 2014; Aspesi and Pinna, 2019; Locci and Pinna, 2019a; Raber et al., 2019).

$GABA_A$ Receptor Subunit Expression and Benzodiazepine Inefficacy in Socially Isolated Mice

Altered corticolimbic GABAergic neurotransmission, including $GABA_A$ receptor subunit composition have been linked with a number of mental disorders (Akbarian et al., 1995; Dean et al., 1999; Lewis, 2000; Ishikawa et al., 2004). Affinity for the benzodiazepine binding at $GABA_A$ receptors is strongly dependent on α1–3,5 and γ2 subunits (Rudolph et al., 1999; Rudolph and Möhler, 2004). Intriguingly, $GABA_A$ receptor subunit expression is highly susceptible to stress effects, pharmacological interventions, as well as, alcohol and substance abuse (Impagnatiello et al., 1996; Pinna et al., 2006a; Bohnsack et al., 2017, 2018; Locci and Pinna, 2017). Protracted stress induces profound changes in the expression of $GABA_A$ receptors that alters the receptor sensitivity to endogenous modulators and synthetic agonists (reviewed in Locci and Pinna, 2017). In socially isolated mice, the mRNA and protein expression of α1, α2, and γ2 of the $GABA_A$ receptor subunits were found reduced by 50% when compared to those of control group-housed mice (Pinna et al., 2006a; Nin et al., 2011b). The expression of α4 and α5 subunits was instead over-expressed by 130% (Pinna et al., 2006a). Protein expression of α1 and α5 in frontal cortices and hippocampal synaptic membranes were likewise decreased and elevated, respectively (Pinna et al., 2006a; reviewed in Locci and Pinna, 2017). Studies at the cortical layer- and cell-specific levels showed that in laser microdissected frontocortical layer I, expression of α1 subunit was decreased by 50% and it was unchanged in the layer V pyramidal neurons following social isolation (Pinna et al., 2006a).

Behavioral pharmacological studies showed that socially isolated mice exhibit a robust resistance to the sedative and anxiolytic pharmacological properties of diazepam and zolpidem. These synthetic agonists act at $GABA_A$ receptor-containing α1–3, 5 subunits (Pinna et al., 2006a). Thus, α1 and 2 subunit downregulation *per se* may explain the decreased responsiveness of socially isolated mice to sedative and anxiolytic benzodiazepines. These results further suggest that γ2 subunit downregulation may have originated a switch with γ subunits that are largely expressed in extrasynaptic $GABA_A$ receptors with a loss of benzodiazepine binding sites that was determined in cortical synaptosomes (Pinna et al., 2006a). Hence, prolonged stress may be associated with formation of benzodiazepine-insensitive $GABA_A$ receptors in cortical neurons that modulate anxiolytic responses (Rudolph et al., 1999; Rudolph and Möhler, 2004; Nin et al., 2011b).

Intriguingly, increases in α4 and δ-subunits in frontocortical membranes from socially isolated rodents (Pinna et al., 2006b; Serra et al., 2008) may originate $GABA_A$ receptors for which endogenous modulators, including allopregnanolone, show a stronger affinity (Belelli and Lambert, 2005; Belelli et al., 2005). Actually, allopregnanolone administered to socially isolated mice induces anxiolytic effects (Pinna et al., 2008).

Translationally, $GABA_A$ receptor expression in the socially isolated mouse shows several commonalities with PTSD patients. Indeed, stress-induced remodeling of $GABA_A$ receptors in PTSD patients results in loss of benzodiazepine pharmacological actions due to decreased benzodiazepine-binding sites to cortex, hippocampus, and thalamus (Geuze et al., 2008). These preclinical and clinical findings provide support for the observation that treatment with benzodiazepine is ineffective for PTSD treatment and prevention. Furthermore, risks associated with their administration generally outweighs the short-term benefits. Benzodiazepine use in the general population is associated with adverse effects (tolerance, dependence and

withdrawal symptoms), in patients with PTSD side effects are even more severe and a study showed significantly increased risk of developing PTSD with their use after recent trauma, worse psychotherapy outcomes, aggressiveness, depression symptoms, and substance use (Deka et al., 2018). In another study, veterans with PTSD administered with benzodiazepines showed higher rates of health care utilization and were more likely to attempt and complete suicide (Guina et al., 2015). Benzodiazepines are, thus, contraindicated for patients with PTSD or recent trauma, evidence-based treatments for PTSD should be favored.

Allopregnanolone Downregulation and Fear Circuitry in Socially Isolated Mice

Allopregnanolone biosynthesis has been found altered in several mood disorders, including depression, anxiety, PTSD, post-partum depression and premenstrual syndrome (Romeo et al., 1998; Uzunova et al., 1998; Rasmusson et al., 2006, 2019; Nemeroff, 2008; Lovick, 2013; Dichtel et al., 2018; Pineles et al., 2018). This deficit was more recently observed in the fronto-cortical pyramidal neurons of the Broadman area 9 (BA9) of male patients affected by major depression (Agis-Balboa et al., 2014). As previously mentioned, therapeutically, elevating the down-regulated allopregnanolone levels in patients with mood disorders also correlated with improved patients' symptoms (Romeo et al., 1998; Uzunova et al., 1998; Agis-Balboa et al., 2014; Kanes S. J. et al., 2017; Kanes S. et al., 2017).

In socially isolated rodents the responsiveness of the HPA axis is decreased. Levels of corticosterone and release of CRH are decreased in the blood flow (Sanchez et al., 1998; Chida et al., 2005; Malkesman et al., 2006). The HPA axis hypo-function is even more evident when socially isolated rodents are exposed to acute stressors. This finding underlies an overall reduced sensitization of the HPA axis to acute stressful stimuli (Sanchez et al., 1998). In rodents, corticolimbic neurons express the biosynthetic enzymes, 5α-reductase type I and 3α-HSD that synthesize allopregnanolone (Agís-Balboa et al., 2006, 2007). Consistently, socially isolated rodents show a time-dependent impairment of neurosteroidogenesis, including the levels of the GABAergic neurosteroid, allopregnanolone. This deficit has been associated with appearance of a number of behavioral dysfunctions, such as delayed and incomplete fear extinction and reemergence of fear upon fear recall (Pibiri et al., 2008; Pinna and Rasmusson, 2014) that resemble behavioral deficits showed in patients affected by anxiety, depressive disorders, and PTSD (Matsumoto et al., 1999; Pinna, 2010; Schüle et al., 2011).

For over a decade, investigating the neurochemical and behavioral deficits expressed by socially isolated rodents, this laboratory, as well as other colleagues, have established that either rats or mice that undergo individual caging, which results in a form of prolonged stress for several weeks, express a downregulation of allopregnanolone levels in corticolimbic areas. This is maintained and results from a downregulation of the expression of 5α-reductase type I, a rate-limiting enzyme in allopregnanolone biosynthesis (Matsumoto et al., 1999; Serra et al., 2000; Pinna et al., 2003; Bortolato et al., 2011; reviewed in Matsumoto et al., 2007). The biosynthesis rate of allopregnanolone and its precursor, 5α-DHP in socially isolated is decreased by 70% when compared to that of group-housed mice (Dong et al., 2001; Pinna et al., 2003). New finding also shows that socially isolated mice express a downregulation of P450scc, another rate-limiting enzyme involved in the inner mitochondrial membrane metabolism of pregnenolone from cholesterol (Locci and Pinna, 2019b). Across several brain areas analyzed, 5α-reductase largest expression decrease was observed in the amygdala and hippocampus (Agís-Balboa et al., 2007). The olfactory bulb and the frontal cortex expressed a moderate downregulation in the neurosteroid biosynthetic enzymes. Importantly, 5α-reductase type I expression did not change in the cerebellum and striatum (Agís-Balboa et al., 2007). As revealed by *in situ* immunohistochemical experiments, 5α-reductase was specifically downregulated in layers V–VI cortical pyramidal neurons, in hippocampal CA3 pyramidal neurons and in dentate gyrus glutamatergic granular cells as well as pyramidal-like neurons of the basolateral amygdala (Agís-Balboa et al., 2007). Importantly, 5α-reductase expression was not decreased in GABAergic long-projecting neurons of the reticular thalamic nucleus, central amygdala, cerebellum, and in the striatum medium spiny neurons. This enzymatic expression decrease was paralleled by a decreased allopregnanolone in discrete corticolimbic areas that was quantified by GC-MS, characterized by unsurpassed structural selectivity and sensitivity (Pibiri et al., 2008; Locci and Pinna, 2019b).

These findings underlie and sustain a dysfunction in corticolimbic circuits that in socially isolated mice is responsible for behavioral deficits (**Figure 3**). Indeed, amygdala pyramidal-like neurons are involved in the regulation of the strength of the inhibitory function of the intercalated inhibitory spiny GABAergic interneurons (ITC) that mediate the connectivity between the basolateral amygdala (BLA) and the central amygdaloid nucleus (CeA; Agís-Balboa et al., 2007). One of the most replicated traits of PTSD connectivity studies is the typical exaggerated amygdala hyperactivity, which results from functional deficits of projections from the prefrontal cortex and hippocampus (Akirav and Maroun, 2007). These glutamatergic neurons located in the prefrontal cortex and hippocampus extend and synapse on GABAergic neurons of the amygdala and regulate an inhibitory input to these amygdala neurons (depicted in **Figure 3**). In normal individual or in resilient subjects, fear following traumatic events can be suppressed by the regulatory role exerted by the prefrontal cortex and hippocampus projections that directly synapse with the amygdala and shut down its hyperactivity. In maladaptive conditions following a traumatic event, this process can be impaired and the cortical inhibitory function on the amygdalar nuclei may be weakened, which results in amygdala hyperactivity and inappropriate and exaggerated fear response and impaired fear extinction, a core neurobiological trait observed in PTSD (Liberzon and Sripada, 2008). Hence, prefrontal cortex regulation of the amygdala ITC neurons dictates the responsiveness to stress and fear (Pare et al., 2004). These GABAergic outputs exert a pivotal role in emotion regulation following stress and directly influence fear extinction learning and regulate the CeA output that mediates responses to conditioned fear (Likhtik et al., 2008). Several lines of evidence have shown that ITC neuron

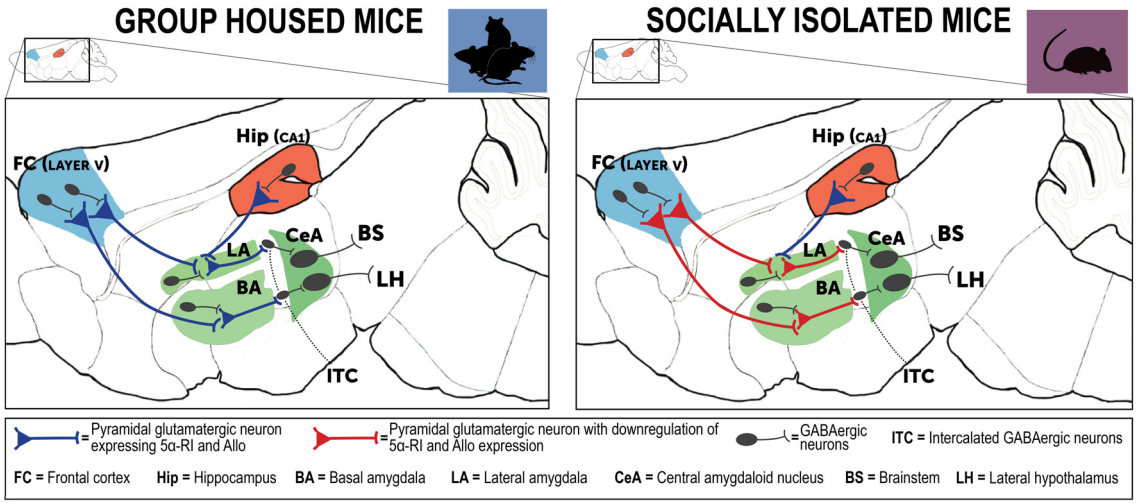

FIGURE 3 | Neurocircuitry underlying the PTSD-like phenotype expressed by socially isolated mice. This is a simplified schematic representation of mouse brain neurocircuitry regulating emotional behavior under physiological (group-housed) and stress-induced deficits (social isolation). The prefrontal cortex and hippocampus directly project to the amygdaloid nuclei to regulate their hyperactivity following traumatic events (Herry et al., 2008). In susceptible individuals, a stressful experience is associated with impairment of cortical inhibitory activity directed to the amygdala, which results in exaggerated hyperactivity and inappropriate fear responses (Akirav and Maroun, 2007; Raber et al., 2019). In PTSD, amygdala hyperactivity is part of a maladaptive emotional processing resulting from exposure to traumatic events. The neural substrates of these behavioral deficits may result from decreased GABA release (downregulated allopregnanolone concentrations (Rasmusson et al., 2006, 2019), in participation with changes in $GABA_A$ receptor subunit subtypes (Geuze et al., 2008). Collectively, these neurobiological alterations may explain emergence of PTSD symptoms (Pinna, 2018). In the socially isolated mice, a stress-induced model of PTSD-like behavioral traits, cortical and hippocampal projections directed to the basolateral amygdala (BLA) show a downregulation of allopregnanolone biosynthesis and behavioral correlates, including increased fear responses and impairment of fear extinction (Agís-Balboa et al., 2007; Pinna et al., 2009). In socially isolated mice (*right panel*), allopregnanolone downregulation in cortical and hippocampus pyramidal glutamatergic neurons and in pyramidal-like neurons of the BLA may represent the molecular underpinnings that recapitulate an increased excitability of the neuronal pathway that converges to the intercalated GABAergic neurons (ITC) and central amygdala (CeA) GABAergic spiny neurons (Agís-Balboa et al., 2007; Pinna et al., 2008). Collectively, reduction of allopregnanolone biosynthesis in corticolimbic glutamatergic neurons may impair cortico-hippocampal-amygdaloid circuits by inhibiting the GABAergic output neurons of the CeA, which project to the hypothalamus and brainstem and may explain the excessive fear responses and other behavioral deficits observed in socially isolated mice (Pinna et al., 2008, 2009). Allo, allopregnanolone; 5α-RI, 5α-reductase type I.

lesions impair fear extinction memory, while activation of these neurons facilitates extinction learning (Jüngling et al., 2008; Likhtik et al., 2008). ITC GABAergic and CeA projections to brainstem and hypothalamus modulate fear responses and fear extinction following stressful events (Pinna et al., 2009). Altogether, the corticolimbic circuits that in socially isolated mice express downregulated allopregnanolone levels, which include the prefrontal cortex, hippocampus and amygdala are directly responsible for the expression of emotional behaviors, including aggressive behavior, fear responses, and anxious behavior, which are commonly observed in PTSD patients (LeDoux, 2000; Milad et al., 2007). In socially isolated mice, these deficits in allopregnanolone biosynthesis and the behavioral dysfunction have been associated with a decrease of corticolimbic BDNF expression (Nin et al., 2011a).

It is important to note that most of the studies in humans with major depressive disorder and PTSD have determined levels of allopregnanolone in the periphery (serum, plasma, CSF) and only a few have quantified levels of allopregnanolone in the post-mortem brain (Agis-Balboa et al., 2014; Cruz et al., 2019). Oppositely, animal studies have for the most part focused on allopregnanolone levels in specific brain regions (Pibiri et al., 2008; Pinna et al., 2008; Locci and Pinna, 2019b). Brain levels of allopregnanolone may also influence the HPA and HPG axes. For instance, the HPA axis can be modulated by the neuronal inhibition initiated by GABAergic neurons within the hypothalamus. Corticosteroids exert a negative feedback on the HPA axis by acting on the hippocampus and the medial prefrontal cortex, which triggers a spike-dependent elevation in GABA release from inhibitory synapses thus stimulating the function of GABAergic neurotransmission. By this mechanism, allopregnanolone may also induce a potent inhibition on the HPA axis activity, which attenuates plasma ACTH and corticosterone increase induced by stress. Thus, locally brain produced allopregnanolone may contribute to regulating neuronal function by modulating HPA axis activity (reviewed in Biggio et al., 2014).

TRANSDIAGNOSTIC BEHAVIORAL FEATURES OF THE SOCIALLY ISOLATED MOUSE

PTSD/Suicide

The decrease of allopregnanolone in socially isolated mice has been associated with behavioral deficits, including anxiety-like

FIGURE 4 | Representation of the main PTSD phenotypes in socially isolated mice. The figure shows the phenotypes in socially isolated mice that recapitulate to deficits in PTSD patients. The circles at the top of the figure show the behavioral phenotype of socially isolated mice that includes aggression, impaired fear memory, impulsivity and anxiety, which are all core behavioral symptoms of PTSD. In the center of the figure are reported the abnormalities in $GABA_A$ receptors and neurosteroid levels. The last circles at the bottom report the altered pharmacological response to benzodiazepines that has been reported both in socially isolated mice and in humans with PTSD. Allo, allopregnanolone; PA, pregnanolone; 5α-RI, 5α-reductase type I.

behavior and aggression. Further, socially isolated mice show impairment of fear extinction and spontaneous reemergence of fear following passage of time and determined during a recall session (Pibiri et al., 2008; Pinna and Rasmusson, 2014; Locci and Pinna, 2019b). Probably, one of the most remarkable behavioral deficits of socially isolated mice regards the heightened aggressive behavior of a resident socially isolated mouse towards a same-sex intruder (Pinna et al., 2003). It is intriguing to note that the expression of aggressive behavior is one prominent behavioral phenotype used to model behavioral traits of suicide occurring in men. If one considers that PTSD is often complicated by comorbid suicidal ideation and suicide attempts, the socially isolated mouse may entail important transdiagnostic features to model aspects seen in the spectrum of PTSD-associated with suicide risk, often observed in veterans (this aspect was recently reviewed in Locci and Pinna, 2019b).

PTSD/AUD

AUD has a general high prevalence in the American population and has even higher abuse rates within PTSD patients (Blanco et al., 2013; Debell et al., 2014; Shorter et al., 2015). Alcohol consumption in subjects with psychiatric conditions is often practiced as a form of self-medication. While substance use disorder is reported to be about double among PTSD patients, AUD reached a 4-fold higher prevalence than the general population, which makes alcohol the most abused substance between PTSD individuals (Jacobsen et al., 2001). Studies in children victims of sexual, psychological and physical abuse have evidenced the higher lifetime prevalence of AUD and PTSD symptoms (Khoury et al., 2010). Comorbidity of PTSD with AUD is even more increased among military personnel (Gates et al., 2012). Progress in understanding the neurobiology of this severe and impactful comorbidity has generally been impeded by the paucity of animal models of PTSD/AUD.

Social isolation in rodents has been often used as a model to predict risk factor for both PTSD and AUD (recently reviewed in Gilpin and Weiner, 2017). Indeed, the social isolation protocol steadily increases ethanol self-administration in a number of methodological procedures, including consumption of ethanol vs. sucrose in a limited-access intermittent two-bottle choice paradigm. Alcohol intake and preference were reported to increase up to 8 weeks (Skelly et al., 2015). Another research team that has used social isolation in male Sprague-Dawley rats during PD 21–42 observed the same results. In this model, conditioned place preference for alcohol was increased (Whitaker et al., 2013), but this model failed to lead to long-lasting anxiety-like behavior or elevated alcohol drinking in females Long Evan rats (Butler et al., 2014). While most experiments were conducted using rats, similar behavioral patterns were noted when mice were isolated in adolescence, which is also associated with more prominent

emotional behavioral deficits, such as aggression, sensory gating and fear deficits (Pibiri et al., 2008; Koike et al., 2009; Gan et al., 2014; Kumari et al., 2016; Locci et al., 2017). Home-cage elevated alcohol consumption and preference that lasted even 1 month during adulthood was primarily observed in male socially isolated mice (Advani et al., 2007; Lopez et al., 2011; Talani et al., 2014).

Several lines of evidence suggest vulnerability to comorbid PTSD and AUD results from sensitization of the dopaminergic mesolimbic system and specifically, decreased dopamine in nucleus accumbens and elevated responsivity of the dopaminergic circuitry connecting VTA–NAc may underlie comorbidity of PTSD and AUD (reviewed in Gilpin and Weiner, 2017). However, other findings have shown that hippocampus allopregnanolone levels are associated with downregulation in hippocampal synaptic excitability and LTP in socially isolated rats (Serra et al., 2000; Pibiri et al., 2008; Sanna et al., 2011; Talani et al., 2016; Locci and Pinna, 2017).

Collectively, social isolation during adolescence appears as a critical period to increase susceptibility to both traumatic stress-induced alcohol drinking and emotional deficits relevant with symptoms of PTSD in humans (Pibiri et al., 2008; Pinna et al., 2008; McCool and Chappell, 2009; Skelly et al., 2015; Locci and Pinna, 2017). Furthermore, these behavioral effects are heightened in male rodents (Butler et al., 2014).

CONCLUSION

PTSD rodent models are far from optimal because they only partially reproduce phenotypic expression of PTSD neurobiology. The symptoms overlap and comorbidity among several mental disorders (e.g., PTSD, depression, anxiety, AUD and suicide) make even more challenging assessing a PTSD preclinical model. The socially isolated mouse model recapitulates several aspects of PTSD neurobiology, including downregulated corticolimbic allopregnanolone concentrations, changes in $GABA_A$ receptor subunit composition, lack of benzodiazepine pharmacological action, and altered neurocircuitry of fear (summarized in **Figure 4**). These neurochemical alterations are associated with a number of behavioral dysfunctions that are core traits of PTSD, including heightened fear responses and impaired fear extinction, as well as, transdiagnostic behavioral features such as, elevated aggressiveness, a behavioral trait that predicts suicide, depressive- and anxiety-like behavior and increased alcohol consumption. Thus, the socially isolated mouse may reproduce a two-hit PTSD/AUD model as well as a PTSD/suicide model (Locci and Pinna, 2019a), comorbidities, which are consistently observed in PTSD patients. These features make the socially isolated mouse a suitable model to study new pharmacological approached as well as establishing a *biomarker axis* for PTSD and PTSD with comorbid AUD or suicide.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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