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The infralimbic cortex and mGlu5 mediate the effects of chronic intermittent ethanol exposure on fear learning and memory

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Abstract

Rationale and Objectives—Alcohol use disorder (AUD) and post-traumatic stress disorder (PTSD) often occur comorbidly. While the incidence of these disorders is increasing, there is little investigation into the interacting neural mechanisms between these disorders. These studies aim to identify cognitive deficits that occur as a consequence of fear and ethanol exposure, implement a novel pharmaceutical intervention, and determine relevant underlying neurocircuitry. Additionally, due to clinical sex differences in PTSD prevalence and alcohol abuse, these studies examine the nature of this relationship in rodent models.

Methods—Animals were exposed to a model of PTSD+AUD using auditory fear conditioning followed by chronic intermittent ethanol exposure (CIE). Then, rats received extinction training consisting of multiple conditioned stimulus presentations in absence of the shock. Extinction recall and context induced freezing were measured in subsequent tests. CDPPB, a metabotropic glutamate receptor 5 (mGlu5) positive allosteric modulator, was used to treat these deficits, and region-specific effects were determined using microinjections.

Results—These studies determined that CIE exposure led to deficits in fear extinction learning and heightened context induced freezing while sex-differences emerged in fear conditioning and extinction cue recall tests. Further, using CDPPB, these studies found that enhancement of infralimbic (IfL) mGlu5 activity was able to recover CIE induced deficits in both males and females.

Conclusions—These studies show that CIE induces deficits in fear-related behaviors and that enhancement of IfL glutamatergic activity can facilitate learning during extinction. Additionally, we identify novel pharmacological targets for the treatment of individuals who suffer from PTSD and AUD.

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Keywords

Alcohol use disorder; post-traumatic stress disorder; prefrontal cortex; glutamate; treatment

Introduction

Alcohol use disorder (AUD) is one of the most common psychiatric disorders affecting over 18 million Americans (NIAAA 2013). It is characterized by deficits in a number of cognitive and behavioral domains including decision making, executive functioning, and impulsivity (Fitzpatrick et al. 2008; Stavro et al. 2013). Another increasingly common condition, post-traumatic stress disorder (PTSD), occurs when a traumatic experience results in persistent re-experiencing of the event leading to anxiety, avoidance of trauma-associated environmental cues, and increased negative and intrusive thoughts that begin after the event (American Psychiatric Association 2013). Additionally, PTSD is one of the most frequently occurring disorders in individuals seeking treatment for substance abuse (Pietrzak et al. 2011). PTSD and alcohol abuse are highly comorbid, such that individuals with PTSD also have co-occurring AUD with rates as high as 41–79% (Pietrzak et al. 2011). Studies indicate that the co-occurrence of AUD and PTSD is associated with greater clinical and functional impairment than with either disorder alone (reviewed in Lehavot et al. 2014). Thus, there is a critical need to better comprehend the complex neuronal interactions between these disorders to develop more effective treatment strategies.

The Role of Learning and Memory in AUD and PTSD

The interactions between AUD, PTSD, and cognition are complex leading to the characterization of AUD and PTSD as disorders of learning and memory (Hyman 2005; Vanelzakker et al. 2013). A simple explanation for this comorbidity is that alcohol, by functioning as an anxiolytic, is abused by PTSD patients to help alleviate their symptoms. Theoretically, a cycle occurs where alcohol is used to alleviate PTSD symptoms but instead renders the fear memory resistant to extinction. This ultimately leads to increased alcohol consumption, the development of AUD, and the progression of cognitive deficits that exacerbate these disorders. This theory is in accordance with the self-medication hypothesis and is further supported by both clinical (Kessler et al. 1995; Sundin et al. 2014) and preclinical (Meyer et al. 2013) studies. Retrospective and prospective studies on time-course indicate that PTSD symptoms generally precede and predict onset of AUD, which, in turn, interferes with PTSD treatment and leads to further functional impairment (Gaher et al. 2014). Importantly, alcohol abuse worsens PTSD symptoms and affects PTSD memories by inducing cognitive deficits that can interfere with the extinction of trauma associated cues (Back et al. 2006) Therefore, these two disorders may share similar neurocircuitry underlying extinction learning which can serve as a potential treatment target for comorbid PTSD/AUD.

Glutamatergic Modulation and Extinction Learning

The fundamental principles of learning and memory play a major role in the development and maintenance of PTSD and AUD. Behavioral therapies that incorporate these principles

(e.g. extinction-based exposure therapy) have been used as a treatment for both PTSD (Kessler et al. 1995) and drug addiction (Conklin and Tiffany 2002), but have shown limited efficacy. However, renewed attention has been placed on the facilitation of extinction and the neural mechanisms underlying this type of inhibitory learning. While extinction behavior appears to reflect the “removal” of a learned memory, a large body of evidence now supports the idea originally suggested by Pavlov (Pavlov 1927) that extinction is “new” and “active” learning. In this line of research, our lab has previously used pharmacological manipulation of the glutamate system through metabotropic glutamate receptor 5 (mGlu5) to successfully enhance the extinction of drug-seeking behavior (Cleva et al. 2011; Gass et al. 2014; Gass and Olive 2009a). Other studies highlight the role of mGlu5 in the infralimbic cortex (IfL) in the retrieval of fear extinction memories and have explored the use of pro-extinction pharmaceuticals (André et al. 2015; Mao et al. 2013; Sethna and Wang 2014; Xu et al. 2009). These studies show that CDPPB, the mGlu5 positive allosteric modulator used in our present studies, has the ability to enhance fear memory extinction in mouse models following exposure to a single foot-shock (Sethna and Wang 2014). The relationship between mGlu5 and fear memory was further explored in studies that used mGlu5 knock-out mice and determined the ability to extinguish auditory and contextual fear was eliminated in these animals (Xu et al. 2009). Multiple studies have examined the ability of mGlu5 modulation to enhance plasticity in the medial prefrontal cortex (mPFC) (Fontanez-Nuin et al. 2011; Sepulveda-Orengo et al. 2013) and show that mGlu5 mediated plasticity in the IfL region of the PFC is needed for the blockade of fear expression during fear extinction and recall. These data further reinforce the hypothesis that the IfL is essential for fear extinction learning and memory (Milad and Quirk 2002; Orsini and Maren 2012; Quirk and Mueller 2008). Additionally, the role of chronic alcohol exposure and resulting plasticity changes in the PFC have been an emerging topic of research (Bertotto et al. 2006; Holmes et al. 2012; Lattal 2007; Ripley et al. 2003). These studies suggest that the mPFC is an important area of interest when examining co-morbid fear and anxiety disorders since alcohol has the ability to alter plasticity through changes in dendritic arborization in this region (Holmes et al. 2012). Overall, the literature makes it clear that there is an interaction between alcohol exposure, fear extinction learning, and glutamate signaling in the IfL, but the exact nature of the relationship between these topics is not clear and will be further addressed in these studies.

Sex Differences in Fear Behaviors

An additional area of research concerning the AUD/PTSD comorbidity is the behavioral differences observed between men and women who suffer from these disorders. Clinical findings indicate that the prevalence of PTSD is twice as high in women as in men (Lebron-Milad and Milad 2012), and PTSD more often preceded AUD in women than in men (Sonne et al. 2003). Additionally, the severity of women’s, but not men’s, PTSD symptoms was associated with increased alcohol consumption (Lehavot et al. 2014). Therefore, it is crucial to better understand the extent of the sex differences that occur between these types of patients to differentially tailor treatment regimens for men and women. Since there are clear clinical differences between the sexes in fear related disorders, this topic has become increasingly important in preclinical studies. Animal models of PTSD often rely on classical conditioning in order to evoke fear behaviors in response to specific cues. While the

circuitry that is responsible for these behaviors is highly conserved between the human and rodent species, there are sexual dimorphisms observed in heritability, receptor expression, neuronal structure, and fear circuit functionality (Ramikie and Ressler 2017). Further, the molecular mechanisms that are responsible for the encoding of fear memories has been shown to differ between males and females (Velasco et al. 2019). These sex differences have been shown to affect all phases of fear learning including conditioning, extinction, and recall. For example, when exposed to discrimination training using auditory threat and safety cues, female rats exhibit escalated learning and were able to discriminate between the cues faster. But, in the following days, females were shown to generalize fear behaviors and did not discriminate between the threat and safety cue like the males. Further, this generalization was shown to be a result of impaired learning of safety cues in females that did not occur in male animals (Day et al. 2016). Additional studies show that female rodents express reduced levels of fear behavior during recall in a model of chronic stress that included both restraint and auditory fear conditioning (Baran et al. 2009). Due to the established structural, functional, and behavioral sex differences in fear learning, it is essential to include comparisons between the sexes in experiments that involve fear-related behavioral assessments.

Rodent Models of PTSD and AUD

The models used for these experiments are commonly used in animal studies to mimic the characteristics of both AUD and PTSD. Many investigators use the chronic intermittent ethanol (CIE) vapor exposure paradigm to model AUD (Ewin et al. 2019; Holmes et al. 2012; Sanna et al. 2002; Singewald and Holmes 2019), and it has become the standard model used to induce alcohol dependence in rats. Furthermore, individuals with AUD often achieve blood ethanol concentrations (BECs) well above the legal limit of intoxication similar to the levels achieved in CIE exposure (Becker 2013; Griffin 2014). The fear-conditioning paradigm is also widely used by investigators to study PTSD (Klodzinska et al. 2004; Singewald et al. 2014; Singewald and Holmes 2019; Vanelzakker et al. 2013). However, using animals to study PTSD is particularly challenging because many of the psychological symptoms that characterize this disorder, including flashbacks, nightmares, and anxiety in response to complex stimuli, are nearly impossible to reproduce outside the human species (Miller and McEwen 2006). While the fear conditioning model may not entirely account for all aspects of PTSD in humans, it does produce many similar characteristic symptoms including increased anxiety behavior, cued fear responses, and context-induced fear expression (Brewin 2001). Importantly, the fear conditioning paradigm lends itself well to these studies since the model is well known, widely used, and it can be employed to study interactions between drug exposure and fear learning. Thus, the purpose of this set of studies was to examine the impact of CIE vapor exposure on the extinction and recall of fear-related behaviors and to determine if manipulation of prefrontal glutamatergic function could serve as a potential treatment target. Additionally, given the substantially different rates of prevalence of these disorders between the sexes, we also investigated potential differences between males and females in these behaviors using our model of AUD/PTSD comorbidity.

Materials and Methods

Drugs

3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) was custom synthesized by Chemir Analytical Services (Maryland Heights, MO) according to previously published methods (Kinney et al. 2005; Lindsley et al. 2004), purified to >95% purity by liquid chromatography-mass spectrometry, and suspended in 10% v/v Tween-80 (Sigma-Aldrich). CDPPB was given as a subcutaneous injection at a dose of 30 mg/kg. This dosage comes from our previous studies showing that 30 mg/kg CDPPB can facilitate extinction learning without resulting in any motor effects (Cleva et al. 2011; Gass et al. 2014; Gass and Olive 2009a). 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP) hydrochloride was purchased from Abcam Inc. (Cambridge, MA) and dissolved in sterile artificial cerebrospinal fluid (aCSF) at a concentration of 5 μg/μl. The MTEP dose of 5 μg/μl was chosen to maximize blockade of mGlu5 receptor activity and is based on our previously published findings (Cannady et al. 2017; Gass et al. 2014; Sinclair et al. 2012). Additionally, similar doses of MTEP have been shown to have no effect on locomotor behavior in rodent models (Klodzinska et al. 2004; Martin-Fardon et al. 2009).

Animals

Male and female Wistar rats [postnatal day (PD) 50 and 250-275 g upon arrival, Harlan, Indianapolis, IN] were housed individually in standard polycarbonate cages. Access to food and water in the home cage was continuous throughout the experiment except during behavioral testing. The animal colony was maintained on a 12:12 reverse light-dark cycle with lights off at 09:00 am, and experimental testing was performed during the dark portion of the cycle. All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee at the Medical University of South Carolina and within guidelines set forth by the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003). Overall, there were a total of 96 animals across seven treatment groups for each sex that were used in total for Experiments 1 and 2. For the behavioral trials done in Experiment 1, there were three groups per sex that consisted of the following treatment groups: Air+Vehicle (n=8/sex), CIE +Vehicle (n=8/sex), and CIE+CDPPB (n=8/sex). Microinjection studies in Experiment 2 consisted of four groups that received either CIE+Vehicle (n=6/sex), CIE+CDPPB (n=6/sex), CIE+CDPPB+MTEP in the IfL (n=6/sex), and CIE+CDPPB+MTEP in the prelimbic cortex (PrL) (n=6/sex). The overall experimental design timeline is depicted in Figure 1.

Fear Conditioning Protocol

Fear conditioning and related paradigms were completed in accordance with previously published methods (Cain et al. 2002; Holmes et al. 2012; Izquierdo et al. 2006; Sinclair et al. 2012; Wellman et al. 2007). Briefly, the conditioning procedure consisted of a 120 second acclimation period, followed by four pairings of the tone (conditioned stimulus, CS, 30 sec, 80 dB, 3 kHz tone) with the footshock (unconditioned stimulus, US, 2 sec, 0.75 mA scrambled footshock) presented during the last 2 seconds of the CS. Each tone/shock pairing was separated by a 10 second inter-stimulus interval. These 5-minute sessions occurred daily for 3 days and served to model characteristics of PTSD (Cain et al. 2012; Vanelzakker et al.

2013). Rats were considered to have reached “conditioning criteria” when they displayed freezing behavior at least 80% of the time during the presentation of the CS. Freezing behavior (based on studies Milad and Quirk 2012; Sierra-Mercado et al. 2011; Quirk and Mueller 2008; Quirk et al. 2010) was determined from digitized videos using FreezeScan software (Clever Systems, Inc.). Treatment groups were behaviorally matched to ensure that all groups had similar levels of freezing before drug manipulation during extinction.

Chronic Intermittent Ethanol (CIE) Exposure

CIE exposure commenced the week following fear conditioning and was used to model alcohol dependence that begins following the experience of a traumatic event. This procedure involved repeated cycles of exposure to binge-like levels of ethanol through vapor inhalation. This model has been previously characterized (Sanna et al. 2002) and has been used by our lab (Gass et al. 2017; Trantham-Davidson et al. 2014) and others (Ewin et al. 2019; Holmes et al. 2012; Singewald and Holmes 2019) to study how chronic exposure to ethanol alters neuronal function. Specifically, this model involved placing rats into an ethanol vapor chamber for 14 consecutive days with each exposure day consisting of 14 hrs. in the chambers and 10 hrs. of abstinence. The rats were kept on a reverse 12 hr. light/dark cycle with lights on at 9:00 pm and off at 9:00 am, and CIE occurred mostly during the lights on phase (6 pm to 8 am). Water intake and body weight were monitored during each intoxication/withdrawal cycle. The control rats were treated equivalently by transferring their home cages into the lab during the same time period but remained outside of the ethanol chambers. The level of intoxication was measured per rat at the end of each vapor exposure period using a 5-point motor intoxication rating scale (Nixon and Crews 2002). The target range of intoxication using this scale was slight-to-moderate motor intoxication (e.g. a rating of 2 to 3, respectively). BECs were determined from tail blood using a standard colorimetric assay at the end of exposure day 2, 6, 10, 15, using methods routinely performed in our lab (Gass and Glenn et al. 2014; Gass and Trantham-Davidson et al. 2014; Trantham-Davidson et al. 2017). This CIE exposure paradigm is particularly useful because it models the typical alcoholic drinking pattern of repeated episodes of binge intoxication and withdrawal with high blood alcohol levels (200-300 mg%) that are difficult to achieve with voluntary alcohol consumption in rodents. Furthermore, our previous findings have shown that ethanol exposure resulting in a BEC that ranges from 200-300 mg% results in PFC dysfunction (Trantham-Davidson et al. 2014). Rats from all groups did not begin fear extinction testing until at least 72 hours after CIE/air exposure to avoid alcohol withdrawal effects.

Fear Extinction Testing

Extinction training began the week following CIE exposure, and an “ABA” experimental design was incorporated to better analyze the impact of CIE exposure on the extinction of fear associated cues and context. Initial fear conditioning occurred in one context (A) and cue extinction occurred in a novel, visually distinct context (B) that consisted of different colored walls and flooring. Black paneling was placed over the clear walls of the operant chamber and the flooring was changed from grid to solid plastic. Extinction training in context B consisted of a 120 second acclimation period followed by 10 presentations of the CS (tone), each lasting 30 seconds and separated by a 10 second inter-stimulus interval. Fear

expression was considered extinguished when the rats froze, on average, less than 30% in response to the CS for 3 consecutive presentations of the CS. Based on group assignment, rats received CDPPB (30 mg/kg, subcutaneously) or vehicle (10% Tween 80, subcutaneously) 20 minutes prior to each extinction session while all further testing was done drug free.

Fear Extinction Recall and Context Renewal

Two days after freezing behavior was extinguished, rats were tested for extinction recall by placing them in the cue extinction environment (context B) and presenting the tone (CS) one time. These trials lasted the duration of the tone (30 seconds) and freezing behavior was measured. Two days following the extinction cue recall test, animals were placed in the original fear context A in the absence of any additional cues (i.e. the tone) to measure context-induced renewal of freezing behavior (Elias et al. 2010; Tovote et al. 2015). Notably, both tests occurred in a drug free state where animals did not receive CDPPB or vehicle injections, and these assessments provided information on how CIE affects extinction of both discrete fear-related cues and contextual environments.

Stereotaxic Microinjection Cannula Implantation

For Experiment 2, a separate group of 48 rats were assigned to microinjection groups to test the ability of local administration of MTEP (an mGlu5 negative allosteric modulator) in the IfL or PrL to prevent the facilitating effects of CDPPB on fear extinction. Surgeries occurred between PD 50 and 56 and were followed by two weeks of recovery before fear conditioning commenced on PD 70. Rats were anesthetized with isoflurane vaporized in medical grade breathing air at a flow rate of 0.4 L/min and placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA). Bilateral microinjection guide cannulae (26 ga O.D., Plastics One, Roanoke, VA) were aimed to terminate 1 mm dorsal to the IfL or PrL cortex. The stereotaxic coordinates used for the IfL cortex were (in mm from bregma and skull surface) anterior/posterior + 3.24, medial/lateral ± 0.6, and dorsal/ventral − 3.8 and for the PrL cortex were anterior/posterior + 3.24, medial/lateral ± 0.6, and dorsal/ventral −2.2 (Paxinos and Watson 2005). Microinjection cannulae were secured to the skull with stainless steel screws and dental cement. Removable obturators (33 ga O.D.) were inserted in the full length of the guide cannulae to limit obstruction by tissue and contamination by external debris. The wound was treated with topical 2% xylocaine and 2% triple antibiotic ointments and sutured closed using 3–0 Vicryl sutures. After surgery, all rats were given carprofen (2.5 mg/kg, s.c. daily for 5 days) for post-operative pain management. Following surgical recovery, rats were exposed to the fear conditioning and CIE exposure paradigms described above.

Microinjection Procedures

After fear conditioning and CIE exposure, rats were assigned to either an MTEP (5 μg/μl) or Vehicle (aCSF) treatment group (n = 6 for each brain region/treatment; 48 rats total). This dose of MTEP was chosen to maximize blockade of mGlu5 receptor activity and is based on our previously published findings (Cannady et al. 2017; Gass et al. 2014; Sinclair et al. 2012). Bilateral microinjections were performed 25 min prior to each extinction session. First, rats were lightly restrained, and obturators were removed. Then, sterile 33-gauge microinjection needles (Plastics One) were connected via microbore tubing to two 100 μl

syringes (Hamilton, Reno, NV). Syringes were mounted on a micro-infusion pump (Harvard Apparatus, Holliston, MA) set to deliver fluids at a flow rate of 0.5 μl/min. Microinjection needles were inserted bilaterally to a depth 1 mm beyond the ventral tip of the guide cannula. Drug solutions were infused at a volume of 0.5 μl/side over a 1 min period. Microinjection needles were left in place for an additional minute to allow for drug diffusion. Next, injectors were removed, and obturators were replaced. Each rat received an injection of CDPPB (30 mg/kg, s.c.) immediately after the microinjection. After 20 minutes, rats were placed in the operant chamber and the extinction session began according to the protocol described above.

Histological Verification of Microinjection Sites

Verification of cannula placement was determined using previously published methods (Gass et al. 2011). Following behavioral procedures, rats were anesthetized with isoflurane and euthanized by decapitation. Brains were then removed, immersed in 10% v/v formalin for at least 1 week at 4°C, transferred to a 30% (w/v) sucrose solution for at least 72 hours at 4°C, and then immersed in 15% (w/v) sucrose for at least 72 hours at 4°C. Brains were then cut into 40-mm coronal sections on a cryostat (Leica CM 1900, Leica Microsystems, Bannockburn, IL), mounted onto microscope slides, and stained with cresyl violet for histological verification of cannula placement under light microscopy.

Determination of Estrous Cycle

Fear extinction has been shown to be affected by estrous cycle (Milad et al. 2009) and, thus, we conducted a small pilot study to assess the impact of the estrous cycle on fear extinction in female rats (n = 6/group). Vaginal swabs [based on the PI's previous experience (Gass et al. 2007) and other published methods (Ford et al. 2002; Roberts et al. 1998)] occurred after each extinction session to prevent the invasiveness of the procedure from disrupting behavior. Findings from this pilot indicate that the rate of fear extinction was not altered by phase of the estrous cycle (Figure 2).

Statistical Analysis

Behavioral data were analyzed using SPSS version 23.0 software (SPSS Inc., Chicago, IL) and Prism version 8 (GraphPad software, La Jolla, CA). A p value of less than 0.05 was considered statistically significant for all tests. Experiments that analyzed fear conditioning responses used independent samples t-tests to compare the number of tone shock pairings needed to meet criteria between males and females. All experiments involving extinction behavior were analyzed using multiple two-way ANOVAs with Tukey's multiple comparisons test done between each treatment group on each day of extinction to compare behavioral responses to each individual conditioned stimulus presentation. An additional two-way ANOVA was done to analyze overall freezing throughout extinction to determine a main effect of treatment group on extinction learning. Animals were removed from analysis once individual freezing was indicative of successful extinction if it occurred prior to day five of extinction. Analysis of freezing during each recall session used one-way ANOVAs with multiple comparisons using Holm-Sidak post-hoc testing to determine differences in freezing in response to the conditioned stimulus presentation between multiple treatment groups.

Results

All animals achieved comparable BECs during CIE vapor exposure

This study used a well-characterized ethanol vapor exposure paradigm to induce alcohol dependence through daily episodes of ethanol vapor exposure and withdrawal over a period of two weeks. To monitor the level of intoxication after each 14-hour ethanol exposure period, a 5-point behavioral intoxication rating scale (Nixon and Crews 2002) was used with a target level of slight-to-moderate intoxication which corresponds to a rating of 2-3, respectively. For all treatment groups across Experiment 1 and 2, a total of 80 male and female animals underwent CIE exposure and the ratings were averaged for each rat across all exposure periods. The intoxication score average across all days and all rats was 2.2 ± 0.05. As a complementary measure to the behavioral rating of intoxication, BECs were obtained from tail vein puncture following exposure periods on days 2, 6, 10, and 15. The target range for BEC was between 200 – 300 mg%, and our analysis revealed average BEC mg% values for males of 249.6 ± 24.5 (Day 2), 240.3 ± 20.6 (Day 6), 230.2 ± 19.8 (Day 10), and 221.3 ± 29.54 (Day 15) with an overall grand average across all 4 days of 235.4 ± 23.6. For the females, our analysis revealed average BEC mg% values of 259.2 ± 16.39 (Day 2), 279 ± 26.4 (Day 6), 251.9 ± 12.4 (Day 10), and 235.4 ± 18.57 (Day 15) with an overall grand average across all 4 days of 256.4 ± 18.4.

CIE exposure results in deficits in fear extinction learning that are treated with CDPPB

Experiment 1 used a total of 48 male and female animals to determine how CIE exposure affects fear learning during extinction and how CDPPB treatment impacts CIE induced deficits in fear extinction. The experimental groups consisted of the following: Males and Females: Air+Vehicle vs. CIE+Vehicle vs. CIE+CDPPB (n=8/group, 6 groups total).

In order to test for fear extinction learning, animals must first meet criteria for fear conditioning. Regardless of subsequent CIE or air treatment, all animals were freezing 80% of the time in response to the tone (Figure 3a), but females required significantly fewer tone/shock pairings to meet this criteria when compared to males [$t_{(14)} = 9.431$, $p < 0.0001$, n=16/sex] (Figure 3b). This indicates that females acquired fear learning at a significantly faster rate than males. Due to the novelty and unexpected nature of the shock on day one of conditioning, freezing levels were lower on day one when compared to day two of conditioning.

Following fear conditioning, animals were put into CIE exposure for two weeks before receiving five days of fear extinction training. As shown in Figure 4a, males exposed to CIE exhibited significantly higher average rates of freezing to the CS across multiple days of extinction training when compared to air-exposed controls. Specifically, there was a main effect of CIE on extinction learning such that CIE exposed animals exhibited increased levels of freezing when compared to air treated animals across the total duration of extinction training [$F_{(49, 489)} = 24.25$, $p < 0.0001$, n=8/group]. Furthermore, females exposed to CIE also showed significantly higher rates of cue-induced freezing during extinction training when compared to air exposed controls throughout extinction [$F_{(49, 500)} = 34.58$, $p < 0.0001$, n=8/group] (Figure 4b). Notably, treatment with CDPPB was effective at attenuating

CIE induced deficits in extinction learning. There was a significant effect of CDPPB on extinction learning such that CDPPB treatment reduced freezing during extinction in CIE exposed animals across the entirety of extinction for both males [$F_{(49, 481)} = 30.58$, $p < 0.0001$, n=8/group] (Figure 4a) and females [$F_{(49, 500)} = 31.19$, $p < 0.0001$, n=8/group) (Figure 4b). Additional analyses were done to compare the rate of freezing in response to each of the 50 individual CS presentations that occurred throughout extinction, and significant differences are noted throughout Figure 4 where *$p < 0.05$ comparing Air +Vehicle to CIE+Vehicle and +$p < 0.05$ comparing CIE+Vehicle to CIE+CDPPB.

A clear sex difference emerged when the rats were tested for extinction cue recall in Context B two days after meeting extinction criteria. When presented with a single tone in the extinction context, there was a significant effect of CIE, where animals exposed to CIE prior to extinction exhibited an increased rate of freezing when compared to both air exposed controls and CIE exposed animals treated with CDPPB [$F_{(2,21)} = 35.74$, $p < 0.0001$, n = 8/group] (Figure 5a). This effect was not seen in female animals, where animals exposed to CIE did not differ in freezing behavior from either air exposed controls or CDPPB treated animals [$F_{(2,21)} = 0.7977$, $p = 0.4636$, n = 8/group] (Figure 5b). The extinction recall test was followed by an examination of context-induced freezing behavior in Context A. This behavioral test consisted of placing the rat back into the original conditioning environment in the absence of any tone presentation in order to assess freezing in response to the context only and not the discrete CS tone. CIE-exposed male rats showed a significant increase in context-induced freezing compared to both air-exposed controls and CIE-exposed animals that were treated with CDPPB prior to each extinction session [$F_{(2,21)} = 9.66$, $p = 0.001$, n = 8/group] (Figure 6a). Additionally, this treatment effect was seen in females as well, where there was a significant increase in freezing to the context see in CIE treated animals compared to air exposed controls and CIE+CDPPB animals [$F_{(2,21)} = 22.85$, $p < 0.0001$, n = 8/group] (Figure 6b). It is important to note that all rats were tested in this procedure in a drug-free state, unlike extinction training where CDPPB or vehicle was administered prior to each session.

The IfL, and not PrL, cortex is involved in the facilitation of extinction learning by CDPPB

These behavioral studies indicate that CDPPB can recover deficits in fear extinction learning that occur following CIE exposure in both male and female rats. Our previously published findings examining the extinction of drug-seeking behaviors found that facilitation of extinction by CDPPB was associated with the formation of calcium permeable AMPA receptors specifically in the IfL region of the PFC (Gass et al. 2014). Therefore, in Experiment 2, we examined whether CDPPB's effects were dependent upon activation of mGlu5 receptors selectively in the IfL cortex using the following treatment groups: Males and Females: CIE+Vehicle vs. CIE+CDPPB vs. CIE+CDPPB+MTEP in the IfL vs. CIE +CDPPB+MTEP in the PrL (n=6/group, 8 groups total).

There was a significant main effect of treatment on freezing behavior during extinction in male rats where CIE+CDPPB+MTEP treated animals exhibited a return to increased levels of freezing when compared to CIE+CDPPB animals across all of extinction training [$F_{(49, 495)} = 24.54$, $p < 0.0001$, n=6/group] (Figure 7a). Essentially, local administration of

MTEP into the IfL cortex prior to CDPPB treatment blocked the enhancing effect of CDPPB on extinction in male rats with a history of CIE. The same experimental design was applied to of female CIE-exposed rats with similar outcomes. Again, infusion of MTEP into the IfL cortex just prior to systemic CDDPB administration prevented the facilitating effects of CDPPB on fear extinction learning, shown by significantly different rates of freezing in the CIE+CDPPB+MTEP animals compared to CIE+CDPPB treated animals across all of extinction [$F_{(49, 495)} = 24.67$, $p < 0.0001$, n=6/group] (Figure 7b). Further analyses revealed differences in responses to each individual CS presentation during extinction which are noted throughout Figure 7 where *$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB and +$p < 0.05$ CIE+CDPPB vs. CIE+CDPPB+MTEP.

When looking at extinction cue recall, CIE+CDPPB treated animals that received MTEP in the IfL displayed increased levels of freezing behavior when compared to CIE + CDPPB treated animals and exhibited freezing similar to CIE exposed animals [$F_{(2,15)} = 27.62$, $p < 0.0001$, n = 6/group] (Figure 8a). This suggests that blockade of mGlu5 in the IfL blocked CDPPB's ability to reduce freezing heightened freezing during extinction recall caused by CIE. CIE exposed females did not show heightened freezing during recall, and there were no differences observed between this group and any other treatment group [$F_{(2,15)} = 0.5237$, $p = 0.6028$, n = 6/group] (Figure 8b). Similar results were observed during context recall. There was a significant effect of MTEP when given in the IfL, where CIE+CDPPB+MTEP treated animals no longer exhibited a reduction in freezing to the context like the CIE+CDPPB animals for both males [$F_{(2,15)} = 12.04$, $p = 0.0008$, n =6/group] (Figure 9a) and females [$F_{(2,15)} = 17.16$, $p = 0.0001$, n = 6/group] (Figure 9b). Essentially, CIE+CDPPB+MTEP treated animals exhibited freezing similar to CIE+Vehicle animals, signifying a lack of treatment effect of CDPPB when mGlu5 is blocked in the IfL. Histological examination of the injection site demonstrated that all microinjections were within the IfL cortex or its boundary regions (Figure 10).

The same experimental procedure was completed in the PrL cortex, but, when MTEP was given in this region, CDPPB treatment was still effective at attenuating CIE-induced deficits in extinction learning. For males, there was still a significant effect of CDPPB treatment on freezing during extinction even when MTEP was given when compared to CIE treated animals [$F_{(49,494)} = 18.4$, $p < 0.0001$, n=6/group] (Figure 11a). This effect was also seen in females, where CIE+CDPPB+MTEP treated animals maintained significantly different levels of freezing when compared to CIE treated animals [$F_{(49, 493)} = 29.78$, $p < 0.0001$, n=6/group] (Figure 11b). Basically, blockade of mGlu5 activity in the PrL with MTEP was not sufficient to stop CDPPB's treatment effects on extinction learning in CIE exposed animals. Differences in responses to each individual CS presentation during extinction are noted throughout Figures 11 where *$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB, +$p < 0.05$ CIE +CDPPB vs. CIE+CDPPB+MTEP, #$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB+MTEP.

When looking at extinction cue recall, blockade of mGlu5 in the PrL was not able to prevent the treatment effect of CDPPB. There was still a significant effect of treatment where CIE exposed animals exhibited an increased rate of freezing to the CS when compared to both CIE+CDPPB and CIE+CDPPB+MTEP treated animals [$F_{(2,15)} = 21.96$, $p < 0.0001$, n = 6/group] (Figure 12a). Essentially, when mGlu5 was blocked in the PrL, CDPPB was still able

to act as an effective treatment on CIE induced heightened freezing during cue recall. In female animals, CIE did not cause any changes in freezing to the CS during cue recall, and there were no differences in freezing between any of the treatment groups [$F_{(2,15)} = 1.196$, $p = 0.3296$, n = 6/group] (Figure 12b). Further, in context recall testing, CIE+Vehicle treated animals exhibited significantly higher rates of freezing to the context when compared to those that received CIE+CDPPB and CIE+CDPPB+MTEP treatment in males [$F_{(2,15)} = 12.7$, $p = 0.0006$, n = 6/group] (Figure 13a) and females [$F_{(2,15)} = 8.358$, $p = 0.0036$, n = 6/group] (Figure 13b). This indicates that when mGlu5 is blocked in the PrL, CDPPB is still able to work as a treatment to prevent heightened freezing in response to the context for both sexes. Histological examination of the injection site demonstrated that all injections were within the PrL cortex or its boundary regions (Figure 14). Together, these findings indicate that mGlu5 activity in the IfL cortex, but not the PrL cortex, is required for CDPPB to attenuate deficits in fear extinction and recall that result from chronic ethanol exposure.

Discussion

The results of the present experiments indicate that fear exposure followed by chronic alcohol leads to IfL mGlu5 dependent behavioral changes in fear extinction learning and memory recall. In this rodent model designed to mimic an increase in alcohol consumption following exposure to a stressor, chronic alcohol exposure led to a deficit in the ability to extinguish fear-related memories and impairments in the recall of extinction memories. Importantly, some of these effects were sex-dependent, suggesting that deficits resulting from alcohol and stress exposure differentially affect males and females. Both male and females exposed to chronic alcohol displayed heightened freezing in context-induced recall testing. However, when tested for extinction cue memory recall, a behavioral deficit was only observed in ethanol-exposed males. An additional sex difference was observed during the fear conditioning phase of testing where female animals escalated freezing behaviors during conditioning compared to males. CDPPB treatment given directly before each extinction session was able to facilitate extinction learning and prevent heightened freezing during cue and context recall. By microinjecting the mGlu5 negative allosteric modulator MTEP in specific PFC subregions regions in combination with systemic CDPPB administration, we were able to determine that the IfL cortex is necessary for CDPPB's effects on extinction learning. The current set of studies provide newly discovered beneficial effects of CDPPB treatment on fear extinction and attenuation of heightened cue- and context-induced freezing following chronic alcohol exposure.

Increasing evidence has implicated the PFC in the extinction of both fear and drug-seeking behaviors. These converging lines of evidence from the fields of fear and drug research suggest that the PrL cortex serves as an "on-switch" for fear expression and drug-seeking, while the IfL cortex functions as an "off -switch" to allow for the expression of extinction behavior (Quirk et al. 2010; LaLumiere and Kalivas 2008; LaLumiere et al. 2010; Peters et al. 2009). While the majority of studies investigating the role of the PrL and IfL subregions support our current findings, there are a small number of published manuscripts that suggest alternative roles of these structures in extinction learning. For example, Marek and colleagues (Marek et al. 2018) have highlighted the impact of PrL to IfL projections. Specifically, the PrL has been shown to send excitatory afferents to neurons in the IfL, some

of which project to the amygdala. Using c-Fos expression as a measure of neuronal activity, they found that PrL neurons that project to the IfL were active during extinction learning and optogenetic stimulation of these PrL to IfL afferents resulted in facilitated extinction learning. While these findings provide a greater role for the PrL in extinction learning, they still highlight the importance of these PFC subregions in extinction-related behaviors. Although not directly examined in the current set of studies there has been a substantial amount of research implicating areas of the amygdala in the extinction of fear conditioning (Quirk et al. 2010; Sierra-Mercado et al. 2011; Meyers and Davis 2002; Meyers and Davis 2007). In addition to extinction learning, a number of studies have also shown that subregions of the amygdala are involved in fear memory reconsolidation (Parsons and Gafford et al. 2006; Parsons, Gafford, and Baruch et al. 2006; Nader et al. 2000; Debiec et al. 2006; Doyere et al. 2007). Given the substantial impact that alcohol abuse has on both the PFC (Abernathy et al. 2010; Tu et al. 2007) and the amygdala (Koob 2009; Silberman et al. 2009; McCool et al. 2010), it is logical to assume that CIE alters the neurocircuitry between these brain regions, potentially causing the changes in extinction learning and memory recall that were observed in these studies.

From a translational perspective, the glutamatergic system is one of the most investigated neurotransmitter systems involved in memory-based treatment approaches. Manipulation of both ionotropic and metabotropic glutamate receptors alters extinction learning of fear and drug-seeking behaviors as well as the reconsolidation of these memories (reviewed in Gass and Chandler 2013; Cleva et al. 2010; Gass and Olive 2008; Meyers et al. 2011; Sorg 2012). However, given the negative side effects associated with direct NMDA enhancement, a focus has been placed on investigating the impact of mGluR manipulation on fear and drug memories. Generally, enhancement of mGluR activity has been shown to facilitate extinction learning for fear (Sethna and Wang 2014) and drug-seeking behaviors (Gass and Chandler 2013; Cleva et al. 2010; Singewald et al. 2014). Additionally, our lab has shown that positive allosteric modulation of mGlu5 facilitates the extinction of cocaine-(Gass and Olive 2009a; Cleva et al. 2011) and alcohol-seeking (Gass et al. 2014). Additional studies have focused on the role of mGlu5 in the prefrontal cortex on fear extinction. It has been shown that the consolidation of fear extinction memories is dependent on mGlu5 activity in the IfL and enhancement of this activity can contribute to fear extinction (Fontanez-Nuein et al. 2011). Furthermore, genetic deletion of mGlu5 leads to impairments in the acquisition and extinction of fear behaviors (Xu et al. 2009). In total, these studies all highlight the necessity of mGlu5 signaling in fear learning and extinction and utilize this function for the treatment of comorbid PTSD/AUD.

Fear learning and memory processes have also been shown to differ between the sexes. Multiple lines of inquiry have found sex differences in fear responses that involve the medial prefrontal cortex. Further, these differences have been found in both behavioral and molecular studies. Behaviorally, sex differences have been established during fear conditioning (Fenton et al. 2016; Keiser et al. 2017), extinction (Voulo and Parsons 2017; Velasco et al. 2019; Matsuda et al. 2015), and recall (Baran et al, 2009; Keiser et al. 2017, Shvil et al. 2014). Females have also been shown to exhibit more active fear avoidance strategies, such as darting rather than freezing, which could account for the behavioral differences observed in these studies (Gruene et al. 2015). Furthermore, while females in

different estrous phases have been shown to extinguish fear behavior at the same rate, these same animals exhibit lower levels of freezing in recall testing when in proestrus compared to metestrus (Maeng et al. 2015). The determination of differential fear response strategies between the sexes is an important factor when looking at behavioral data and would be a valuable analysis for further fear related studies. Additionally, there are a number of differences between the sexes in the circuitry and biological mechanisms responsible for fear learning (Ramikie and Ressler, 2017). When looking at auditory fear memories, it has been shown that females exhibit enhanced learned fear expression due to elevated gamma oscillations in the medial prefrontal cortex (Fenton et al. 2016). Gamma oscillations have been shown to play a role in memory processing in the prefrontal cortex and have been shown to be involved in fear learning in both preclinical (Fitzgerald et al. 2014) and clinical populations (Mueller et al. 2014). Therefore, innate signaling differences between males and females in the mPFC could be playing a role in the sex differences we observed during fear conditioning. The PFC is a sexually dimorphic brain area with females having large populations of estrogen receptors in this region (Almey et al. 2014). Estrogen has been shown to affect long term potentiation and synapses in the mPFC which could be involved in the underlying cause of multiple sex differences exhibited during fear learning processes (Galvin and Ninan 2014; Gupta et al. 2001; Shanmugan and Epperson 2014; Zeidan et al. 2011). Sex differences have also been established in glutamatergic systems where females exhibit higher levels of glutamate when compared to males in a variety of brain regions, and there is differential glutamate expression across the estrous cycle (Wickens et al. 2018). Additionally, there are known interactions between estrogen receptors and metabotropic glutamate receptors in females (Tonn Eisinger et al. 2018; Kasten et al. 2019) that could affect the prefrontal cortex and therefore affect responses to fear stimuli.

Due to the complexity of modeling neuropsychiatric disorders in rodents, there are a number of limitations that should be taken into account when examining this data. One minor limitation in these studies comes from the potential impact of the estrus cycle on extinction and the fact that we chose to simply compare the behavior of rats starting in metestrus/diestrus vs. proestrus/estrus. Due to the fact that the within group variability is low and the rate of extinction is very similar between the groups, this gives us initial support that cycle phase is not altering extinction behavior. Furthermore, other studies support this conclusion by showing that the rate of fear extinction does not differ between females in proestrus vs. metestrus, and, further, females given estrogen injections before extinction displayed freezing behavior similar to those that received vehicle (Maeng et al. 2015). However, there are multiple ways to examine the impact of cycle phase on fear behaviors, and we plan to further explore them in future studies. An additional limitation comes from the fact that these studies do not include animal cohorts that received only CDPPB or MTEP in absence of any other experimental manipulation. In our previous studies that examined CDPPB's effects on learning during the extinction of ethanol seeking behavior following CIE exposure, we found that CDPPB given in the absence of CIE was able to enhance extinction compared to air exposed controls that did not receive CDPPB. It is important to note that while these animals did not receive CIE, they were still exposed to ethanol during self-administration (Gass et al 2017). While the literature regarding CDPPB is mainly focused on drug seeking behaviors, it has been reported in the literature that CDPPB can enhance fear

extinction learning and recall in the absence of a drug insult (Sethna and Wang 2014). As such, for these experiments, while the focus was on using CDPPB to reverse CIE induced deficits, we would expect animals treated with CDPPB in the absence of CIE to extinguish fear behaviors faster than all other groups. With regards to MTEP's effects on behavior, a majority of the studies done using MTEP have been done systemically (Simonyi et al. 2010; Pietraszek et al. 2005; Schulz et al. 2001). In the present studies, MTEP was microinjected in specific brain subregions in order to block mGlu5 activity, and, therefore, the behavioral effects that are observed when MTEP is given systemically are not expected to occur in these studies. Additionally, these experiments used a single dose of CDPPB and MTEP. Multiple previous studies done in our lab using CDPPB and MTEP have tested various doses in order to optimize CDPPB's effects on extinction learning (Gass and Olive 2009a; Gass and Olive 2009b; Gass et al. 2014; Gass et al. 2017; Widholm et al. 2001; Cleva et al. 2011). Using 0.3, 3, or 30 mg/kg CDPPB these studies found that 30 mg/kg was necessary to induce significant effects on extinction learning that were prevented with MTEP given intraperitoneally. At this dose, CDPPB did not affect locomotor behavior or cause any neurotoxic effects (Gass and Olive 2009a). Earlier studies using microinjections of MTEP in the nucleus accumbens at a dose of 1, 3, and 10 ug/uL found that while 1 ug/uL was not sufficient to alter behavior and 10 ug/uL caused a reduction in locomotor activity, the 3 ug/uL concentration was able to reduce ethanol seeking without altering locomotor activity (Gass and Olive, 2009b). Therefore, the single doses of both CDPPB and MTEP used in these studies have been thoroughly validated to have optimal effects on learning without causing deleterious side effects.

Together, these studies establish multiple behavioral deficits that occur as a result of stress and alcohol exposure, successfully treated these deficits with CDPPB, and determined the necessity of IfL mGlu5 activity in this treatment effect. Additionally, the current findings identify potential brain mechanisms that underlie the detrimental effects of comorbid alcohol and stress exposure and strongly support the therapeutic potential of the prefrontal glutamatergic system.

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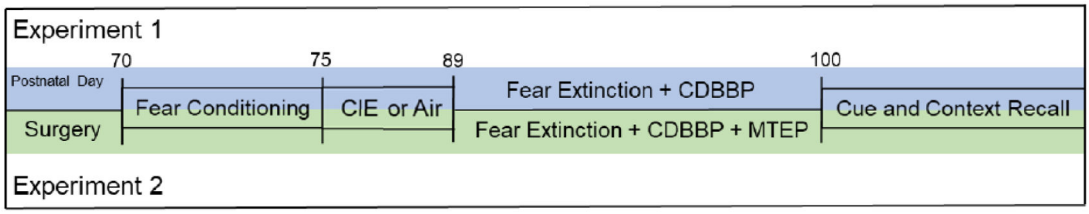

Fig. 1. Experimental Design.

For Experiment 1, animals started fear conditioning on PD 70 followed by two weeks of CIE or air exposure before being tested in fear extinction. A treatment group was included that received CDPPB injections 20 minutes prior to each extinction session. Testing ended with cue and context recall sessions. For Experiment 2, surgery to implant microinjection canulae started on PD 50 and was followed by fear conditioning and ethanol exposure. Then, fear extinction testing occurred and included a group that received MTEP in the IfL or PrL directly before each CDPPB injection. Experiments concluded with fear recall and context induced freezing tests.

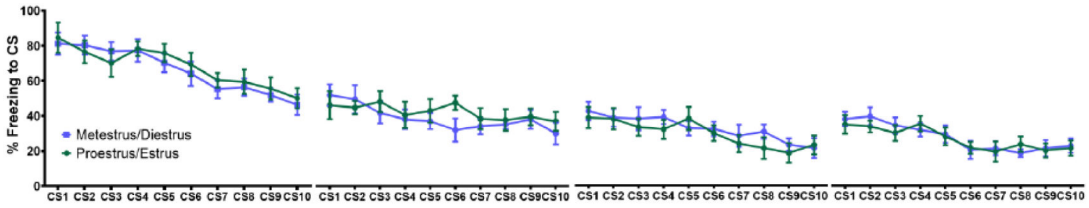

Fig. 2. Estrous phase does not affect fear extinction.

Female animals in metestrus/diestrus exhibited similar fear extinction freezing rates when compared to those in the proestrus/estrus phase.

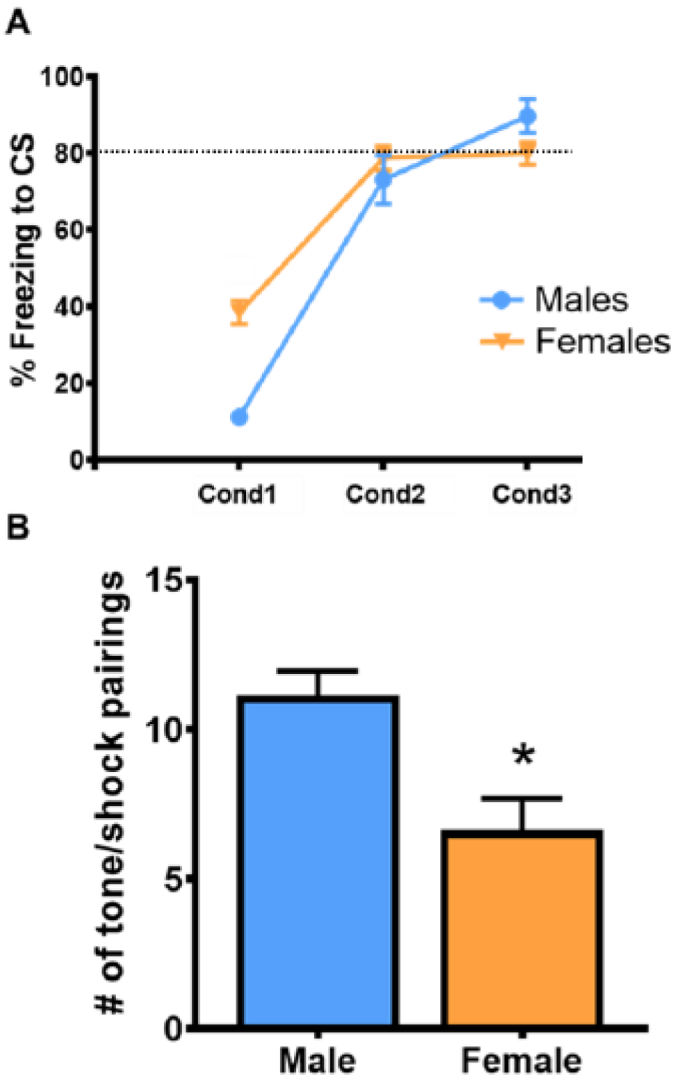

Fig. 3. Female animals meet fear conditioning criteria faster than males.

A) Regardless of subsequent air or CIE treatment, all animals met fear conditioning criteria of 80% freezing by conditioning Day 3. **B)** Female animals conditioning faster than males, since they required fewer tone/shock pairings to meet conditioning criteria ($t_{(14)} = 9.431$, *p < 0.0001, n=16/sex).

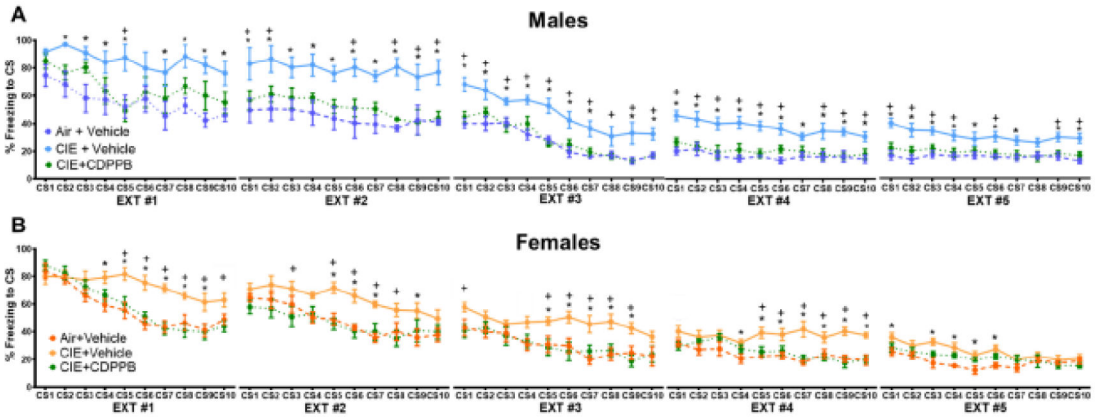

Fig. 4. CDPPB attenuates CIE-induced deficits in extinction learning for both males and females.

While CIE exposed males and females both exhibited impairments in extinction learning, CDPPB given directly before extinction was able to reduce freezing during extinction to levels comparable to controls for CIE exposed males **(A)** and females **(B)** (*$p < 0.05$ comparing Air+Vehicle to CIE+Vehicle, +$p < 0.05$ comparing CIE+Vehicle to CIE+CDPPB, n=8/group).

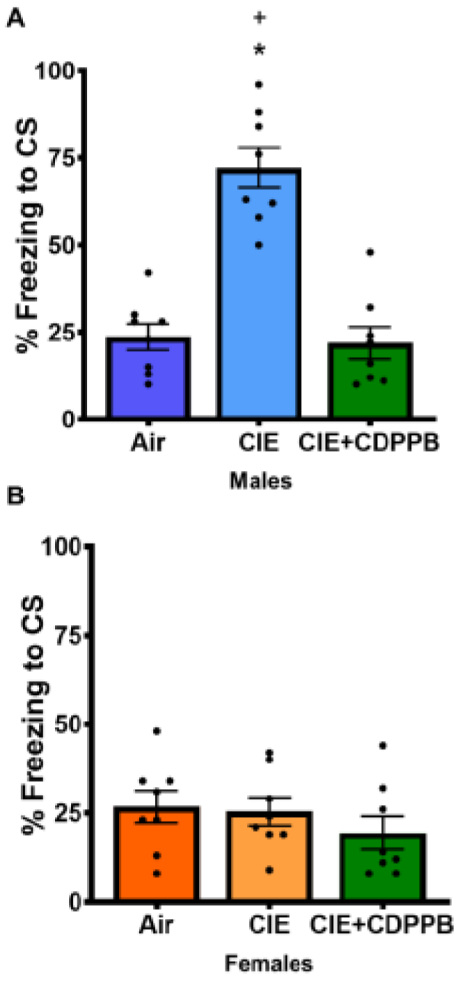

Fig. 5. CIE causes heightened freezing during extinction cue recall in males only, and this is prevented with CDPPB.

(A) CIE exposed males exhibited heightened freezing in the extinction cue recall test when compared to air treated animals, and this effect is attenuated with CDPPB treatment resulting in decreased levels of freezing in CIE treated animals [$F_{(2,21)} = 35.74$, $p < 0.0001$, n = 8/group]. **(B)** Conversely, female CIE animals did not show increased freezing when compared to either air exposed controls or CIE+CDPPB treated animals [$F_{(2,21)} = 0.7977$, $p = 0.4636$, n = 8/group] (*$p < 0.05$ CIE vs. Air, +$p < 0.05$ CIE vs. CIE+CDPPB).

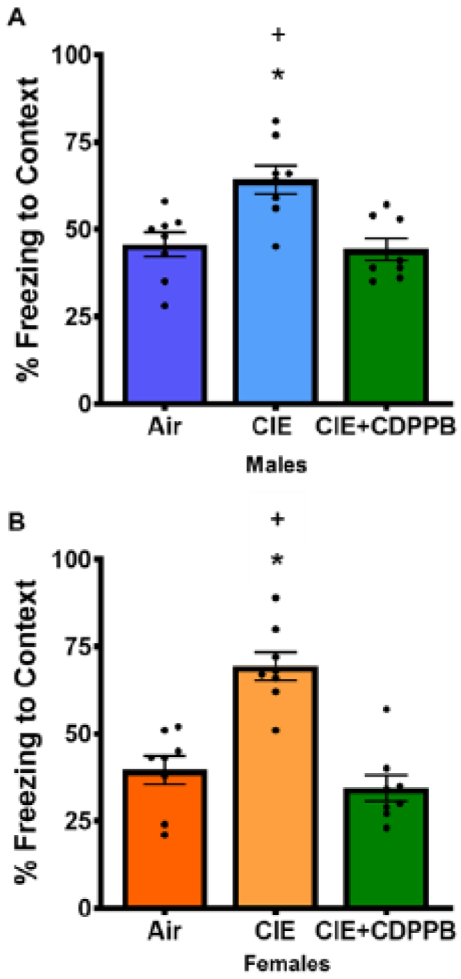

Fig. 6. CIE heightens freezing during fear context recall in both sexes, and CDPPB treatment prevents this effect.

(A) There was a significant effect of treatment during context recall where CIE exposed males exhibited heightened responding in the fear context recall test when compared to both air treated animals and those given CIE+CDPPB [$F_{(2,21)} = 9.66$, $p = 0.001$, n = 8/group]. **(B)** Additionally, female CIE animals exhibited increased freezing when compared to air exposed controls, and freezing was reduced following CDPPB treatment [$F_{(2,21)} = 22.85$, $p < 0.0001$, n = 8/group] (*$p < 0.05$ CIE vs. Air, +$p < 0.05$ CIE vs. CIE+CDPPB).

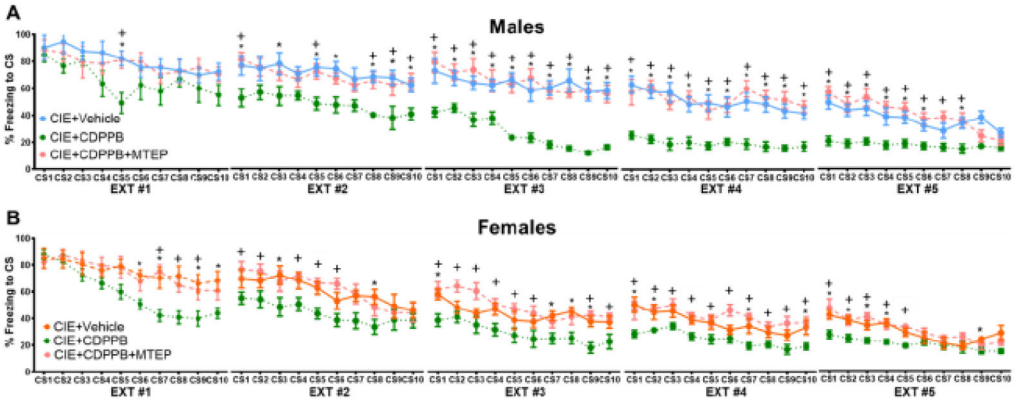

Fig. 7. In both sexes, negative allosteric modulation of mGlu5 in IfL cortex blocks the facilitating action of CDPPB on extinction learning.

(A) Infusion of MTEP into the IfL cortex blocked the ability of CDPPB to reduce freezing throughout extinction in CIE exposed male animals. **(B)** This was also true for females, where there was a significant effect of MTEP injections in CIE and CDPPB treated animals on freezing levels throughout extinction (*$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB, +$p < 0.05$ CIE+CDPPB vs. CIE+CDPPB+MTEP, n=6/group).

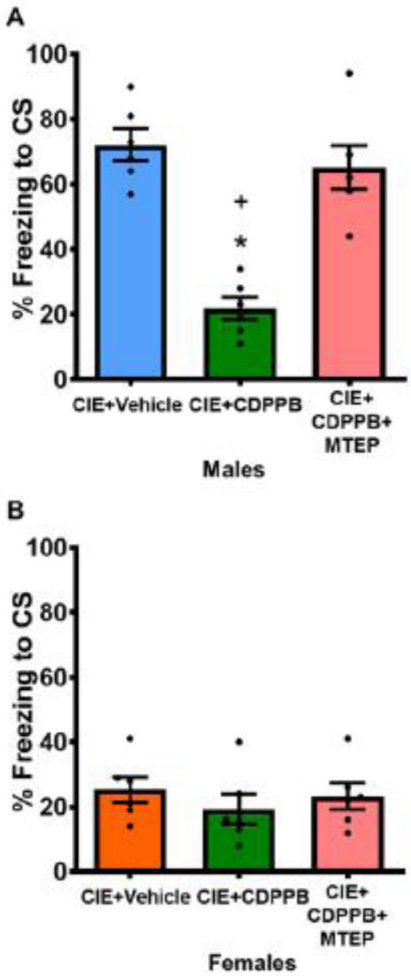

Fig. 8. MTEP infusion in the IfL prior to each extinction session blocks the ability of CDPPB to reduce freezing during cue recall in males.

(A) In males, CIE exposure led to heightened freezing during extinction cue recall and this effect was attenuated with CDPPB while CIE + CDPPB animals that received MTEP in the IfL returned to heightened levels of freezing [$F_{(2,15)} = 27.62$, $p < 0.0001$, n = 6/group]. **(B)** There were no differences observed between any of the female treatment groups in extinction cue recall [$F_{(2,15)} = 0.5237$, $p = 0.6028$, n = 6/group] (*$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB, +$p < 0.05$ CIE+CDPPB vs. CIE+CDPPB+MTEP)

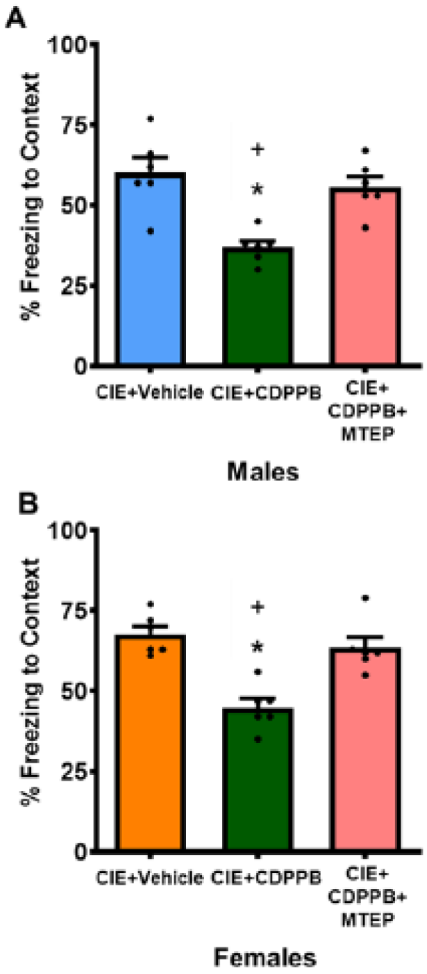

Fig. 9. MTEP given in the IfL prevents the treatment effect of CDPPB on heightened freezing during context recall tests.

A) While CDPPB treatment reduces freezing during context recall in CIE exposed male animals, MTEP given in the IfL blocks this effect of CDPPB and these animals return to heightened levels of freezing [$F_{(2,15)}$ = 12.04, p = 0.0008, n =6/group]. **B)** This was also seen in female animals, where CIE+CDPPB+MTEP treated animals exhibited freezing behavior comparable to CIE animals that received vehicle [$F_{(2,15)}$ = 17.16, p = 0.0001, n = 6/group] (*p < 0.05 CIE+Vehicle vs. CIE+CDPPB, +p < 0.05 CIE+CDPPB vs. CIE+CDPPB +MTEP).

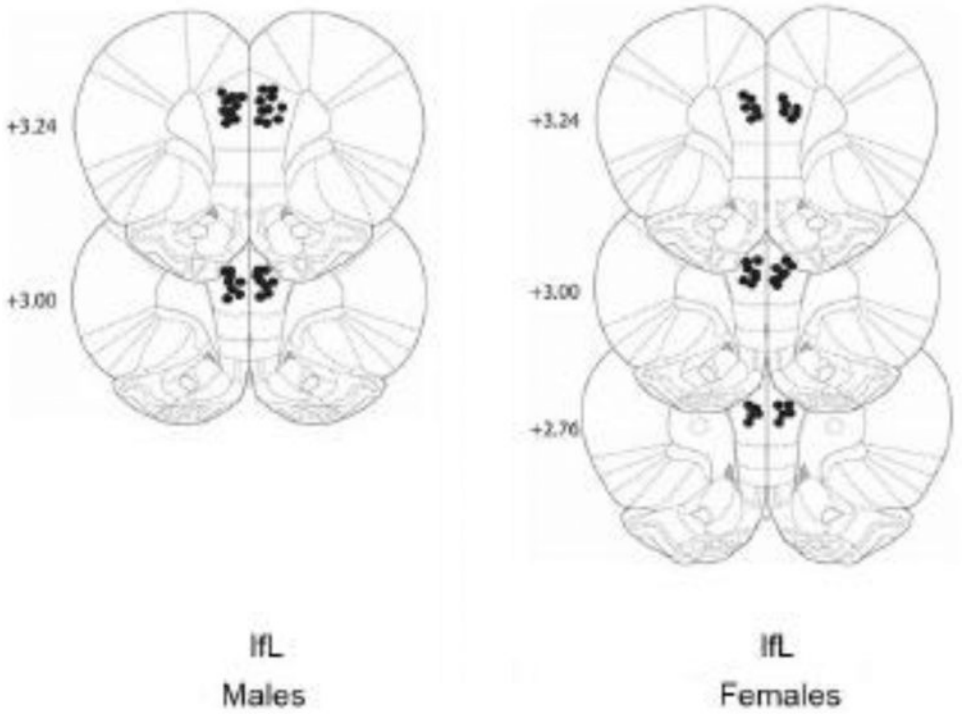

Fig. 10. Histology, IfL.

Histological verification of microinjection placements for male and female IfL infusions.

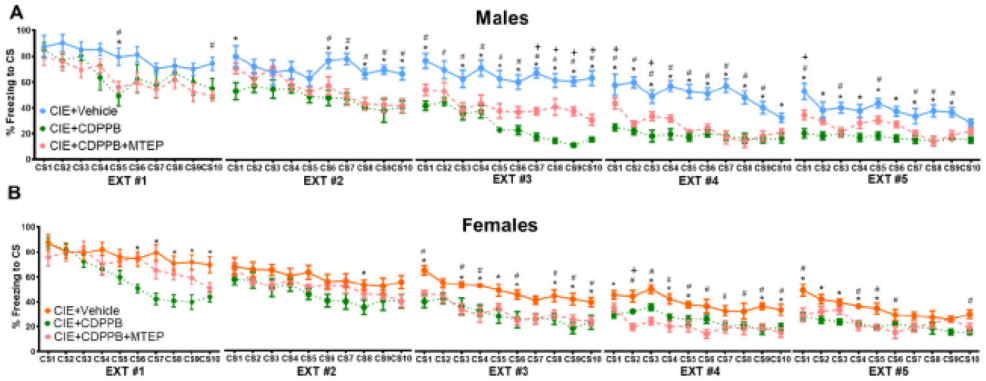

Fig. 11. MTEP infusion in the PrL prior to each extinction session does not alter CDPPB's facilitation of extinction learning.

(A) Negative allosteric modulation of mGlu5 through MTEP infusion into the PrL cortex did not change CDPPB effects on extinction learning following CIE, since these animals exhibited levels of freezing comparable to CIE+CDPPB treated animals. **(B)** This was also true for females, where there was no effect of MTEP injections in CIE and CDPPB treated animals on freezing levels throughout extinction (*$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB, +$p < 0.05$ CIE+CDPPB vs. CIE+CDPPB+MTEP, #$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB +MTEP, n=6/group).

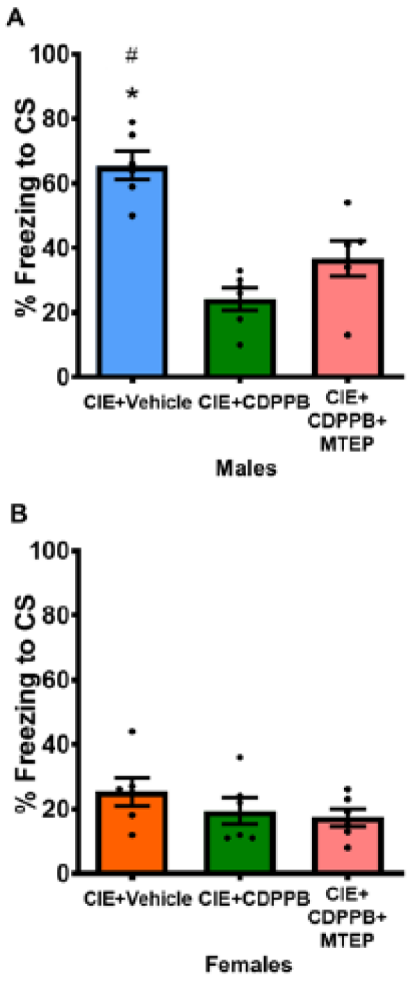

Fig. 12. MTEP given in the PrL does not alter CDPPB's ability to reduce freezing during cue recall in CIE exposed animals.

(A) In males, CIE+CDPPB treatment reduced freezing when compared to CIE+Vehicle animals [$F_{(2,15)}$= 21.96, $p < 0.0001$, n = 6/group] and CIE+CDPPB+MTEP animals exhibited freezing similar to those that received CIE+CDPPB. **(B)** In female animals, CIE +CDPPB+MTEP treatment did not cause any significant differences when compared to CIE +Vehicle and CIE+CDPPB treated animals [$F_{(2,15)}$ = 1.196, p = 0.3296, n = 6/group] (*$p <$ 0.05 CIE+Vehicle vs. CIE+CDPPB, #$p < 0.05$ CIE+Vehicle vs. CIE+CDPPB+MTEP).

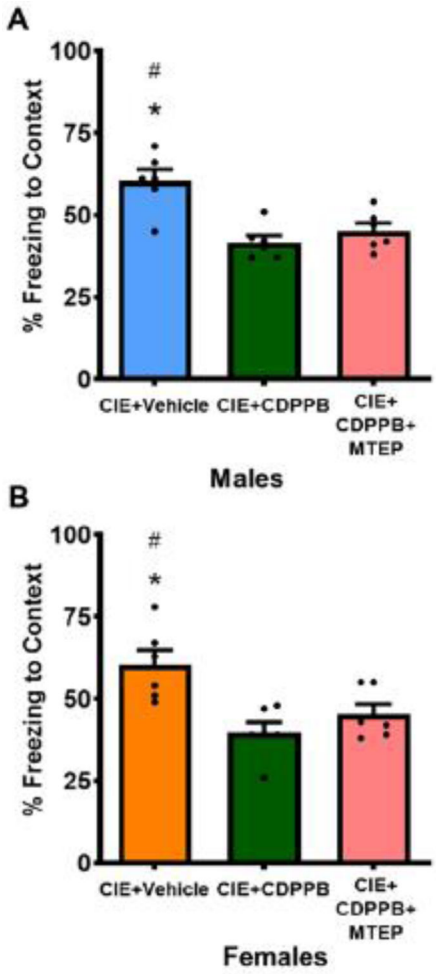

Fig. 13. PrL MTEP infusion does not alter the ability of CDPPB to reduce freezing during context recall for both sexes.

A) Male animals treated with CIE+CDPPB and CIE+CDPPB+MTEP exhibited similar levels of freezing during context recall while CIE+Vehicle animals displayed heightened freezing [$F_{(2,15)}$ = 12.7, p = 0.0006, n = 6/group]. **B)** This effect was also seen in females, where CIE+CDPPB+MTEP and CIE+CDPPB animals both showed reduced levels of freezing when compared to CIE+Vehicle animals [$F_{(2,15)}$ = 8.358, p = 0.0036, n = 6/group] (*p < 0.05 CIE+Vehicle vs. CIE+CDPPB, #p < 0.05 CIE+Vehicle vs. CIE+CDPPB+MTEP).

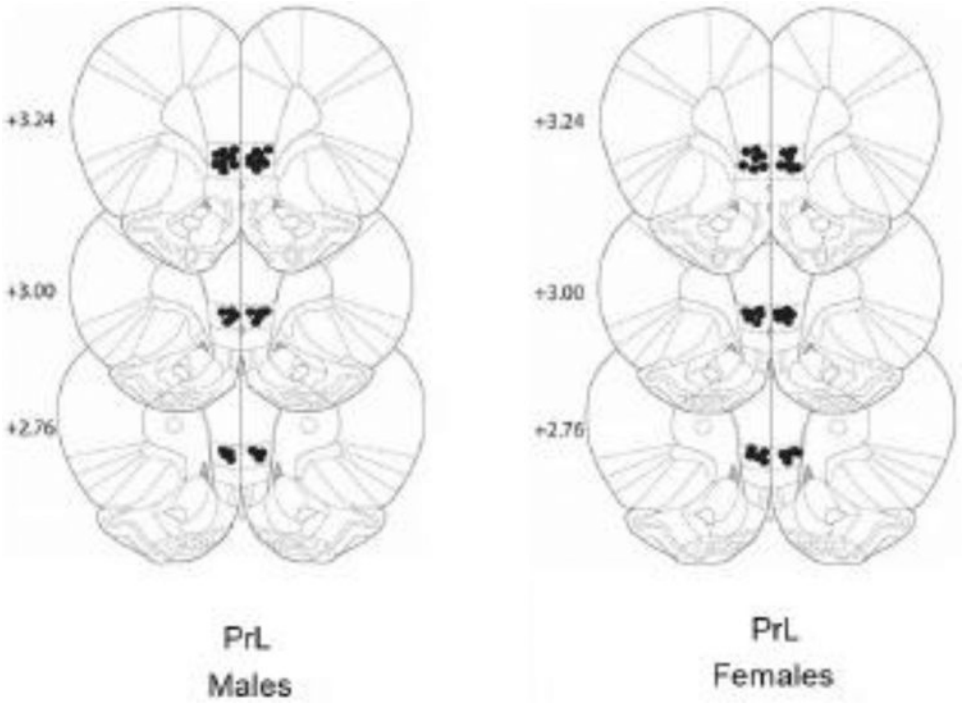

Fig. 14. Histology, PrL.

Histological verification of microinjection placements for male and female PrL infusions.