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Comorbid PTSD and binge drinking: a mouse model of individual differences in fear memory expression and ethanol consumption

Kate Lawson
Vassar College

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Post traumatic stress disorder (PTSD) and substance use disorders are common psychiatric diagnoses in the US and are often comorbid. However, relatively little work has used animal models to explore comorbid PTSD and alcohol use disorders (AUD). Here, we conduct multiple studies with mice that test whether voluntary binge-like ethanol (EtOH) consumption alters the expression of a previously established and extinguished aversive conditioned memory, or c-fos expression in the prelimbic cortex (PL), infralimbic cortex (IL), or central nucleus of the amygdala (CeA). These areas are associated with fear and drug seeking, extinction of fear and drug memories, and conditioned fear responses respectively. We analysed sex differences in EtOH drinking levels and whether individual differences in drinking were correlated with cued fear expression. Following behavioral quantification, we analysed c-fos expression for sex differences and differences between EtOH and control animals in addition to correlating individual differences in c-fos expression to individual differences in either volume of EtOH consumed or cued fear expression. We focused on individual differences because of the relevance to clinical models. However, not all people who binge drink will become addicted to alcohol, not all people who experience trauma will develop PTSD, and not all people who binge drink and experience trauma will develop both an AUD and PTSD. Finally, we looked into whether social isolation or exposure to fear conditioning would change patterns of EtOH consumption. We found sex differences in EtOH consumption. Additionally, animals that consumed higher levels of EtOH expressed more fear following extinction training. We predict a reduction in c-fos expression in all areas analysed in the EtOH group compared to the control group, and a negative correlation between c-fos expression and volume EtOH consumed in the IL and PL, with a positive correlation between c-fos expression and cued fear expression in the CeA. The findings of this mouse model teach us that binge-like EtOH consumption impairs the recall of an extinguished fear memory, keeping fear expression elevated to a stimulus that is no longer aversive. While in humans alcohol consumption may stem from self-medication of symptoms of PTSD, chronic binge drinking may reduce the efficacy of prolonged exposure, the leading treatment of PTSD. Retention of PTSD symptoms may lead to increasing EtOH consumption, worse treatment outcomes, and a continuing cycle of addiction.

Introduction

Post traumatic stress disorder (PTSD) is the fourth most common psychological disorder in the United States with a lifetime prevalence around 7% (Kessler, Berglund, & Demler, 2005), and is often comorbid with alcohol use disorders (AUD) (Kessler et al., 1997). However, frequent binge drinking episodes do not necessarily indicate an AUD, and many people who experience a traumatic event will not develop any symptoms of PTSD; combining frequent binge drinking with traumatic experience does not necessarily indicate comorbid AUD and PTSD (Esser et al., 2014; CDC, 2018). Individuals may be more or less resilient to developing an AUD or PTSD based on genetic and behavioral risk factors, in addition to socioeconomic risk factors such as income and education (Kendler et al., 2012; Keyes et al., 2012; Grant, Goldstein, & Saha, 2015; Sareen, 2014; Török et al., 2019). The study of individual difference in the resilience and susceptibility to PTSD and AUD is an important research focus with implications for clinical therapeutic treatments. Using a mouse model to explore the interactions between voluntary binge-like ethanol (EtOH) consumption and cued fear expression can elucidate the impact of chronic alcohol consumption on neuronal circuits underlying classical fear conditioning.

A PTSD diagnosis requires a person to have been exposed to a traumatic event, or experience repeated exposure to aversive details of the traumatic event. PTSD symptoms include intrusive thoughts, nightmares, flashbacks, and emotional distress after traumatic reminders, in addition to numbing of all trauma related stimuli, negative alteration in mood and cognition, and irritability, aggression, or destructive behavior, among others (APA, 2013). PTSD often co-occurs with other psychiatric disorders, and this co-occurrence is stronger among women than men (Kessler et al., 1997). Anxiety and affective disorders make up the largest proportion of lifetime co-occurring cases among women, while substance disorders, conduct disorder, and antisocial personality disorder is largest among men (Kessler et al., 1997). Currently, we are unsure why these psychiatric disorders occur so often together. The comorbidity may be due to a common causal pathway, one disorder may be causally secondary to the other, such as when alcohol is used to mask other psychiatric symptoms, or there may selection bias in clinical studies (Kessler et al., 1997). While PTSD is often caused by a single stressful life event, long-term, chronic stress can also induce changes to both brain and behavior. Chronic stress is associated with long-lasting changes in neurotransmitter systems implicated in the stress response, and has also been linked to a reduction in amygdala volume (Peltier et al, 2019).

The amygdala is linked to physiological and behavioral responses to fear, stress, and substance abuse, and PTSD is associated with both structural and functional changes to the amygdala (Suh & Ressler, 2018). Impaired amygdala functioning can inhibit fear extinction, making it harder to learn that a formerly aversive stimulus is no longer harmful. This neural circuitry involved in fear and anxiety is essential to the development of PTSD in humans, and is highly conserved throughout evolution (Flandreau & Toth, 2017). This conservation makes

animal models of chronic stress and fear expression more analogous to human development of PTSD.

Animal models of fear learning and extinction use acute or chronic stressors to induce PTSD-like symptoms. In order to induce PTSD-like symptoms, animal models include severe trauma over a relatively short duration. This trauma can be in the form of foot shock, tail suspension, exposure to predator odor, and more (Peltier et al., 2019). In reliable models of PTSD, the intensity of the trauma predicts the severity of the outcome and there are significant individual differences in outcomes (Flandreau & Toth, 2017). If the trauma is too severe, all animals will develop PTSD-like symptoms; this can conceal the discovery of individual differences and risk factors. Development of PTSD-like symptoms depends on the presence of various risk and protective factors in both animals and humans, which is why the same trauma affects different people in different ways (Török et al., 2019). Animal models have shown amygdala responsivity to be positively correlated to PTSD symptoms, while medial prefrontal cortex (mPFC) and hippocampus are structurally smaller and show hypo-functionality (Török et al., 2019). Within the mPFC are the infralimbic cortex (IL) and prelimbic cortex (PL). The PL is believed to maintain fear responses and drug seeking behavior, while the IL controls memory extinction and reduction of fear response (Peters, Kalivas, & Quirk, 2009; Török et al., 2019). The PL projects excitatory connections to the basal amygdala, while the IL sends excitatory connections preferentially to GABAergic, inhibitory, neurons in the basal lateral amygdala and central nucleus of the amygdala (CeA; Peters, Kalivas, & Quirk, 2009). These projections from the IL drive down activity in the CeA, a major site of fear memory storage, in response to extinction training.

In the United States, 30 million adults have an AUD and alcohol consumption is the third leading cause of preventable morbidity and mortality (Grant, Goldstein, & Saha, 2015; Peltier et al., 2019). In order to meet the criteria for the AUD diagnosis, a person must present at least 2 of the following symptoms within a 12 month period: hazardous use, social or interpersonal problems related to use, major life roles neglected due to use, withdrawal, tolerance, more or longer use than intended, repeated attempts to control use, large amounts of time spent using, physical or psychological problems related to use, activities given up in order to use, and craving (APA, 2013).

Genetic risk factors for AUD can predispose people to tolerance and heavy use in addition to withdrawal and continued use despite problems (Kendler et al., 2012). Genetics may also play a role in the many comorbidities associated with AUD, as a majority of people with an AUD have at least one other psychiatric disorder (Kessler et al., 1997). These include significant associations between 12-month and lifetime AUD and other substance use disorders, major depressive disorder, bipolar I disorder, specific phobia, antisocial and borderline personality disorders, persistent depressive disorder, PTSD, panic disorder, and generalized anxiety disorder (Grant, Goldstein, & Saha, 2015). Adverse health effects associated with binge drinking include unintentional injuries, alcohol poisoning, meningitis, and many others (Naimi et al., 2003).

Many consequences of binge drinking have especially high social and economic costs, including interpersonal violence, unintended pregnancy, and lost productivity (Naimi et al., 2003). In the United States, rates of AUD are higher among men than women (Grant, Goldstein, & Saha, 2015), which is not what we see in animal literature (Adams, 1995; Sneddon, White, & Radke, 2019, Yoneyama et al., 2008). Most studies in rodents, including the C57Bl/6N strain used in the current study, report higher EtOH consumption and higher blood ethanol concentrations (BEC) in female animals (Adams, 1995; Sneddon, White, & Radke, 2019, Yoneyama et al., 2008).

"Drinking in the dark" (DID) is a paradigm that allows animals to voluntarily consume binge-like levels of EtOH (Thiele, Crabbe, & Boehm, 2015; Thiele & Navarro, 2013; Rhodes et al., 2005). In most DID paradigms, animals are single-housed and provided free access to a 20% v/v EtOH solution for 2 hours starting 3 hours into their dark cycle. Mice are nocturnal, and this two hour period during the dark cycle is when the animals tend to consume the most calories (Thiele, Crabbe, & Boehm, 2015; Thiele & Navarro, 2013). One of the major downsides to this paradigm is that the animals need to be single-housed, which is inherently stressful for social animals and not analogous to humans who binge drink in social settings. To ameliorate this added stressor, we designed a novel social binge drinking paradigm ("Social drinking in the dark"; sDID) so animals could remain pair-housed and still voluntarily drink to pharmacologically significant binge-like levels.

During fear extinction and fear extinction recall, top-down control via the mPFC and hippocampus project to the amygdala to modulate previously conditioned fear responses (Suh & Ressler, 2018). Top-down effects on fear expression can be at this level of specific brain regions, but can also be at the level of cognition. Changes in cognition brought about by cognitive therapy are associated with changes in brain activity (Clark & Beck, 2010; Hofmann et al., 2013). This suggests a relationship not only between cognition and behavior, but also that conscious regulation of emotions can directly alter patterns of brain activity (Hofmann et al., 2013). Support for this idea of top-down regulation also comes from studies that show changes to the pattern of activity elicited by a familiar stimulus following exposure to a novel stimulus. Freeman (1991) reported this finding in the olfactory cortex of rabbits, where the pattern of EEG activation for the familiar smell of sawdust changed after the animal was exposed to the smell of a banana. The same stimulus, sawdust, presented before and after a novel stimulus, banana, may give rise to different patterns of activity or a difference conscious experience despite the stimulus itself not having changed (Freeman, 1991). The top-down effects of cognition on perception have been called into question in some cases (Firestone & Scholl, 2016). However, the complex and dynamic network of feedback between brain regions supports the idea that changes to cognition and attention can shift perception towards certain goals and away from others (Beck & Clevenger, 2018). This could be why many addictions rely on cognitive rehabilitation as treatment, instead of utilizing pharmacological treatments (Sofuoglu et al., 2013). Additionally,

prolonged exposure, a form of cognitive behavioral therapy, is one of the most commonly used treatments for PTSD (Powers et al., 2010).

In order to explore the relationship between cognition, behavior, and neuroscience, we began this series of studies with DID Fear in an attempt to replicate a previous experiment in the lab that used intraperitoneal (i.p.) injections of an EtOH solution instead of a voluntary paradigm. Using a similar behavioral paradigm, results from this experiment showed that mice administered EtOH following cued extinction training expressed a greater amount of freezing (fear) in the cued fear recall test (Scarlata et al., *in press*). Additionally, the EtOH group froze more to a novel tone in a generalisation test (Scarlata et al, *in press*). These data showed that chronic EtOH administration augments cued fear memory generalisation while impairing fear extinction retrieval. Here, we sought to replace i.p. injections with the single-housed DID paradigm to eliminate the stress of the injection and allow for us to see individual differences in levels of the animals' EtOH consumption. We moved from DID Fear into sDID Fear to reduce the stress of social isolation. From there, we tested the effects of stress on voluntary binge-like EtOH consumption by providing animals with open access to an EtOH solution without fear conditioning in sDIDSS. To follow up these studies, we will use adolescent mice to assess the developmental differences in acquisition and extinction of fear memories, and in voluntary binge-like EtOH consumption. Social drinking is important for studying neurodevelopmental questions because younger mice are differentially sensitive to stress as compared to older mice.

We developed a novel social binge-like drinking paradigm to highlight individual and sex differences in EtOH consumption between animals. We hypothesised that voluntary binge-like drinking would replicate the effects of EtOH on cued fear extinction and cued fear generalization that were observed previous studies (Scarlata et al., *in press*). Additionally, the voluntary drinking aspect of our experimental design allowed for an analysis of how individual differences in EtOH consumption impact fear memory expression. We hypothesised that the more EtOH an animal consumed, the more it would freeze in the cued recall test following extinction training. Because we expect to see neuroadaptations induced by EtOH, not general consummatory behavior, this correlation should only hold true for the animals in the EtOH group. Nonspecific patterns of consumption should not influence fear memory performance, so there should be no correlation between water consumption and fear expression. We also predicted a relationship between fear expression in fear conditioning and cued fear expression and binge-like levels of EtOH consumption, so that the more an animal froze in either fear conditioning or cued fear extinction, the more it would drink when provided with open access to EtOH. Finally, we hypothesised that the animals that consumed EtOH would show impairments in cued fear extinction compared to controls.

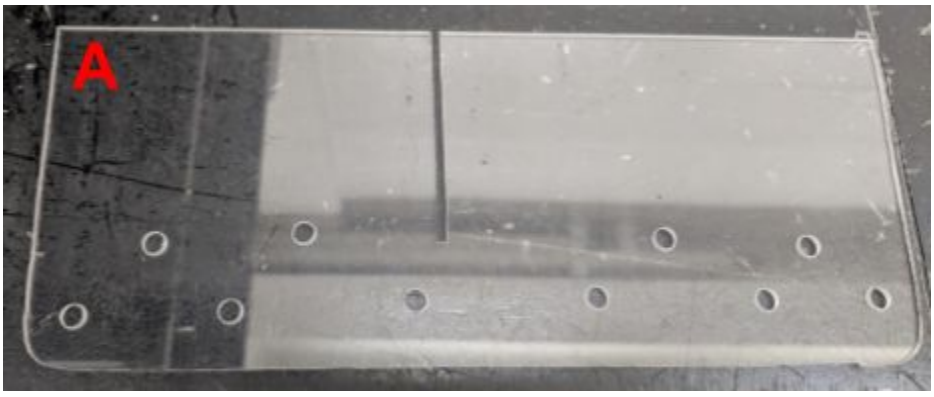
Following behavioral and EtOH consumption analysis, the expression of c-fos was mapped in the IL, PL, and CeA (both lateral and medial). C-fos is a commonly used activity marker, and here we used c-fos expression to quantify EtOH induced neuroadaptations following the cued fear recall test (Alberini, 2005; Dragunow & Faull, 1989; Strekalova et al., 2003). The

IL, PL, and CeA were chosen for analysis in order to replicate previous findings that reported reductions in expression of Arc, another activity marker, in these areas. The IL and PL both exert top-down effects on the CeA, modulating its activity through excitatory projections (Peters, Kalivas, & Quirk, 2009). We hypothesised that we would find less c-fos expression in all regions in the EtOH group, and a negative correlation between c-fos expression and volume EtOH consumed in the IL and PL, with a positive correlation between c-fos expression and cued fear expression in the CeA.

Methods

Animals - Adult (71-184 days old) male and female C57BL/6N (B6) mice were used in all experiments. Mice derived from a common stock (Charles River Laboratory, Kingston, NY) and were bred at Vassar College over multiple generations. Mice were group-housed (at least 2 per cage) in standard cages (minimal enrichment) in a temperature (21.1 °C), humidity (65%), and light/dark cycle (lights on 0600h EST) controlled vivarium. Food and water were available *ad libitum* and cages were cleaned twice a week. All experimental procedures were conducted in accordance with the National Institutes of Health guidelines on the Care and Use of Animals in Research and received prior approval by the Vassar College Institutional Use and Animal Care Committee (protocol 16-03P, approved with a continuation in 2018)

Housing - Mice in sDID Fear and sDIDSS were transported from their home cages into 'bar' cages on five consecutive days during the dark cycle, and remained in the bar cage for 2 hours. Bar cages were constructed from standard cage bottoms and bedding with a plexiglass divider and modified wire cage top (Figure1). The plexiglass dividers separated one half of the cage from the other so that two animals could be housed together but retain access to separate food and water bottles. The dividers were perforated to permit olfactory, auditory, visual and potentially tactile interactions between cagemates. The wire cage top was fitted with a second opening that could accommodate 130 mL sipper tube style bottles (Petco Animal Supplies, San Diego, CA).



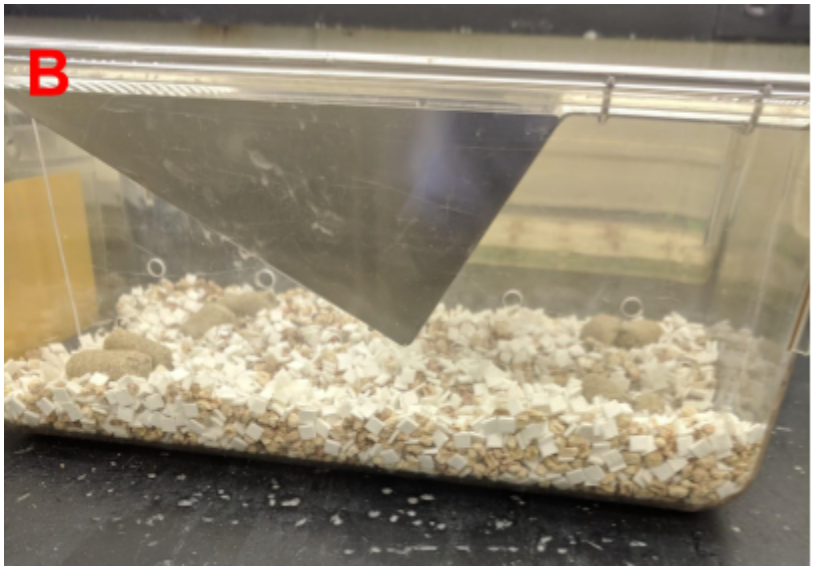


Figure 1. (A) Plexiglass divider that separated animals during the 2 hours of open access to EtOH in the sDID protocol. (B) Cage with divider and wire cage top in place. Food pellets were distributed into the bedding so that mice still had *ad libitum* access to food during sDID.

Fear Conditioning - All experiments were conducted during the light cycle. Home cages of mice were carried from the colony room to a dimly lit room and allowed to habituate for 30 minutes. Fear conditioning was conducted in unaltered commercial chambers (20 x 30 x 18 cm; Context A) located within sound-attenuating cabinets (58 x 61 x 45 cm) using Graphic State software for controlling and delivering the tone and shock stimuli (Coulbourn Instruments, Holliston, MA). Prior to all training and testing, the decibel level for the auditory tone frequency was measured in each chamber using a sound level meter (R8050, REED Instruments, Wilmington, NC) and calibrated to 75 dB. Mice were placed in the fear conditioning chamber and habituated for 180 seconds prior to three pairings of a 20 sec, 5-kHz, 75 dB auditory tone conditioned stimulus (CS) that co-terminated with an electric foot shock as an unconditioned stimulus (US; 0.5 sec, 0.6 mA). The CS/US pairings were separated by a variable inter-trial intervals (ITI; 20, 80, and 60 seconds). Mice were removed from the chamber 60 seconds after the final CS/US pairing. The total training time was 6 minutes and 40 seconds. The chambers were thoroughly cleaned with a 70% EtOH solution between mice. Detailed protocols for all experimental procedures can be found in the OSF repository at https://osf.io/unfb9/

Contextual Cues - In order to isolate cued fear learning from contextual fear learning, mice were fear conditioned in Context A (Figure 2A) and tested for recall of that memory in Context B (Figure 2B). Following fear conditioning, animals were exposed to Context A in order to reduce contextual fear. Mice were then exposed to Context B to reduce freezing to a novel context. In Context A, every home cage was carried from the colony room to a dimly lit habituation room neighboring the conditioning room. Mice were transported from the habituation room into the brightly lit conditioning room in a clean cage. The conditioning

chambers had clear walls and metal shock bars as the floor. In between animals, the chambers were cleaned with a 70% EtOH solution. In Context B, the animals' home cages were carted from the colony room into a brightly lit habituation room neighboring the conditioning room. Mice were transported from the habituation room into the conditioning room in a cage with a thin layer of clean bedding. A small fan was always running in Context B to provide background noise. The conditioning chambers in Context B consisted of Context A masked visually, tactilely, and olfactorily. The shock bars were replaced with white plexiglass and a thin layer of clean bedding, the walls of the testing chamber were covered with black and white striping, and the testing chambers were cleaned between mice using a 1% acetic acid solution instead of the 70% EtOH used in Context A. To amplify this olfactory cue, a paper towel sprayed with the 1% acetic acid solution was included inside the cabinet that housed the testing chamber.

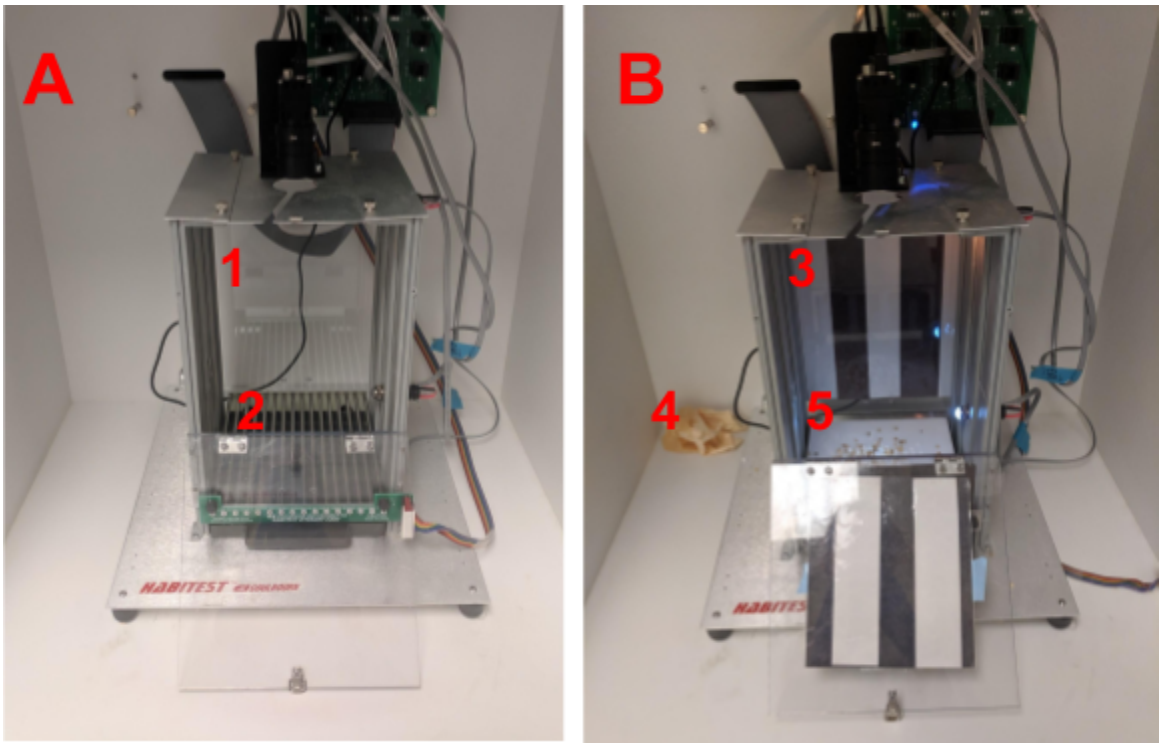


Figure 2. Conditioning chamber setup for (A) Context A and (B) Context B. In Context A, the walls of the chamber are clear (1), the floor is metal shock bars (2), and the chamber is cleaned with 70% EtOH between animals. In Context B, the walls of the chamber are black and white stripes (3), the chamber is cleaned with 1% acetic acid (4), and the floor is white plexiglass with a thin layer of clean bedding (5).

Context Extinction - To extinguish contextual fear to Context A, animals' home cages were carried from the colony room and habituated in the same dimly lit room for 30 minutes before mice were placed back into Context A for 24 minutes and 45 seconds. During this time, no tones or shocks were presented.

Context Habituation - To habituate animals to Context B and reduce baseline freezing to a novel context, animals' home cages were transported from the colony room using a cart and were allowed to habituate for 30 minutes in a brightly lit room before being placed into Context B for 24 minutes and 45 seconds. During the habituation, no tones or shocks were presented.

Cued Extinction - Animals' home cages were transported using a cart from the colony room to the same brightly lit room and allowed to habituate for 30 minutes. The testing room was set up in accordance with Context B. After habituation, animals were placed into the chamber and following a 180 second habituation, mice were presented with either 25 or 50 temporally massed (5s ITI) CS presentations (20 sec, 5-kHz, 75 dB) (Cain et al., 2003). The total training time for the 25 CS presentation was 14 minutes and 20 seconds and the total time for 50 CS presentation was 24 minutes and 45 seconds. No shocks were presented during this time.

"Drinking in the dark" - Mice were weighed and tail-marked every day in the evening. A 20% v/v solution of EtOH was prepared every night of the five night paradigm by diluting 95% EtOH stock solution (Pharmco Aaper, Brookfield, CT) with tap water. Sipper tube style bottles were filled to the top with either the EtOH solution or water in order to reduce leakage from insufficient vacuum seals. Bottles were transported into the colony room on a cart at 2100 EST, 3 hours into the dark cycle. Experimenters wore a red light headlamp and kept white light to a minimum in order to disturb the animals as little as possible. In the single housed paradigm, the animals' home cages were placed on the countertop in the colony room and their water bottles were removed. Replacement bottles were weighed and flipped over before being placed into the cage so that the sipper tube rested at a 90° angle to the cage bars. During this process, the experimenter monitored for drips. One cage, called a 'dummy' cage, was constructed to be identical to all other cages, but there was no animal inside. The dummy served to quantify how much liquid was lost when the bottles were flipped over and when the cages with the bottles were transported. Animals had open access to the EtOH solution for 2 hours. At 2300 EST the experimenter returned to the colony room and removed the replacement bottles. Replacement bottles were weighed and the original water bottles were returned to the cages from which they were removed. Replacement bottles were washed in the wet lab to limit noise and time spent in the colony room during the dark cycle.

When animals were pair housed, a "social drinking-in-the-dark" paradigm (sDID) was employed. Just as in the single-housed paradigm, sipper tube bottles were filled to the top with a 20% v/v solution of EtOH. At 2100 EST bottles were transported into the colony room. A home cage was placed on the countertop next to its associated bar cage. One mouse was placed on one side of the divider and the other mouse was placed on the opposite side (Figure 3A). After both animals were inside the cage, both replacement bottles (either both water or both EtOH) were weighed, flipped over, and placed into the cage so that the sipper tube rested at a 90° angle to the cage bars (Figure 3B). The bar cage was replaced on the shelf next to the home cage and was undisturbed until 2300 EST (Figure 3C). At 2300 EST both the bar cage and the home cage

were placed on the counter. Both bottles were removed and weighed before the animals were placed back into the home cage. On the first, third, and fifth days of the protocol, mice with one tail-marked were placed on the left side of the cage. On the second and fourth days of the procol, mice with two tail-marks were placed on the left side of the cage. This alternation was performed in order to avoid a side preference. Regardless of which side of the cage an animal was placed on, every animal had the same bottle for all five nights.

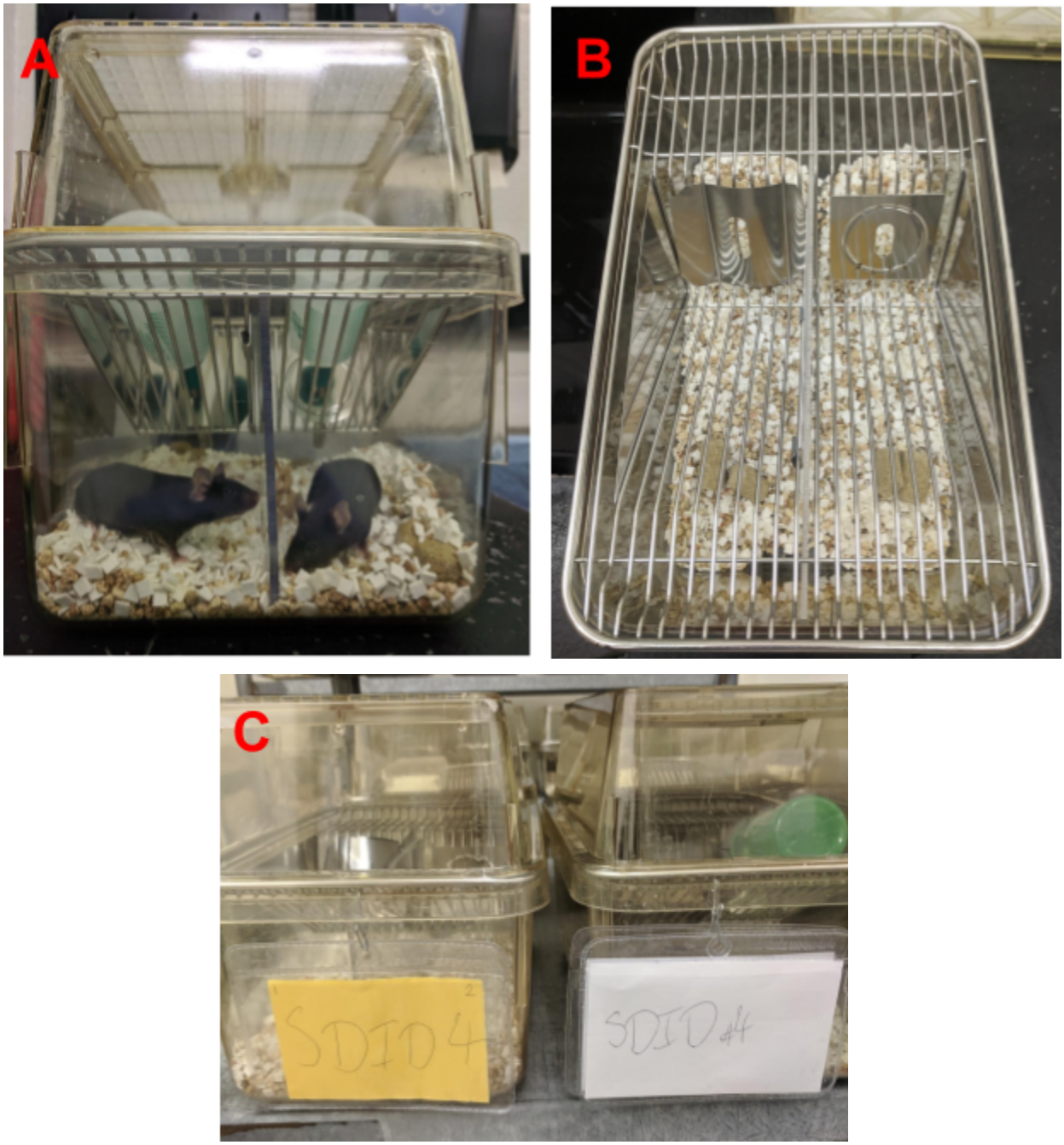


Figure 3. (A) Front view of the modified cages with animals and bottles in place (B) Top view of the modified cages. One animal from the home cage was placed on one side of the divider,

and the other mouse was placed on the other side. Then replacement bottles were weighed and placed into the cage top. (C) Bar and home cages on the shelf of the colony room. The bar cage was right next to the home cage so that the appearance of the colony room would be minimally changed when the animals were moved from the home cage into the bar cage.

Cued Recall - Following 3 drug-free days where the animals underwent no experimental manipulations, the animals' fear memory was tested. The conditioning chambers were set up in accordance with Context B and animals' home cages were transported from the colony room using a cart and habituated for 30 minutes in a brightly lit room. Once placed into the chambers, mice habituated for 180 seconds before the CS was replayed (20 sec, 5-kHz, 75 dB) three times separated by a variable ITI (80, 20, and 60 seconds). The total training time was 6 minutes and 40 seconds. During this time, no shocks were presented.

c-fos Immunohistochemistry - Exactly 90 minutes after the cued recall test, mice were administered a ketamine/xylazine cocktail solution intraperitoneally (100:10 mg/mL) and transcardially perfused with ice cold 1X PBS, followed by ice cold 4% paraformaldehyde in 1X PBS (Schafe, 2011; Ploski et al., 2008). Brains were extracted and stored in 4% PFA overnight then transferred to 1X PBS and stored at 4°C until vibratome sectioning. In DID Fear, brains were mistakenly left in PFA for 20 days before being transferred to 1XPBS. Brains were sectioned coronally (40 μm thick) on a vibratome (VT1200, Leica Biosystems Inc., Buffalo Grove, IL). Every fourth section was collected in a well plate (no more than 12 sections/well) in 1X PBS for immunohistochemistry. Sections were rinsed in 1X PBS three times, before incubation in a 1X PBS/1% bovine serum albumin (BSA)/ 0.2% Triton-X solution for 30 min to reduce non-specific binding. Sections were rinsed three more times then incubated overnight in c-fos 1:500 primary rabbit polyclonal antibody (EnCor Biotechnology Inc., Gainesville, FL) at room temperature. The following day, sections were rinsed in 1X PBS five more times before a 1-hour incubation in Goat anti-Rabbit IgG Secondary Anti-body, Alexa Fluor 488 (1:250) (ThermoFisher Scientific, Waltham, MA) at room temperature. Sections were rinsed three more times in 1X PBS and mounted onto gel-coated slides. When slides were dry they were coverslipped with fluromount then allowed to dry again in a covered slidebox.

c-fos expression analysis - For all neuronal counting, the experimenter was blind to experimental group and sex of the sample. Cell counts were conducted in six separate counting frames per region of interest (ROI) in each mouse. All counting was conducted using fluorescent microscopy and a 250 X 250 μm counting frame. Each ROI was first identified in reference to anatomical landmarks under a 4X objective lens using a reference atlas (Allen Institute for Brain Science, 2011), then photographed under a 20X objective lens using SPOT software (version 5.2, Sterling Heights, MI). Cell counting was conducted in the CeA and both shallow and deep layers of the IL and PL. In the PL and IL, the main anatomical landmark used was the corpus callosum, while identification of the CeA relied on the external capsule and amygdalar capsule (Figure 4).

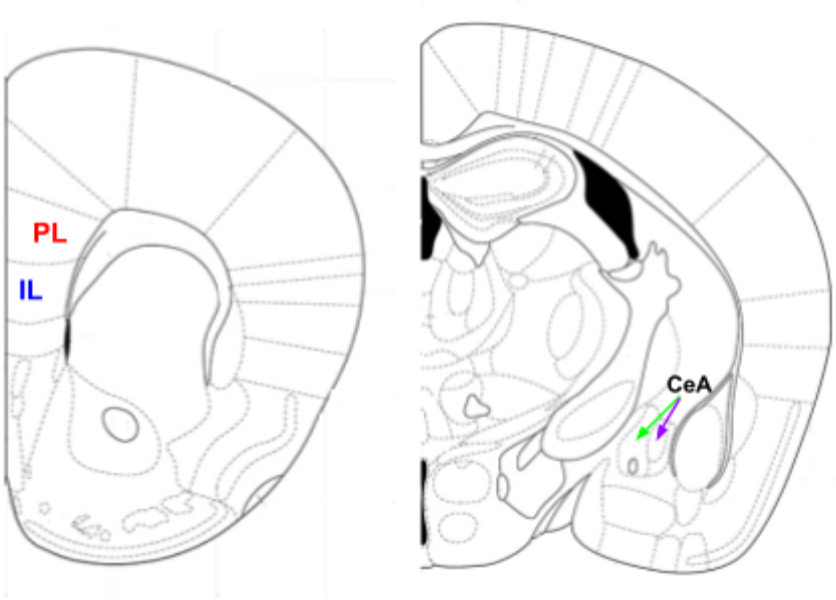


Figure 4. (A) Atlas image with PL labeled in red and IL in blue at bregma 1.54. (B) Atlas image with medial CeA labeled in green and later CeA labeled in purple at bregma -1.22. Both images are right, transverse sections adapted from Paxinos and Franklin (2012).

Behavioral quantification - A camera positioned over the fear conditioning chambers recorded digital video. The video was analyzed offline using a video tracking system that quantified immobility (SMART v3.0, Panlab, Harvard Apparatus, Barcelona, Spain). For every animal, a blind experimenter ensured that the software was correctly marking bouts of immobility. Freezing was defined as bouts of immobility lasting longer than 1 second. Freezing was used as a behavioral measure of a conditioned fear response. The freezing duration during the 180 second habituation phase and during the CSs or ITIs were averaged and converted into a percentage of time spent immobile in habituation, every CS, and every ITI.

Statistical Analyses

Behavior - For all analyses, the independent variable was treatment (EtOH, no EtOH control), sex (F, M), or experiment (levels: DID Fear, sDID Fear, and sDIDSS). The dependent variable was mean percent freezing for habituation or CSs. Mixed model analysis of variance (ANOVA) was used to analyze how percent freezing changes over time (within-group factor), and between treatment groups, experiments, and sex (between-group factors). Follow-up comparisons of significant findings were conducted using t-tests. All statistical analyses were conducted in R notebooks (RStudio, Boston, MA) and graphing was done in Origin (OriginLab, Northampton, MA).

EtOH consumption - Bottle weights were recorded immediately before being placed into cages and again immediately upon removal. The final weight was subtracted from the initial

weight for all bottles, including the dummy bottle. Then, the change in the dummy bottle weight was subtracted from the change in all other bottles. This eliminated the weight lost from flipping the bottles over and from transporting cages, and left only weight lost due to activity of the animals. However, while 1 gram of water equates to a 1 mL volume, this does not hold for EtOH. The weight to volume ratio depends on the concentration of EtOH in the solution, so weight of EtOH (0.789g/mL) and the concentration of the solution (20%) can be used to calculate the weight in relation to water. Thus to quantify the weight of EtOH lost, the change in bottle weight was divided by 0.958 ($\frac{(0.7893\times20)+80}{100}$). Every animal's weight was recorded during the light cycle, and here the weight was divided by 1000 to determine the weight in kilograms. The final self administered value also took into account the specific gravity of EtOH, and was calculated as $\frac{0.789\times0.20\times(\textit{change in bottle weight}\times0.958)}{\textit{body weight in kg}}$ (Homes lab, 2019).

Pattern analyses of c-fos expression - A multivariate ANOVA was used to analyse the relationship between c-fos expression in the IL, PL, and CeA and experimental group and sex. Significant findings were followed up with Bonferroni corrected t-tests. Correlations were also used to determine the relationship between c-fos expression and both fear expression and volume of EtOH consumed in the DID paradigm.

Procedures

Experiment 1: "DID Fear"

One week prior to beginning the experimental procedures, animals were single-housed and given the sipper style bottle as a water bottle. After this week of habituation, animals were subjected to a 13 day experimental paradigm. On Day 1, animals were fear conditioned according to the protocol outlined above. Day 2 and Day 3 were context extinction in Context A and context habituation in Context B respectively. Day 4 consisted of the temporally massed 25 CS administration in cued fear extinction. Day 5-9 were the DID paradigm, where animals were provided open access to a 20% v/v EtOH solution during the natural dark cycle for 2 hours a night for 5 nights. Following three days with no experimental manipulation, mice were placed back into Context B for cued recall on Day 13 (Figure 5). Ninety minutes after completing the cued recall protocol, animals were perfused and their brains were taken. Brains were mistakenly left in 4% PFA for 3 weeks instead of 24 hours before being placed into 1XPBS. Four brains were sliced and c-fos immunohistochemistry was run to ensure that the brains were not over-fixed. Following these test immunos, 10 more brains were sliced and c-fos immunohistochemistry was run. Ethanol consumption data was analysed to find possible sex differences or a correlation between age and volume of EtOH consumed. Behavioral data was analysed to assess differences in fear expression between EtOH and control groups, and a correlation between amount of EtOH consumed and fear expression in either cued extinction or cued recall. A median split analysis was run on behavioral data from the cued recall test and all animals were classified as either high drinkers, low drinkers, or control. Following completion

of neuronal counting, the c-fos data will be analysed to assess differences in c-fos expression between EtOH and control groups. Additionally, correlations will be performed to find a relationship between activity in the IL and PL and EtOH consumption, and activity in the CeA and fear expression.

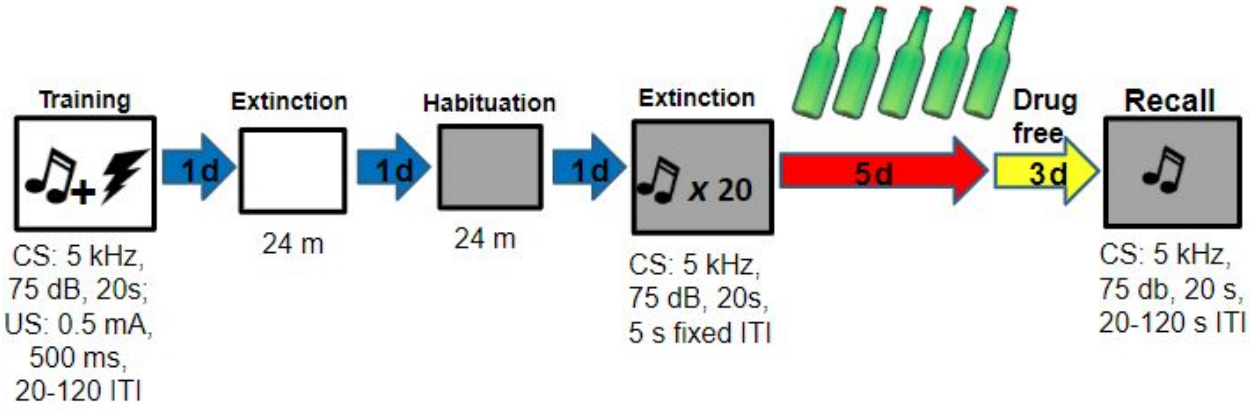


Figure 5. Experimental paradigm of DID Fear. On consecutive days, mice underwent fear conditioning, Context A extinction, Context B habituation, and cued fear extinction. Then animals were given open access to a 20% v/v EtOH solution for 2 hours a night for 5 nights in the DID paradigm. DID was followed by 3 drug-free days and finally the cued fear recall test. After the recall test, animals were sacrificed and their brains were taken for c-fos immunohistochemistry.

Experiment 2: "sDID Fear"

Three weeks prior to beginning the experimental procedures, animals were pair housed and given the sipper style bottle as a water bottle. After this period of habituation, animals were subjected to a 13 day experimental paradigm. On Day 1, animals were fear conditioned according to the protocol outlined above. Day 2 and Day 3 were context extinction in Context A and context habituation in Context B respectively. Day 4 consisted of a temporally massed 50 CS administration in cued fear extinction. Days 5-9 were the social drinking in the dark paradigm (sDID). In preparation, 10 cages were constructed with plastic dividers and a cage top that was modified to accommodate two sipper tube style bottles. These "bar" cages were assigned to one home cage, so that every night the same pair of animals was placed into the same bar cage. Additionally, each sipper bottle was assigned to one animal, so the same bottle would be given to the same animal every night. Mice were placed on alternating sides of the bar cage on subsequent nights to account for side preferences. Following three days with no experimental manipulation, mice were placed back into Context B for cued recall on Day 13 (Figure 6). Ninety minutes after completing the cued recall protocol, animals were transcardially perfused and their brains were taken, though no c-fos immunohistochemistry was performed on these brains. Ethanol consumption data was compared to data from DID Fear to quantify whether animals consumed more EtOH when housed with a cagemate than while alone. As before, EtOH

consumption data were analysed to find possible sex differences or a correlation between age and amount consumed. Behavioral data were analysed to assess differences in fear expression between EtOH and control groups, and a correlation between amount of EtOH consumed and fear expression in either cued extinction or cued recall.

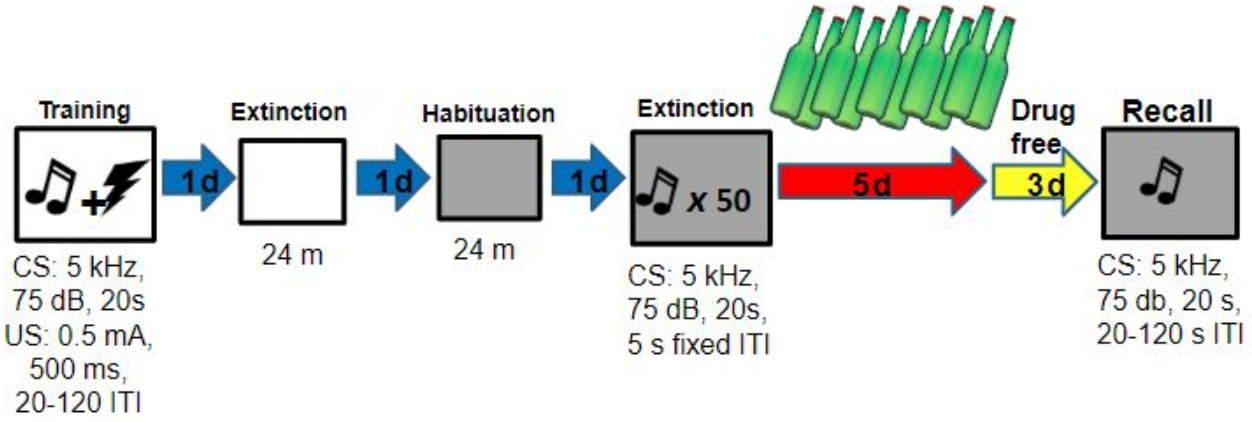


Figure 6. Experimental paradigm for sDID Fear. The key difference between sDID Fear and DID Fear is that animals were pair-housed throughout and were permitted olfactory, auditory, visual and potentially tactile interactions with their cagemate during the two hour sDID protocol.

Experiment 3: "sDIDSS"

One week prior to beginning the experimental procedures, animals were pair-housed and given the sipper style bottle as a water bottle. After this week of habituation, animals were subjected to a 13 day experimental paradigm. On Day 1, animals were placed into Context A and exposed to the fear conditioning protocol while the shock bar was unplugged. Day 2 and Day 3 were context extinction in Context A and context habituation in Context B respectively. Day 4 consisted of the temporally massed 50 CS administration that mimicked cued fear extinction. Day 5-9 were the sDID paradigm, where animals were provided open access to a 20% v/v EtOH solution for 2 hours a night for 5 nights in a bar cage along with their home cagemate. Following three days with no experimental manipulation, mice were placed back into Context B for cued recall on Day 13 (Figure 7). Ninety minutes after completing the cued recall protocol, animals were transcardially perfused and their brains were taken, though no IHC was performed on these brains. Ethanol consumption data was compared to data from sDID Fear to quantify whether animals consumed more EtOH after receiving shocks during fear conditioning. As before, EtOH consumption data was analysed to find possible sex differences or a correlation between age and amount consumed. No behavioral data was analysed, because the absence of the US in fear conditioning meant that these animals did not encode a fear memory that we could test.

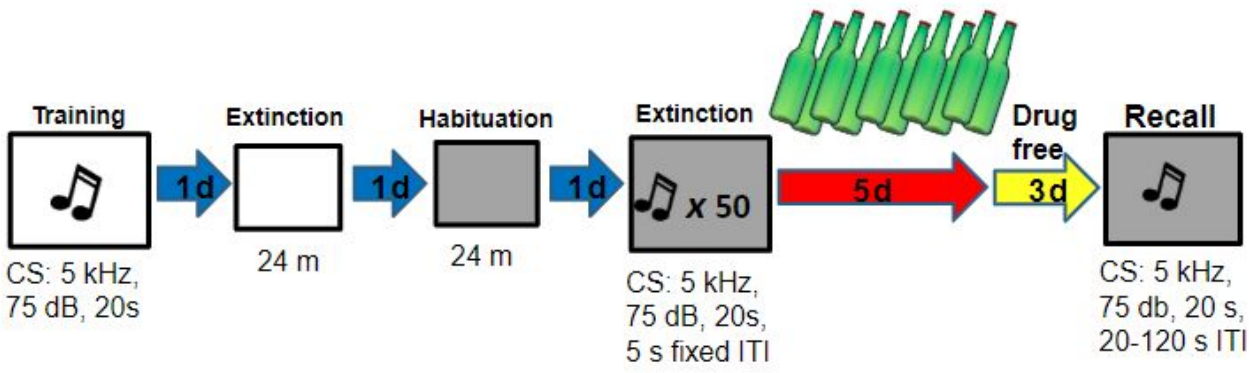


Figure 7. Experimental paradigm for sDIDSS. The only differences between sDIDSS and sDID is that animals were not exposed to the US during fear conditioning and thus did not learn to associate the tone with a shock.

Exploratory experimentation - Following this series of experiments, we modified the protocol to include a 45 minute habituation to the bar cages preceding EtOH access during the 5 days of sDID (Szumlinski et al, 2019). For this habituation, animals were placed into the bar cage at 2015 EST and were provided with new water bottles in both sides of the cage top. At 2100 EST, the water bottles were weighed and returned to the cage for the water group, or replaced with EtOH bottles for the EtOH group. Mice in this exploratory experiment underwent the same 13 day paradigm that was used in sDID Fear. Protocols, analysis scripts, and data from this experiment will be available on the OSF repository at https://osf.io/495cj/ after the cohort completes the cued fear recall test on 5/5/19.

Results

Experiment 1: "DID Fear"

On consecutive days following fear conditioning, mice (n=34, 17 female) were placed into Context A and B for 25 minutes. The following day, mice were placed into Context B and presented with the CS 25 times in a temporally massed (5 s ITI) fashion (Cain et al., 2003). They received 5 days of the DID paradigm (n=20 EtOH) followed by 3 days drug-free before being replayed the CS in the cued recall test. One animal was excluded (male, EtOH) from all analyses due to a leaky bottle. We found that all animals acquired the fear memory as shown by enhanced freezing during cue presentation ($F_{2,60}$=95.5, p=2.27e-19; Figure 8). There were no statistically significant sex differences and no baseline differences between the EtOH and control groups.

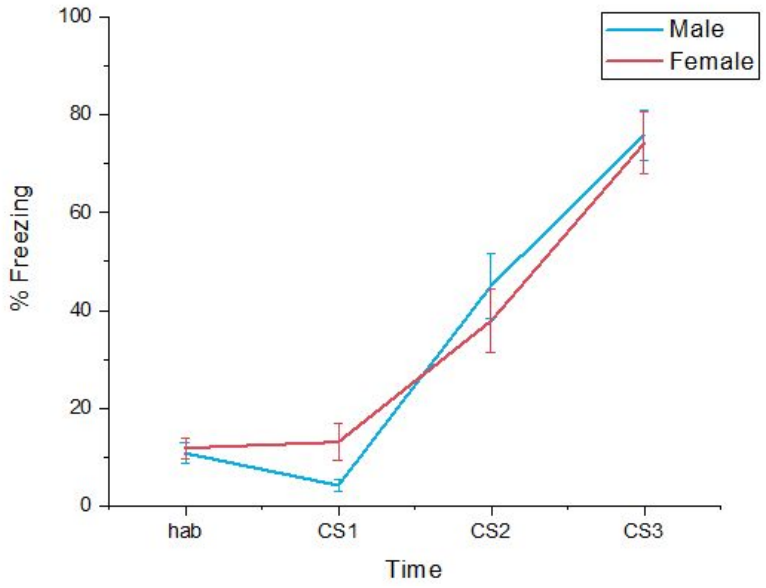


Figure 8. Fear conditioning in DID Fear (n=33). There were no statistically significant sex differences, nor differences between experimental conditions ($p>0.05$). There was an increase in freezing over the CS presentations ($F_{2,60}=95.5$, $p=2.27e-19$). Data presented as mean ± SEM.

Following fear conditioning, Context A extinction, and Context B habituation, animals were exposed to a temporally massed presentation of 25 CS. All animals extinguished the fear memory during cued extinction as shown by reduced freezing over time ($F_{24,720}=5.03$, $p=1.45e-13$; Figure 9). There were no statistically significant sex differences and again no baseline differences between the experimental conditions.

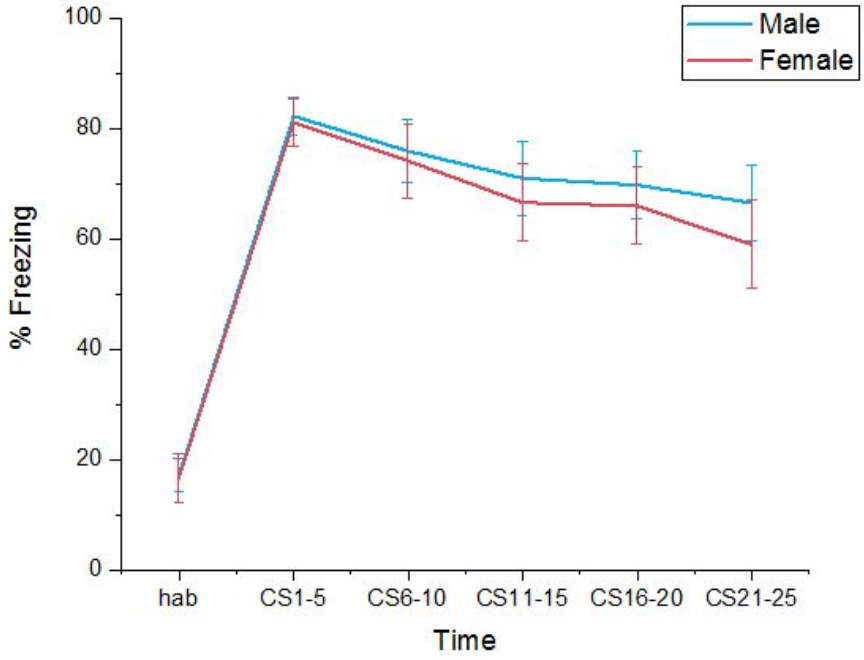


Figure 9. Cued extinction in DID Fear (n=33). No statistically significant effect of sex or group was detected ($p>0.05$). There was a decrease in freezing as the CS was presented ($F_{24,720}=5.03$, $p=1.45e-13$). Data presented as mean ± SEM.

Following cued extinction, mice began the DID paradigm in which they had open access to a 20% v/v EtOH solution for 2 hours a night for 5 consecutive nights. Day 1 was excluded from statistical analyses due to problems with the scale. There was no statistically significant main effect of sex or day (male average over 5 days =1.49, female average over 5 days =1.80); but a statistically significant interaction of sex X day ($F_{3,51}=2.98$, $p=0.0395$; Figure 10) was detected.

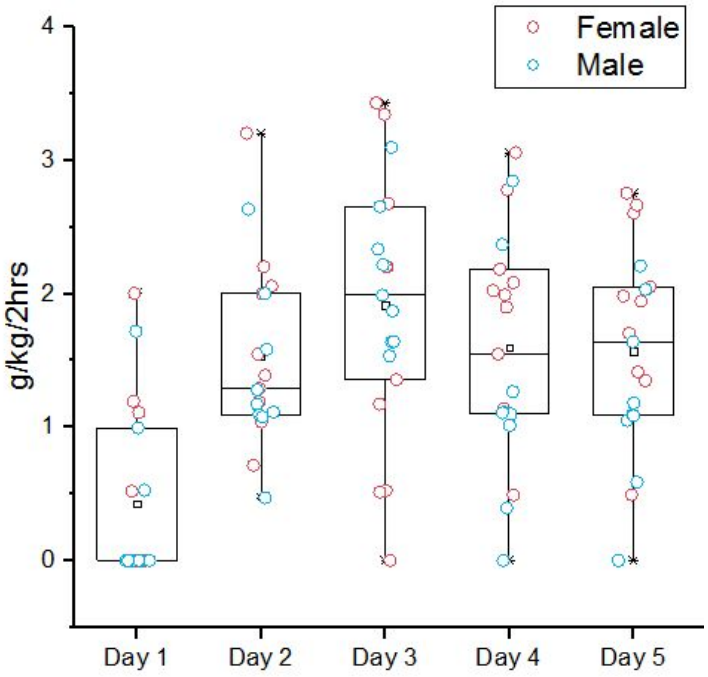


Figure 10. Ethanol consumed in DID Fear (n=19). Day 1 was excluded from analysis due to issues with the scale in cohort 1. There were no sex differences ($F_{1,17}=0.245$, $p=0.245$) nor differences over the 4 days included in analysis ($F_{3,54}=1.384$, $p=0.257$). The box displays values within one interquartile range (IQR) around the mean (small square) with the median shown as a line through the box. Whiskers display values 1.5 IQR from the mean.

After completing the DID paradigm, mice were undisturbed for 3 drug-free days. Then they were presented with 3 CS with a variable ITI in the cued recall test. During the recall test, mice in the EtOH group did not show distinct freezing levels from mice in the control group. There were neither statistically significant sex differences, nor changes in freezing over the course of the CS presentations (Figure 11).

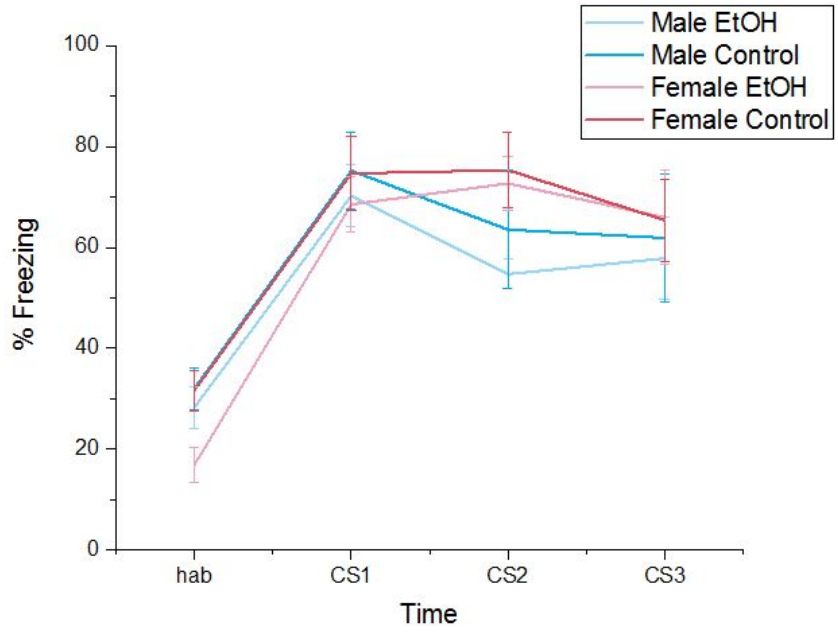


Figure 11. Cued recall test in DID Fear (n=33). No effect of sex ($F_{1,32}$=0.652, p=0.4250), group ($F_{1,32}$=0.195, p=0.661), nor changes over time ($F_{2,66}$=2.862, p=0.0642). Data presented as mean ± SEM.

Within the freezing data from the cued recall test, a median split (median = 1.557 g/kg/2hr; Figure 12A) revealed higher freezing among mice that consumed more EtOH. No main effect of drinking level was observed but an effect of time ($F_{2,60}$=3.39, p=0.0404) and an interaction of drinking level X time ($F_{4,60}$=3.90, p=0.00701) emerged. Post hoc tests revealed higher freezing among animals that consumed more EtOH at CS3 (p<0.05; Figure 12B).

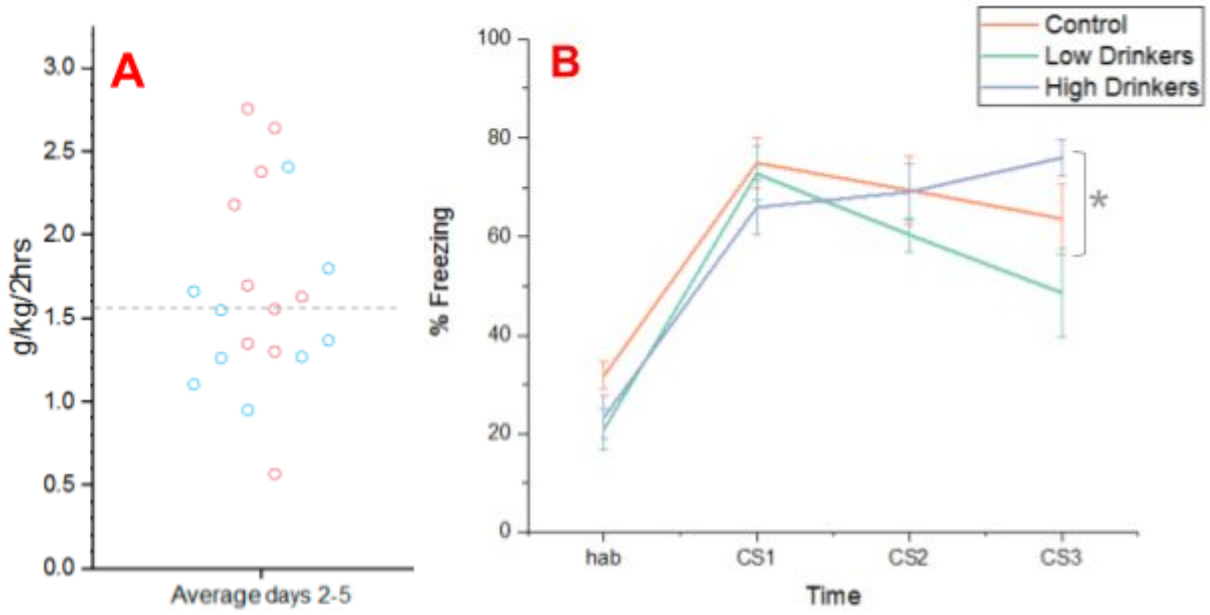


Figure 12. Separation of high and low drinkers in DID Fear. (A) Average EtOH consumption on days 2-5 of DID with the median split shown in gray (n=19). Animals above the gray line were considered high drinkers, while animals below the line were labeled low drinkers. (B) Percent freezing in the cued recall test analysed by a median split separating the group into high and low EtOH drinkers (n=33). High drinkers froze more as the test progressed ($F_{4,60}$=3.90, p=0.00701) but there was no main effect of drinking level on freezing. Data presented as mean ± SEM.

In the EtOH group, EtOH consumed correlated with freezing in CS3 of the cued recall test (r=0.538, p=0.0176; Figure 13). There was no statistically significant correlation in the control group (r=0.06).

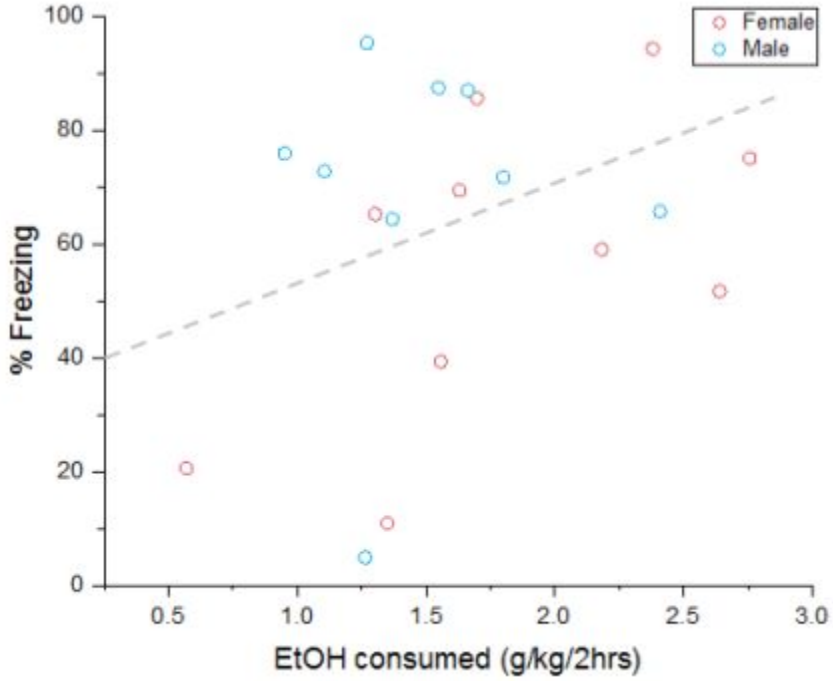


Figure 13. Fear expression and EtOH consumption. Positive correlation between average EtOH consumed in days 2-5 of DID and percent freezing in CS3 of recall (n=19; r=0.538, p=0.0176). Best fit line shown in gray.

Exactly 90 minutes after the cued fear recall test, mice were transcardially perfused with 4% PFA and brains were extracted. Later, brains were sliced to 40 microns on a vibratome and c-fos immunohistochemistry was performed. Slices were then mounted on gel coated slides and c-fos expressing cells were quantified in the IL, PL, and CeA using fluorescent microscopy. For every animal, six samples were collected bilaterally for every ROI. In order to test whether EtOH consumption led to a reduction in c-fos expression in all ROI and as an exploratory analysis to examine sex differences, we used a multivariate ANOVA to find differences based on sex or experimental group. We predict to find fewer c-fos expressing cells in the EtOH group in

the PL, IL, and CeA based on previous studies in the lab (Scarlata et al., *in press*). Also, we predict that there will be a negative correlation between c-fos expression and volume EtOH consumed in the IL and PL, with a positive correlation between c-fos expression and cued fear expression in the CeA. Data from the c-fos expression will be available on the OSF repository at https://osf.io/495cj/ after 5/9/19.

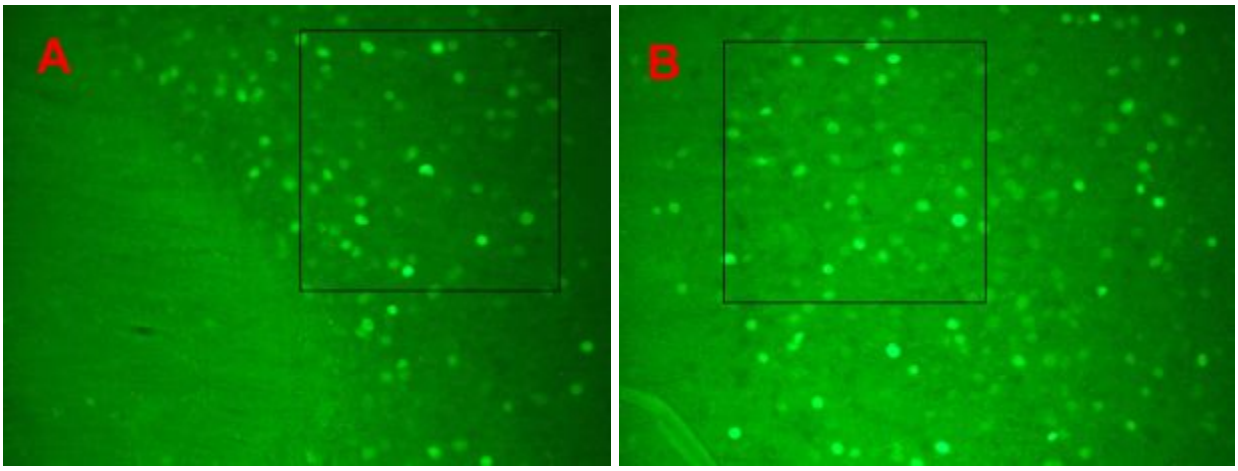


Figure 14. Sample c-fos expression in the IL (A) A 250 x 250 µm counting frame superimposed upon a 20X magnified image of the deep IL in the left hemisphere. Within this counting frame, 9 cells were counted. (B) A 250 x 250 µm counting frame superimposed upon a 20X magnified image of the shallow IL in the right hemisphere. Within this counting frame, 10 cells were counted.

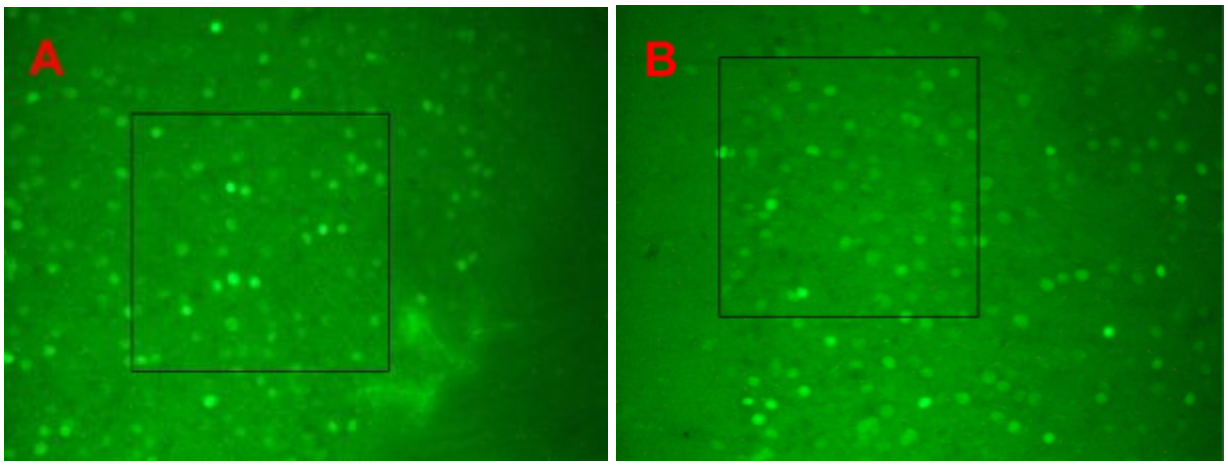


Figure 15. Sample c-fos expression in the PL (A) A 250 x 250 µm counting frame superimposed upon a 20X magnified image of the deep PL in the right hemisphere. Within this counting frame, 6 cells were counted. (B) A 250 x 250 µm counting frame superimposed upon a 20X magnified image of the shallow PL in the right hemisphere. Within this counting frame, 4 cells were counted.

Experiment 2: “sDID Fear”

On consecutive days following fear conditioning, mice (n=20, 10 female) were placed into Context A and B for 24 minutes. The following day, mice were placed into context B and presented with the CS 50 times in a temporally massed (5 s ITI) fashion (Cain et al., 2003). They received 5 days of the sDID paradigm (10 EtOH) and 3 days drug-free before being replayed the CS in the cued recall test. We found that all animals acquired the fear memory during fear conditioning as evidenced by increased freezing as the test progressed ($F_{2,38}$=52.5, p=1.17e-11; Figure 16). There were no statistically significant sex differences and no baseline differences between the experimental groups.

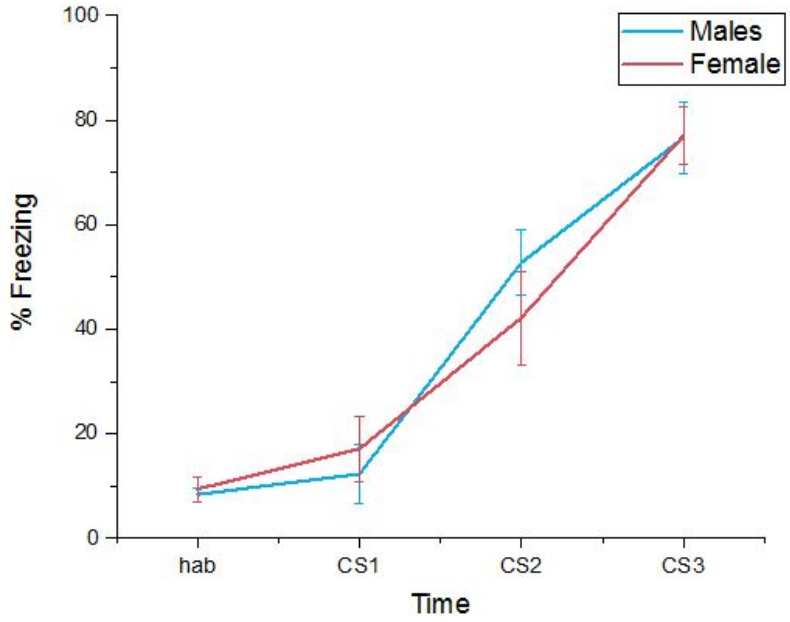


Figure 16. Fear conditioning in SDID Fear (n = 20). There was an increase in freezing over time ($F_{2,38}$=52.5, p=1.17e-11) but no statistically significant differences based on sex or experimental group. Data presented as mean ± SEM.

After contextual extinction and habituation, all animals extinguished the fear memory during cued extinction ($F_{49,882}$=7.58, p=4.55e-41), with a significant interaction between sex and time ($F_{49,882}$=1.42, p=0.0319) but no main effect of sex (Figure 17).

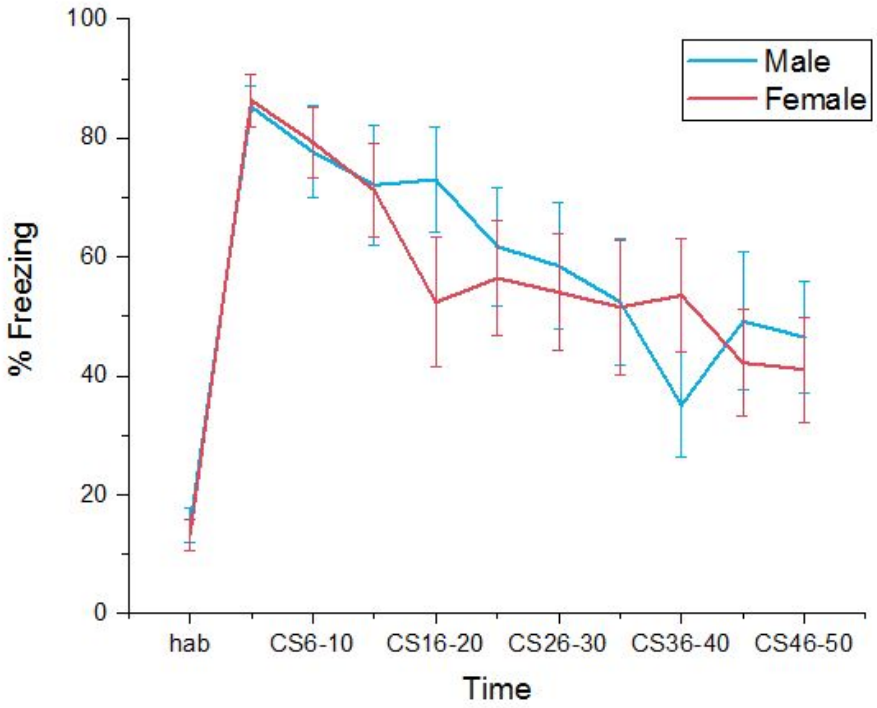


Figure 17. Cued extinction in DID Fear (n=20). All animals froze less as the test progressed ($F_{49,882}$=7.58, p=4.55e-41). There was no main effect of sex, but females reduced their freezing faster ($F_{49,882}$=1.42, p=0.0319). Data presented as mean ± SEM.

Following cued extinction, mice began the sDID paradigm in which they had open access to a 20% v/v EtOH solution for 2 hours a night for 5 consecutive nights while accompanied by their cagemate. Ethanol consumption increased over the five day sDID paradigm ($F_{4,36}$=2.86, p=0.0371) but there were no statistically significant sex differences in terms of EtOH consumption (Figure 18). Drinking levels were consistently lower for sDID than DID ($F_{1,27}$=26.1, p=2.29e-5).

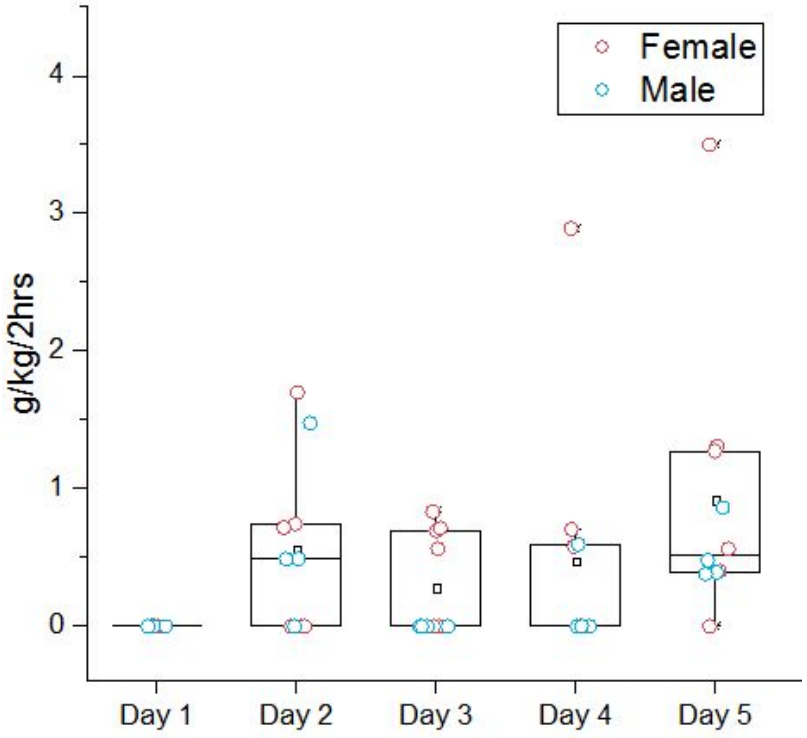


Figure 18. Ethanol consumed in sDID Fear (n=10). All animals drank more later in the paradigm ($F_{4,36}$=2.86, p=0.0371), but less than in the DID Fear paradigm ($F_{1,27}$=26.1, p=0.0000229). The box displays values within one interquartile range (IQR) around the mean (small square) with the median shown as a line through the box. Whiskers display values 1.5 IQR from the mean.

After three drug-free days in which all mice were undisturbed, animals underwent the cued fear recall test in which they were exposed to the CS an additional three times. During the recall test, mice in the EtOH group did not differ from mice in the control group though unlike in DID Fear, animals expressed less fear as the test progressed ($F_{2,36}$=3.54, p=0.0383) with strong trends for interactions between both sex X time ($F_{2,36}$=2.76, p=0.0764) and group X time ($F_{2,36}$=2.94, p=0.0653; Figure 19).

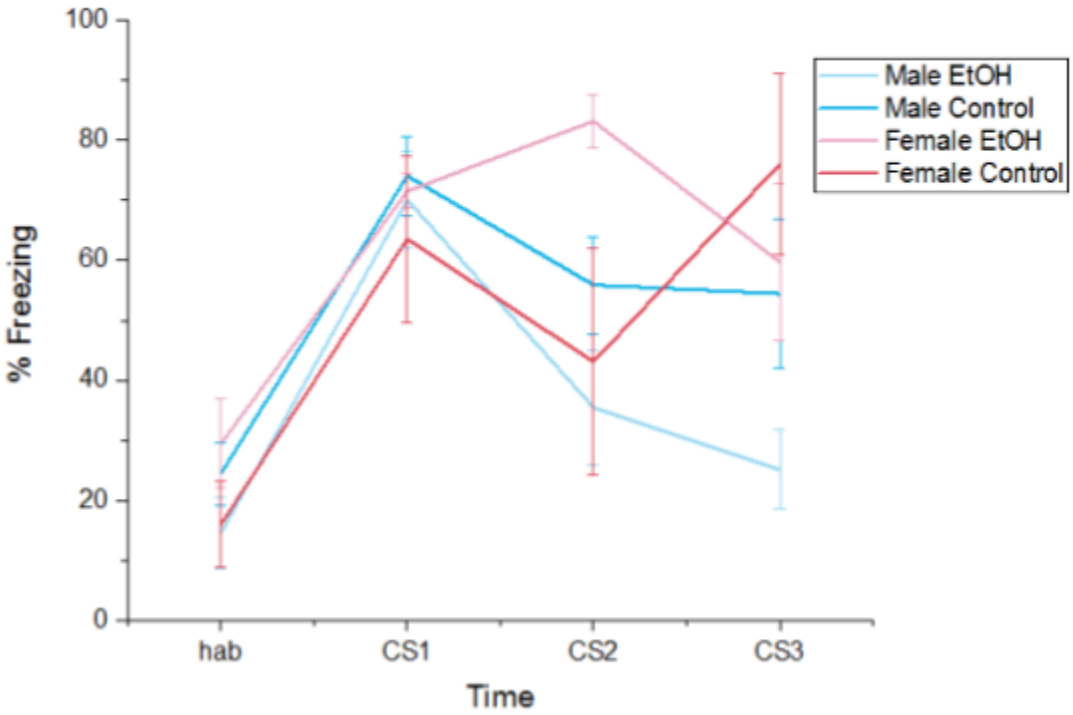


Figure 19. Cued recall test in sDID Fear (n=20). Animals froze less with additional CS presentations ($F_{2,36}$=3.54, p=0.0383), but just as in DID Fear there were no main effects of sex or experimental group. There were trends for males to reduce their freezing faster than females ($F_{2,36}$=2.76, p=0.0764) and for EtOH animals to reduce their freezing faster than control animals ($F_{2,36}$=2.94, p=0.0653). Data presented as mean ± SEM.

Experiment 3: "sDIDSS"

Mice (n=20, 10 female) underwent the fear conditioning protocol while the shock bars were unplugged, and were exposed to the CS without US. On consecutive days 24 hours after fear conditioning, mice were placed into Context A and Context B for 24 minutes. The following day, mice were placed into context B and presented with the CS 50 times in a temporally massed (5 s ITI) fashion (Cain et al., 2003). They received 5 days of the sDID paradigm (n=17 EtOH) and 3 days drug-free before being replayed the CS in a recall test. Three animals (3 female) were excluded from analysis due to leaky bottles during the sDID paradigm. We did not analyse behavioral data from fear conditioning nor from cued fear extinction because the animals did not learn about the CS without a paired US. Ethanol consumption increased over the five day paradigm ($F_{4,64}$=5.21, p=0.0106) with a strong trend for a main effect of sex ($F_{1,15}$=4.02, p=0.0632; Figure 20). There was a strong trend for higher drinking levels in sDIDSS than in sDID Fear ($F_{1,25}$=3.95, p=0.0579).

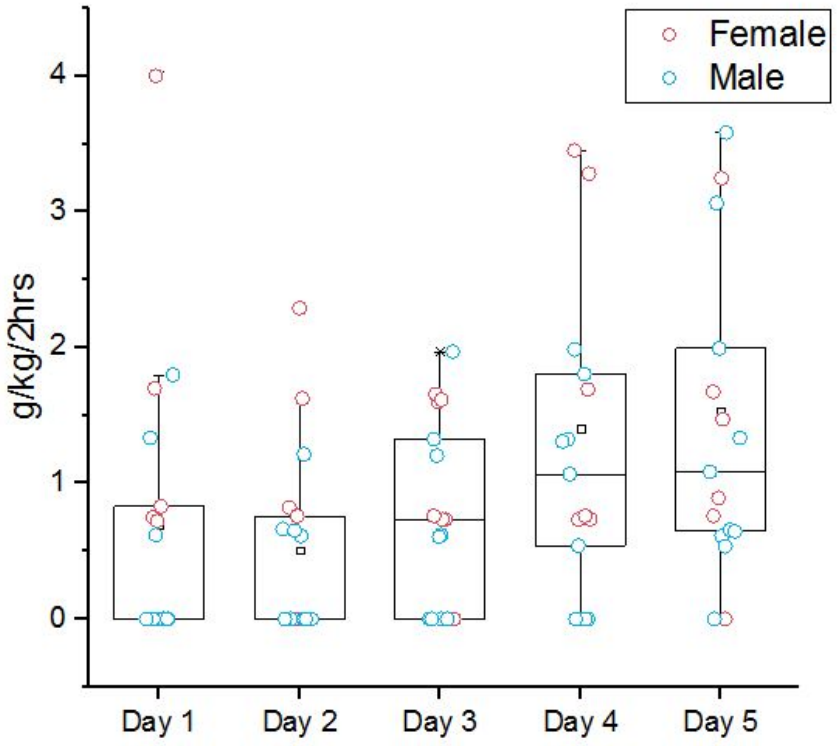


Figure 20. Ethanol consumed in sDIDSS (n=17). Ethanol consumption increased over the course of the paradigm ($F_{4,64}$=5.21, p=0.0106) with strong trends for females to drink more than males ($F_{1,15}$=4.02, p=0.0632) and for all animals to consume for EtOH than the sDID Fear paradigm ($F_{1,25}$=3.95, p=0.0579). The box displays values within one interquartile range (IQR) around the mean (small square) with the median shown as a line through the box. Whiskers display values 1.5 IQR from the mean.

Comparing the DID Fear pilot and two experiments revealed that females consumed more ($F_{1,40}$=6.44, p=0.01529), animals consumed more on later days ($F_{3,120}$=3.66, p=0.0145), and the different experimental protocols caused different levels of drinking ($F_{2,40}$=11.0, p=0.000156). Additionally, there was a significant interaction of both sex X time ($F_{3,120}$=2.91, p=0.0376) and experimental condition X time ($F_{6,120}$=3.83, p=0.00159; Figure 21).

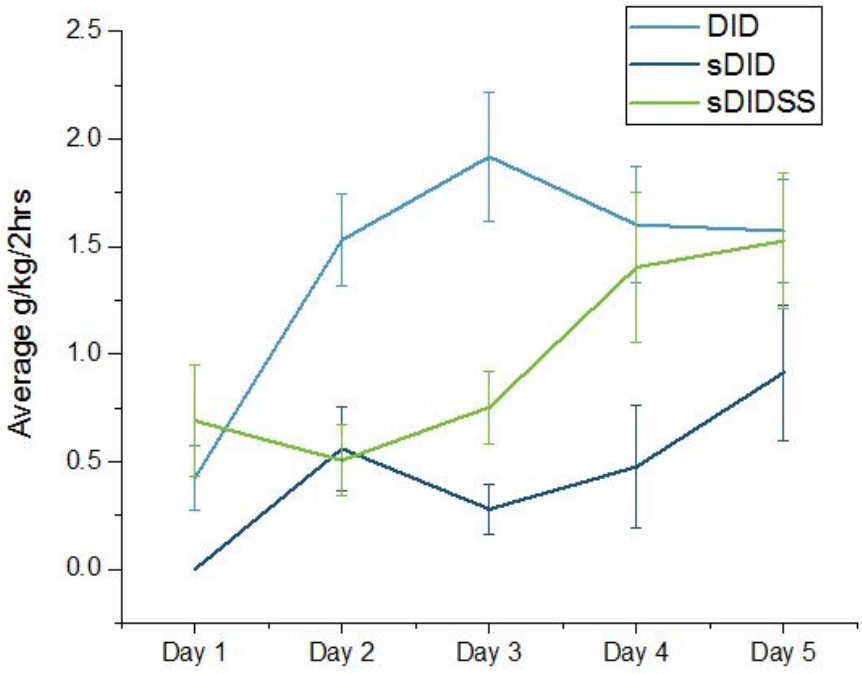


Figure 21. Comparison in drinking levels among the three experimental conditions (n=70). Females consumed more than males ($F_{1,40}$=6.44, p=0.01529), all animals consumed more on later days of the paradigm ($F_{3,120}$=3.66, p=0.0145), and animals in DID Fear drank more than either of the other experimental paradigms ($F_{2,40}$=11.0, p=0.000156). Females increased their consumption faster than males ($F_{3,120}$=2.91, p=0.0376), and animals in all three experiments increased their consumption over time at different rates ($F_{6,120}$=3.83, p=0.00159). Data presented as mean ± SEM.

Exploratory data analysis:

Voluntary EtOH consumption is not associated with the weight loss seen with i.p. injections of a 20% v/v EtOH solution, with animals gaining weight at the end of the paradigm ($F_{9,414}$=18.6, p=5.02e-26). There were also significant interactions of both sex X day ($F_{9,414}$=2.11, p=0.0277) and EtOH or Control group X day ($F_{9,414}$=1.62, p=0.0108) within the voluntary consumption paradigms. Ethanol injections showed more weight loss than the voluntary consumption paradigms ($F_{1,60}$=17.3, p=0.000104), with animals losing more weight as the experiment progressed ($F_{5,300}$=16.3, p=3.29e-14), and an interaction of experiment type X time ($F_{5,300}$=23.6, p=5.17e-20; Figure 22).

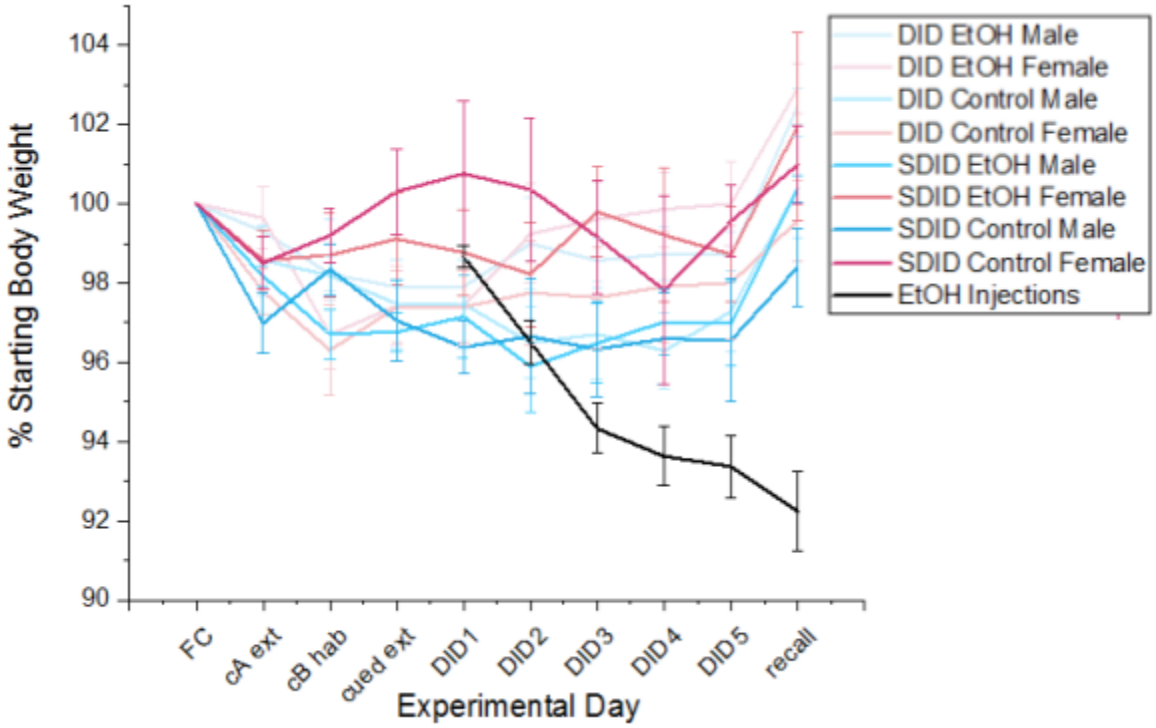


Figure 22. Percent starting body weight over the course of the 13 day experimentation (n=63). Ethanol injections are only included for the days of EtOH administration and the cued recall test because injection protocol ordered the behavioral days differently. Animals in the voluntary consumption paradigms gained weight as the paradigm progressed ($F_{9,414}$=18.6, p=5.02e-26) with females gaining weight faster than males ($F_{9,414}$=2.11, p=0.0277) and EtOH animals gaining weight faster than control animals ($F_{9,414}$=1.62, p=0.0108). Animals in the injection group lost more weight than the animals in the voluntary consumption paradigms ($F_{1,60}$=17.3, p=0.000104), losing weight as the experiment progressed ($F_{5,300}$=16.3, p=3.29e-14). Data presented as mean ± SEM.

Discussion

A median split on drinking levels in DID Fear revealed that high levels of EtOH consumption led to higher levels of freezing in the cued fear recall test following extinction training. This result replicates previous findings in the lab that animals exposed to EtOH showed higher freezing levels following extinction training than the control injection group (Scarlata et al., *in press*). This could be because the administration of EtOH within 24-36 hours after cued extinction training inhibits the consolidation of the new memory. Ethanol is thought to impair memory functioning at the cellular level by facilitating inhibitory receptors and impairing excitatory receptors in regions like the hippocampus that are important for memory consolidation (Nestler, 2001). Administration of EtOH while either encoding or consolidating a memory can thus prevent the cellular activity and strengthening of synapses necessary for new memory

formation. Cued extinction is not erasure of a previously consolidated fear memory, but instead a form of learning that suppresses a previously conditioned response (Peters, Kalivas, & Quirk, 2009). If EtOH inhibits this cued extinction learning, mice that consume high levels of EtOH will retain their previously acquired conditioned fear response while mice that do not consume EtOH will retain the extinction training. There were no significant sex differences in either the acquisition or extinction of fear memory, but we did find support for sex differences in EtOH consumption with females drinking more than males (Adams, 1995; Cozzoli et al, 2014; Yoneyama et al, 2008).

While mice that consumed the most EtOH froze the most in the cued fear recall test, the group of animals that consumed low levels of EtOH trended towards freezing less than the control animals with no access to EtOH. This could be a symptom of hypoanxiety, as is sometimes seen following low levels of EtOH consumption (Szumlinski et al., 2019). To better test the effects of low level consumption of EtOH, future work could lengthen the cued fear recall test to 10CS instead of 3CS to assess how a second extinction learning session is impacted by EtOH. Additionally, behavioral measures such as an open field test could be used in order to determine baseline differences in anxiety-like behavior between low- and high-drinkers. We predicted that mice in DID Fear would consume more EtOH than animals in sDID Fear because of the added stress of single housing. Although the data supports that hypothesis, we did not take into account the potential stressor of transferring the mice between cages during the dark cycle in sDID protocol. Moving the animals between cages may have been stressful, causing the mice to explore the bar cage less and consume less EtOH (Szumlinski et al., 2019). One solution to this problem is to allow the animals to habituate to the bar cage before providing access to the EtOH. We will be amending the sDID protocol to include this 45 minute habituation period in later studies, and data from the first of these studies will be available on the OSF repository at https://osf.io/495cj/ after 5/5/19.

In humans, alcohol consumption tends to increase following traumatic experiences (Kline et al., 2014; Nickerson et al., 2014). Thus we hypothesised that fear conditioned mice in sDID Fear would consume more EtOH than non-shocked mice in sDIDSS. Instead, we saw that mice in sDIDSS consumed more EtOH than animals in sDID Fear, but less than animals in DID Fear. Some studies in rodents have shown that certain types of stressors may actually reduce EtOH consumption (Cozzoli et al., 2014). Footshock, used in DID Fear and sDID Fear, is one of the stressors that drives freezing down. This may be why animals in sDID Fear consumed less EtOH than animals in sDIDSS. In order to replicate this study with higher EtOH consumption, we could use predator odor as a stressor that drives drinking up by using dirty bedding from rats as bedding for the experimental mice. Another limitation of foot shock as a model of PTSD is that there is little individual variability in terms of sensitivity and resiliency (Flandreau & Toth, 2017). The majority of animals exposed to electric shock will go on to display behavioral consequences to the stress, while not all humans exposed to trauma will develop symptoms of PTSD (Flandreau & Toth, 2017).

Additionally, measuring a cued fear response following fear conditioning does not include any of the many other symptoms required for a PTSD diagnosis. This is why we are not really examining a mouse model of PTSD, but instead are using fear memory learning and extinction to uncover processes underlying PTSD symptoms. To more closely examine the similarities between the current study's fear learning paradigm and human symptoms of PTSD, we could also test avoidance-like symptoms with behavioral assays such as a plus maze or social avoidance, or test negative alterations in mood and cognition with a forced swim test, or test hyperarousal with sleep fragmentation or marble burying assays (Flandreau & Toth, 2017).

Interestingly, our exploratory analysis of the weight data showed a trend of female animals retaining more weight than males. This may suggest that females are more resilient to the stress of fear conditioning than males, or that the additional EtOH consumption enabled better weight retention. The sex X day and experimental group X day interactions provide support for females gaining more weight as paradigm went on, and for EtOH animals to gain more weight than controls as the paradigm went on. As was predicted, all animals in the voluntary consumption paradigms retained more weight than the animals that underwent injections of EtOH. This supports the notion that DID and sDID protocols are less stressful for the mice than the injection paradigm.

We have not yet completed the analysis of the c-fos data we collected from DID Fear. Based upon previous work in the lab that used a different molecular activity marker, we expect to find a reduction in c-fos expression in all ROI in the EtOH group compared to the control group (Scarlata et al., *in press*). We expect to see decreased activity in the IL and PL among animals that consumed higher levels of EtOH, within the EtOH group. This reduction of activity in the mPFC is commonly associated with high risk, impulsive behavior seen in both drug addicts and PTSD patients (Peters, Kalivas, & Quirk, 2009). In animals that expressed more fear than average, we expect to see elevated levels of c-fos expression in the CeA. Amygdala responsivity is shown to be positively correlated to PTSD symptoms, while mPFC is structurally smaller and shows hypo-functionality (Török et al., 2019). Finally, we also hypothesise that there will be a positive correlation between c-fos expression and fear expression in the CeA, but a negative correlation between c-fos expression and EtOH consumption in the IL and PL. A significant correlation in c-fos expression would support the individual differences we saw at behavioral and cognitive levels of EtOH consumption and cued fear expression.

Implications

Not all people who binge drink will become addicted to alcohol, not all people who experience trauma will develop PTSD, and not all people who binge drink and experience trauma will develop both an AUD and PTSD. Using a mouse model to explore the interactions between binge-like EtOH consumption and cued fear expression can elucidate the impact of chronic EtOH consumption on fear learning and extinction of fear learning. PTSD is sometimes thought of as an inability to extinguish a fear memory, so uncovering the mechanisms behind the

failure to extinguish a memory is an important therapeutic goal (Suh & Ressler, 2018). In humans, individuals may be more or less resilient to developing either of these disorders based on genetic, behavioral, and socioeconomic risk factors (Kendler et al., 2012; Keyes et al., 2012; Grant, Goldstein, & Saha, 2015; Sareen, 2014; Török et al., 2019). Here, we studied the individual differences in the cellular, behavioral, and cognitive levels of EtOH consumption and fear expression in a mouse model in order to better understand the interactions of alcohol and fear memory. We replicated previous findings of sex differences in EtOH consumption and high EtOH consumption leading to impaired fear extinction recall. We found that high EtOH consumption correlates with high freezing in the cued recall test following extinction training, but these findings may be difficult to extrapolate to humans.

In clinical settings, baselines must be set for determining both who is binge drinking and how much alcohol must be consumed to be considered binge drinking. Commonly, binge drinking is the consumption of a sufficiently large amount of alcohol to place the drinker at increased risk of experiencing alcohol related problems and to place others at increased risk of experiencing secondhand effects (Wechsler & Nelson, 2001). This amount of alcohol is typically set at five drinks for men and four for women (Wechsler & Nelson, 2001). Questionnaires and interviews are often used to assess levels of binge drinking within a population, but these measures are not always accurate because participants can lie about their consumption or may simply not be aware of how much they drink (Midanik, 1988). This makes it very difficult to quantify levels of binge drinking in humans.

The sex differences in consumption found in non-human mammals is the opposite of that found for humans. The reasons for this difference are unclear. In studies of rodents, females voluntarily consume more EtOH than males (Adams, 1995; Sneddon, White, & Radke, 2019, Yoneyama et al., 2008). But in humans, the reverse is seen (Grant et al., 2017). Also interesting for this study is that the C57BL/6N strain of mouse used in the current experiments are heavy drinkers (Sneddon, White, & Radke, 2019; Yoneyama et al., 2008). Replicating the current study using wild type or a different inbred strain could ensure that our findings are not strain specific for animals that tend to consume the most EtOH. Replicating this work with a wild type strain may make our findings more relevant to humans with AUD, though there is a strong genetic component to AUDs (Kendler et al., 2012, Kessler et al., 1997).

While some people may be genetically predisposed to be more likely to develop either an AUD or PTSD, the high comorbidity between this disorders may also be explained by their shared circuits. Shared circuitry is not enough to explain the comorbidity, but just as classical conditioning links previously unassociated stimuli through a shared circuit, a similar process may be responsible for the co-occurrence of AUD and PTSD. Classical conditioning can associate two stimuli through repeated temporal pairing, such as when a tone and electrical shock co-terminate. After a few pairings, exposure to the tone will not only activate auditory regions, but will also activate the amygdala to mount a fear response regardless of whether the shock is present. Some PTSD symptoms related to fear expression are mediated by the PL and its

projections to the amygdala (Peters, Kalivas, & Quirk, 2009). Drug seeking behavior seen in AUD takes this same path from the PL into the amygdala (Peters, Kalivas, & Quirk, 2009). It's possible that in individuals that already have developed one of these disorders, the repeated stimulation of the PL and amygdala makes the other disorder more likely to develop. For example, if someone has PTSD and casually drinks alcohol, the stimulation of the PL due to fear expression from PTSD may drive them to drug seeking behavior that increases their consumption of alcohol (Peters, Kalivas, & Quirk, 2009). This association between fear and alcohol may function the same way as classical conditioning, where although these stimuli were never explicitly linked, exposure to one stimuli activates a response to the other. Alternatively, an individual may increase their alcohol consumption as a form of self-medication to conceal symptoms of PTSD.

The shared circuitry between AUD and PTSD links drug use and fear learning at a neuroanatomical level. Increased neuronal activity in the amygdala is associated with both renewal and spontaneous recovery of extinguished fear and reinstatement of drug seeking behavior (Goode & Maren, 2010). The amygdala is implicated in fear learning and stimulus associations, but some regions within the amygdala can have more specific roles. For example, projections to the PL from the basal lateral amygdala are necessary for cued reinstatement of drug-seeking, while projections to the IL mediate fear extinction (Goode & Maren, 2019). The IL and PL can also regulate activity in the amygdala in a top-down manner. Drug relapse can occur with PL stimulation of the basal lateral amygdala, while extinction recall requires projection from the IL to the amygdala (Peters, Kalivas, & Quirk, 2009). Additionally, the CeA has been implicated in stress-induced drug relapse (Goode & Maren, 2019).

The most commonly used therapy for PTSD is prolonged exposure, which involves an individual being exposed to aspects of their traumatic experience over multiple sessions (Powers et al., 2010). This type of therapy mimics extinction training often used in animal models of fear learning. Over time, the memory will weaken in strength because it is not being reinforced (Powers et al., 2010). One of the problems with prolonged exposure, however, is that traumatic memories can be spontaneously recovered upon exposure to associated contexts or stimuli. This relapse in extinction of fear is difficult to combat with cognitive rehabilitation, and there are not approved pharmacological treatments for PTSD (Powers et al., 2010). The ideal treatment for PTSD would augment inhibitory control and executive functioning (Powers et al., 2010). Similarly, many cognitive therapies for AUD and other substance use disorders function independently of pharmacological therapies (Sofuoglu et al., 2013). Chronic drug use has been associated with significant cognitive impairments, especially in attention, working memory, and inhibition, so improving cognitive functioning in these areas may enhance treatment outcomes for AUD (Sofuoglu et al., 2013).

Substance use disorders are sometimes thought of as being chronically relapsing brain disorders (Hsiang et al., 2014). But it is possible that at one point in our evolutionary history, there was an advantage to the cycle of binge, withdrawal, and craving that commonly

accompanies drug addictions (Nestler, 2001). Addictions can form memories that are resistant to extinction learning and can persist through relapses even after years of sobriety (Hsiang et al., 2014). These highly persistent memories may have once led people to remember sources of food or other biologically salient cues with minimal reinforcement. The strength of the memory associated with drugs of abuse may be due not just to the appetitive nature of the drug, but the aversive nature of the withdrawal process. Symptoms of withdrawal can include irritability, nausea, vomiting, insomnia, tremor, hyperalgesia, hyperthermia, hyperventilation, tachycardia, anxiety, and hyper-excitation such as hallucinations, delusions, and grand mal seizures (George & Koob, 2017; Metten et al., 2017).

The relationship between the appetitive drug and the aversive withdrawal represents a balance between short term reward and longer term consequences outlined in both opponent processing theory and systems of reinforcement learning. Opponent processing theory outlines an A process, or positive mood state, and a B process, or negative mood state (George & Koob, 2017). The B process responds to the A process in order to return the agent to homeostasis, and can be seen in the cycle of intoxication and withdrawal. However, after a point the B process can overpower the A process, leading to an allostatic state and preventing a return to homeostasis (Figure 23). For every agent, there are individual differences that define the magnitude of the A process in response to an appetitive stimulus and the size of the subsequent B process.

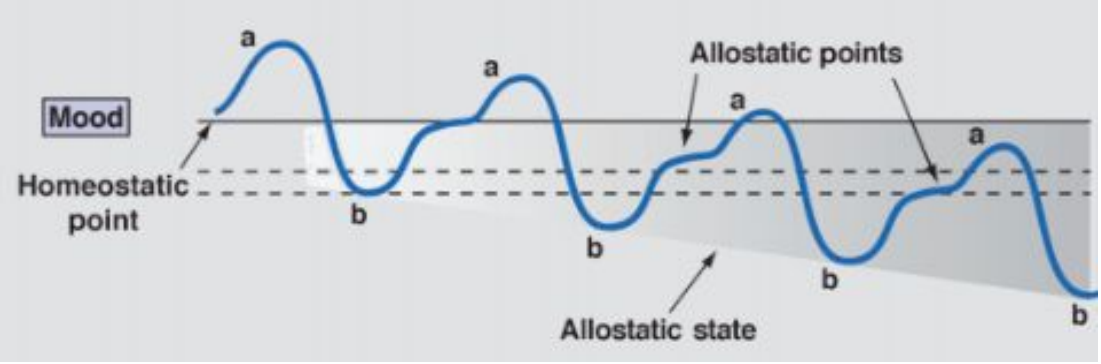


Figure 23. Opponent processing theory. The A process reflects the appetitive feelings associated with intoxication, while the B process represents the aversive feelings of withdrawal and cravings. Image from George & Koob (2017).

In systems of reinforcement learning, computational models aim to balance short and long term payoff in order to learn how to accomplish long term goals (Sutton & Barto, 2018). These models would likely learn from the accumulation of the B process on the allostatic state that the A process is not appetitive enough to outweigh the B process. Without drastically augmenting the A process, as is the case when drug dose is increased, the reward does not outweigh the drawbacks. And when the drug dose is increased, the withdrawal also increases, leading to another iteration in the cycle of addiction (George & Koob, 2017).

Conclusions

Future work will use the sDID procedure to measure age (adolescent) and sex differences in EtOH drinking levels, cued fear expression, and cued fear generalization in C57BL/6N mice. We plan to study adolescents because young mice are differentially sensitive to stress and thus may consume more EtOH following fear conditioning than adult animals. Additionally, emerging adulthood in humans is becoming an increasingly vulnerable period for AUD onset and therefore is worth further research (Grant, Goldstein, & Saha, 2015). Previous work in the lab has shown that mice administered EtOH following cued extinction training express more fear in the cued fear recall test and to a novel tone in a generalisation test (Scarlata et al, *in press*). This research showed that chronic EtOH administration impaired fear extinction retrieval while elevating overgeneralisation. Future work could aim to find a relationship between levels of EtOH consumption and fear expression to a novel tone. Additionally, we could add behavioral tests of withdrawal in order to quantify a greater range of individual differences. Withdrawal is one of the symptoms of AUD, and in humans AUD relapses can be caused by anhedonia and cravings experienced in withdrawal (APA, 2013; Metten et al., 2017). Establishing a relationship between fear expression and severity of withdrawal symptoms, as measured using temperature regulation, anhedonia and depression-like symptoms, ataxia, or anxiety-like symptoms, may provide more information about risk factors for high levels of EtOH consumption. Finally, follow-up studies may choose to measure blood ethanol concentration (BEC) to get a more accurate value for EtOH consumed by every animal. Quantifying BEC would involve taking blood from the animals, which may be an added stressor. We may be able to ameliorate some of that stress by taking blood during the day when the animals are more accustomed to being handled, and back calculating using the mouse's metabolism to establish BEC during the night.

Here, we replicate previous findings of our lab, and further support that chronic EtOH intake impairs fear extinction retrieval and augments cued fear memory generalisation through neuroadaptations in top-down processing. While we plan to continue working with mice, there is a need to connect animal models with clinical therapies. More work should be done in individuals with comorbid AUD and PTSD to see the effects of reducing EtOH consumption on PTSD symptoms. As we saw here, chronic EtOH administration can impair recall of extinction training. Eliminating EtOH consumption for the duration of prolonged exposure treatments for PTSD could enhance therapeutic outcomes. Reducing the severity of PTSD symptoms may then drive down the consumption of EtOH as a form of self-medication, easing the cycle of addiction.

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