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Interactions Between Stress, Ethanol, and THC Exposure and Effects on Prefrontal Reliant Signaling and Behavior

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by

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ABSTRACT

CORA ERIN SMILEY. Interactions between stress, ethanol, and THC exposure and effects on prefrontal reliant signaling and behavior. (Under the direction of JUSTIN T. GASS and L. JUDSON CHANDLER).

Post-traumatic stress disorder (PTSD) and substance use disorder (SUD) are two highly prevalent and highly debilitating psychological conditions that often occur comorbidly. In accordance with the self-medication hypothesis, drugs like alcohol and cannabis are used following exposure to a traumatic event to acutely reduce anxiety, but, in the long term, these substances cause impairments in learning and memory processes in the prefrontal cortex and reduce the effectiveness of therapeutic treatments for these disorders. As such, the overarching hypothesis of this dissertation is that exposure to stress, alcohol, and cannabis lead to alterations in learning and memory due to modifications in prefrontal cortex signaling, and it is further hypothesized that these impairments can be reversed by normalizing glutamatergic function in this region. These experiments were divided into three main branches of study using behavioral pharmacology as well as optogenetics and fiber photometry to investigate the deleterious effects of stress, cannabis, and alcohol exposure on cognitive functioning and glutamate signaling in the prefrontal cortex. In the first set of studies, restraint stress was followed by set-shifting and alcohol self-administration behavioral tasks to examine stress-induced changes in cognitive flexibility and drug seeking behavior respectively. These experiments established stress induced

increases in ethanol seeking and relapse like behavior as well as impairments in cognitive flexibility. The second set of studies established the detrimental effects of alcohol exposure on fear learning during extinction training. Further, this effect was shown to be dependent on glutamatergic activity in the prefrontal cortex using microinjection and optogenetic studies. In the third set of studies, THC vapor and chronic ethanol administration led to deficits in prefrontal cortex reliant behaviors including cognitive flexibility, ethanol seeking, and responses to fear stimuli, and fiber photometry was used to measure effects of these drugs on prefrontal cortex glutamate signaling. Additionally, the pharmaceutical compounds CDPPB and N-acetylcysteine (NAC) were used to pharmacologically reverse the behavioral and signaling impairments that occur as a result of alcohol and cannabis exposure respectively. Taken together, the data presented within this dissertation highlight the effects of alcohol and cannabis on prefrontal cortex-reliant signaling and behavior and use clinically available therapeutics to treat these deficits in a model of PTSD/SUD comorbidity.

Chapter 1

BACKGROUND AND SIGNIFICANCE

Post-Traumatic Stress Disorder and Comorbidities

Post-traumatic stress disorder was first recognized as a psychiatric disorder in the DSM-III published in 1980, but its symptomology has been ascribed to a number of different syndromes that have been defined throughout history (Marc-Antonie Crocq 2000). When examining sample populations, lifetime prevalence of traumatic event exposure reaches approximately 70-89% with instances of physical or sexual assault, witness of a violent or accidental death, and experience

of a disaster or accident being the most commonly reported traumas (Kilpatrick et al. 2013, Benjet et al. 2016). While a high percentage of the population reports exposure to a traumatic event, approximately 25-30% of these patients will develop PTSD (Spottswood et al. 2017). The criterion for a PTSD diagnosis spans 20 symptoms that include hyperarousal, negative mood, avoidance, and re-experiencing the trauma that last for a least a month following exposure to a stressor (Miao et al. 2018, Kilpatrick et al. 2013, Sareen 2014). Thus, the lifetime prevalence of PTSD has been reported to reach ~11% in the general population and 25% in the veteran population (Spottswood et al. 2017). PTSD also has a high level of impact on the community surrounding the patient, since PTSD is associated with difficulty parenting, a reduction in income, and children of patients with PTSD exhibit increased levels of impairments in psychosocial behavior (Miao et al. 2018, Sareen 2014).

There are a variety of factors that lead to an increased chance of developing PTSD including female sex, having an adverse childhood experience, previous drug use, and veteran status (Spottswood et al. 2017, Atwoli et al. 2015, Christiansen and Elklit 2008, Carlson et al. 2016, Muller et al. 2017). Women are found to not only develop PTSD at more than twice the rate of men (Olff 2017), but they develop the disorder at a younger age (Christiansen and Elklit 2008, Smith and Cottler 2018). These sex differences observed in PTSD are consistent with the hypothesis that there are innate sex differences in brain activity in response to stress. Females have been shown to exhibit increased amygdala activation in

response to negative stimuli and maintain stronger interconnectivity between the frontal cortex and the limbic system when compared to men (Helpman et al. 2017). 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.

There are three main neuronal circuits that have been shown to be implicated in the initiation of PTSD and, thus, have become targets for the treatment of this disorder (Sheynin and Liberzon 2017). First, the fear learning circuitry, with a focus on the amygdala and medial prefrontal cortex, has been highly implicated in PTSD with the general hypothesis being that alterations in PFC signaling results in hyperactivation of the amygdala and exaggerated responses to fear stimuli (Markowitz and Fanselow 2020, Sheynin and Liberzon 2017, Kelmendi et al. 2016). Additionally, PTSD is associated with irregularities in the circuitry that is responsible for context processing including the hippocampus with connections to the mPFC that are involved in contextual regulation of fear responses (Kelmendi et al. 2016, Sheynin and Liberzon 2017). The third circuit of interest includes the emotional regulation pathway from the PFC and amygdala that, when impaired following stress exposure, leads to the improper regulation of emotions and contributes to PTSD symptoms (Sheynin and Liberzon 2017). The only FDA-approved pharmacological treatment for PTSD are selective serotonin reuptake inhibitors (SSRIs) while the first line psychological treatment is exposure therapy (Kelmendi et al. 2016, Sheynin and Liberzon 2017). Notably, SSRIs do not

treat stress induced alterations in the three circuits described above and have found to be effective as a PTSD treatment in less than 30% of patients (Alexander 2012, Kelmendi et al. 2016). Recently, further studies have shown that these medications to not have any effect in the treatment of PTSD in military veterans when compared to placebo (Kelmendi et al. 2016). Additionally, exposure therapies have been found to lack efficacy with up to 77% of patients still qualifying for PTSD diagnosis, ~50% not reporting any change in their symptom severity, and some patients exhibiting a worsening of symptoms following treatment with exposure therapy (Markowitz and Fanselow 2020). While exposure-type therapies are used with the intention to affect plasticity in these fear-associated circuits, these psychotherapies are often not effective in PTSD patients due to impairments in the circuitry that allows for extinction learning (Markowitz and Fanselow 2020, Kelmendi et al. 2016, Sheynin and Liberzon 2017). Therefore, since our first line treatments for PTSD are often ineffective, recent research has focused on developing novel treatments to restore normal activity in these circuits to allow for more effective therapy outcomes (Sheynin and Liberzon 2017, Kelmendi et al. 2016).

Alcohol Use Disorder

PTSD often does not occur in isolation, with approximately 90% of patients with PTSD reporting at least one comorbid psychological disorder (Miao et al. 2018) with the most prevalent including depression, anxiety, or substance use disorder (Sareen 2014, Muller et al. 2017, Carlson et al. 2016). In patients with PTSD, alcohol is one of the most commonly used substances to decrease the feelings of heightened anxiety and arousal experienced with PTSD symptoms (Smith and Cottler 2018). The common occurrence of these symptoms in PTSD patients can often lead to alcohol use disorder (AUD), which is recognized when drinking reaches a rate of over five drinks per day for males and four for females. This disorder is marked by a series of physiological and neurological symptoms that are generally a result of the effects of ethanol on cellular toxicity in the brain and periphery (Kranzler and Soyka 2020, Wackernah et al. 2014). Alcohol is one of the most commonly used illicit substances, and the 12-month prevalence of AUD has been recently shown to reach levels as high as 13.9% with lifetime prevalence of ~36% (Grant et al. 2017). Importantly, rates of AUD differ between the sexes, with males reporting double the rate of AUD when compared to females (Kranzler and Soyka 2020).

While alcohol use disorder alone is one of the most common psychiatric disorders, AUD in PTSD patients is observed at rates up to 79% (Pietrzak et al. 2011). Broadly, there are two main theories regarding the bidirectional relationship between comorbid PTSD and AUD with evidence to support both hypotheses.

First, it has been widely reported that AUD develops following PTSD in accordance with the self-medication hypothesis where alcohol is being used to decrease anxiety (Smith and Cottler 2018). This hypothesis has been observed in both epidemiological research, where a PTSD diagnosis predicts the later development of AUD (Breslau et al. 2003), and in clinical studies, where patients with PTSD report increased alcohol cravings following the presentation of trauma related cues (Coffey et al. 2002). More recently, an alternative hypothesis has been suggested under which patients with substance use disorders are more likely to experience a traumatic event and, thus, develop PTSD than non-users (Cottler et al. 1992). Additional epidemiological studies have also found sex differences in this effect, where drug use has been shown to be initiated prior to trauma exposure in males but not females (Cottler et al. 2001).

PTSD and AUD interact in a complex manner to affect cognition and has led to the characterization of AUD and PTSD as disorders of learning and memory (Hyman 2005; Vanelzakker et al. 2013). As mentioned above, alcohol can be used as an anxiolytic by PTSD patients to acutely reduce the intense anxiety experienced following PTSD symptoms such as nightmares or re-experiencing the trauma. Theoretically, a cycle occurs where alcohol is used to alleviate PTSD symptoms but instead renders the fear memory resistant to extinction **(Figure 1-1**). This ultimately leads to increased alcohol consumption, the development of AUD, and the progression of cognitive deficits that exacerbate these disorders. This theory follows 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. Essentially, chronic and high-volume alcohol intake can affect cognitive functioning and worsens PTSD symptoms by interfering with the learning and memory processes that are responsible for the extinction of trauma associated cues (Back et al. 2006) Therefore, it is important to examine the underlying circuitry that overlaps between these two disorders to establish potential targets for the treatment of comorbid PTSD/AUD.

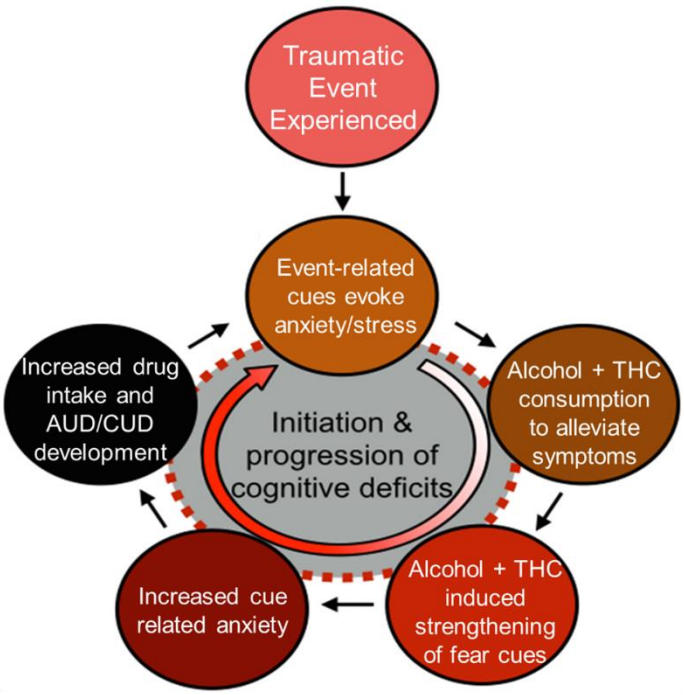

Figure 1-1: The Alcohol/THC and Stress Cycle. The studies under this dissertation are based on this cycle of trauma exposure followed by drug use in order to treat the anxiety symptoms that come from experiencing trauma related cues in the future. As this cycle progresses, repeated drug use following cue-induced anxiety leads to impaired plasticity in the prefrontal cortex that allow for the development of a substance use disorder along with the progression of cognitive deficits.

Due to the pervasive effects of alcohol exposure on the brain, the neurocircuitry underlying the initiation and maintenance of AUD is widespread. The glutamatergic system is highly involved in the reinforcing effects of alcohol through downstream activity in key brain regions including the nucleus accumbens and amygdala (Gilpin and Koob 2008, Koob and Volkow 2018). Additionally, the PFC has been highly studied with regards to addiction due to its functions in executive control and ability to modulate dopamine from the reward circuits originating in the striatum (Volkow et al. 2011, Koob and Volkow 2018). Glutamatergic signaling at both NMDA as well as metabotropic glutamate receptors subtype 5 (mGlu5) is also involved in the addiction process due to their role in increasing plasticity throughout the brain (Gilpin and Koob 2008). Additionally, alcohol has been shown to have effects on glutamatergic signaling throughout neuronal stress systems which leads to alterations in both behavioral responses to stressors as well as dysregulation of this circuitry (Gilpin and Koob 2008, Volkow et al. 2011, Koob and Volkow 2018).

While AUD is highly prevalent and one of the leading causes of preventable deaths, there is a three-pronged barrier to treating this disorder since there is a lack of effective treatments, an extremely low 8.3% rate of treatment seeking, and an under prescription of pharmacological treatments (Kranzler and Soyka 2020, Ray et al. 2020, Witkiewitz et al. 2019, Swift and Aston 2016). Additionally, the symptoms that are experienced during alcohol withdrawal are highly aversive including nausea, anxiety, tremors, and life-threatening seizures (Swift and Aston 2016, Witkiewitz et al. 2019). Currently, there are four pharmaceutical treatments

approved by the FDA for the treatment of AUD. Generally, the therapeutic approach behind these treatments depends on lessening the rewarding properties of alcohol use by inducing deleterious side effects as a consequence of alcohol use (Swift and Aston 2016). Over time, the pairing of alcohol with these side effects leads to an aversive response to alcohol and decreased use (Witkiewitz et al. 2019, Swift and Aston 2016). Due to the unpleasant nature of these drug treatments, adherence and long-term use of these medications are difficult to achieve in the AUD population (Swift and Aston 2016). With regards to psychological treatments, cue-exposure therapy is often used for the treatment of substance use disorders (Ray et al. 2020, Chambless and Ollendick 2001, Mellentin et al. 2017). Such treatments rely on the presentation of alcohol-associates cues and stimuli in the absence of any alcohol reinforcer so that over time patients lose their enhanced responses to alcohol related cues (Byrne et al. 2019, Mellentin et al. 2017, Ray et al. 2020). This method of therapy is theoretically sound but does not take into account the fact that patients with AUD have deficits in plasticity and cognition as a result of alcohol intake and, therefore, are not able to fully respond to cue exposure therapies (Ray et al. 2020, Conklin and Tiffany 2002). Thus, recent research has been focused on using pharmacological agents to enhance cognition and increase the efficacy of cue exposure therapies for the treatment of AUD (Ressler et al. 2004, Ray et al. 2020, Vengeliene et al. 2008).

Cannabis Use Disorder

In addition to alcohol, cannabis use is also highly prevalent in patients with PTSD (Hasin et al. 2016). As public perception of cannabis remains positive, with trends towards legalization growing across the country, cannabis use has been steadily rising with rates of drug use at 4.1% in 2002 increasing to 9.5% in 2013 (Hasin et al. 2015). Not only has cannabis become one of the most commonly abused substances, concentrations of delta-9 tetrahydrocannabinol (THC), the psychoactive component of the drug, have increased as well. In the 1990's, the concentration of THC in common cannabis strains was ~2%, but more recently THC concentration has been recorded to be 17-28% in the most popular strains in Colorado dispensaries (Stuyt 2018, Lafaye et al. 2017, Licata et al. 2005). Importantly, while the THC concentration has been sharply increasing, the cannabidiol (CBD) concentration in cannabis has decreased from 0.5% to 0.09-0.2% (Stuyt 2018, Lafaye et al. 2017). This is especially concerning considering the fact that the determinantal effects of cannabis are directly related to the amount of THC while CBD can have a protective effect on the adverse effects of THC (Niesink and vanLaar 2013, Lafaye et al. 2017). Chronic use of high-THC cannabis has been shown to cause deficits in cognition, increased anxiety and psychosis, and a higher chance of developing cannabis use disorder (CUD) (Volkow et al. 2014, Lafaye et al. 2017, Fergusson and Boden 2008). While 9% of people who use cannabis will develop CUD, this rate increases to 25-50% in those who start using in adolescence (Volkow et al. 2014). Earlier cannabis use is also associated

with further adverse effects including comorbid psychiatric disorder, reduced life satisfaction, and cognitive impairment (Fergusson and Boden 2008, Rey et al. 2002), most likely due to ongoing brain development, including the maturation of synaptic connectivity and the endocannabinoid system, that continues throughout mid- to late-adolescence (Tortoriello et al. 2014, Volkow et al. 2014, Berghuis et al. 2007, Keimpema et al 2011).

While cannabis is one of the most commonly used elicit substances, it has only recently been recognized to have the potential to lead to CUD (Brezing and Levin 2017, Panlilio and Justinova 2017). Therefore, research dedicated to the treatment and reduction of harm in CUD has not been fully examined. Although research regarding cannabis use has been growing since the early 2000s, there are currently no FDA-approved treatments for CUD (Brezing and Levin 2017). While CUD alone is associated with adverse consequences, this disorder is also highly comorbid with other substance use and psychiatric disorders that lead to further impairments in cognition. Those with CUD have been shown to have up to a 10x greater chance of developing AUD and a 6x greater chance of developing PTSD than those without CUD (Hasin et al. 2015, Hasin et al. 2016). Additionally, there is a bidirectional relationship observed with PTSD and CUD and, in states in which cannabis is legalized, up to 38.5% of their users cite PTSD symptoms as their reason for obtaining the drug (Yarnell 2015, Mizrachi Zer-Aviv et al. 2016, Wilkinson et al. 2015). Additionally, CUD rates are especially high among veterans who have developed PTSD following trauma exposure (Khoury et al. 2010).

Acutely, cannabis can have an anxiolytic effect for PTSD patients, but prolonged and frequent cannabis use also leads to an increased risk of developing CUD along with impairments in prefrontal cortex reliant cognition. This is observed especially in adolescents, where marijuana is the most commonly used illicit substance by adolescents with PTSD (Yarnell, 2015), and an adolescent with PTSD has a two-fold increase in the likelihood of developing CUD (Bujarski et al., 2012). While cannabis acutely acts to decrease anxiety, use of this drug is often associated with worse symptoms outcomes in patients with PTSD, potentially due to long term effects on learning and memory through impairments in the developing prefrontal cortex (Wilkinson et al. 2015). While the presence of comorbid CUD, AUD, and PTSD is firmly established, there has been a lack of pre-clinical research into the underlying brain mechanisms that contribute to the maintenance and treatment of these co-morbid disorders.

Animal Models of PTSD, AUD, and CUD

While the prevalence of AUD, PTSD, and CUD is extremely high, the current therapies and pharmaceutical treatments for these disorders do not often have a high rate of efficacy (Kelmendi et al. 2016). Therefore, it is especially important to complete preclinical research using translational methods to investigate the underlying mechanisms responsible for the initiation and maintenance of these disorders. As such, a variety of different animal models have been developed to mimic these disorders in rodents. While there are multiple appropriate models that could be used to depict AUD and PTSD in rats, the models used for these studies were specifically selected due to their ability to longitudinally measure changes in fear learning and memory.

To employ a valid model of PTSD, it should depend on the same underlying neurocircuitry that is involved in fear responses between humans and rodents, induce behavioral responses to fear associated cues, and mimics the initiation of the disorder following exposure to a pivotal experience that induces extreme levels of stress (Flandreau et al. 2017, Goswami et al. 2013). A preclinical model of PTSD in rodents should allow for longitudinal studies of symptoms induced by stress exposure and require only a short duration of exposure to a stressful event to induce PTSD-like symptoms (Siegmund and Wotjack 2006, Goswami et al. 2013). A variety of tasks have been developed to induce a PTSD-like phenotype in rodents including restraint stress, single prolonged stress, social defeat stress, and fear conditioning (Borghans and Homberg 2015, Flandreau and Toth 2018,

Goswami et al. 2013, Whitaker et al. 2014). While each model has individual strengths and weaknesses, fear conditioning is the model that best allows us to address the hypotheses involved in these experiments regarding long-term relationships between chronic drug exposure and fear related learning and memory (Ursano et al. 2007; Borghans et al. 2015; Lissek et al. 2015; Singewald et al. 2019; Mahan et al. 2012; Amstadter et al. 2009). Since the establishment of conditioning relationships by Pavlov almost one hundred years ago, the ability to manipulate cue responses has been largely used to study psychiatric disorders preclinically (Pavlov 1927, VanElzakker et al. 2013). Briefly, conditioning studies rely on the formation of associations between an unconditioned stimulus (US) paired with a conditioned stimulus (CS) where subsequent presentations of the CS will allow the originally unconditioned response (UR) to become a conditioned response (CR) (Lissek and van Meurs 2014, VanElzakker et al. 2013). This premise underlies the method of fear conditioning used to induce behavioral phenotypes similar to PTSD, where a tone and footshock serve as the CS and US respectively, and the freezing response is the UR that develops into the CR over time (Lissek and van Meurs 2014). Thus, we can measure the CR over time during both the conditioning, extinction, and recall phases as well as monitor responses during these behaviors following chronic drug exposure.

Fear conditioning was followed by binge-like exposure to alcohol in these experiments to translationally model exposure to a discrete trauma followed by drug use as self-medication. For our model of AUD, certain aspects of the disorder

in humans need to be considered, including the level of alcohol consumption, alcohol dependence, compulsive alcohol seeking, and relapse behavior (Crabbe 2014, Helms et al. 2015, Goltseker et al. 2019). While many different drug self-administration paradigms have been developed that address the seeking and relapse aspects of AUD, these protocols will only allow for blood ethanol concentrations to reach moderate levels since animals will not drink to excess (Augier et al. 2014, Goltseker et al. 2019). Therefore, a more intensive model has to be used for studies examining the effects of a high and sustained levels of ethanol exposure. As such, many investigators now 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 which could not be established in rodents with voluntary drinking paradigms alone (Becker 2013; Griffin 2014). As such, CIE exposure was used for the experiments involved in this dissertation to model the binge-like levels of alcohol exposure commonly achieved in patients with AUD.

In recent years, cannabis use has been more intensively examined in preclinical studies. While previously the effects of cannabis have been studied by testing the effects of CB1 agonists on the brain, more translational models of cannabis exposure have been developed recently as government regulations

regarding the use of THC have changed (Panlilio and Justinova 2017). An additional challenge with modeling cannabis use in animals is that laboratory animals would not establish self-administration behavior for THC (Lefever et al. 2014, Panlilio and Justinova 2017). Recently, a novel and reliable method THC self-administration has been established that uses a combination of THC and CBD as well as a passive vapor exposure to temper the aversive and anxiogenic aspects of THC administration (Spencer et al. 2019). Not only will rats acquire self-administration behavior using this model, but they achieve the physiological and neurological alterations that are often observed as a result of cannabis use (Spencer et al. 2019). Therefore, the experiments discussed in this dissertation involve this THC vapor administration model to mimic frequent cannabis intake in a translationally relevant method.

In summary, while there are many valid animal models that could be used to replicated these disorders in rodents, we incorporated fear conditioning, CIE, and THC+CBD vapor exposure as our models of stress, alcohol, and cannabis exposure to generally study long-term relationships between stress, alcohol, and cannabis and their impact on the neurocircuitry involved in learning and memory.

The Prefrontal Cortex

The prefrontal cortex (PFC) is highly involved in both addiction and anxiety disorders and, therefore, is the target region of study for these experiments. In humans, the PFC makes up one third of the cortex and has complex structural and functional organization that allow for its involvement in executive function and top-down control of behavior (Siddiqui et al. 2008, Abernathy et al. 2013). This region is located in the frontal cortex area anterior to the primary motor cortex and includes the orbitofrontal, lateral, and medial/cingulate PFC subregions which maintain reciprocal connections (Abernathy et al. 2013, Radnikow and Feldmeyer 2018). The PFC also sustains long-range projections throughout the brain from cells across six cortical cell layers (Abernathy et al. 2013). These layers can be differentiated through distinct anatomical and cellular features and display layer-specific gene expression of different cell types and receptors (Radnikow and Feldmeyer 2018). While over 80% of the cortex is comprised of glutamatergic cells, there is layer specific cellular diversity in terms of morphology and signaling properties that allow these layers to serve distinct functions (Song and Moyer 2018, Little and Carter 2012, Dembrow et al. 2010, Abernathy et al. 2013). For example, layer V excitatory pyramidal cells are the primary source of PFC output while layers 1 and 2/3 receive and process signals from axons originating across a wide range of other brain regions including the thalamus, amygdala, and hippocampus (Little and Carter 2012, Dembrow et al. 2010). Additionally, although they make up a smaller percentage of total cells throughout this region, inhibitory

GABA interneurons are found throughout the PFC and serve to modulate signaling from the primary pyramidal cells (Abernathy et al. 2013). The diffuse projections into and out of the PFC allow for control over a wide range of higher order functions including the planning and direction of behavioral output and attention, active memory and encoding, and language (Siddiqui et al. 2008, Abernathy et al. 2013). The control of these functions is distributed throughout the lateral, orbitofrontal, and medial PFC subregions (Siddiqui et al. 2008, Abernathy et al. 2013). Generally, the lateral PFC has been shown to be highly involved in the control of working memory, attention, and cognitive flexibility (Siddiqui et al. 2008, Abernathy et al. 2013, Szczepanski and Knight 2014), while the orbitofrontal region is known for its functions in emotional and inhibitory control, decision making regarding rewards, and reversal learning (Siddiqui et al. 2008, Abernathy et al. 2013, Szczepanski and Knight 2014). While the functions of the lateral and orbitofrontal PFC are broadly involved in the circuitry of addiction and anxiety disorders, the medial PFC (mPFC) is especially important due to its ability to control fear and drug-seeking behaviors (Siddiqui et al. 2008). Additionally, this region has been shown to be essential for the exposure-based therapies previously mentioned as the first-line psychological therapy for conditions like PTSD and AUD (Groblewski and Stafford 2010). Therefore, in this dissertation, the mPFC and its subregions are the focus of study due to their established ability to modulate behavioral responses to drug and fear cues and involvement in the pathophysiology of PTSD/AUD.

Prelimbic and Infralimbic Subregions

This dissertation's focus on mPFC subregions originates from the fact that patients with PTSD and AUD exhibit alterations in these regions as a result of stress and alcohol exposure (Suh and Ressler 2018). It is important to note that mPFC subregions are differentially referenced in humans versus rodents, where the human dorsal anterior cingulate cortex (dACC) is a correlate of the rodent prelimbic cortex (PrL) while the human ventro-medial PFC is the infralimbic cortex (IfL) in the rodent (Milad and Quirk 2012). Generally, the PrL region has been shown to function in the promotion of fear behaviors while the IfL is involved in extinction learning by reducing responses to fear stimuli (Milad and Quirk 2012, Peters et al. 2009). There is a wide range of studies that investigate the PrL that demonstrate its ability to control responses to fear cues, including signaling studies where the PrL exhibits increased activity in response to fear cues (Burgos-Robles et al. 2009) and activation studies where PrL activation or inactivation increases or decreases the expression of conditioned fear behaviors respectively (Laurent and Westbrook 2009, Sierra-Mercado et al. 2006, Sierra-Mercado et al. 2011, Corcoran and Quirk 2007, Vidal-Gonzalez et al. 2006). Additional studies have shown that there is conditioning induced plasticity exhibited in the PrL (Burgos-Robles et al. 2007, Corcoran and Quirk 2007, Mahan and Ressler 2012, Song et al. 2015) and time-course studies determine that activity in the PrL mirrors the freezing activity of the animal during conditioning (Gilmartin and Helmstetter 2010, Milad and Quirk 2016, Burgos-Robles et al. 2009). The PrL has also been shown

to be highly involved in the top-down control of drug-seeking behaviors (Lasseter et al. 2010). The contributions of the PrL to drug seeking behavior has been shown with regards to a wide variety of drugs of abuse including cocaine (Cornish and Kalivas 2000, McFarland et al. 2003, West et al. 2015), MDMA (Ball and Slane 2012), heroin (LaLumiere and Kalivas 2008, Lasseter et al. 2010), and alcohol (Palombo et al. 2017, Kroener et al. 2012). These studies highlight the fact that the PrL is especially important in both the acquisition of drug seeking behavior as well as cue-induced relapse (Lasseter et al. 2010). Additionally, this function of the PrL has been shown to involve glutamatergic signaling from this region to the nucleus accumbens core (NAc), since inactivation of this pathway prevents relapse like behavior (LaLumiere and Kalivas 2008). Thus, due to the highly overlapping role of the PrL in both fear expression and promotion of drug seeking behavior, this region is an important target of study regarding comorbid PTSD and SUD.

Alternately, the IfL has been shown to be involved in opposing the PrL to allow for reduced responses to fear stimuli by controlling extinction learning (Milad and Quirk 2016). Initial lesion studies showed that the loss of IfL activity leads to an inability to retrieve extinction memories (Quirk et al. 2000) while recording studies demonstrate that IfL signaling is involved during extinction retrieval (Milad and Quirk 2002), and stimulation of this region reduces the expression of fear behaviors (Herry and Garcia 2002, Milad and Quirk 2002). Specifically, it has been shown that extinction memories rely on NMDA activation in the IfL (Burgos-Robles et al. 2007, Chang et al. 2010) and increased plasticity in this region is required to

express extinction behavior (Santini et al. 2008). Parallel to its function in the extinction of fear behaviors, the IfL is also highly involved in the extinction of drug-seeking behaviors. IfL projections to the nucleus accumbens shell (NAs) have been shown to be involved in the suppression of drug seeking behavior (Peters et al. 2009), stimulation of the IfL has the ability to reduce relapse in reinstatement tests, and inactivation of the IfL following extinction allows for a return to drug seeking behavior (Ovari and Leri 2008, Peters et al. 2008). Therefore, as a pivotal region for the control of extinction of fear and drug-seeking behaviors, the IfL is an important region to study in terms of the treatment of comorbid PTSD/SUD.

Downstream Signaling Targets

There are a wide variety of downstream regions to which the PrL and IfL signal to control the expression and suppression of fear and drug-seeking behaviors (**Figure 1-2**) (Peters et al. 2009). With regards to the expression of fear behavior, the PrL is known to signal to multiple subregions of the amygdala through excitatory glutamatergic projections (Peters et al. 2009, Brinley-Reed et al. 1995, Vertes 2004, Gabbott et al. 2005). The basolateral amygdala (BLA) is one of the primary sites of projection from the PrL to control the expression of conditioned fear behavior (Anglada-Figueroa and Quirk 2005, Herry et al. 2008). Additionally, there are multiple internal signaling pathways within the amygdala from the lateral amygdala, the main area responsible for the storage of fear memories, and the central amygdala, the main source of output from the amygdala, to allow for

increased amygdala output to downstream regions such as the brainstem and hypothalamus to increase the presentation of the physiological and behavioral fear responses (Blair et al. 2001, Repa et al. 2001, Wilensky et al. 2006, Zimmerman et al. 2007). Alternatively, the IfL also sends glutamatergic projections to the amygdala, but instead targets the GABA interneurons in the intercalated cell region of the amygdala that allow for a reduction of activity from the central amygdala (Berretta et al. 2005, Peters et al. 2009, Jüngling et al. 2008, Likhtik et al 2008). These amygdala subregions have also been shown to exhibit NMDA-dependent plasticity as a result of sustained IfL activity during extinction learning (Royer and Pare 2002). Additionally, these regions have differential projections to the nucleus accumbens to control addiction related behaviors (Peters et al. 2009). The PrL is known to signal to the NAc and studies have shown that increased glutamate release in this pathway can serve as a trigger for the relapse of drug seeking behavior (Voorn et al. 2004, McFarland et al. 2003, LaLumiere and Kalivas 2008). Alternatively, the IfL projections to the NAs are responsible for extinction of drug seeking behaviors shown the correlation of increased glutamatergic plasticity in this region with extinction behavior as well as inactivation of this circuitry leading to a return of conditioned drug seeking (Peters et al. 2008, Peters et al. 2009, Sutton et al. 2003). These regions have been shown to be clinically relevant in disorders such as PTSD and AUD, where these patients exhibit extinction learning deficits and hyperactivity in response to fear and drug related cues due to alterations in these regions (Phelps et al. 2004, Milad et al. 2007, Milad et al. 2008, Garavan et al. 2000, Coffey et al. 2002). Thus, while this dissertation focuses on

the contribution of the PrL and IfL to fear- and drug-related behaviors, these downstream regions are further targets of interest that could be involved in the results reported herein.

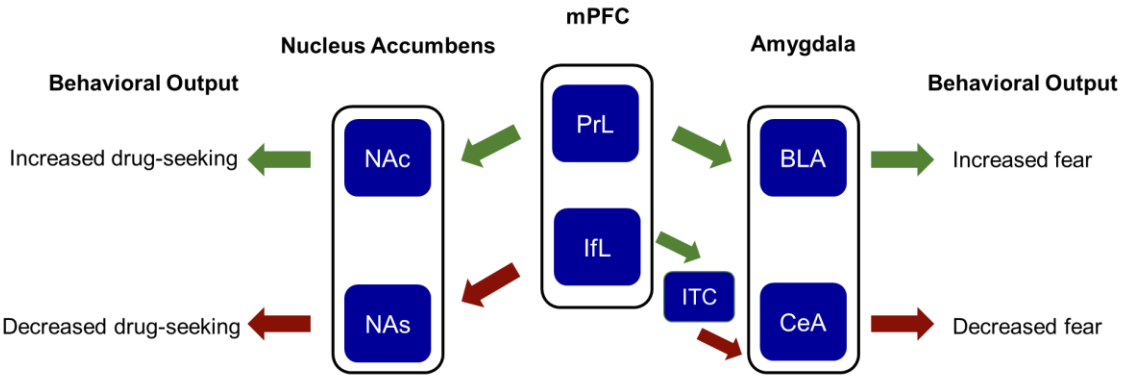

Figure 1-2: Downstream Targets of the Medial Prefrontal Cortex. The PrL and IfL subregions of the mPFC project to differential targets in order to control both fear promotion and drug seeking behaviors. The PrL projections to the BLA increase activity in this region to increase behavioral responses to fear cues while the IfL projects to the ITCs that decrease activity in the CeA which decreases the expression of fear and allows for extinction behavior. Alternatively, these regions project to separate regions of the accumbens to control drug-seeking behavior, with the PrL to NAc pathway increasing drug seeking and the IfL to NAs projections decreasing this behavior. Abbreviations: prelimbic, PrL; infralimbic, IfL; medial prefrontal cortex, mPFC; basolateral amygdala, BLA; intercalated cells of the amygdala, ITCs; central amygdala, CeA; nucleus accumbens core, NAc; nucleus accumbens shell, NAs.

Effects of THC, Ethanol, and Stress on PFC Glutamate Function

Since the PrL and IfL have been shown to be extensively involved in the control of fear and drug-seeking behaviors, it is important to examine how these regions are affected by stress and drug exposure to cause the disordered behavioral responses found in PTSD/SUD (McEwen and Gianaros 2011). Both acute and chronic stress exposure has been shown to affect glutamatergic transmission in the prefrontal cortex, and therefore cognitive functioning, through

increased glucocorticoid release that occurs following stress exposure (Popoli et al. 2011, Liston et al. 2006). The major glucocorticoid in rodents, corticosterone (CORT), has been shown to affect baseline glutamate release in the PFC and acute stress exposure or CORT administration will cause a rapid increase in glutamate in this region (Karst et al. 2005). Similarly, exposure to chronic stressors will cause long-term increases in glutamate in the PFC (Moghaddam 1993, Bagley and Moghaddam 1997). These alterations in glutamate signaling due to stress exposure will also cause detrimental changes in plasticity in the PFC to amygdala pathway that lead to lasting changes in behavioral responses to stress (Musazzi et al. 2015). Additionally, drastic increases in glutamate as a result of stress exposure can lead to excitotoxicity and cell death in the PFC which further impairs cognitive function (Popoli et al. 2011).

Alcohol has the ability to affect a wide variety of receptors throughout the brain to cause pervasive effects on neural function (Abernathy et al. 2010). In the prefrontal cortex, alcohol has been shown to affect glutamate signaling following both acute and long-term exposure (Abernathy et al. 2010, Burnett et al. 2015). Additionally, the severity of alcohol dependence has been corelated with glutamate levels recorded in cerebrospinal fluid in humans (Umhau et al. 2010). Alterations in glutamate signaling due to prolonged alcohol exposure have also been shown to be associated with deficits in cognitive functioning that can last even following prolonged abstinence periods of over a year (Burnett et al. 2015, Stavro et al. 2013) and the degree of cognitive impairments is correlated with an increased risk

for relapse (Abbott and Gregson 1981, Bowden-Jones et al. 2005). These alcohol dependent cognitive deficits have been shown to be a result of maladaptive changes in glutamate function in the PFC as a result of alcohol exposure (Burnett et al. 2015, Chandler et al. 1993, Qiang et al. 2007, Kroener et al. 2012). Often, these neuroadaptations lead to impairments in behaviors such as impulse control and cognitive flexibility, which lead to deficits in top down control and contribute to compulsive and high-volume alcohol consumption (Burnett et al. 2015, Kroener et al. 2012).

In addition to the glutamatergic alterations induced by stress and alcohol exposure, cannabis has also been shown to affect this system in the PFC especially when used during adolescence (Cass et al. 2014, Renard et al. 2017, Schneider et al. 2008). The PFC is highly remodeled during adolescence, including endocannabinoid and CB1 mediated synaptic pruning to maintain the excitatory/inhibitory signaling balance (Renard et al. 2017, Caballero et al. 2014, Thomases et al. 2013). Thus, if CB1 activity is altered through external sources, such as cannabis, this refinement process can be disrupted leading to long-term changes in excitatory signaling in the PFC as a result of cannabis exposure (Cass et al. 2014). Essentially, increased CB1 activity during the adolescent phase of development impairs the maturation of GABA synapses and subsequently allows for a hyperactivity of the glutamate synapses in the PFC (Cass et al. 2014, Renard et al. 2017). Therefore, cannabis use during adolescence could have long-term

effects on behaviors that depend on the PFC including responses to stress and drug cues (Meier et al. 2012, Solowij et al. 2002, O'Shea et al. 2004).

Glutamatergic Modulators as PTSD/SUD Treatment

The glutamate system in the PFC has increasingly become a treatment target for PTSD/SUD due to the high level of involvement of glutamatergic functioning in this region in the disordered behavioral regulation found with these disorders. A variety of glutamatergic modulators that target both ionotropic NMDA receptors as well as metabotropic mGlu5 receptors have been studied as a treatment for both PTSD and AUD (Averill et al. 2017). Ketamine, an NMDA antagonist, has recently been explored as a treatment for PTSD and has been shown to cause significant reductions in the severity of PTSD symptoms when compared to midazolam, an anxiolytic medication commonly used to treat PTSD related anxiety (D'Andrea and Sewell 2013, Feder et al. 2014). Additional therapeutics include d-cycloserine (DCS), a partial agonist at NMDA receptors, which has been used clinically with variable effects (Difede et al. 2013, Attari et al. 2014). DCS has been used as an adjunct to psychological treatments, including exposure therapy, to modulate NMDA receptors and plasticity to improve extinction learning (Averill et al. 2017, Norberg et al. 2008). Similarly, glutamatergic modulators have been used in the treatment of addictive disorders (Joffe et al. 2018). DCS has also been used for its effects on drug-seeking and has been shown to facilitate the extinction of this behavior (Botreau et al. 2006). Along with

targeting glutamate receptors to enhance extinction and treat AUD, pharmacotherapies have been used to target glutamate reuptake at glutamate transporter 1 (GLT1) to decrease the glutamate spillover often associated with this disorder (Rao et al. 2015). Specifically, ceftriaxone has been used to increase GLT1 and has been shown to decrease ethanol consumption as well as attenuate withdrawal and relapse (Rothstein et al. 2005, Rao and Sari 2012, Sari et al. 2011). Therefore, due to the role of glutamate in mediating the symptomology of PTSD and AUD, the studies completed in this dissertation use glutamatergic modulators to alter the effects of stress and drug exposure on prefrontal cortex reliant signaling and cognition.

One pharmacotherapy used for these studies is CDPPB (3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl) benzamide). CDPPB is a positive allosteric modulator of mGlu5 receptors that has been shown to affect glutamate signaling in the PFC and behavioral responses to fear and drug related learning paradigms following drug exposure in preclinical models of PTSD and AUD (Gass et al. 2018, Smiley et al. 2020). Specifically, CDPPB has been shown to enhance the extinction of cocaine and ethanol seeking behaviors, and these behavioral changes are associated with enhanced plasticity in the mPFC (Gass and Olive 2009, Cleva et al. 2011, Gass et al. 2018). In these studies, the effects of CDPPB were shown to be mGlu5 dependent, since infusions of an mGlu5 antagonist into the mPFC prevented the effects of CDPPB on the extinction of alcohol seeking behavior (Gass et al. 2014). Thus, due to the highly overlapping underlying circuitry responsible for drug

seeking and fear expression, further studies covered under this dissertation utilized CDPPBs ability to enhance extinction by targeting fear extinction learning.

N-acetylcysteine (NAC) is a clinically available therapeutic that has recently become a popular treatment for a variety of psychological disorders, including PTSD and AUD, due to its ability to affect glutamate homeostasis in the brain (Reissner and Kalivas 2010). Both clinically and preclinically, NAC has been shown to affect drug seeking behavior, clinically through a reduction in drug use when administered to veterans with comorbid PTSD/SUD (Back et al. 2017) and preclinically by reducing stress-cue induced relapse in alcohol self-administration models (Garcia-Keller et al. 2019). Preclinical studies have also sought to determine the method through which NAC is exerting these effects. Both stress and alcohol exposure have been shown to alter the levels of GLT1 and NAC has been shown to restore GLT1 levels while reducing drug seeking behaviors (Kalivas and Volkow 2011, Brown et al. 2013). Therefore, due to its ability to normalize glutamatergic functioning in the brain and effects on treating the deleterious effects of stress exposure and drug exposure, NAC is a promising candidate for the treatment of co-morbid PTSD/AUD and needs to be further tested in animal models to discern the mechanism of action through which it is working to impart these effects.

Summary

PTSD and SUD are disorders of learning and memory that involve deficits in overlapping circuitry in the prefrontal cortex. These disorders often occur comorbidly due to the anxiolytic properties of drugs such as cannabis and alcohol. While acutely these substances will decrease the feeling of anxiety, they also lead to long term alterations in prefrontal glutamate function that can cause a progression of cognitive deficits that impair the effectiveness of pharmacological and psychological treatments of PTSD. Therefore, it is increasingly important to study the prefrontal cortex to determine how stress and drug exposure are affecting this region and to determine glutamate functioning in this region could serve as a target for the treatment of PTSD altered by substance abuse.

Statement of Problem and Specific Aims

Post-traumatic stress disorder and comorbid substance use disorder is a highly prevalent and debilitating reality for many patients. As a result of stress and drug exposure, there are a variety of deleterious effects on the prefrontal cortex in terms of signaling and activity as well as behavioral and cognitive consequences. This is reflected in both clinical and preclinical studies that demonstrate that the presence of both PTSD and SUD result in worst symptomology as well as decreased efficacy of both pharmacological and psychological treatment options. Therefore, it is increasingly important to examine the bidirectional relationship

between stress exposure and drug use to determine optimal targets for treatment. The experiments presented within this dissertation use fear conditioning as well as chronic intermittent ethanol exposure and THC vapor exposure to model PTSD, alcohol use, and cannabis use to answer questions regarding the effects of drug exposure on fear learning as well as the effects of stress exposure on drug intake. The overall hypothesis for these studies is that exposure to THC and ethanol alters signaling in the prefrontal cortex to affect fear learning and memory and that, conversely, stress exposure escalates responses to drug related cues through a similar mechanism. The following aims were designed to test this hypothesis.

SPECIFIC AIM 1: TEST THE HYPOTHESIS THAT STRESS EXPOSURE CAUSES ALTERATIONS IN ETHANOL SEEKING AND COGNITION THAT CAN BE PREVENTED WITH N-ACETYLCYSTEINE.

Following exposure to a traumatic event, patients that develop PTSD often use substances like alcohol to self-medicate symptoms of anxiety that are often experienced when exposed to trauma-related cues. Additionally, these patients experience the development of cognitive deficits that impairs the effectiveness of psychological treatment options and worsens symptomology. Therefore, to study this relationship in preclinical models, we used a model of PTSD/AUD using restraint stress followed by ethanol self-administration to see how prior exposure to stress effects ethanol seeking during acquisition and reinstatement. Further, animals were tested in a strategy set-shifting task to assess how stress exposure

affects cognition. Finally, we tested N-acetylcysteine in a variety of different treatment schedules to optimize the use of this drug for the prevention of stress-induced alterations in ethanol seeking and cognition.

SPECIFIC AIM 2: TEST THE HYPOTHESIS THAT CHRONIC ETHANOL EXPOSURE IMPAIRS FEAR LEARNING THROUGH PREFRONTAL GLUTAMATERGIC MODULATION.

Due to the bidirectional relationship between stress exposure and alcohol abuse, we next wanted to test the effects of chronic ethanol on fear learning using an alternate model of PTSD/AUD using fear conditioning and chronic intermittent ethanol exposure (CIE). First, we tested the effects of CIE on fear extinction learning and responding during recall testing and then used CDPPB to enhance extinction learning in these animals. Microinjection experiments were then completed to determine the neuronal localization of both CIE and CDPPB's effects on extinction learning by implanting canulae into either the prelimbic (PrL) or infralimbic cortex (IfL). Next, we wanted to further investigate the relationship between chronic ethanol exposure and fear learning by using optogenetics in this PTSD/AUD model to inhibit either the PrL or IfL during fear memory reconsolidation to alter fear extinction learning.

SPECIFIC AIM 3: TEST THE HYPOTHESIS THAT THC INTERACTS WITH ETHANOL AND STRESS TO INDUCE FURTHER COGNITIVE DEFICITS DUE TO IMPAIRMENTS IN PREFRONTAL CORTEX GLUTAMATERGIC FUNCTIONING.

Along with alcohol, cannabis is the most highly abused substance by PTSD patients to acutely reduce anxiety. Additionally, there is an inverse relationship where cannabis abuse is associated with a higher chance of developing PTSD and AUD in the future. As such, we then wanted to determine how THC and ethanol exposure interact to cause deficits in prefrontal cortex reliant behavioral paradigms including cognitive flexibly, drug seeking, and responses to fear stimuli. Vapor exposure to both THC and ethanol was followed by testing in strategy set-shifting procedures, ethanol self-administration, and fear conditioning paradigms to assess how drug exposure affects behavioral responses to these mPFC dependent tasks. Further, calcium imaging through fiber photometry was used to assess the activity of the PrL following exposure to THC and ethanol.

Chapter 2

N-ACETYLCYSTEINE PREVENTS STRESS INDUCED ESCALATIONS IN ETHANOL SEEKING AND DEFICITS IN BEHAVIORAL FLEXIBILITY

Background and Significance

Post-traumatic stress disorder (PTSD) and alcohol use disorder (AUD) are two psychiatric disorders that have become increasingly prevalent and debilitating in terms of symptomology and cost to society (Smith and Cottler 2018). Not only do these conditions cause a variety of incapacitating symptoms, but they often occur co-morbidly with even worse symptoms and treatment outcomes (Dworkin et al. 2018; Petrakis et al. 2017). Some of the most common PTSD symptoms include re-experiencing the trauma through events like flashbacks and nightmares

that are induced following exposure to cues that are reminiscent of the original trauma event (Ralevski et al. 2014). In both preclinical and clinical research, it has been shown that exposure to chronic ethanol can cause deficits in fear cue extinction learning and memory which can further fear cue associations and allow for continued maladaptive cue responding in PTSD patients that also have AUD (Nosen et al. 2015; Vujanovic et al. 2019). Additionally, there are few methods of treatment available for these disorders and they are often not effective (Flanagan et al. 2018). Thus, there is a need for further research into the establishment and maintenance of these disorders to develop pharmacological treatment options for comorbid PTSD and AUD.

Recent studies have focused on the glutamate system as a target for treatment of both addictive (Kalivas and Volkow et al. 2011; Gass and Olive 2007) and anxiety disorders (Averill et al. 2017; Petrakis et al. 2017) as well as their comorbidity (Verplaetse et al. 2018; Smiley et al. 2020). Exposure to both trauma and drugs of abuse can have long term effects on plasticity and homeostasis that can lead to dysregulation of fear and alcohol seeking behaviors in the future (Averill et al. 2017; Rao et al. 2015; Hwa et al. 2017, Gonzales et al. 1997). In addition to glutamatergic modulators such as ketamine and D-cycloserine, N-acetylcysteine (NAC) has become a popular therapeutic to target this dysregulated plasticity to treat PTSD and AUD (Averill et al. 2017; Tomoko et al. 2018; McClure et al. 2014; Flanagan et al. 2016). NAC has been used in recent studies that examined the effect of NAC on PTSD and SUD symptoms in veterans, and NAC treated patients

reported significant improvements in both drug craving as well as PTSD symptoms compared to placebo (Back et al. 2016).

Thus, the present experiments examined this relationship in animal models using restraint stress followed by alcohol self-administration to mimic a discrete stressor event followed by compulsive alcohol use. NAC was used to reduce stress cue-induced reinstatement of alcohol seeking, which serves as a preclinical model of trauma cue exposure induced relapse. These experiments found that exposure to acute restraint stress contributed to increased alcohol seeking during acquisition, extinction, and reinstatement and that NAC treatment administered either before, after, or surrounding the stressor was able to prevent this increased drug seeking.

Materials and Methods

Animals

Male Wistar rats (PD 52, 250 g on arrival; Charles River Laboratories, Raleigh, North Carolina) were double housed and habituated to a reverse light/dark cycle with lights off at 9 a.m. Food and water was available *ad libitum* throughout all experiments, except for during behavioral testing (~1-2 hours per day). Animals had 1 week of acclimation in the colony before any testing or treatment commenced. All experiments were approved by the Animal Care and Use Committee of the Medical University of South Carolina and were in

accordance with guidelines set forth by the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003). A total of 120 animals were used across four main experiments and were broken up into the treatment groups referenced in **Table 2-1**.

Table 2-1. Treatment Groups For Experiments 1-4

Treatment Groups			
Experiment 1 n = 6/group	**Experiment 2** n = 8/group	**Experiment 3** n = 8/group	**Experiment 4** n = 6/group
Sham Stress + Vehicle	Sham Stress + Vehicle	Sham Stress + Vehicle	Sham Stress + Vehicle
Sham Stress + NAC @ Ext & Rst	Sham Stress + NAC @ Stress	Sham Stress + NAC	Sham Stress + NAC @ Stress
Stress + Vehicle	Stress + Vehicle	Stress + Vehicle	Stress + Vehicle
Stress + NAC	Stress + NAC @ Stress	Stress + NAC Pre-Stress	Stress + NAC @ Stress
		Stress + NAC Post-Stress	

Acute Restraint Stress

Animals were exposed to an acute stressor in the form of a 2-hour restraint stress exposure session. Each animal was placed into a flat-bottomed Plexiglas restrainer measuring 3.25 inches in diameter and 8 inches in length and placed into a new clean cage for the duration of the test. A conical tube cap containing 3 mL of an essential oil was included within each of the cages to be used as a stress-related cue in further testing. For Experiment 1, a crossover design was used with regards to stress-paired odor cues where half of the rats were exposed to a sandalwood scent while the other half received lemon odor exposure while restrained. Following Experiment 1, which showed that responding was specific to

the stress paired and not the unpaired-odor cue, all animals were exposed to sandalwood odor in further experiments. Sham animals were transferred to new cages and kept there for two hours in the presence of the odor but were not restrained. Animals were monitored for differences in body weight and coat texture in the following weeks to ensure that the stress exposed animals did not differ from sham animals with regards to heath.

Alcohol Self-Administration

Prior to operant training, all animals were exposed to a two-bottle choice paradigm that involved a bottle of 20% ethanol along with a bottle containing water placed on the home cage for three days per week for two weeks to familiarize animals with the sensory aspects of the drug. Two days following the last two-bottle choice session, animals were placed into operant chambers and trained to lever press for 20% ethanol on an FR-1 schedule of reinforcement based on our previously published methods (Gass 2014a). Sessions occurred three times per week and lasted for one hour with each press on the active lever delivering 20% ethanol in water for 1.5 seconds with the stimulus light illuminated and a tone presented (2900 Hz, 65 dB). Each active lever press was followed by a 4 second timeout during which presses were recorded but did not lead to reinforcer delivery. Once response criteria of 30 lever presses was met (~8-10 sessions), ethanol concentration was lowered to 10% and criteria of 60 lever presses was met (~12 sessions). Animals completed extinction training once self-administration behavior

was established (~20 sessions),. During extinction sessions, pressing on the active lever occurred in the absence of any light/tone cues or ethanol reinforcer. Extinction criteria was met when lever pressing for each rat was below 20% the rate of lever pressing recorded on the last two days of self-administration training (~8 sessions).

Stress Cue-Induced Reinstatement

Animals were put into reinstatement testing following the extinction of lever pressing behavior. During each 30-minute session, lever presses were recorded but did not result in light/tone presentation or ethanol delivery. Two reinstatement tests occurred, one in the presence of the stress paired odor and the second in the presence of the non-paired order. Using a random crossover design, half of the animals received their paired odor first while the other half received the non-stress paired odor during the first reinstatement test. Each reinstatement test was separated by two subsequent extinction sessions to reduce lever pressing prior to the second reinstatement test. Experiment 1 established that reinstatement effects were specific to the stress paired odor and, therefore, all following experiments used sandalwood odor as the stress paired cue and, thus, only required a single reinstatement test (experimental design shown in **Figure 2-1**).

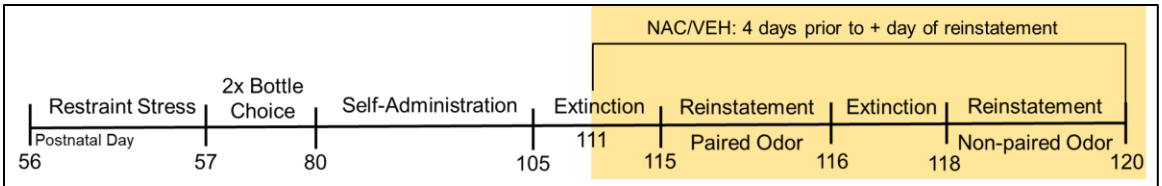

Figure 2-1: Experimental Design for Experiment 1. Animals were first exposed to a single acute restraint stress session before returning to their home cage where they were exposed to two-bottle choice for either water or 20% ethanol over the next two weeks. Self-administration commenced once animals became familiar with the ethanol solution. Following successful acquisition of self-administration behavior, lever pressing was extinguished over the next week. Extinction sessions were followed by both stress-paired odor reinstatement and non-paired odor reinstatement. NAC or Vehicle treatment was given for four days prior to and on the day of reinstatement. This design corresponds to data in figure 2-4 and 2-5.

NAC Administration and Schedules

For all experiments, NAC was administered at a dose of 100 mg/kg and was prepared the day of injection in 27 mg/mL NaOH in saline and pH was adjusted to 7.2. Vehicle of 0.9% saline was administered to all other animals and injections were delivered intraperitonially (IP). A variety of different injection schedules were tested to optimize the timeline of treatment and the effects on the glutamate system. For Experiment 1, NAC was administered for 4 days prior to and on the day of reinstatement testing. Experiment 2 involved NAC treatment for 4 days prior to, the day of, and four days following the restraint stress exposure session, while Experiment 3 included treatment groups that received either NAC for 4 days prior to the stressor or for 4 days following the stressor (**Figure 2-2**).

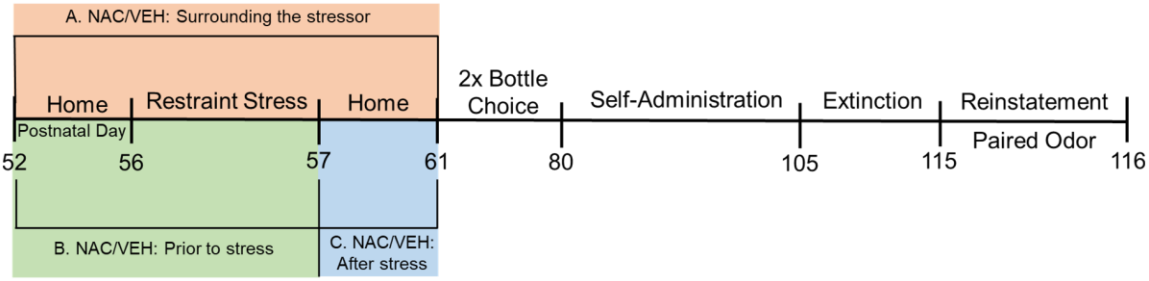

Figure 2-2: Experimental Design for Experiment 2 and 3. For Experiment 2, NAC was given for four days prior to the stressor, the day of stress, and for four days following the stressor (A). In Experiment 3, NAC was given either for four days prior to the stressor and on the day of stress (B) or for the four days following the stressor (C). After 2-bottle choice, self-administration, and extinction training, animals were exposed to a single reinstatement session in the presence of the stress paired odor. This design corresponds to data in figure 2-6, 2-7, 2.8, and 2.9.

Set-Shifting Analysis of Behavioral Flexibility

A between-session strategy set-shift test was used to assess the effects of stress and NAC exposure on learning, behavioral flexibility was examined using (**Figure 2-3**). A separate group of 24 animals were trained to press both the right and left levers to receive 10% sucrose followed by retractable lever training and determination of a side preference. Animals were then trained under a first rule which required animals to respond to the lever that was cued by illumination of the associated stimulus light and criteria for this rule was met when animals pressed 8 times in a row correctly in a minimum of 30 trials. Once this criterion was met, animals were tested in a single session under a second rule that required animals to press on a single lever location regardless of where the light appears. Animals that take longer to learn the second rule and continue responding for the original rule are considered to have impaired behavioral flexibility. We also recorded the type of errors that animals were making during this trial, mainly perseverative

errors which are presses on the incorrect lever before the animal has made five correct presses under the second location rule.

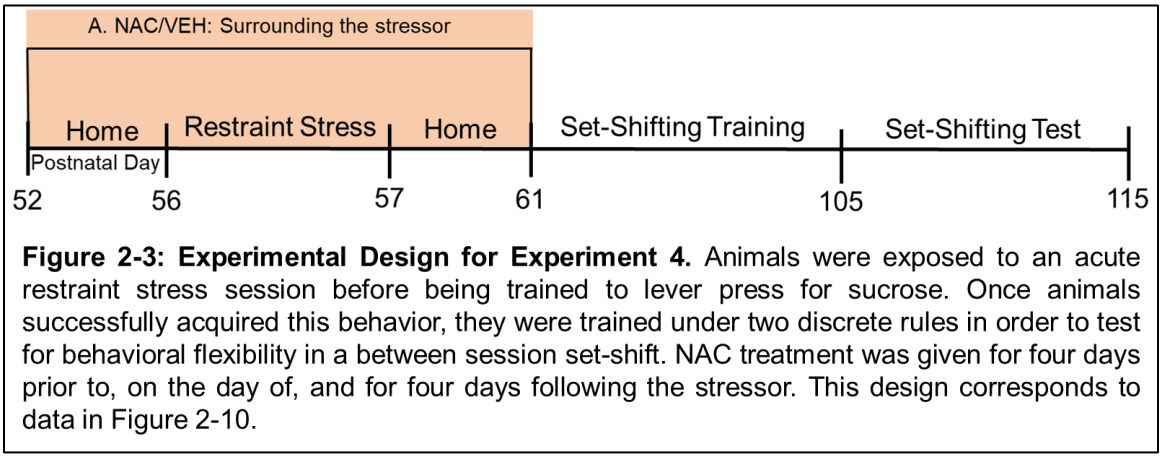

Figure 2-3: Experimental Design for Experiment 4. Animals were exposed to an acute restraint stress session before being trained to lever press for sucrose. Once animals successfully acquired this behavior, they were trained under two discrete rules in order to test for behavioral flexibility in a between session set-shift. NAC treatment was given for four days prior to, on the day of, and for four days following the stressor. This design corresponds to data in Figure 2-10.

Statistics

All analyses were performed using Prism version 8.0 (GraphPad Inc). Power analyses were completed to determine optimal group sizes with a significance level of $p < 0.05$. For single comparisons, analyses used a two-tailed Student's t-test while multiple comparisons were completed using two-way ANOVAs. Repeated measures ANOVAs were used for within subject comparisons. A Bonferroni post-hoc analysis was performed if the interactions found in the ANOVAs were significant.

Results

Exposure to acute restraint stress leads to increases in ethanol seeking

First, we determined the long-term effects of a single acute restraint stress exposure on ethanol seeking behavior during self-administration, extinction, and stress-cue induced reinstatement (**Figure 2-1**). Animals were exposed to a 2-hour restraint stress or sham stress in the presence of either a lemon or sandalwood scent to serve as a conditioned odor cue during later phases of training. Following stress exposure, all animals underwent 2 weeks of two bottle choice for 20% ethanol before being trained in operant self-administration followed by extinction. There was no difference between stress and sham animals during two-bottle choice (**Figure 2-4A**) or during the first 10 sessions of ethanol self-administration where animals were still learning ethanol seeking behavior (**Figure 2-4B**).

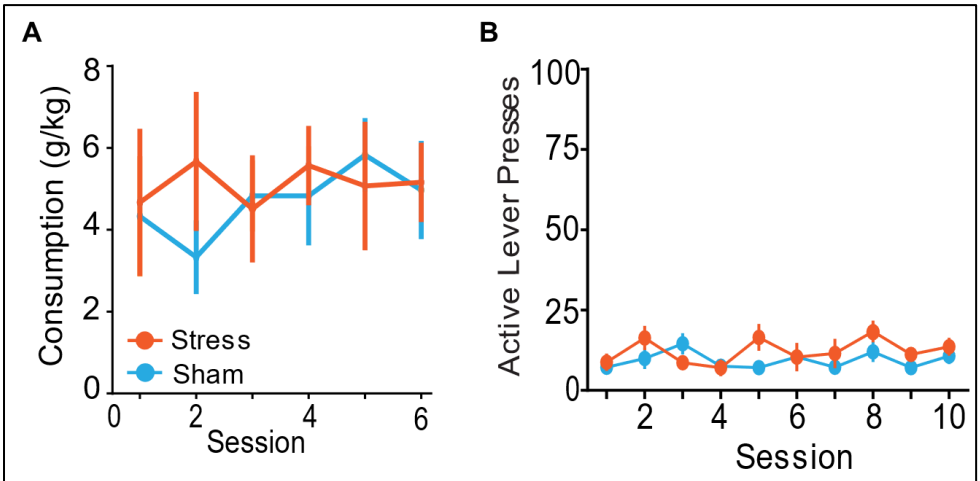

Figure 2-4: Stress and sham animals did not differ in ethanol intake during the initial phase of alcohol exposure. A) During the 2-bottle choice that preceded self-administration training, there was no difference between the groups in ethanol consumption. **B)** Similarly, there was no difference in lever pressing during the first ten self-administration sessions when animals were still acquiring ethanol seeking behavior (n=12/group).

Animals that were previously exposed to restraint stress exhibited increased ethanol seeking during self-administration following acquisition. These animals increased lever pressing when compared to sham animals during the last ten self-administration sessions (**Figure 2-5A**, two-way RM-ANOVA time $F(5, 115) = 69.97$, $p < 0.001$, stress vs. sham $F(1,22) = 68.54$, $p < 0.001$, interaction $F(9,198 = 5.803$, $p < 0.001$). Additionally, stress exposed animals also sought more ethanol reinforcers during these sessions when compared to sham animals (**Figure 2-5B**, two-way RM-ANOVA time $F(5,115) = 80.64$, $p < 0.001$, stress vs. sham $F(1,22) = 58.38$, $p < 0.001$, interaction $F(9,198) = 5.512$, $p < 0.005$). This stress-induced increase in ethanol seeking was also present when examining at the total number of reinforcers received across the entirety of self-administration (**Figure 2-5C**, two-way ANOVA stress vs. sham $F(1,20) = 347.7$, $p < 0.001$). Following self-administration, animals were exposed to 8 extinction sessions and stress exposed animals exhibited increased lever pressing during this phase of training as well (**Figure 2-5D**, two-way RM-ANOVA time $F(5,105) = 8.35$, $p < 0.001$, stress vs. sham $F(1,22) = 70.83$, $p < 0.001$, interaction $F(9,198) = 8.35$, $p < 0.001$).

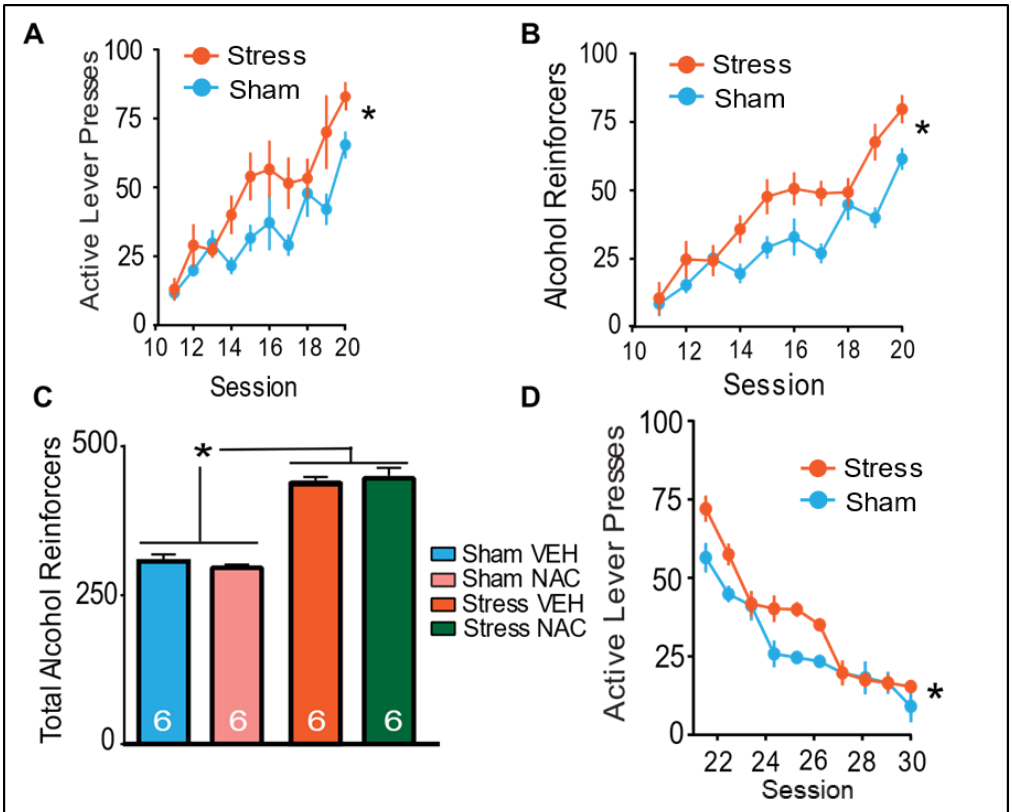

Figure 2-5: Exposure to restraint stress increased ethanol seeking during self-administration acquisition and extinction. A) Stress exposure increases active lever pressing during the last ten sessions of self administration. **B)** When compared to sham groups, stress exposed animals sought an increased number of reinforcers during multiple self-administration sessions. **C)** When looking at overall reinforcers received throughout self-administration, stress exposed animals received more reinforcers than sham animals. Both the stress and sham groups were broken up evenly to reflect similar levels of reinforcers received for groups that were to receive NAC or Vehicle later in testing. **D)** During extinction, stress exposed animals retained heightened lever pressing longer than sham animals (Stress vs. sham, *$p < 0.001$, n=12/group).

Escalations in ethanol seeking during reinstatement following stress exposure are prevented with NAC treatment

Following the extinction of lever pressing behavior, animals were tested for reinstatement of alcohol seeking behavior. For 4 days prior to and on the day of the reinstatement test, animals were administered I.P injections of either 100 mg/kg NAC or Vehicle (0.9% saline). Rats within each exposure group were evenly distributed into NAC and Vehicle treatment groups so that animals did not differ in ethanol seeking behavior prior to treatment (two-way ANOVA NAC vs. Veh $F(1,20) = 0.57$, $p = 0.46$). Stress exposed animals exhibited increased lever pressing in response to the stress paired odor cue during reinstatement when compared to sham animals, and NAC treatment prior to reinstatement resulted in a decrease in active lever presses when compared to stressed animals that received vehicle (**Figure 2-6A**, three way RM-ANOVA Ext vs. Rst $F(1,20)$, 56.78, $p < 0.001$, stress vs. sham $F(1,20) = 55.33$, $p < 0.001$, NAC vs. Vehicle $F(1,20) = 47.57$, $p < 0.0001$, interaction $F(1,20) = 82.07$, $p < 0.001$). There were no differences in lever pressing behavior between any of the treatment groups when examining reinstatement sessions completed in the presence of the odor that was not paired with the stressor (**Figure 2-6B**, three-way RM-ANOVA Ext vs. Rst $F(1,20) = 2.32$, $p =0.144$, sham vs. stress $F(1,20) = 0.35$, $p = 0.559$, NAC vs. Vehicle $F(1,20) = 0.55$, $p = 0.466$, interaction $F(1,20) = 0.69$, $p = 0.416$) or in inactive lever presses during the stress paired (**Figure 2-6C**) and unpaired odor (**Figure 2-6D**) reinstatement sessions.

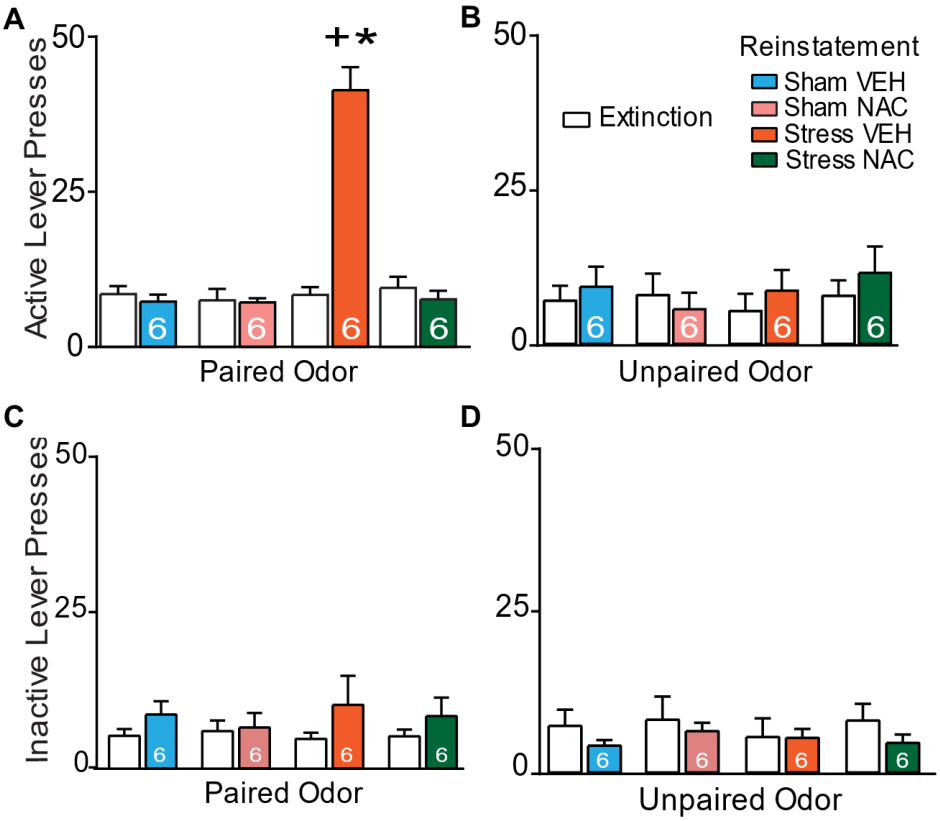

Figure 2-6: Exposure to the stress paired odor increases ethanol seeking during reinstatement and this effect is prevented with NAC treatment. A) NAC given to previously stressed animals during extinction and reinstatement reduced active lever pressing during stress-paired odor cue reinstatement when compared to stressed animals that did not receive NAC (*p < 0.05 comparing stress + Veh vs. stress + NAC, +p < 0.05 comparing within group). **B)** When reinstatement occurred in the presence of the odor that was not paired with the stressor, there were no changes in active lever pressing. Additionally, there were no differences in inactive lever presses during either the paired **(C)** or unpaired **(D)** odor reinstatement sessions.

NAC treatment surrounding, prior to, and following the stressor all served as effective treatments

Since NAC can have differential effects on the glutamate system depending on the timing of treatment, two additional experiments were completed to determine the treatment schedule that was the most effective at reducing responding during reinstatement (**Figure 2-2**). The first experiment involved NAC administration for 4 days prior to, on the day of, and for 4 days following the stressor to prevent stress induced changes in glutamatergic functioning. There were no differences found between any groups in terms of ethanol consumption during 2-bottle choice (**Figure 2-7A**) or the first ten sessions of self-administration (**Figure 2-7B**). Once animals acquired self-administration behavior, stress exposed animals exhibited an increase in active lever pressing compared to both sham exposed animals and those that had been exposed to restraint stress and treated with NAC surrounding the stressor (**Figure 2-7C**, two-way RM-ANOVA NAC vs. Vehicle $F(3,28) = 13.72$, $p < 0.001$, time $F(9,52) = 248.9$, $p < 0.001$, interaction $F(27,252) = 2.575$, $p < 0.001$). This increase in ethanol seeking was also reflected by an increased number of reinforcers received across the entirety of self-administration training by stress exposed animals when compared to Sham + Veh and Sham + NAC animals as well as stress exposed animals that were treated with NAC (**Figure 2-7D**, one-way ANOVA $F(3,28) = 226.7$ $p < 0.001$).

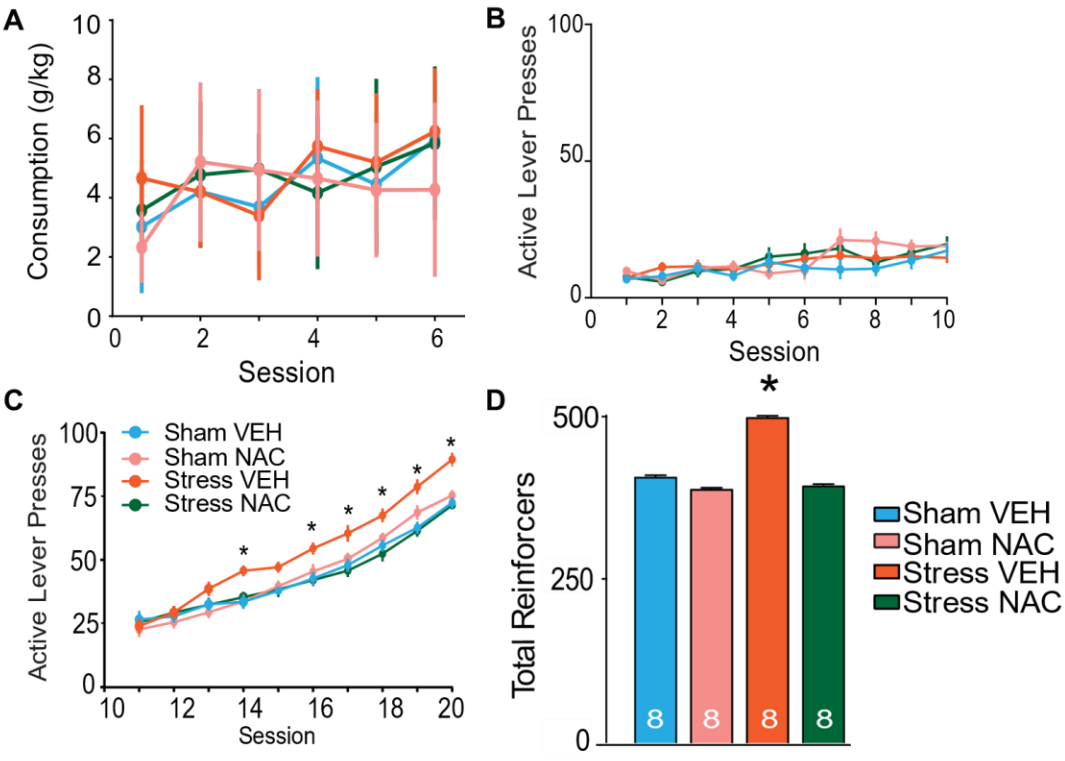

Figure 2-7: Stress exposure leads to increased ethanol seeking during acquisition and this is prevented with NAC treatment surrounding the stressor. A) During 2-bottle choice, there were no differences between any treatment group in terms of ethanol consumption. **B)** Additionally, there were no differences in active lever pressing in the first ten sessions during the acquisition phase of self-administration. **C)** During the last ten sessions of self-administration, stressed animals completed an increased number of active lever presses when compared to both the sham groups as well as the stressed animals that received NAC surrounding the stressor. **D)** This increase in ethanol seeking was also apparent when examining total reinforcers received throughout self-administration, where stress exposed animals sought significantly more reinforcers when compared to all other treatment groups (*$p < 0.05$).

Following self-administration, lever pressing behavior was extinguished before animals were put into stress cue-paired reinstatement testing. Again, stress exposure served to increase active lever pressing during reinstatement in the presence of the stress paired odor, while NAC treatment administered surrounding the stressor was able to prevent this heightened ethanol seeking (**Figure 2-8A**).

Additionally, there was no differences between any treatment groups in inactive lever pressing during this reinstatement session (**Figure 2-8B).** Taken together, these data suggest that NAC treatment surrounding a stressor is effective at preventing stress-induced increases in ethanol seeking.

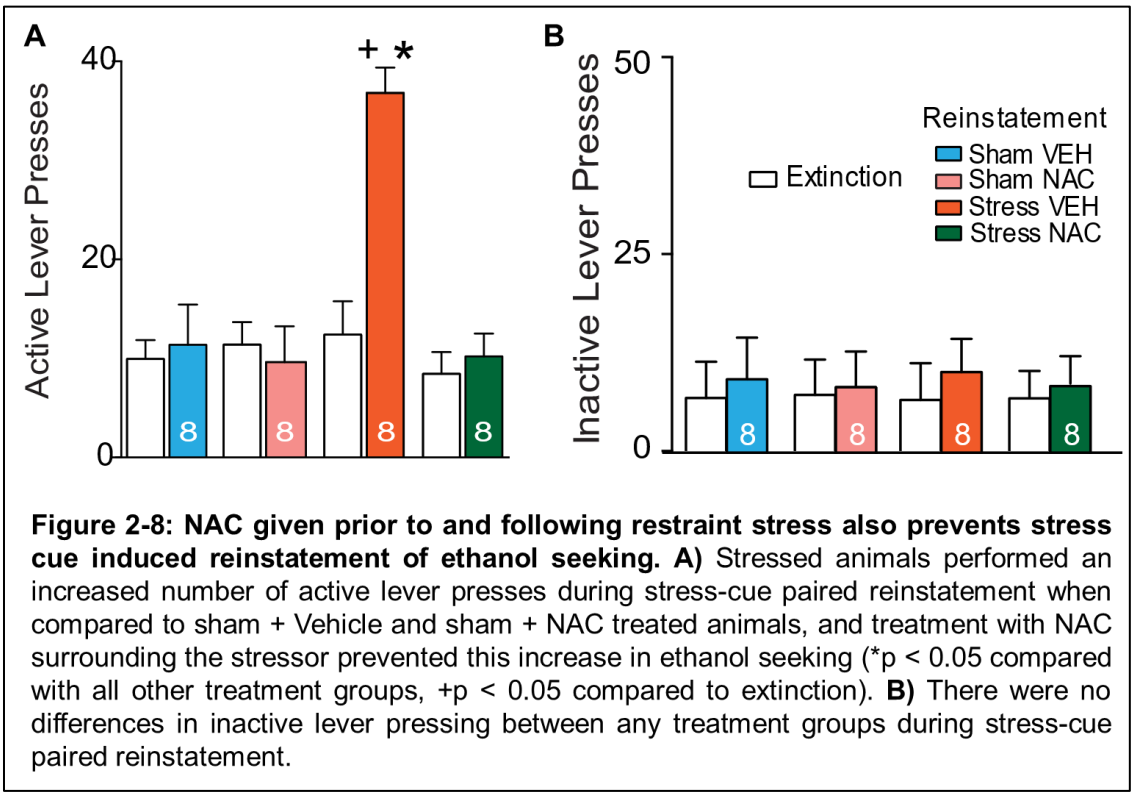

Figure 2-8: NAC given prior to and following restraint stress also prevents stress cue induced reinstatement of ethanol seeking. A) Stressed animals performed an increased number of active lever presses during stress-cue paired reinstatement when compared to sham + Vehicle and sham + NAC treated animals, and treatment with NAC surrounding the stressor prevented this increase in ethanol seeking (*$p < 0.05$ compared with all other treatment groups, +$p < 0.05$ compared to extinction). **B)** There were no differences in inactive lever pressing between any treatment groups during stress-cue paired reinstatement.

Once NAC was established as an effective treatment when administered surrounding the stressor, our next experiment was designed to further narrow down the necessary treatment regimen for NAC. Two different NAC administration schedules were implemented to determine if NAC treatment either prior to or

following restraint stress was able to prevent stress induced escalations in ethanol seeking during self-administration and reinstatement. As such, NAC was administered for either 4 days before stress and the day of stress or for 5 days after the stress occurred (**Figure 2-2**). Again, there were no changes in ethanol seeking during either 2-bottle choice (**Figure 2-9A**) or the first ten sessions of self-administration (**Figure 2-9B**). Additionally, we were able to replicate the ability of stress exposure to increase lever pressing during self-administration and NAC treatment both prior to or following the stressor were effective at preventing increased ethanol seeking (**Figure 2-9C**, two-way RM ANOVA treatment $F(4,350)$, $P < 0.0001$, time $F(9,350) = 179$, $p < 0.001$, interaction $F(36,350) = 1.83$, $p < 0.001$). This effect was also reflected in the increased number of reinforcers sought by stress exposed animals that was prevented with NAC treatment either before or after the stressor (**Figure 2-9D**, one-way ANOVA $F(4,35) = 231.2$, $p < 0.001$).

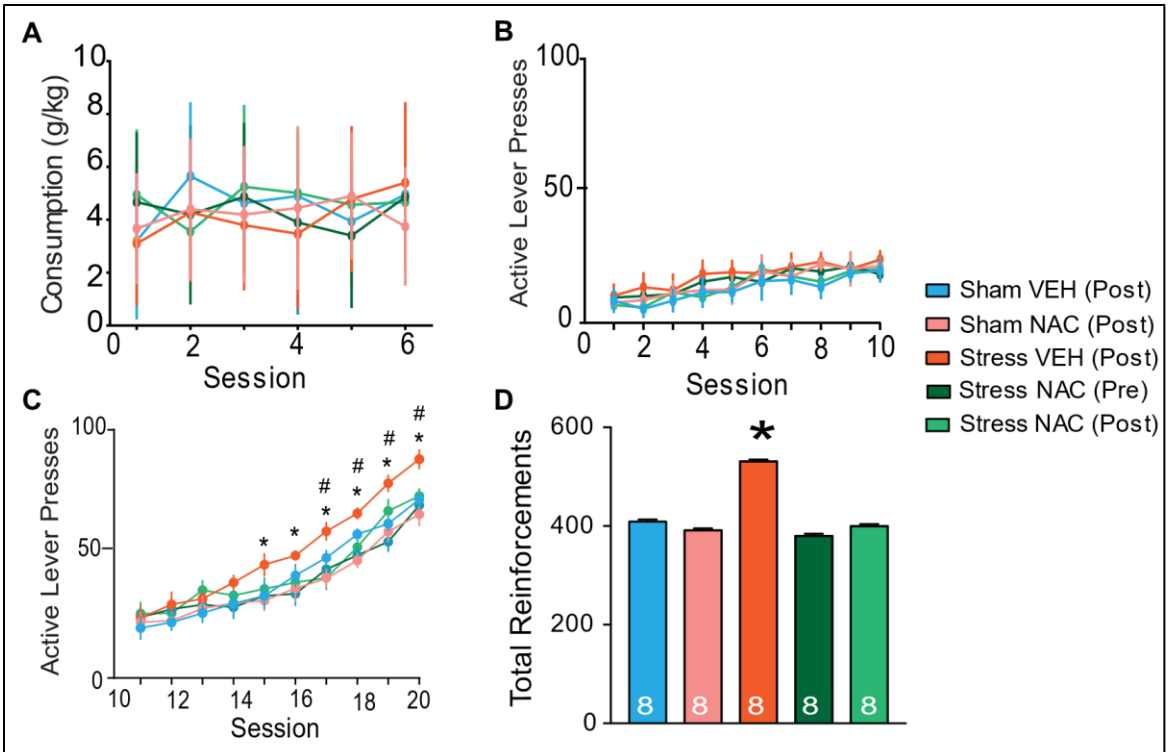

Figure 2-9: NAC given either prior to or following the stressor prevents stress induces escalations in ethanol seeking during self-administration. A) There were no differences between any treatment groups with regard to ethanol consumption during 2-bottle choice, or **(B)** during the first ten sessions of self-administration. **C)** While stress exposure led to increased active lever pressing during the last ten sessions of self-administration, both NAC given pre-stress and NAC post-stress prevented this increase in ethanol seeking (*p < 0.05 comparing stress and sham groups, #p < 0.05 comparing stress + Veh and stress + NACpre and NACpost groups). **D)** Stress exposed animals exhibited significantly more reinforcers than sham groups, and NAC given either pre- or post-stress was able to prevent this increase in ethanol seeking (*p < 0.05 compared to all other treatment groups.

Following the extinction of lever pressing behavior, animals were tested in stress-cue induced reinstatement. During this session, animals that were previously exposed to restraint stress exhibited increased lever pressing behavior when compared to all other groups, and animals administered NAC either prior to or following the stressor did not exhibit this relapse-like behavior (**Figure 2-10A,** two-way RM-ANOVA Ext vs. Rst $F(1,35) = 57.42$, $p < 0.0001$, treatment $F(4,35) =$

28.37, p < 0.0001, interaction F(4,35) = 29.28, p < 0.0001). This behavior was specific to active lever presses, since there were no differences between any treatment group in inactive lever pressing (**Figure 2-10B**).

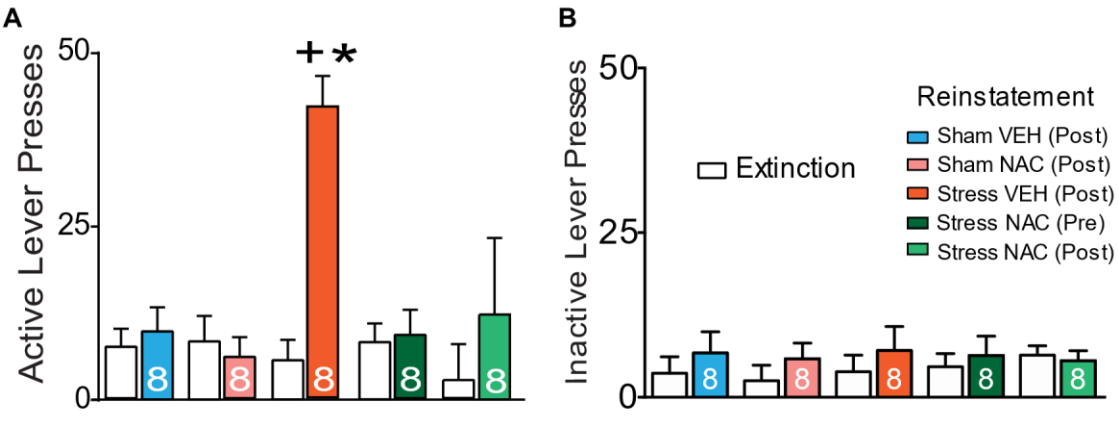

Figure 2-10: Stress cue induced reinstatement is prevented by NAC treatment given either before or after the stressor. A) Stress exposure lead to increases in active lever pressing during stress-cue paired reinstatement and this is prevented by NAC treatment given either before or after the restraint stress exposure session (*p < 0.05 compared to all other groups, +p < 0.05 compared to extinction). **B)** There were no differences between any treatment group in inactive lever presses during this session.

Stress exposure causes deficits in behavioral flexibility that are prevented with NAC

An operant set-shifting task was used to determine if stress exposure or NAC treatment had any effects on operant learning. NAC (100 mg/kg) or vehicle was delivered for 4 days before, on the day of, and for 4 days following the stressor and set-shifting training commenced the following week (**Figure 2-3**). Once animals were trained to lever press for sucrose under a light cue specific rule, the

rule was changed to a location specific rule and trials to criteria were measured. All animals were required to meet criteria for the visual rule in a minimum of 30 trials before being tested in the location rule, and neither NAC treatment or stress exposure had an effect on initial learning (**Figure 2-11A** $F(3,4) = 0.038$, $p = 0.989$). Once the rule was switched, stress exposed animals took more trials to meet criteria when compared to sham exposed animals, signifying an impairment in cognitive flexibility, and NAC treatment surrounding the stressor prevented this learning deficit (**Figure 2-11A,** $F(3,20) = 5.77$, 0.005). Additionally, we found that stress exposed animals exhibited an increased number of perseverative errors when compared to all other treatment groups, and the increase in trials was not due to animals omitting lever presses (**Figure 2-11B,** $F(3,20) = 4.46$, $p < 0.015$). Additionally, NAC treatment in the absence of a stressor did not cause any changes in trials to criteria or perseverative errors. Taken together, these data indicate that NAC alone does not cause changes in operant learning and can prevent stress induced deficits in cognitive flexibility.

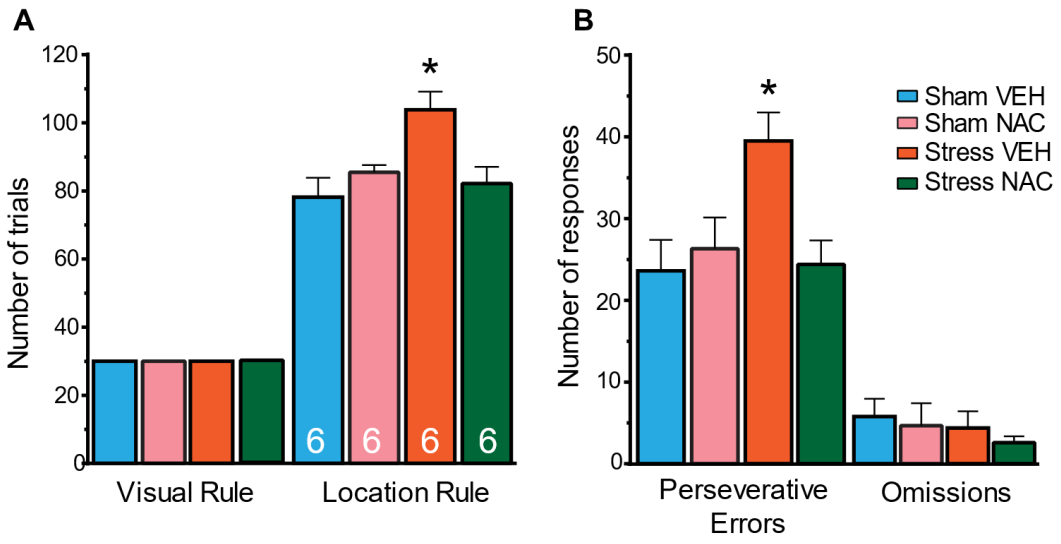

Figure 2-11: Restraint stress exposure induces deficits in cognitive flexibility that are prevented with NAC treatment. A) All animals successfully met criteria for 30 trials under the initial visual rule before being tested in the between session set-shift that altered the lever pressing requirements. Under this second rule, animals that were previously exposed to a stressor took an increased number of trials to meet criteria when compared to all other treatment groups (*$p < 0.05$ compared to all other treatment groups). **B)** This increased number of trials resulted from an increased number of perseverative errors performed during this session (*$p < 0.05$ compared to all other treatment groups) and not from changes in omissions.

Discussion

These experiments conclude that exposure to acute restraint stress causes escalations in ethanol seeking behavior during self-administration acquisition, extinction, and stress cue induced reinstatement. Additionally, stress exposure induces deficits in cognitive flexibility which could be contributing to the impairments observed in reinstatement testing. Further, NAC was successful at preventing both escalations in ethanol seeking as well as deficits in cognitive flexibly when administered either prior to stress, following the stressor, or at both

time points. These data support the relationship between stress exposure and increased ethanol seeking and establish NAC as a pharmacological modulator that is affective at preventing stress induced relapse.

While there are a multitude of brain circuits involved in the establishment and maintenance of disorders like PTSD and AUD, the prefrontal cortex and its projections to the nucleus accumbens are especially important for relapse-like behaviors (Chen et al. 2010). These circuits are also altered following exposure to drugs of abuse, and this causes a variety of adaptations that can, in turn, reinforce the maladaptive behaviors that underly substance use (Lüscher and Malenka 2011). In an addiction state, these projections have impaired plasticity which, in part, is induced by impairments in the regulation of glutamate homeostasis (Kalivas 2009). Importantly, the connections between these regions have been shown to be involved in the ability to alter behavioral responses to a changing environment which is involved in the cognitive flexibility processes required for the extinction of drug-seeking and fear-related behaviors (Spencer et al. 2016). Additionally, prefrontal plasticity, glutamatergic function, and behavioral output have all been shown to be affected by exposure to an intensely stressful event (McEwen and Morrison 2013, Arnsten 2009). In animal models, dendritic morphology in the prefrontal cortex has been shown to be reduced following exposure to both pharmacological and behavioral models of extreme stress (Cook and Wellman 2004), and this reduction in structural plasticity results in impaired functional plasticity as well (Jackson and Moghaddam 2006, Radley et al. 2015). These

underlying changes in plasticity and glutamatergic functioning in the projections from the prefrontal cortex to the accumbens could play a role in the behavioral affects observed on drug seeking and reinstatement following exposure to restraint stress in these studies.

NAC has been shown to modulate the brain through a number of different mechanisms. Primarily, the ability of NAC to affect the glutamatergic system has been the focus of its use for the treatment of addiction and anxiety disorders (Ooi et al 2018; McClure et al. 2014; Kupchik et al. 2012; Tomko et al. 2018; Zhou et al. 2008). NAC has been shown to affect glutamate homeostasis through multiple mechanisms including regulation of the cysteine-glutamate and glia glutamate transporters (Kupchik et al. 2013; Ooi et al 2018; Zhou and Kalivas 2009). Both of these glutamatergic modulators are impaired followed drug exposure and reduced expression of these transporters is associated with the reinstatement of drug-seeking (Ooi et al. 2018). Specifically, NAC could be acting through these glutamatergic mechanisms to prevent stress cue induced relapse in these studies. Additionally, NAC has the ability to regulate antioxidant tone and multiple pro- and anti-inflammatory mediators (Minarini et al. 2017; Ooi et al. 2018; Šalamon et al. 2019; Tardiolo et al. 2018; Mokhtari et al. 2017; Uraz et al. 2013; Sadowska et al. 2007). Specifically, NAC has the ability to reduce levels of pro-inflammatory mediators such as TNF-a (Peristeris et al. 1992), IL-1b (Palacio et al. 2011), nitric oxide synthase (Bergamini et al. 2011), and activated microglia (Karalija et al. 2012), and increases anti-inflammatory factors (Bavarsad-Shahripour et al. 2014).

Since exposure to chronic ethanol (Fernandez-Lizarbe et al. 2009; Marshall et al. 2016) and stress (Walker et al. 2013; Maydych 2019; Voorhees et al. 2013) has been shown to have the opposite affect by increasing pro-inflammatory factors, NAC acting as an antioxidant and anti-inflammatory agent could work to prevent the deleterious behavioral consequences that occur as a result of PTSD and AUD.

While these experiments examined the behavioral effects of restraint stress exposure on ethanol seeking behavior and cognitive flexibility as well as the established the ability of NAC to treat these deficits, further studies need to be completed to determine the mechanism of action though which NAC is working to impart these effects.

CHAPTER 3

INFRALIMBIC MGLU5 SIGNALING MEDIATES THE DELETERIOUS EFFECTS OF CHRONIC INTERMITTENT ETHANOL EXPOSURE ON EXTINCTION LEARNING

Background and Significance

Post-traumatic stress disorder (PTSD) is one of the most highly observed conditions that occurs in patients that are in treatment for substance abuse and, as such, the rates of comorbidity between PTSD and alcohol use disorder (AUD) have been recorded to reach rates as high as 41–79% (Pietrzak et al. 2011). Additionally, there is greater clinical and functional impairment observed in those with both PTSD and AUD when compared to either disorder alone (Lehavot et al.

2014), and there is evidence that PTSD symptoms are a significant risk factor for AUD which, in turn, interferes with PTSD treatment (Gaher et al. 2014). An additional area of interest with regards to comorbid PTSD/AUD focuses on differences between the sexes since these disorders often have different clinical profiles between men and women (Lebron-Milad and Milad 2012, Sonne et al. 2003). Along with these clinical observations, preclinical examinations of sex differences in PTSD models have observed sexual dimorphisms in heritability, receptor expression, neuronal structure, and fear circuit functionality (Ramikie and Ressler, 2017). Therefore, for the purpose of these studies, an examination of sex differences was included to determine any differences in responses to fear stimuli following chronic ethanol.

AUD and PTSD can often be classified as disorders of learning and memory (Hyman 2005; Vanelzakker et al. 2013) due to the complex interactions between alcohol and stress exposure in the prefrontal cortex. While alcohol can be abused by PTSD patients to help acutely alleviate their anxiety symptoms, in the long-term, alcohol abuse worsens PTSD symptoms and induces cognitive deficits that interfere with the extinction of trauma associated cues (Back et al. 2006). Additionally, these data reveal that anxiety disorders such as PTSD are affected by repeated alcohol use (Tipps et al. 2013), and chronic alcohol use also affects PTSD memories. Importantly, the first line behavioral therapies for both PTSD (Kessler et al. 1995) and drug addiction (Conklin and Tiffany 2002), including extinction-based exposure therapies, depend on these basic principles of learning and memory. Therefore, due to impairments in these processes associated with

chronic alcohol exposure following stress, these therapies often have limited efficacy for these patients. Thus, targeting these learning and memory impairments with pharmacological modulators could serve to increase the effectiveness of these therapies in the treatment of comorbid PTSD and AUD. Following this line of research, previous studies in our lab have targeted the extinction phase of memory using cognitive enhancers that increase metabotropic glutamate receptor 5 (mGlu5) activity to facilitate learning during this time (Cleva et al. 2011, Gass et al. 2014a, Gass and Olive 2009a). Additional studies have used mGlu5 modulators to enhance plasticity in the medial prefrontal cortex (mPFC) and demonstrate that mGlu5 mediated plasticity in the infralimbic subregion is necessary to block the expression of fear behaviors to allow for successful extinction (Fontanez-Nuin et al. 2011, Sepulveda-Orengo et al. 2013).

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 this relationship in comorbid PTSD/AUD is not clear. Therefore, the purpose of these experiments was to determine the effects of chronic intermittent ethanol vapor exposure on the extinction and recall of conditioned fear responses and to utilize manipulation of prefrontal glutamatergic systems to reverse these effects. An additional area of study within these experiments focuses on potential differences between males and females in responses to fear stimuli given the substantially different rates of prevalence of these disorders between the sexes.

Materials and Methods

Animals

These experiments used male and female Wistar rats [postnatal day (PD) 50 and 250-275 g upon arrival, Harlan, Indianapolis, IN] who were individually housed in standard polycarbonate cages. Throughout the experiment, access to food and water in the home cage was continuous except during behavioral testing. Animals were habituated to a 12:12 reverse light-dark cycle with lights off at 09:00 am in order for experimental testing to be 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). A total of 96 animals across seven treatment groups for each sex were used in total for Experiments 1 and 2. Experiment 1 consisted of behavioral experiments completed using fear conditioning followed by CIE exposure and required three groups per sex that received the following treatments: Air+Vehicle, CIE+Vehicle, and CIE+CDPPB (n=8/sex, 48 animals total). Experiment 2 replicated these behavioral studies and also included treatment groups that received surgery to implant a microinjection canula for further manipulation, and required four groups that received the following treatments: CIE+Vehicle, CIE+CDPPB, CIE+CDPPB+MTEP in the IfL, and

CIE+CDPPB+MTEP in the PrL (n=6/sex, 48 animals total). The overall experimental design timeline is depicted in **Figure 3-1**.

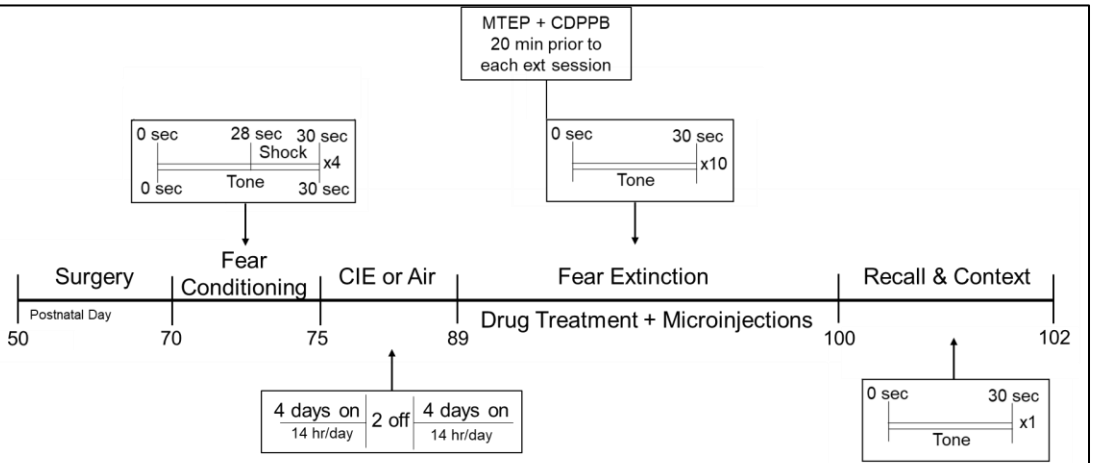

Figure 3-1: Overview of the PTSD/AUD model experimental design. Outline of the procedure and timeline for all experiments. Briefly, surgery to implant microinjection canulae was followed by fear conditioning and ethanol exposure. Then, fear extinction testing occurred along with the injection of any drug treatments. Experiments concluded with fear recall and context induced freezing tests.

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 administered at a dose of 30 mg/kg and all injections were delivered subcutaneously. Previous studies in our lab have shown that 30 mg/kg CDPPB has the ability to facilitate extinction learning without

resulting in any motor effects (Cleva et al. 2011, Gass et al. 2014, Gass and Olive 2009a). MTEP [3-((2-Methyl-4-thiazolyl) ethynyl)pyridin hydrochloride] was purchased from Abcam Inc. (Cambridge, MA) and dissolved in sterile artificial cerebrospinal fluid (aCSF) to reach a final concentration of 5 µg/µl. This dosage is based on our previously published findings and was chosen to maximize blockade of mGlu5 receptor activity (Cannady et al. 2017, Gass et al. 2014, Sinclair et al. 2012). Additionally, there have been no locomotor effects reported using similar doses of MTEP (Klodzinska et al. 2004, Martin-Fardon et al. 2009).

Fear Conditioning Protocol

Fear conditioning paradigms were completed following the protocol used in previously published works (Cain et al. 2002, Holmes et al. 2012, Izquierdo et al. 2006, Sinclair et al. 2012, Wellman et al. 2007). Conditioning trials occurred once a day for a total of three days and lasted approximately five minutes per session to preclinically model characteristics of PTSD (Cain et al. 2012, Vanelzakker et al. 2013). Each session 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 tone. A 10 second inter-stimulus interval separated each tone/shock pairing. Conditioning criteria was met when rats displayed freezing behavior at least 80% of the time during the presentation of the CS. FreezeScan software (Clever Systems, Inc.) was used to determine freezing

behavior from digitized videos (Milad and Quirk 2012, Sierra-Mercado et al. 2011, Quirk and Mueller 2008, Quirk et al. 2010). Treatment groups were behaviorally matched to ensure that all groups had similar levels of freezing prior to further manipulation.

Chronic Intermittent Ethanol (CIE) Exposure

CIE exposure was started following the completion of fear conditioning to model alcohol dependence that starts in response to the experience of a traumatic event and required repeated cycles of exposure to binge-like levels of ethanol through vapor inhalation. This is a highly characterized model of ethanol exposure that has been used by our lab (Gass et al. 2017, Trantham-Davidson et al. 2014) and others (Sanna et al. 2002, Ewin et al. 2019, Holmes et al. 2012, Singewald and Holmes 2019) to study the effects of chronic ethanol on the brain and behavior. For CIE exposure, animals were placed into an ethanol vapor chamber for 14 hours a day for 14 consecutive days which left 10 hrs. of abstinence per day. As the rats were on a reverse 12-hour light/dark cycle with lights on at 9:00 pm and off at 9:00 am, CIE was planned to occur mostly during the lights on phase (6 pm to 8 am). To make sure that CIE did not have deleterious effects on the health of the animals, body weight and water intake were monitored throughout exposure. Animals in the control groups were transferred to the lab to remain in their home cages during the time period in which CIE animals were in the chambers. Intoxication levels were measured per rat at the end of each vapor exposure period

using a 5-point motor intoxication rating scale (Nixon and Crews 2002) with a target of slight-to-moderate motor intoxication, or a rating of 2 to 3, respectively. BECs were determined from tail blood using a standard colorimetric assay at the end of exposure days 2, 6, 10, 15, using previously published methods (Gass and Glenn et al. 2014, Gass and Trantham-Davidson et al. 2014, Trantham-Davidson et al. 2017). Fear extinction paradigms began 72 hours after CIE exposure ended to avoid any effects of alcohol withdrawal.

Fear Extinction Testing

An “ABBA” experimental design was used to determine the effects of CIE exposure on the extinction of fear responses to both the cue and context. The initial fear conditioning trials occurred in the original fear box with silver metal walls and silver grate flooring (context A) and cue extinction occurred in a novel, visually distinct context (B) that consisted of black paneled walls and solid plastic flooring. Each extinction session 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. Extinction criteria was met when animals froze, on average, less than 30% of the time in response to the CS for 3 consecutive presentations of the CS. For those in the drug treatment groups, animals received CDPPB (30 mg/kg, subcutaneously) or vehicle (10% Tween-80, subcutaneously) 20 minutes prior to each extinction session while all further testing was completed in a drug free state.

Extinction Cue Recall and Context Recall

Following the successful extinction of freezing behavior, animals were tested for extinction cue recall by placing them in the extinction environment (context B) and exposing them to a single tone presentation. These trials lasted for a total of 150 seconds, including a minute prior to and a minute following the 30 second tone, and freezing behavior was measured in response to the tone. Subsequently, animals were tested for context-induced recall of freezing behavior by placing them in the original fear context A without presentation of the tone (Elias et al. 2010, Tovote et al. 2015). These tests were both included to determine the effects of CIE exposure on the retention of extinction learning for fear-associated cues and environments.

Stereotaxic Microinjection Cannula Implantation

For Experiment 2, a separate group of 48 rats performed the same behavioral paradigms, but with the addition of treatment groups that received microinjections of MTEP, an mGlu5 negative allosteric modulator, in the IfL or PrL to determine if CDPPB induced increases in mGlu5 activity in these regions is responsible for its facilitation of fear extinction learning. Surgeries to implant bilateral microinjection canula commenced between PD 50 and 56 and rats were allowed two weeks of recovery before beginning behavioral testing. Isoflurane was vaporized in medical grade breathing air at a flow rate of 0.4 L/min to anesthetize

animals before placing them in a stereotaxic instrument (Kopf Instruments, Tujunga, CA). The microinjection cannula (26 ga O.D., Plastics One, Roanoke, VA) was implanted 1 mm dorsal to the IfL or PrL cortex, and the following stereotaxic coordinates were used: anterior/posterior + 3.24, medial/lateral ± 0.6, and dorsal/ventral − 3.8 for the IfL and anterior/posterior + 3.24, medial/lateral ± 0.6, and dorsal/ventral −2.2 for the PrL (in mm from bregma and skull surface) (Paxinos and Watson 2005). Stainless steel screws were placed into the skull and were covered with dental cement to secure the microinjection cannula in place. To prevent tissue obstruction and contamination from debris, removable obturators (33 ga O.D.) were inserted in the full length of the guide cannula. Following surgery, the incision was sutured using 3-0 Vicryl sutures and treated with 2% xylocaine and 2% triple antibiotic ointments topically. Post-operative care consisted of daily injections of carprofen (2.5 mg/kg, s.c. for 5 days) for pain management. Once animals were allowed at least two weeks for surgical recovery, they were exposed to our model of PTSD/AUD through fear conditioning followed by CIE.

Microinjection Procedures

Microinjections of 5 μg/μl MTEP into either the PrL or IfL were completed 25 minutes prior to each extinction session. Animals were lightly restrained to remove the obturators and insert sterile 33-gauge microinjection needles (Plastics One) connected through microbore tubing to two 100 μl syringes (Hamilton, Reno, NV).

A micro-infusion pump held both syringes (Harvard Apparatus, Holliston, MA) and was set to deliver either MTEP or aCSF at a flow rate of 0.5 µl/min for a total of one minute. The microinjection needles were inserted to a depth 1 mm beyond the ventral tip of the guide cannula and were left in place for an additional minute following drug injection to allow for diffusion. Obturators were replaced following the removal of the microinjection needles, and animals in the CDPPB groups received subcutaneous injections (30 mg/kg) directly after each microinjection. Twenty minutes following the completion of both injections, animals were placed in the operant box for completion of the extinction training sessions as described above.

Histological Verification of Microinjection Sites

To obtain brain tissue for verification of cannula placement, rats were anesthetized with isoflurane and euthanized by decapitation following previously published methods (Gass et al. 2011). Following extraction, brains were placed into 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. A cryostat (Leica CM1900, Leica Microsystems, Bannockburn, IL) was used to section brains into 40-mm coronal slices and mounted onto microscope slides. These slides were stained with cresyl violet for histological verification of cannula placement using light microscopy.

Determination of Estrous Cycle

A small pilot study was conducted in a separate group of animals (n = 6/group) to determine the impact of estrous cycle on fear extinction learning, since these processes have been shown to be altered based on cycle phase (Milad et al. 2009) To minimize any effects of the testing on behavioral outcomes, vaginal swabs were completed at the end of each extinction session (Gass et al. 2007, Ford et al. 2002, Roberts et al. 1998). This pilot experiment determined that there were no changes in fear extinction learning between females at different phases of the estrous cycle (**Figure 3-2**) and, thus, swabs were not completed on any other groups within this study.

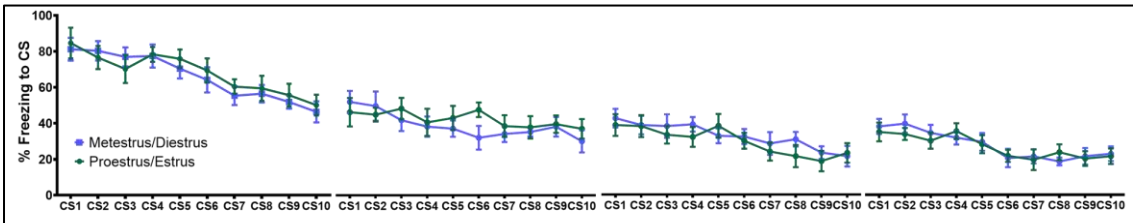

Figure 3-2: Estrous cycle phase does not alter fear extinction rates. Female animals in metestrus/diestrus exhibited similar fear extinction freezing rates when compared to those in the proestrus/estrus phase.

Statistical Analysis

Software including SPSS version 23.0 (SPSS Inc., Chicago, IL) and Prism version 8 (GraphPad software, La Jolla, CA) were used to analyze the behavioral data from these experiments. All tests required a p value of less than 0.05 for

statistical significance. Fear conditioning data was analyzed using independent samples t-tests to compare the number of tone shock pairings needed to meet criteria between males and females. Extinction behavior data were analyzed using multiple two-way ANOVAs with Tukey's multiple comparisons test performed 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 completed 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. Behavioral responses during recall testing were analyzed using a one-way ANOVA with multiple comparisons using Holm-Sidak post-hoc testing to determine differences in freezing between multiple treatment groups.

Results

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).

Females meet fear conditioning criteria at a faster rate than males

All animals must first meet criteria for fear conditioning in order for further analysis of fear extinction learning to be completed. All animals were freezing 80% of the time in response to the tone regardless of subsequent CIE or air treatment (**Figure 3-4A**), 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 3-4B**). Although, while freezing rates differed between the sexes in the earlier phases of conditioning, all animals were freezing at a similar rate by the end of conditioning which allows for between group comparisons to be made during extinction learning.

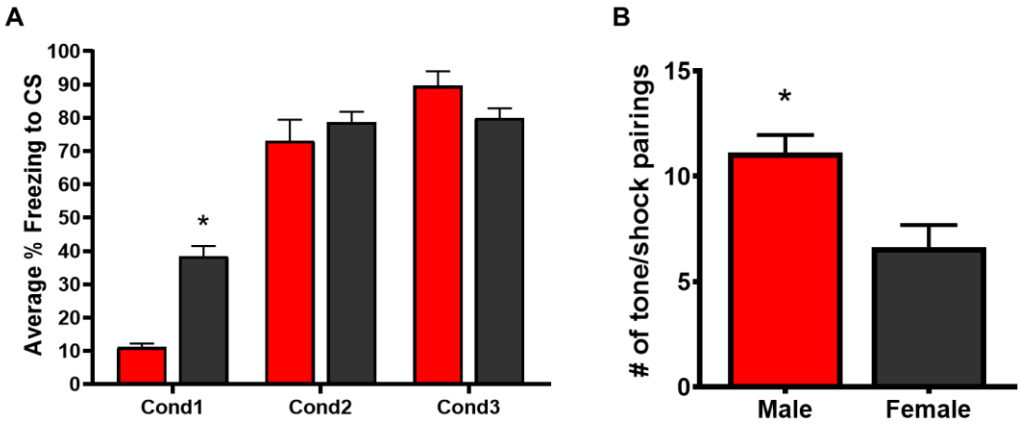

Figure 3-3: Female animals freeze more during day one of conditioning and meet fear conditioning criteria faster than males. Regardless of subsequent air or CIE treatment, female animals exhibited an increased rate of fear conditioning when compared to males, shown by **(A)** increased levels of freezing on the first day of conditioning along with **(B)** fewer sessions required to meet conditioning criteria (*p < 0.0001, n=16/sex).

CIE exposure resulted in moderate levels of intoxication for all animals

Following fear conditioning, animals were put into CIE exposure for a total of two weeks. Throughout CIE, a 5-point behavioral intoxication rating scale (Nixon and Crews 2002) was used to monitor the level of intoxication achieved following each 14-hour ethanol exposure period. The goal for these experiments was a behavioral intoxication rating of 2-3 which correspond to moderate levels of intoxication. Throughout both Experiment 1 and Experiment 2, there were a total of 80 male and female animals in CIE treatment groups and intoxication ratings were averaged for all animals across exposure sessions. An overall average intoxication rating of 2.2 ± 0.05 was achieved across all exposure periods and animals. Blood ethanol concentrations were determined from tail vein puncture directly following CIE exposure on days 2, 6, 10, and 15 to serve as a complementary measure to the behavioral intoxication ratings. To achieve moderate intoxication ratings, BEC target values were between 200 – 300 mg%. Average BEC mg% values for males were recorded to be 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. Females exhibited 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 (**Figure 3-3**)

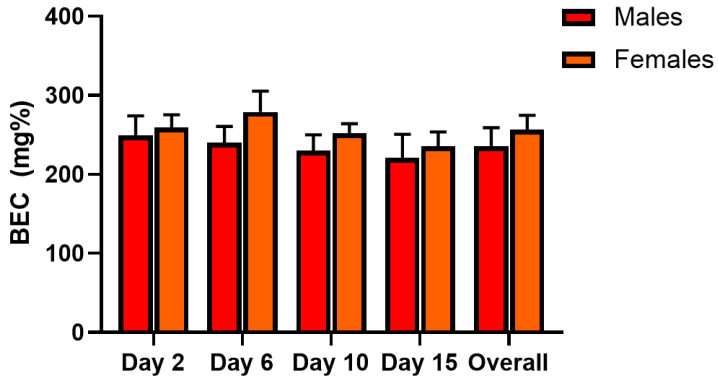

Figure 3-4: Average BEC measured for all animals in this study. BECs were obtained from analysis of tail vein puncture from each rat following exposure periods on days 2, 6, 10, and 15 (n = 80/day).

Deficits in fear extinction learning are established following CIE exposure and are treated with CDPPB

Following CIE exposure, animals were subjected to extinction training for a total of five days. When examining the average freezing response on day of extinction (**Figure 3-5**) there was a main effect of CIE on extinction learning such that CIE exposed animals showed increased levels of freezing when compared to air treated animals across the total duration of extinction training. Specifically, 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 (**Figure 3-5A**) [$F(49, 489) = 24.25$, $p < 0.0001$, n=8/group]. The same effect was observed in females as well, where animals exposed to CIE also exhibited significantly higher rates of cue-induced freezing when compared to air

exposed controls throughout extinction [F (49, 500) = 34.58, p < 0.0001, n=8/group] (**Figure 3-5B**). Notably, treatment with CDPPB was effective at attenuating CIE induced deficits in extinction through the facilitation of 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 3-5A**) and females [F(49, 500) = 31.19, p < 0.0001, n=8/group) (**Figure 3-5B**).

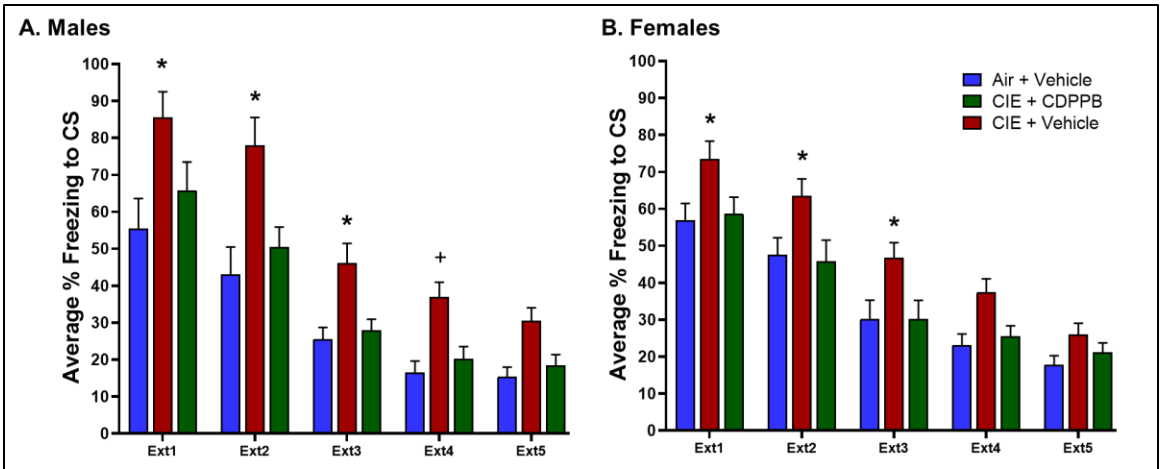

Figure 3-5: CDPPB attenuates CIE-induced deficits in extinction learning for both males and females. While CIE exposed males and females both exhibited impairments in extinction learning, CDPPB given directly before extinction was able to reduce freezing during extinction to levels comparable to controls for CIE exposed males **(A)** and females **(B)** (*p < 0.05 CIE + Vehicle vs. Air + Vehicle and CIE + CDPPB, +p < 0.05 Air + Vehicle vs. CIE+Vehicle, n=8/group).

CIE differentially affects extinction cue and context recall between the sexes

Two days following the completion of extinction training, animals were tested for extinction cue recall through the presentation of a single tone in Context B. A clear sex difference emerged during this test, since there was a significant effect of CIE on cue recall in male, but not female, animals. Those exposed to CIE prior to extinction exhibited an increased rate of freezing when compared to both air exposed controls and CIE exposed male animals treated with CDPPB [$F(2,21) = 35.74$, $p < 0.0001$, n = 8/group] (**Figure 3-6A**). This effect was not observed in female animals, where those 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 3-6B**).

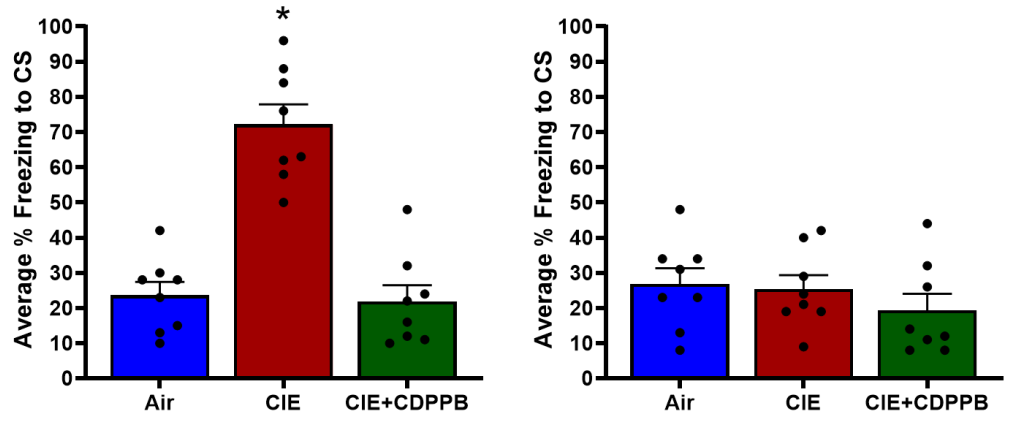

Figure 3-6: CDPPB prevents heightened freezing during cue recall in males. **(A)** CDPPB treatment caused CIE animals to reduce their freezing when compared to CIE animals that received vehicle (*p < 0.0001 CIE vs. Air and CIE + CDPPB, n=8/group). **(B)** Conversely, female CIE animals did not show increased freezing when compared to CIE+CDPPB treated animals (p = 0.6056, n=8/group).

Following the extinction recall test, animals were placed back into the original conditioning environment (context A) in the absence of any tone presentation to assess freezing in response to the context only. Notably, all drug manipulation occurred previously during extinction training, and all recall testing was performed in a drug-free state. During this test, 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 3-7A**). Additionally, this treatment effect was observed 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 3-7B**).

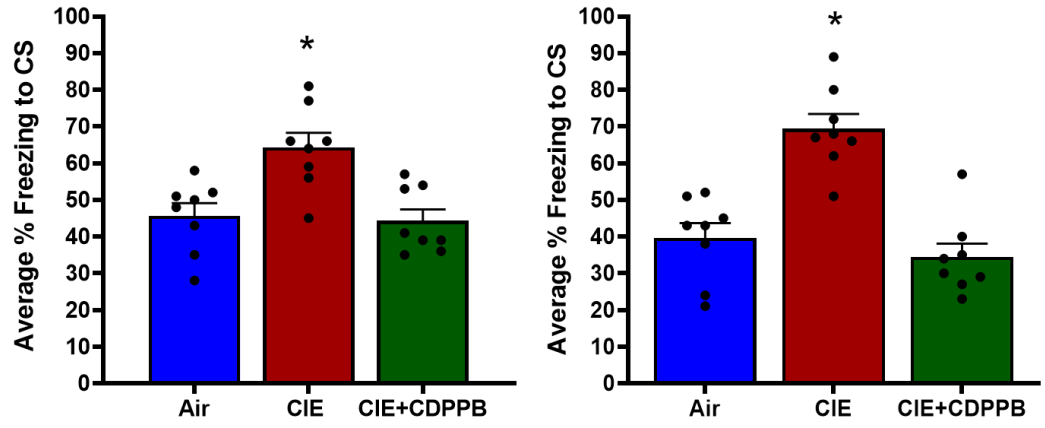

Figure 3-7: CDPPB prevents heightened freezing during fear context recall in both sexes. (A) Male CIE animals treated with CDPPB reduced freezing during context recall when compared to CIE animals that received vehicle. **(B)** For females, freezing was reduced in CIE animals following CDPPB treatment when compared to CIE exposed animals that received vehicle (*p < 0.0001 CIE vs. Air and CIE + CDPPB, n=8/group).

CDPPB facilitates extinction learning through increases in mGlu5 activity in the infralimbic cortex

The behavioral studies in Experiment 1 determined that CDPPB can recover CIE induced deficits in fear extinction learning in both male and female animals. Previous studies carried out in our lab using CDPPB found that the enhancement of extinction of drug-seeking behaviors 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).

During extinction, there was a significant main effect of treatment in male rats where those administered CIE+CDPPB+MTEP exhibited freezing levels similar to CIE animals that did not receive any treatment [$F(49, 495) = 24.54$, $p < 0.0001$, n=6/group] (**Figure 3-8A**). Essentially, microinjections of MTEP into the IfL cortex concurrent with CDPPB treatment blocked its facilitating effects on extinction learning. Additionally, a similar effect was observed in female animals where local administration of MTEP into the IfL cortex just prior to systemic CDDPB administration prevented the facilitating effects of CDPPB on fear extinction learning, since CIE+CDPPB+MTEP treated animals exhibited increased levels of

freezing when compared to CIE+CDPPB treated animals across multiple extinction sessions [F(49, 495) = 24.67, p < 0.0001, n=6/group] (**Figure 3-8B**).

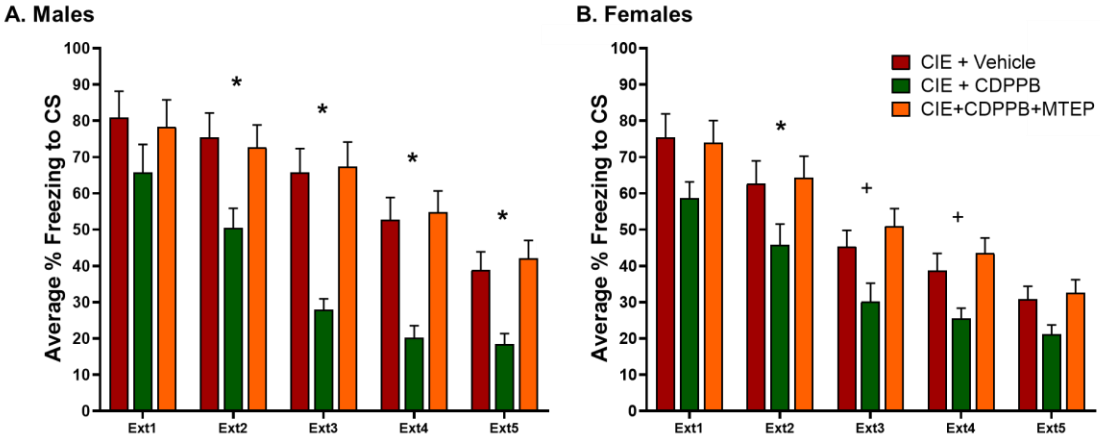

Figure 3-8: In both sexes, negative allosteric modulation of mGlu5 in IfL cortex blocks the facilitating action of CDPPB. (A) Negative allosteric modulation of mGlu5 through MTEP infusion into the IfL cortex blocked the ability of CDPPB to reduced freezing throughout extinction in CIE exposed male animals. **(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+CDPPB vs. CIE + Vehicle and CIE + CDPPB + MTEP, +p < 0.05 CIE+CDPPB vs. CIE+CDPPB+MTEP, n=6/group).

MTEP delivered into the IfL blocks the ability of CDPPB to prevent increases in freezing during recall testing

During cue recall testing in context A, CIE+CDPPB animals that received MTEP microinjected into the IfL exhibited increases in freezing behavior when compared to CIE+CDPPB treated animals. MTEP treated animals also displayed freezing similar to those exposed to CIE that did not receive CDPPB treatment [F(2,15) = 27.62, p < 0.0001, n = 6/group] (**Figure 3-9A**). This data suggests that when mGlu5 activity is blocked in the IfL so is CDPPB's ability to prevent CIE

induced increases in freezing during recall. Additionally, due to a lack of effect of CIE on cue recall in female animals, and there were no differences observed between female treatment groups [F(2,15) = 0.5237, p = 0.6028, n = 6/group] (**Figure 3-9B**).

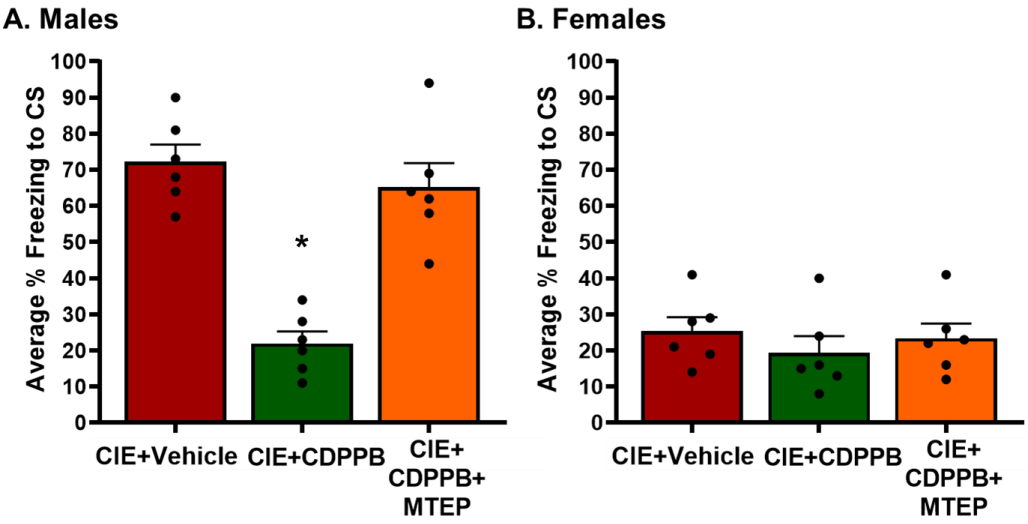

Figure 3-9: MTEP infusion in the lfL prior to each extinction session blocks the ability of CDPPB to reduce freezing during cue recall in males.
(A) Male CIE + CDPPB animals that received MTEP in the lfL returned to heightened levels of freezing when compared to CIE+CDPPB animals (*p < 0.0001 CIE + CDPPB vs. CIE + Vehicle and CIE + CDPPB + MTEP, n=6/group). **(B)** CIE+CDPPB exposed females did not exhibit differences in freezing when compared to CIE+CDPPB+MTEP treated animals (p = 0.7629, n=6/group).

MTEP microinjections resulted in a similar effect on context recall in both sexes. There was a significant effect of treatment on freezing during context recall, since CDPPB treatment resulted in decreased freezing when compared to CIE exposed animals, and MTEP treatment in these animals prevented the reduction

in freezing to the context shown following CDPPB treatment in males [F(2,15) = 12.04, p = 0.0008, n =6/group] (**Figure 3-10A**) and females [F(2,15) = 17.16, p = 0.0001, n = 6/group] (**Figure 3-10B**). 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. Additionally, histological examination of the injection site demonstrated that all microinjections were within the IfL cortex or its boundary regions (**Figure 3-11**).

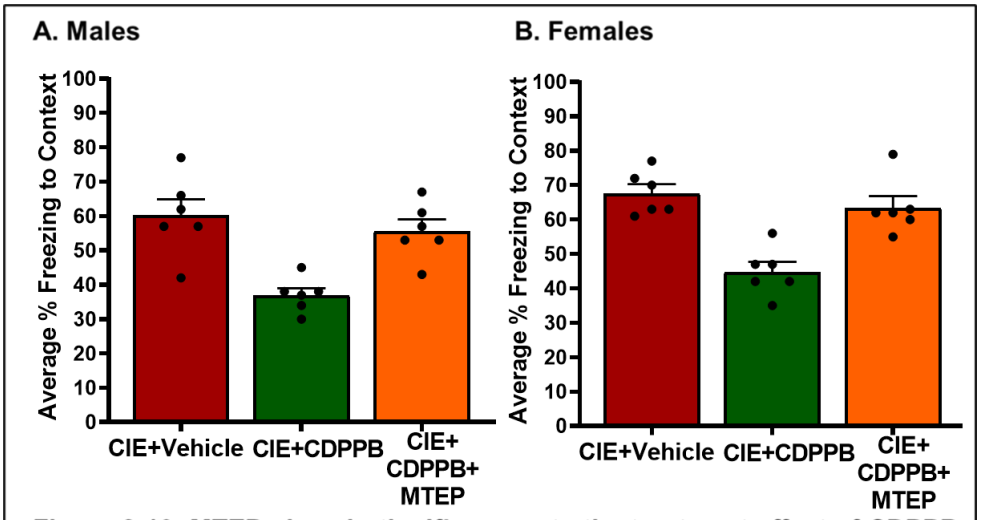

Figure 3-10: MTEP given in the IfL prevents the treatment effect of CDPPB on heightened freezing during context recall tests. A) While CDPPB treatment reduces freezing during context recall in CIE exposed male animals, MTEP given in the IfL blocks this effect of CDPPB and these animals return to heightened 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 and CIE+CDPPB+MTEP)

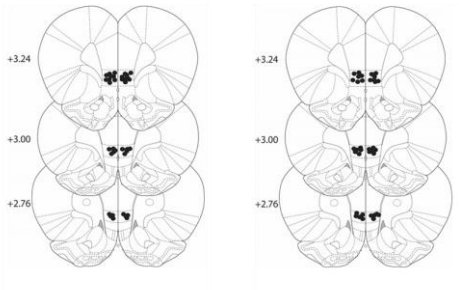

Figure 3-11: Histological verification of microinjection placements for male and female lfL infusions.

Reduction of mGlu5 activity in the prelimbic cortex does not affect CDPPB's ability to reverse CIE induced deficits in extinction learning

Animals were exposed to the same fear conditioning and CIE model followed by CDPPB treatment and MTEP administration during fear extinction, but microinjections occurred in the PrL cortex. Following blockade of mGlu5 activity in the PrL using MTEP, CDPPB treatment was still effective at attenuating CIE-induced deficits in extinction learning for both males [$F(49,494) = 18.4$, $p < 0.0001$, n=6/group] (**Figure 3-12A**) and females $F(49, 493) = 29.78$, $p < 0.0001$, n=6/group] (**Figure 3-12B**). Essentially, the ability of CDPPB to facilitate extinction learning was not dependent on increases in mGlu5 in the PrL cortex.

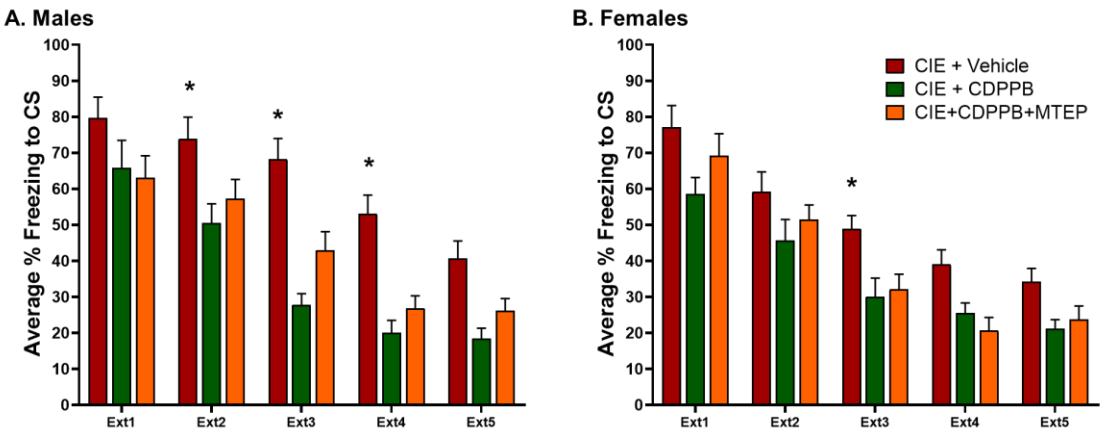

Figure 3-12: MTEP infusion in the PrL prior to each extinction session does not alter CDPPB's facilitation of extinction learning. (A) Negative allosteric modulation of mGlu5 through MTEP infusion into the PrL cortex did not change CDPPB effects on extinction learning in CIE exposed animals. **(B)** 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 and CIE + CDPPB + MTEP, n=6/group).

MTEP negative allosteric modulation of mGlu5 activity in the PrL did not affect CIE induced increases in freezing during recall

In cue recall testing, CIE exposed animals exhibited a significantly increased level of freezing when compared to CIE+CDPPB treated animals, and MTEP treatment did not stop CDPPB from decreasing freezing during this session. There was still a significant effect of treatment where CDPPB was still able to act as an effective treatment on CIE induced heightened freezing during cue recall [$F(2,15) = 21.96$, $p < 0.0001$, $n = 6$/group] (**Figure 3-13A**). Due to the lack of change in freezing behavior as a result of CIE exposure in female animals, and there were no differences in freezing between any of the treatment groups during cue recall testing [$F(2,15) = 1.196$, $p = 0.3296$, $n = 6$/group] (**Figure 3-13B**).

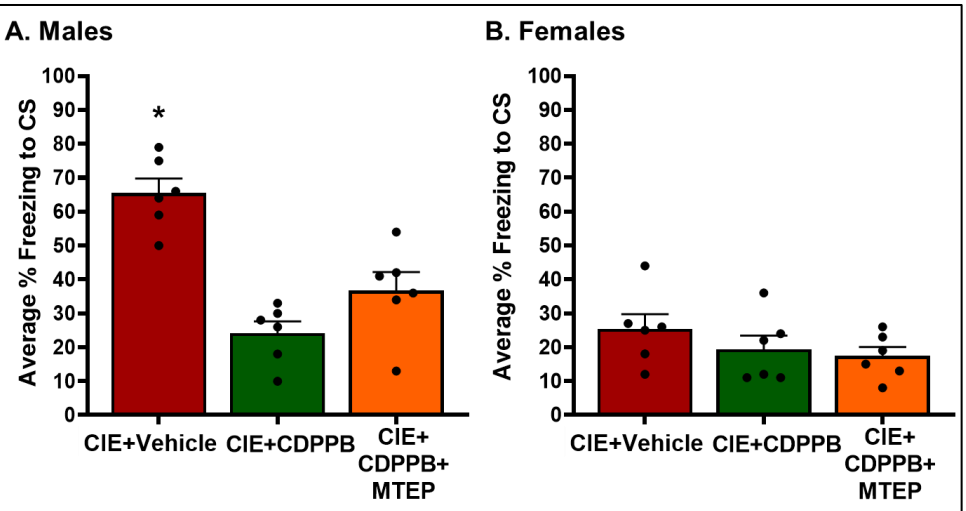

Figure 3-13: MTEP given in the PrL does not alter CDPPB's ability to reduce freezing during recall in CIE exposed animals. (A) In males, CIE+CDPPB treatment reduced freezing when compared to CIE+Vehicle animals. CDPPB was also still able to reduce freezing when administered in the PrL compared to CIE+Vehicle animals (*p < 0.001 CIE + Vehicle vs. CIE + CDPPB and CIE + CDPPB + MTEP, n=6/group) **(B)** In female animals, CIE+CDPPB+MTEP treatment did not cause any significant differences when compared to CIE+Vehicle (p = 0.3251, n=6.group) and CIE+CDPPB treated animals (p = 0.9271, n=6/group).

CIE treated animals also 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 3-14A**) and females [$F(2,15) = 8.358$, $p = 0.0036$, n = 6/group] (**Figure 3-14B).** 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 revealed that all microinjections were found to be contained within the PrL cortex or its boundary regions (**Figure 3-15**). 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.

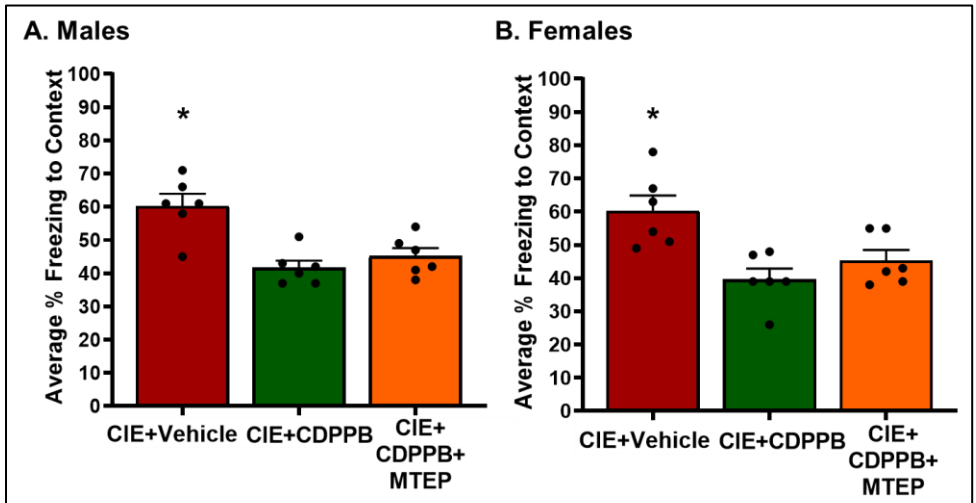

Figure 3-14: Infusion of MTEP into the PrL does not alter the ability of CDPPB to reduce freezing during context recall for both sexes. (A) Male animals treated with CIE+CDPPB and CIE+CDPPB+MTEP exhibited similar levels of freezing during context recall while CIE+Vehicle animals displayed heightened freezing. **(B)** This effect was also seen in females, where CIE+CDPPB+METP and CIE+CDPPB animals both exhibited reduced levels of freezing when compared to CIE+Vehicle animals (*p < 0.001 CIE + Vehicle vs. CIE + CDPPB and CIE + CDPPB + MTEP, n=6/group).

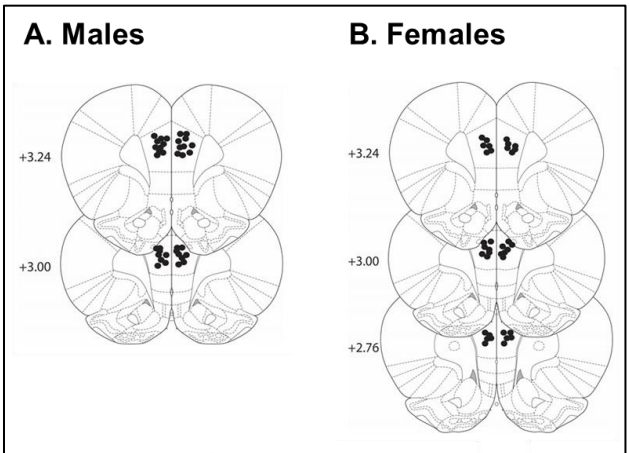

Figure 3-15: Histological verification of microinjection placements for male and female PrL infusions.

Discussion

These experiments indicate that stress exposure followed by chronic ethanol intake leads to impairments in fear extinction and memory recall. Additionally, these studies illustrate that the enhancement of mGlu5 activity in the infralimbic cortex has the ability to reverse these CIE induced deficits in both sexes. In CIE exposed animals, treatment with the mGlu5 positive allosteric modulator, CDPPB, prior to each extinction training session was able to facilitate extinction learning and prevent heightened freezing during cue and context recall. We were able to determine that the IfL cortex is necessary for CDPPB's effects on extinction learning by microinjecting the mGlu5 negative allosteric modulator MTEP in either the prelimbic or infralimbic cortex in combination with systemic CDPPB administration, Further, sex differences were found with regards to initial responses to fear stimuli during conditioning, as well as to extinction cue recall testing. 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.

When examining preclinical data there are always a number of limitations that need to be addressed due to the complexity of modeling neuropsychiatric disorders across species. First, examinations of estrous cycle were limited to a small pilot study and were not completed for all animals included in these experiments. Additionally, this pilot compared the behavior of animals starting in metestrus/diestrus vs. proestrus/estrus instead of separating out all four phases

into different groups. Regardless, due to the fact that the within group variability is low and the rate of extinction is very similar between the groups, this provides initial support that cycle phase is not altering extinction behavior. Further published studies support these conclusions and determined that the rate of fear extinction does not differ between females in proestrus vs. metestrus, and that females administered estrogen prior to extinction displayed freezing behavior similar to those that received vehicle (Maeng et al. 2015). While these studies did include a wide variety of treatment groups, there were no animal cohorts that received only CDPPB or MTEP in the absence of prior ethanol exposure. Previous work has shown that CDPPB has the ability to enhance the extinction of ethanol seeking behaviors during self-administration paradigms, so we would assume that the cognitive enhancing effects would still be present when CDPPB treatment is delivered without prior CIE exposure. Although published studies often focus on the ability of CDPPB to enhance the extinction of drug seeking behaviors, it has been reported that CDPPB can enhance fear extinction learning and recall (Sethna and Wang 2014). While not included within this study, MTEP has been delivered as a systemic treatment and has been found to have off target effects on behavior (Simonyi et al. 2010; Pietraszek et al. 2005; Schulz et al. 2001). Alternatively, these studies microinjected MTEP into select brain regions and, as such, negative allosteric modulation of mGlu5 was restricted to these regions and the behavioral effects that are observed when MTEP is administered systemically are not expected to occur in these studies. An additional limitation in these studies comes from the fact that a single dose of CDPPB and MTEP was used and a dose-

response curve was not examined. Due to the lab's experience using these drugs, multiple doses of CDPPB and MTEP have been tested to optimize CDPPB's effects on extinction learning and this dose-response curve has been previously established (Gass and Olive 2009a; Gass and Olive 2009b; Gass et al. 2014; Gass et al. 2017; Widholm et al. 2001; Cleva et al. 2011). When testing 0.3, 3, or 30 mg/kg CDPPB, 30 mg/kg CDPPB was necessary to induce significant effects on extinction learning that were prevented with MTEP delivered intraperitoneally. There were no effects on locomotor behavior or any changes in neurotoxicity when CDPPB was administered at this dose (Gass and Olive 2009a). Additionally, previous studies were completed that examined MTEP dosing and found that, while 1 ug/uL was not sufficient to alter behavior and 10 ug/uL led to 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 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.

Taken together, the results from these experiments demonstrate multiple behavioral deficits that occur as a result of stress and alcohol dependent alterations in learning and memory due to impairments in infralimbic mGlu5 activity. These studies identify CDPPB as a pharmacological treatment that has the potential to rescue alcohol induced deficits in learning and memory that could be efficacious in treating these deficits often observed in the clinical PTSD/AUD population. 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.

CHAPTER 4

OPTOGENETIC MANIPULATION OF THE PRELIMBIC CORTEX DURING FEAR MEMORY RECONSOLIDATION ALTERS FEAR EXTINCTION IN A PRECLINICAL MODEL OF CO-MORBID PTSD/AUD

Background and Significance

Post-traumatic stress disorder (PTSD) and alcohol use disorder (AUD) are two highly debilitating psychology conditions that do not have many effective treatment options. While there is a lifetime prevalence of 8.3% and 29.1% for PTSD and AUD respectively, the presence of PTSD leads to a three times greater chance of also developing AUD (Gilpin and Weiner 2017). As such, the rate of

comorbidity between PTSD and AUD has been recorded to be as high as 30% - 59% (Jacobsen et al. 2001). This is especially true for the military population, where combat exposure was significantly associated with binge drinking behavior and alcohol-related problems (Jacobson 2008), and alcohol has been established as the most abused drug among veterans with PTSD (Gilpin and Weiner 2017). Not only is there a higher lifetime prevalence of AUD in PTSD patients, but the comorbid presence of these disorders causes more severe symptomology and worse treatment outcomes (Neupane et al. 2017). For specific PTSD symptom subtypes, including trauma reexperiencing and psychiatric distress, those using alcohol reported a higher degree of symptom severity with poorer PTSD/AUD treatment outcomes (Read et al. 2004). Interestingly, PTSD has a higher incidence rate in female patients than male patients and there are sex-specific differences in PTSD incidence, severity of symptoms, and propensity to use alcohol as a coping strategy (Lehavot et al. 2014, Sonne et al. 2003). Since the prevalence of comorbid PTSD and AUD is increasing and leads to worse symptomology and treatment outcomes, there is increasing need for research into the underlying neurobiological processes that are related to the establishment and maintenance of these disorders. Further, these established sex-differences in the clinical population make it increasingly important to conduct preclinical studies in both male and female animals to determine optimal treatment strategies between the sexes.

The establishment and treatment of both PTSD and AUD rely on learning and memory systems that overlap in the prefrontal cortex (PFC). There have been both functional and structural alterations observed in the infralimbic (IfL) and

prelimbic (PrL) regions of the medial PFC (mPFC) in patients with PTSD and AUD (Gilpin and Weiner 2017). Additionally, these regions have important functions in the promotion and suppression of fear and drug seeking behaviors (Peters et al. 2009). Importantly, the most common psychological treatment strategies for PTSD and AUD rely on intact mPFC processes, and impaired functionality of these areas might explain why these treatments do not have a high rate of efficacy (Conklin et al. 2002). Specifically, one common method of AUD and PTSD treatment involves exposure therapy, where exposure to trauma or drug related cues occurs in a safe and controlled environment to allow the patient to form new associations between these cues and their conditioned responses (Watkins et al. 2018). This treatment method involves many facets of learning and memory systems that rely on the PFC including memory reconsolidation and extinction (Cooper et al. 2017). Memory reconsolidation is a process that allows memories that have already been consolidated into long-term storage to be retrieved and updated with new information (Nader et al. 2015). This is also important for extinction learning during which new associations are formed between previously established cues and responses (Quirk et al. 2008). While these learning and memory processes are required for PTSD and AUD treatments to be effective, clinical and preclinical data have found them to be impaired following exposure to alcohol (Bisby et al. 2015, Kroener et al. 2012, Smiley et al. 2020). As such, a novel line of research has emerged that focuses on targeting these learning and memory processes with pharmaceutical treatments to enhance the effectiveness of the extinction learning

reliant therapies used to treat PTSD and AUD (McGuire et al. 2014; Gass et al. 2014b).

Our lab has recently established that both male and female animals exposed to chronic binge-like levels of ethanol exhibit deficits in the ability to extinguish fear cue associations during extinction learning. We also determined that this process was dependent on glutamatergic activity at metabotropic glutamate receptor 5 (mGlu5) receptors specifically in the IfL region of the mPFC (Smiley et al. 2020). The current set of experiments was designed to further examine the relationship between alcohol exposure and fear related learning and memory and the involvement of the PrL and IfL subregions. Rats were first exposed to fear conditioning followed by chronic intermittent ethanol exposure (CIE) to model the clinical pattern of trauma exposure followed by binge ethanol intake. Optogenetics was then used to manipulate the prelimbic and infralimbic regions of the mPFC during fear memory reconsolidation to test the hypothesis that blockade of PrL fear promotion during fear memory reconsolidation would lead to a decrease in the presentation of fear behaviors during extinction. These data provide important information regarding the role of the mPFC in fear learning and memory that is impaired following exposure to chronic ethanol and support the involvement of these regions in the treatment of comorbid PTSD and AUD.

Materials and Methods

Animals

Wistar rats (48 male, 48 female, postnatal day 55 on delivery, Charles River Laboratories) were pair housed in standard polycarbonate cages with food and water continuously available except for during behavioral testing. Prior to experimental manipulation, animals were habituated to a 12:12 reverse light-dark cycle. All behavioral testing was then performed during the dark phase of the cycle. Approval by the Institutional Animal Care and Use Committee at the Medical University of South Carolina was achieved for all experimental testing under the guidelines of the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003). A total of 96 animals were used for these studies and were assigned to the following treatment groups outlined in **Table 4-1**.

Table 4-1. Treatment Groups For Experiments 1 & 2

Treatment Groups	
Experiment 1 M&F, n = 6/group	**Experiment 2** M&F, n = 6/group
CIE-NI = CIE without PrL inhibition	CIE-NI = CIE without IfL inhibition
CIE-I = CIE with PrL inhibition	CIE-I = CIE with IfL inhibition
CIE = CIE without surgical implant	CIE = CIE without surgical implant
Air = air exposed controls, no implant	Air = air exposed controls, no implant

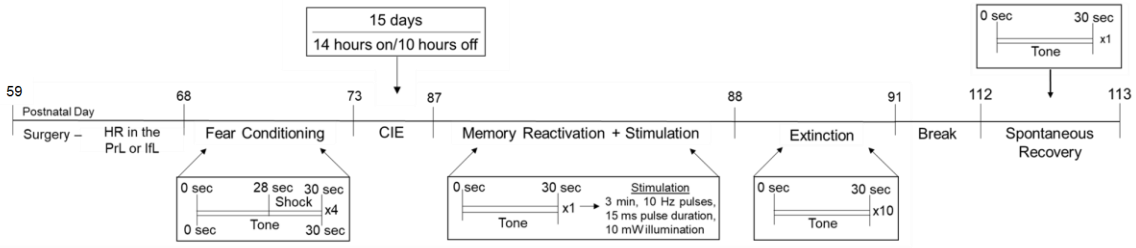

Figure 4-1: Experimental Design and Timeline. Experiments commenced with surgery to inject the inhibitory virus pAAV-CaMKIIa-eNpHR3.0-eYFP into either the PrL or IfL cortex. Following surgical recovery, animals went through three days of auditory fear conditioning consisting of four presentations of the tone (CS) paired with a shock. Then, animals were put into CIE exposure for two weeks followed by fear memory reactivation in the operant chamber and optogenetic inhibition of the PrL or IfL. Five total extinction sessions were given to monitor freezing behavior during extinction learning following blockade of memory reconsolidation. After a three-week break, animals were put back into the fear conditioned environment and presented with a single tone in order to measure the long-term effects of PrL or IfL inhibition on the spontaneous recovery of fear behavior.

Surgical Procedures

Experiments followed the overall design and timeline presented in **Figure 4-1.** For all surgical procedures, rats were anesthetized with isoflurane and oxygen at a flow rate of 0.4 L/min and placed in a stereotaxic device (Kopf Instruments). For Experiment 1, animals received a unilateral injection either 0.5 uL of pAAV-CaMKIIa-eNpHR3.0-eYFP or an EYFP reporter construct (UNC Viral Vector Core, Chapel Hill, NC) injected at a rate of 0.1 uL per minute in to the prelimbic cortex (PrL). The stereotaxic coordinates used for the PrL were as follows: anterior/posterior + 3.24, medial/lateral ± 0.6, and dorsal/ventral -2.2 (in millimeters from bregma and the skull surface). Experiment 2 followed the same procedure, but virus was instead injected in the IfL at the following coordinates: anterior/posterior +3.24, medial/lateral ± 0.6, and dorsal ventral -3.8 (in millimeters from bregma and the skull surface). For all surgeries, the injection needle (Hamilton, 1 uL syringe) was mounted in a micro-infusion pump (Harvard

Apparatus) and kept in place for 10 minutes following the injection to allow for virus diffusion. The fiber optic implant (Thorlabs Inc., Newton, NJ) was then placed directly dorsal to the virus injection site and stainless-steel screws covered in dental cement were used to secure the implant to the skull. The wound was sutured with 3-0 Nylon sutures and treated with 2% triple antibiotic ointment. Rats were post-operatively treated with antibiotics (cefazolin, 0.3 mL, s.c.) and carprofen analgesic (0.4 mL, s.c.) for three days following the surgery. Following the completion of all behavioral procedures, rats were perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Following extraction, brains were kept in 4% PFA for 48 hours before being transferred to 20% sucrose for an additional 72 hours. Brains were frozen and coronal slices were sectioned at 40 um and examined for fluorescence to histologically verify virus and fiber placement.

Fear Conditioning Paradigm

All behavioral testing was performed in standard operant boxes (Med Associates Inc., Fairfax, VT) contained within sound attenuating chambers. Auditory fear conditioning procedures began nine days after surgery to allow for recovery. Animals were operantly conditioned using four pairings of a tone (conditioned stimulus [CS] - 30 sec, 80 dB, 3 kHz) with a 0.75 mA foot-shock that occurred during the last two seconds of the tone. A 60 second habituation period occurred at the start of each session, and each tone/shock pairing was separated by a ten second inter-stimulus interval. This conditioning protocol was repeated for

a total of three days until all animals achieved conditioning criteria of freezing for 80% of tone. Freezing during each session was digitally determined by FreezeScan (CleverSys Inc., Reston, VA) (Milad and Quirk 2012, Sierra-Mercado et al. 2011, Quirk and Mueller 2008, Quirk et al. 2010). This protocol has been used by our lab (Smiley et al. 2020, Gass et al. 2014a, b, Gass et al. 2017, Gass and Olive 2009) and others (Singewald et al. 2014, Singewald and Holmes 2019, VanElzakker et al. 2013) as a preclinical model of stress exposure used to evaluate fear learning and memory (Cain et al. 2012, VanElzakker et al. 2013).

Chronic Intermittent Ethanol Procedure

Once fear conditioning criteria was met, animals were exposed to a two-week chronic intermittent ethanol (CIE) protocol. CIE chambers consisted of clear acrylic boxes that measured 24 × 24 × 14 inches (Plas Labs; Lansing, MI), and pair-housed cage mates were placed in the same chamber for a total of 15 nights from 1800 hours in the evening until 0800 hours the next morning. This schedule resulted in a total of 14 hours of vapor exposure and 10 hours of abstinence each day. A five-point behavioral scale was used to monitor individual levels of intoxication for each animal. Briefly, motor impairment was assigned a rating based on the following behaviors: 1 - no signs of intoxication, 2 - slightly intoxicated (slight motor impairment), 3 - moderately intoxicated (obvious motor impairment but able to walk), 4 -highly intoxicated (dragging abdomen, loss of righting reflex), 5 - extremely intoxicated (loss of righting reflex and loss of eye blink reflex) (Nixon and Crews, 2002). The goal of each exposure session was to achieve moderate

intoxication with a behavioral rating of 2-3, and this level of intoxication has been consistently shown in our lab to correspond to ~200-300 mg/dl blood ethanol concentration (BEC) (Smiley et al. 2020, Trantham-Davidson et al. 2014, Gass et al. 2014a, Gass et al. 2017, Uys et al. 2016). Body weight was monitored throughout this procedure to maintain the health of the animals. Additionally, control animals were treated similarly by bringing them into the lab and placing them outside of the chambers throughout exposure.

Memory Reactivation and Optogenetic Stimulation Parameters

Two days following the end of CIE, animals were exposed to fear memory recall paired with optogenetic inhibition of the PrL or IfL to block fear memory reconsolidation. Fear memories were reactivated through the presentation of the conditioned fear cues including the fear environment as well as the tone that previously served as the conditioned stimulus. Animals received optogenetic inhibition during the memory reconsolidation period in their home cage immediately following the presentation of the fear associated cues. Photoactivation was induced by 532 nm light delivered intracranially for three minutes (10 Hz pulses, 15 ms pulse duration, 10 mW illumination).

Fear Extinction and Spontaneous Recovery

Following this initial fear memory reactivation, animals completed a total of five extinction sessions to monitor freezing behavior in response to the CS to determine the effect of optogenetic inhibition of the PrL or IfL cortex during memory

reconsolidation on extinction learning. All extinction sessions consisted of ten presentations of the tone (CS) for thirty seconds separated by a 10 second inter-stimulus interval. There was no shock delivered during these sessions, and freezing was recorded during each tone presentation. Extinction criteria was met when animals were freezing less than 30% of the time in response to the CS. Once animals successfully met this criterion, there was a three-week break before they were placed back into the operant boxes for a single session during which the CS was presented a single time and freezing was measured for the duration of the tone (30 seconds) to measure the spontaneous recovery of fear behaviors. Importantly, optogenetic inhibition was only administered following the initial fear memory reactivation, and all extinction and recall testing was completed without any photostimulation.

Statistical Analyses

The primary dependent variable measured for the fear conditioning, extinction, and spontaneous recovery sessions was percent freezing in response to the conditioned stimulus (tone). These behavioral data were analyzed using SPSS version 23.0 software (SPSS Inc., Chicago, IL) and Prism version 8 (GraphPad software, La Jolla, CA). Both fear conditioning and extinction data were analyzed using a 2-way ANOVA with Holm-Sidak multiple comparisons as the post-hoc test used to determine differences between each treatment group. Extinction data were analyzed as an average response to all ten CS presentations for each day of extinction. For spontaneous recovery data, an ordinary one-way

ANOVA with Holm-Sidak multiple comparisons test used to compare all four treatment groups. All experiments used an n of 6 animals/treatment group, and a p value of less than 0.05 was considered statistically significant.

Results

These experiments used chronic intermittent ethanol exposure to model alcohol dependence with binge-like levels of ethanol exposure. This highly characterized model has been previously used by our lab to establish deficits in fear- and drug-related learning (Smiley et al. 2020; Gass et al. 2017). A five-point behavioral intoxication scale (Nixon and Crews 2002) was used to gauge intoxication levels in each of the animals with a target of moderate intoxication corresponding to a 2-3 rating. A subset of 72 (36M, 36F) out of the total 96 animals underwent CIE exposure, and the intoxication ratings were averaged between each sex and across all exposure periods. The intoxication score average across all days and all rats was 2.8 ± 0.09. As a complementary measure to the behavioral intoxication rating, blood ethanol content (BECs) was 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 267.36 ± 29.17 (Day 2), 262.4 ± 27.21 (Day 6), 251.8 ± 26.3 (Day 10), and 239.54 ± 24.6 (Day 15) with an overall grand average across all 4 days of 255.3 ± 26.82. For the females, our analysis revealed average BEC mg% values of 261.7 ± 25.45 (Day 2), 265.5 ± 28.76 (Day 6), 257.11 ± 22.52 (Day 10),

and 242.08 ± 19.73 (Day 15) with an overall grand average across all 4 days of 256.6 ± 24.1 (**Figure 4-2**).

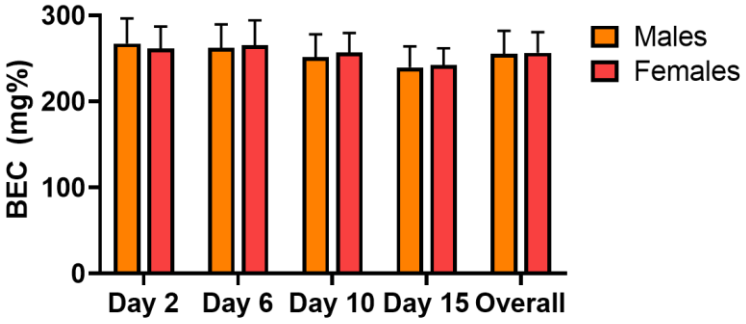

Figure 4-2: CIE ratings for all animals in Experiments 1 & 2. Blood ethanol content was measured on CIE days 2,6,10, and 15 and, in both males and females, was recorded to be between 200-300 mg% which corresponds to a moderate intoxication rating.

Prelimbic inhibition during fear cue memory reconsolidation facilitates extinction learning

In Experiment 1, we examined the effect of PrL inhibition during fear memory reconsolidation on fear extinction learning in a rat model of PTSD/AUD. Experimental procedures were completed following the outline in **Figure 4-1**. The treatment groups used for Experiment 1 included comparisons between animals that received CIE without PrL Inhibition (CIE-NI), CIE with PrL Inhibition (CIE-I), CIE without a surgical implant (CIE), and air (Air) for each sex.

Prior to extinction testing, all animals were required to meet a criteria of freezing 80% of the time in response to the tone (conditioned stimulus, CS) during the fear conditioning phase. Analysis of freezing behavior during this phase of

training was completed to ensure that all animals received the same degree of fear conditioning before subsequent experimental manipulation. In **Figure 4-3**, each data point represents an average of the freezing responses to the four CS presentations that occur on each day of conditioning. For male animals, there was a significant difference in freezing to the CS between treatment groups during the initial CS presentations (**Figure 4-3A**, $F(3, 20) = 14.94$, $p < 0.0001$), but there were no significant differences between the groups by Day 3 ($F(3, 20) = 0.8281$, $p = 0.4939$), and all animals met the 80% criteria. For females, there was no significant difference in freezing behavior throughout conditioning between any treatment groups (**Figure 4-3B**, $F(3, 60) = 1.998$, $p = 0.1239$), and all animals were freezing over 80% by their third conditioning session.

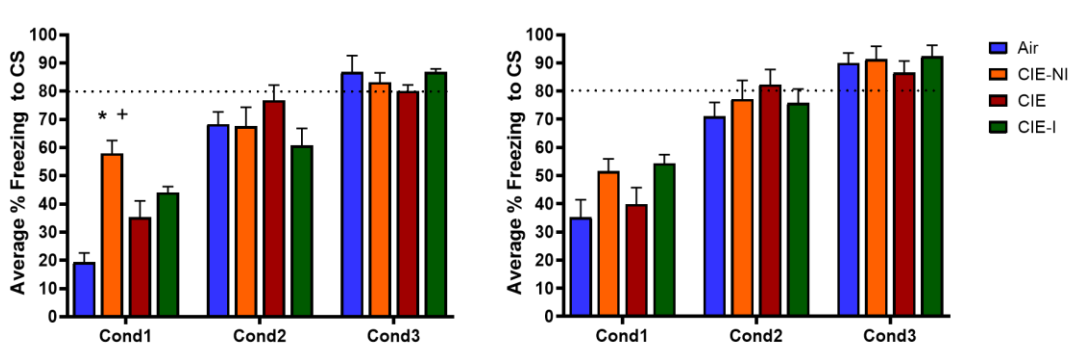

Figure 4-3: Prior to further experimental testing, all animals met fear conditioning criteria in Experiment 1. Significant differences were observed in freezing between the groups for male animals on Day 1 of conditioning, but, regardless of subsequent CIE exposure or optogenetic inhibition, all groups met 80% freezing criteria and there were no significant differences in freezing by the end of conditioning for both males (**A**, $F(3, 20) = 0.8281$, $p = 0.4939$) and females (**B**, $F(3, 20) = 0.3924$, $p = 0.7598$). Individual group differences are noted on the figure where * = CIE-NI vs. CIE and Air, + = Air vs. CIE and CIE-I ($p < 0.05$, n = 6/group).

Animals were permitted a two-day break to allow for fear memory consolidation after conditioning, and were subsequently exposed to two weeks of CIE. The following week, animals were put back into the fear context and exposed to a single presentation of the CS (tone) to induce fear memory recall. Freezing was measured during this fear recall session and analysis revealed no differences between any groups in baseline freezing during this session (**Figure 4-4**). Immediately following the fear memory recall session, optogenetic inhibition of the PrL occurred during the memory reconsolidation phase and freezing to the CS was measured across five subsequent extinction sessions. Each bar depicted on **Figure 4-4** is representative of average freezing to a total of 10 tone presentations on each extinction day. For male animals there was a main effect of treatment on freezing to the CS throughout extinction (**Figure 4-4A**, $F (3, 120) = 25.83$, $p < 0.0001$). As shown in **Figure 4-4A**, CIE exposed animals that did not receive surgical implants exhibited significantly higher levels of freezing when compared to air exposed animals throughout extinction which replicates our recently published studies regarding CIE's effects on extinction learning (Smiley et al. 2020). Additionally, these experiments found that inhibition of the PrL during fear memory reconsolidation prevented CIE induced deficits in extinction learning, since CIE-I animals exhibited significantly lower levels of freezing when compared to the CIE-NI and CIE groups. This was also observed in female animals where there was a main effect of treatment on freezing in response to the tone throughout extinction training (**Figure 4-4B**, $F (3,120) = 21$, $p < 0.0001$). In these animals, CIE exposure led to deficits in extinction learning with CIE exposed animals exhibiting

higher levels of freezing to the CS compared to air exposed animals throughout extinction. Furthermore, these experiments found that blockade of PrL activity during fear memory reconsolidation had the ability to enhance extinction since CIE-I animals exhibited significantly lower rates of freezing when compared to CIE-NI and CIE animals on multiple days of extinction training. Individual differences between all treatment groups on each extinction day are shown in **Figure 4-4** where * = CIE-I vs. CIE and CIE-NI, and + = Air vs. CIE.

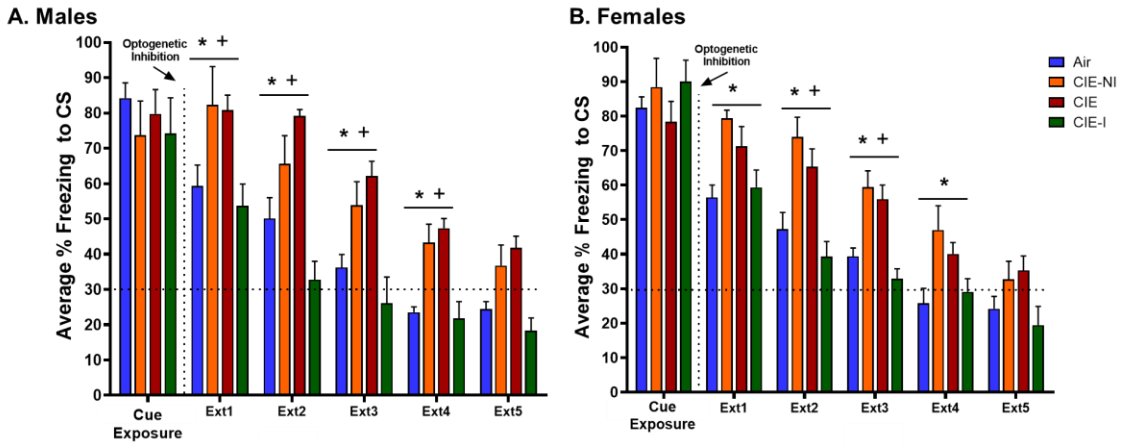

Figure 4-4: Inhibition of the PrL following fear memory reactivation facilitates extinction learning for both males and females. (A) For male animals, CIE exposure led to increased freezing during extinction when compared to both air and CIE-I groups ($F(3, 120) = 25.83$, $p < 0.0001$). **(B)** Female animals exhibited a similar effect, where CIE exposed groups exhibited a higher rate of freezing during extinction when compared to both CIE-I and air exposed animals. ($F(3, 120) = 21.00$, $p < 0.0001$). Individual group differences are noted on each extinction day where * = CIE-I vs. CIE-NI, and + = Air vs. CIE ($p < 0.05$, n = 6/group).

Following extinction, animals were permitted a three-week break before being tested for spontaneous recovery of fear behavior in response to the fear context and a single CS (tone) presentation. Blockade of fear memory reconsolidation, through inhibition of the PrL cortex during the initial fear memory

recall session, prevented the spontaneous recovery of freezing behavior in male and female animals, which supports the hypothesis that PrL inhibition disrupted fear memory reconsolidation. There was a main effect of treatment for males ($F(3, 20) = 8.594$, $p = 0.0007$) where CIE exposed animals exhibited heightened freezing when compared to air exposed controls, and CIE-I animals froze significantly less than the CIE-NI group (**Figure 4-5A**). This was also observed in females, where CIE and CIE-NI exposed animals exhibited a significantly higher rate of freezing when compared to air exposed controls and the CIE-I group (**Figure 4-5B**, $F(3, 20) = 8.493$, $p = 0.0008$). Essentially, manipulation of fear memory reconsolidation through inhibition of the PrL had long term effects on fear behaviors elicited in response to previously conditioned fear cues. Individual differences between the groups are noted on **Figure 4-5** where * = CIE-NI vs. CIE-I and + = CIE vs. Air.

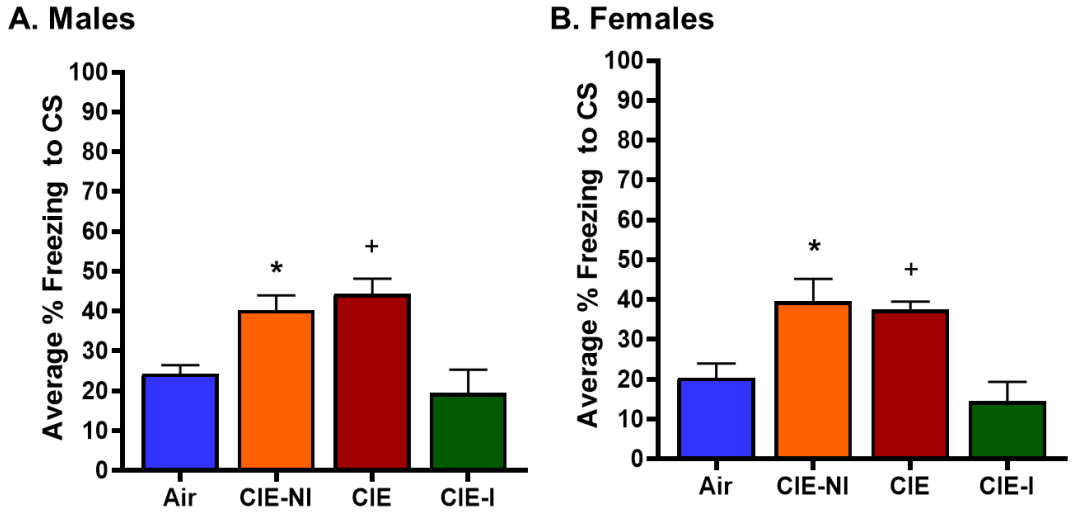

Figure 4-5: PrL inhibition during fear memory reconsolidation results in long term effects on fear cue-induced freezing behavior. (A) After a three-week break following extinction, male animals exposed to CIE exhibited significantly higher levels of freezing in spontaneous recovery when compared to CIE-I and air exposed controls ($F(3, 20) = 4.326$, $p = 0.0167$). **(B)** A similar effect was seen when spontaneous recovery was tested in females, where CIE exposure led to increased freezing in this session when compared to air exposed controls and CIE-I animals ($F(3, 20) = 8.493$, $p = 0.0008$). Individual differences between the groups are noted throughout the figure with * = CIE-NI vs. CIE-I and + = CIE vs. Air.

Infralimbic inhibition following fear cue exposure does not alter extinction learning

For Experiment 2 we used the same methodology described above, but optogenetic inhibition was instead targeted to the IfL cortex. The treatment groups used for Experiment 2 included animals that received CIE without IfL Inhibition (CIE-NI), CIE and IfL Inhibition (CIE-I), CIE without a surgical implant (CIE), and air (Air) for each sex. Prior to any experimental manipulation, freezing behavior during fear conditioning was examined to confirm that the responses to fear stimuli were consistent between treatment groups. For male animals, on Day 1 of

conditioning, there were significant differences between animals who would subsequently receive different treatments (F (3, 20) = 6.085, p = 0.0041), but by Day 3 of conditioning all animals met freezing criteria of 80% and there were no differences between any treatment groups (**Figure 4-6A**, F (3, 20) = 1.414, p = 0.2680). This trend was similar in female groups, where on Day 1 there were significant differences between the groups (F (3, 20) = 4.870, p = 0.0106), but by Day 3 there were no differences between any groups and all animals were freezing above criteria of 80% (**Figure 4-6B**, F (3, 20) = 0.6780, p = 0.5757).

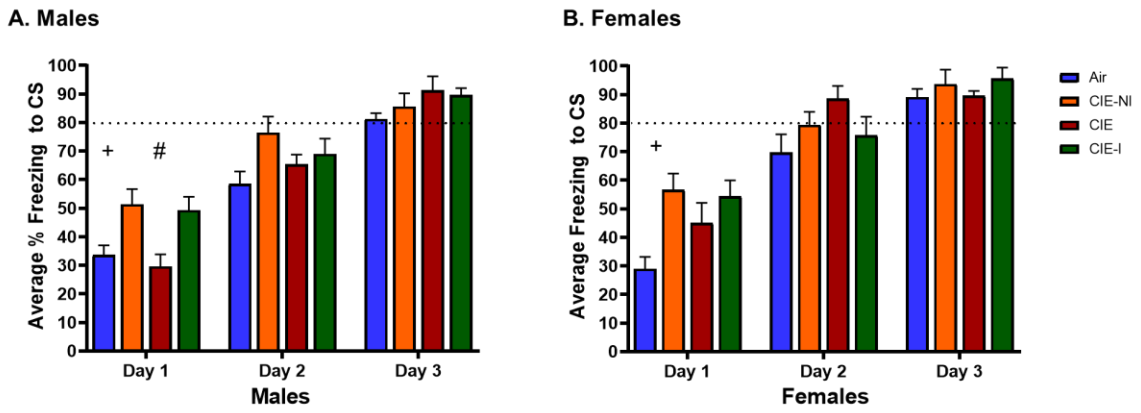

Figure 4-6: In Experiment 2, all animals met fear conditioning criteria prior to experimental manipulation. **(A)** After a three-week break following extinction, male animals exposed to CIE exhibited significantly higher levels of freezing in spontaneous recovery when compared to CIE-I and air exposed controls (F (3, 20) = 4.326, p = 0.0167). **(B)** A similar effect was seen when spontaneous recovery was tested in females, where CIE exposure led to increased freezing in this session when compared to air exposed controls and CIE-I animals (F (3, 20) = 8.493, p = 0.0008). Individual differences between the groups are noted throughout the figure with + = Air vs. CIE-NI and CIE-I, and # = CIE vs. CIE-NI and CIE-I.

Once all animals successfully met conditioning criteria and completed CIE exposure, they were re-exposed to the fear associated environment and cues to induce fear memory recall. Directly after this session, the IfL was inhibited during the memory reconsolidation phase and animals were subsequently exposed to five extinction training sessions during which freezing to the CS was recorded. A main affect was observed with regards to treatment in male animals (**Figure 4-7A**, $F (3, 120) = 11.53$, $p < 0.0001$), but an analysis of multiple comparisons revealed that this treatment effect was specific to CIE animals who exhibited heightened freezing when compared to the air group throughout extinction training. Unlike PrL inhibition groups, there were no significant differences recorded in freezing throughout extinction when comparing the CIE-I and CIE-NI groups. This effect was also absent in female animals, where there was a significant impact of CIE exposure ($F (3, 120) = 5.249$, $p = 0.0019$), but there were no significant differences between the CIE-I and CIE-NI groups (**Figure 4-7B**). Essentially, while inhibition of the PrL during fear memory reconsolidation was able to prevent CIE induced deficits in extinction learning, IfL inhibition during fear memory recall in CIE exposed animals did not alter freezing behavior.

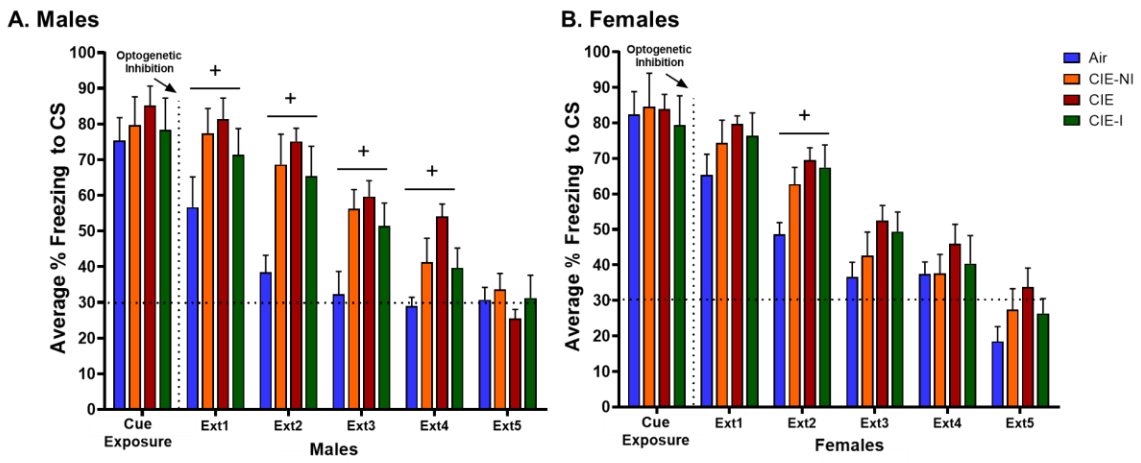

Figure 4-7: Inhibition of the lfL following fear memory recall does not alter fear extinction in either males or females. (A) There was an overall effect of treatment on freezing during extinction for male animals (F (3, 120) = 11.53, p <0.0001), but an analysis of multiple comparisons showed that this difference was only reflected when comparing CIE vs. Air exposed groups. There was no significant difference between animals that received lfL inhibition when compared to those that received sham stimulation. **(B)** There was a similar effect observed in female animals where CIE exposure induced a significant treatment effect (F (3, 120) = 5.249, p = 0.0019), but there was no effect of lfL inhibition during memory reconsolidation on freezing during extinction (+ p < 0.05 Air vs. CIE, n = 6/group).

Following extinction, animals were permitted a three-week break before being tested for the spontaneous recovery of freezing behavior following exposure to the fear-related cues and context. For male animals, there was a significant effect of treatment (**Figure 4-8A**, F (3, 20) = 3.573, p = 0.0323), but multiple comparisons analysis revealed that the only significant difference was between the CIE and Air groups (p = 0.0258). Importantly, there was no difference in freezing to the CS between the CIE-I group and the CIE-NI group. Additionally, for female animals, there were no significant differences between any treatment groups in freezing to the CS during the spontaneous recovery testing session (**Figure 4-8B**, F (3, 20) = 0.9874, p = 0.4187). Following the completion of the behavioral

experiments, animals were transcardially perfused and brains were collected for analysis of virus placement (**Figure 4-9**).

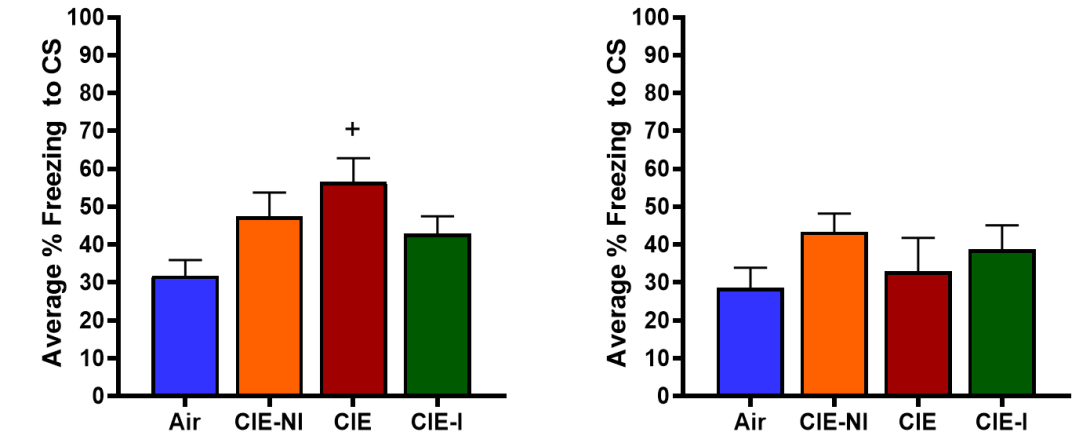

Figure 4-8: IfL inhibition during fear memory recall does not impact the spontaneous recovery of fear behavior. (A) For male animals, CIE exposure led to significantly increased levels of freezing during spontaneous recovery testing when compared to air exposed animals ($F(3, 20) = 3.573$, $p = 0.0323$), but prior inhibition of the IfL during memory reconsolidation did not cause any changes in freezing during this test. **(B)** For females, there were no significant differences between any treatment groups during the spontaneous recovery test ($F(3, 20) = 0.9874$, $p = 0.4187$) (+ $p < 0.05$ CIE vs. Air n = 6/group).

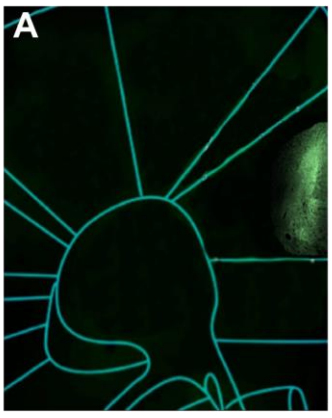

Figure 4-9: Histological verification of virus placement A) Representative image of AAV-CaMKIIa-eNpHR3.0-EYFP infused into layer 5 of the rat PrL cortex.

Discussion

These studies conclude that inhibition of the PrL following fear memory recall blocks fear memory reconsolidation and, thus, facilitate the extinction of fear behaviors and reduce freezing during spontaneous recovery. This was shown to be observed in both male and female animals and was specific to the PrL cortex since repetition of the experiment targeting the IfL cortex instead did not induce the same behavioral response. The rodent model of comorbid PTSD/AUD used in these studies also served to replicate and confirm previous studies completed in our lab that explored deficits in fear learning following binge-like ethanol exposure (Smiley et al. 2020). These experiments provide preclinical evidence that alterations of activity in the prelimbic cortex can serve to alter the presentation of fear behaviors during extinction learning and spontaneous recovery in both males and females. Additionally, these studies support the hypothesis that manipulation of signaling in these regions could serve as a viable treatment option for individuals with PTSD/AUD.

Previously, work in our lab has thoroughly investigated the role of the PrL and IfL cortices in the expression and suppression of alcohol seeking behavior (LaCrosse et al. 2015, Gass et al. 2014a, b, Gass et al. 2017, Gass et al. 2013). Briefly, these studies focused on positive allosteric modulation of the PrL and IfL cortices to treat the structural and functional deficits that occur as a result of chronic alcohol exposure. These experiments also examined the behavioral impairments that can occur as a result of chronic ethanol, including impaired extinction of drug-seeking behaviors induced by plasticity changes in these regions. Due to the

additional function of the PrL and IfL in the expression and suppression of fear-related behaviors (Hayen et al. 2014, Goode et al. 2019, Peters et al. 2009, Moorman et al. 2015, Palombo et al. 2017), current work has focused on manipulation of these regions to treat co-occurring PTSD and AUD. For these experiments, animals are exposed to fear conditioning paradigms before CIE exposure to translationally model the progression of these comorbid disorders. Essentially, this design allowed us to model the human pattern of trauma exposure followed by binge-like levels of ethanol used to self-medicate anxiety symptoms while fear extinction training modeled the exposure-type therapies used in human psychiatric treatment for PTSD/AUD. Recently, we demonstrated the ability of chronic alcohol exposure to cause deficits in fear extinction learning and used glutamatergic manipulation of IfL during fear extinction to treat these deficits (Smiley et al. 2020). The present experiments expanded upon these data by using optogenetics to manipulate prefrontal glutamatergic activity during fear memory recall to affect extinction learning instead of during active extinction learning. These results add to the established literature regarding differential roles for the PrL and IfL cortices and incorporate a novel aspect by using a comorbid model of PTSD/AUD in both male and female animals.

While clinical examinations of PTSD rely on a variety of symptoms that are verbally described, such as re-experiencing the trauma, nightmares, and intrusive thoughts, we cannot rely on these same diagnostic criteria in animal models (Miao et al. 2018). As such, using a preclinical model introduces a variety of limitations that need to be addressed. To utilize a valid model of PTSD, we have to rely on

the underlying neural circuitry that is involved in fear behaviors, behavioral responses that are shared between humans and rodents, and the establishment of the disorder through exposure to a fixed experience of extreme stress (Flandreau et al. 2017). With regard to specific preclinical PTSD models, the model must allow for longitudinal studies of symptomology to assess long term responses to fear stimuli, be induced by exposure to an aversive event with only a short duration necessary to invoke PTSD-like symptoms, and increasing intensity of the stimulus must be able to increase the severity of the outcomes (Siegmund and Wotjack 2006). Established preclinical models of PTSD include restraint stress, single prolonged stress, social defeat stress, and fear conditioning. While each of these models has aspects that meet the above criteria, fear conditioning is the model that best allows us to answer questions regarding longitudinal relationships between the alcohol exposure and fear related learning and memory (Ursano et al. 2007, Borghans et al. 2015, Lissek et al. 2015, Singewald et al. 2019, Mahan et al. 2012, Amstadter et al. 2009). While our preclinical model of PTSD/AUD achieves validity, there are additional limitations that need to be considered when examining these data. First, all extinction training was completed relatively close to the completion of ethanol exposure. The literature regarding the effects of ethanol on fear learning have been mixed with regards to both the level of ethanol exposure that is required to induce changes in fear learning as well as any long-term effects induced by ethanol (Holmes et al. 2012, Quiñones-Laracuente et al. 2015). As such, an important aspect of future studies would be to include groups that receive extinction training at a later date to assess the long-term effects of

ethanol exposure on fear learning. An additional limitation comes from the fact that estrous cycle was not examined in the female treatment groups. While estrous cycle has been shown to alter behavior in certain preclinical models of addiction (Lacy et al. 2016, Anker and Carroll 2011) and cognition (Cordeira et al. 2018, Broestl et al. 2018), data from both our lab (Smiley et al. 2020) and others (Maeng et al. 2015) establishes the fact that estrous cycle phase does not have an effect on freezing behavior during extinction. Therefore, we do not see estrous cycle as a factor that could serve to alter fear behaviors during extinction in these studies.

Taken together, the results from these experiments conclude that inhibition of the PrL, and not the IfL, during fear memory reconsolidation facilitates extinction learning and prevents increased freezing during memory reconsolidation for both male and female animals in a preclinical model of PTSD/AUD. These studies support the use of modulation of memory reconsolidation for the treatment of learning and memory deficits that occur as a result of exposure to stress and chronic alcohol and, further, identify specific time-points and brain regions that are necessary for this treatment effect.

CHAPTER 5

ADOLESCENT THC AND ETHANOL EXPOSURE LEADS TO IMPAIRMENTS IN PREFRONTAL CORTEX RELIANT COGNITION

Background and Significance

Cannabis use disorder (CUD) has doubled in prevalence over the past decade as marijuana becomes increasingly accessible due to a country-wide trend towards legalization. As a result, marijuana is the most commonly used illicit drug in the United States. Importantly, levels of Δ-9-tetrahydrocannabinol (THC), the psychoactive component of the drug, have also steadily increased in recent years. Studies have found that cannabis use has grown the most in the adolescent

population, a group that is increasingly at risk for harmful side-effects of drug use due to ongoing brain maturation, most notably in the prefrontal cortex (PFC). This region is involved in both addiction and anxiety disorders that can result from neurochemical changes due to trauma experience or insults from chronic drug use. These alterations often lead to cognitive deficits that maintain and reinforce both disorders. Clinical studies have found that the age of onset of marijuana use is positively correlated with the development of both CUD as well as long-term impairments in cognition. The main deficits examined in this patient population include behavioral inflexibility and propensity towards both risky and impulsive behaviors. These deficits are common to both substance use disorders and anxiety disorders and serve as barriers to successful treatment outcomes. Increasing evidence indicates that CUD often occurs along with other debilitating conditions including both alcohol use disorder (AUD) and anxiety disorders such post-traumatic stress disorder (PTSD). These disorders have also been shown to induce similar cognitive deficits that could add to the deleterious effects of chronic cannabis exposure. The average age at which CUD develops is around late adolescence to early adulthood, a period in which brain development, especially in prefrontal regions, is still progressing (Khoury et al. 2010). Surveys of high school seniors determined that half of them have tried marijuana while 5% of them participate in daily use (Schweinsburg et al. 2008). Adolescents who begin marijuana use earlier, and adult users who started use in mid-adolescence, have more problems with cognition even following the cessation of marijuana use. Studies completed in both animals and humans indicate that chronic marijuana

exposure causes a greater vulnerability to developing abnormalities in cognition (Schweinsburg et al. 2008, Pope et al. 2003, Patton et al. 2002, Irimia et al. 2015, Hill et al. 2006). There is consensus in the literature that, when comparing early vs. late onset cannabis use, those that started using cannabis earlier in life during adolescence exhibit poorer cognitive performance than those that started using cannabis later and those that did not use cannabis (Pope et al. 2003). Interestingly, there has also been associations found between long term cannabis use and higher rates of developing anxiety and depression later in life, especially for females who begin drug use in adolescence (Patton et al. 2002). While the acute effects of cannabis use on cognition are established, there is less data that examines long term effects on cognitive processing. Studies using animal models are also scarce, but there is some evidence that long term exposure to THC can cause motor impulsivity and perseveration, hallmarks of behavioral disinhibition and impaired cognitive flexibility (Irimia et al. 2015). Other models that have used CB1 agonists have found decreased behavioral flexibility in a model of strategy set shifting (Hill et al. 2006). While the presence of cognitive deficits is being explored in the clinical population, there is further research needed to determine the mechanism by which these deficits are induced to develop new targets for pharmacological treatments. Therefore, these studies examined the impact of THC vapor exposure and chronic intermittent ethanol exposure (CIE) as a model of co-occurring cannabis and alcohol use during adolescence on prefrontal cortex reliant behaviors. Following adolescent exposure to THC/ethanol, animals were tested for

deficits in cognition and alterations in fear and alcohol seeking behaviors were addressed with the use of N-acetylcysteine as a pharmaceutical treatment.

Materials and Methods

Animals

Male Wistar rats were postnatal day (PD) 35 for adolescents and PD 55 for adults upon arrival (Charles River Laboratories) and were pair housed in standard cages in a vivarium that maintained a reverse light-dark cycle. Lights were turned off at 0900 and this allowed for behavioral testing to occur during the dark phase. Animals received continuous access to food and water except during behavioral testing which occurred for up to one hour per day. All experiments had prior approval by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and were completed within guidelines set forth by the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003). A total of 88 animals were used across Experiments 1 and 2, and the treatment groups were assigned based on the outline in **Table 5-1**.

Table 5-1. Treatment Groups Used For Experiment 1 & 2

Treatment Groups		
Experiment 1 n = 8/group	**Experiment 2** n = 8/group	
Air + Air (Air)	Adolescent	Air
THC + Air (THC)		THC
Air + CIE (CIE)		THC + NAC
THC + CIE	Adult	Air
THC + CIE + NAC		THC
		THC + NAC

Adolescent THC Vapor Exposure

THC vapor exposure started at PD 40 for adolescent animals and PD 60 for adults. The method of vapor administration occurred according the protocol previously established by Spencer et. al. 2018. During each exposure session, animals received a total of 20 minutes of vapor exposure per day for 5 days. Out of the total 20 minutes, each THC exposure session is broken up in the following manner to keep THC concentrations steady: in the morning there were two 5-minute exposures followed by a three-hour break and two additional 5-minute exposures. The solution vaporized during each exposure session consisted of THC at a concentration of 200 mg per mL of ethanol and CBD at 20 mg per mL of ethanol. These solutions are mixed at a 1:1:1 ratio with glycerol to obtain a final solution containing THC and CBD at a ratio of 10:1. A Volcano (Storz and Bickel, Oakland, CA) was used to vaporize 150 uL of the THC/CBD solution and the resulting vapor was administered equally among the animals. Animals assigned to the NAC treatment groups received an intraperitoneal (IP) injection of the drug

immediately following each exposure session. Following the five total days of THC exposure, animals were permitted a two-day break before entering chronic intermittent ethanol exposure (CIE).

Chronic Intermittent Ethanol Exposure (CIE)

Chronic intermittent ethanol exposure (CIE) is a model of binge-like alcohol consumption that uses repeated cycles of ethanol vapor exposure and abstinence. This procedure started at ~PD 47 for animals in Experiment 1 and consisted of four nights of vapor exposure from 1800-0800 (14 hours on/10 hours off) followed by a two-day break, and this cycle was completed a total of two times. Intoxication levels were graded on a five-point scale that judges the level of intoxication based on motor behavior. Rats were scored based on the following behaviors: 1: no signs of intoxication and no motor impairment, 2: slight intoxication and slight motor impairment, 3: moderate intoxication with obvious motor impairment but retains the ability to walk, 4: loss of righting reflex, highly intoxicated, 5: loss of righting reflex and eye blink reflex, extremely intoxicated (Nixon and Crews, 2002). The goal of each CIE session was to reach moderate intoxication levels which corresponds to a 2-3 on the five-point scale and ~250 mg/dl BEC. Animals in treatment groups that received NAC received injections two hours before going into the CIE chambers at night and immediately after they came out of the chambers in the morning.

Strategy Set-Shifting Task

To study the effect of adolescent THC and ethanol exposure on behavioral flexibility in adulthood, Experiment 1 utilized a strategy set-shifting paradigm following the techniques established by Stan Floresco (Floresco et al. 2008). To establish lever pressing behavior, there were a total of five training phases prior to the set-shifting test. First, Phase1A consisted of both levers being presented and a press on either lever would result in the delivery of 10% sucrose. Once all animals were able to perform ~50 lever presses within this 30-minute session, they were able to move onto Phase1B (~10 days of training). This phase was identical to Phase 1A with the addition of retraction of the levers following each lever press. Again, animals had to maintain ~50 lever presses during these session before moving on the Phase1C (~8 days of training). Phase1C built upon 1B by presenting the retractable levers for ten second intervals. If animals did not press the lever within the ten seconds, levers were retracted, and the trial restarted. Animals were able to move on from Phase1C once they finished the session with less than 10 omissions (~10 trials). Animals were then tested for a side preference for the test session to be completed using the opposite lever. The following two phases involved pressing a specific lever according to a trained rule to determine changes in cognitive flexibly when the rule was changed between sessions. Rule 1 was established in PhaseCue, during which animals were required to respond to the lever that was cued by illumination of the associated stimulus light. Criteria for learning this rule was met when all animals press 8 times in a row correctly in a

minimum of 30 trials. These experiments tested a between-session set-shift, so, once criteria was met for the first rule, the session on the following day was under a second rule. The second rule required the animals to press on a single lever location regardless of where the light appears. Two days of testing were administered using this criteria, and animals were eliminated from the experiment if they did not meet criteria within this time period. Two different types of errors, perseverative and regressive, were recorded to determine if the ability to either shift to new strategy or maintain the new strategy was affected by drug exposure. Perseverative errors were those that occur before the animal has made five correct presses under the second rule while regressive errors are those that occur after five correct presses have been made. Animals that take longer to learn the second rule and make more errors that cause them to continue responding for the original rule are those considered to have impaired behavioral flexibility.

Operant Ethanol Self-Administration

For Experiment 2, operant self-administration paradigms were used to determine the effects of adolescent THC exposure on ethanol seeking during adulthood. Following THC exposure, animals were placed into operant chambers and trained to lever press for 20% ethanol on an FR-1 schedule of reinforcement. These self-administration sessions each lasted one hour and occurred three days a week. Each lever press on the active lever delivered 20% ethanol v/v in water accompanied by stimulus light illumination and tone presentation (2900 Hz, 65 dB)

and was followed by a 4-second timeout period during which presses were recorded but did not lead to reinforcer delivery. Once response criteria of 30 lever presses was met (~10 sessions), ethanol concentration was lowered to 10% and criteria of 60 lever presses was met (~15 sessions). Animals completed extinction training following the completion of self-administration. During extinction sessions, pressing on the active lever allowed for stimulus light illumination and tone presentation in the absence of any ethanol reinforcer. Extinction criteria was met when lever pressing for each rat was reduced to 80% of the lever presses recorded on last two days of self-administration training (~5 sessions). Once lever pressing was extinguished, there was a break period of 21 days following which spontaneous recovery of lever pressing behavior was tested in a single session where lever presses were recorded across the 30-minute session and resulted in cue presentation alone.

N-acetylcysteine (NAC) Preparation

For animals in the NAC treatment group across both experiments, NAC was delivered at a dose of 100 mg/kg intraperitonially (IP) and was prepared on the day of injection following previously published methods (Garcia-Keller et al. 2019). To achieve this concentration, 5 grams of NAC (Millipore Sigma) was added to 5 mL of 27 mg/mL NaOH in 0.9% saline. Since this solution is acidic, 200 mg/mL NaOH in saline was added in 50 uL increments until the solution reached a pH of ~7.5. On the day of injection, animals were weighed and dosed accordingly.

Statistical Analyses

These behavioral data were analyzed using SPSS version 23.0 software (SPSS Inc., Chicago, IL) and Prism version 8 (GraphPad software, La Jolla, CA). All experiments used an n of 8 animals/treatment group, and a p value of less than 0.05 was considered statistically significant. The primary dependent variable measured for Experiment 1 was either infusions received, well entries completed, or average lever pressing for each of the training and test phases. Behavioral data from Phase1A-1C, Side Preference, and Phase Cue trials were analyzed using a 2-way ANOVA with Holm-Sidak multiple comparisons as the post-hoc test used to determine differences between each treatment group. Trials completed in the set-shifting test were analyzed using an ordinary one-way ANOVA with Holm-Sidak multiple comparisons test used to compare all five treatment groups while errors made during this test were analyzed through a 2-way ANOVA with a Holm-Sidak multiple comparisons test. For Experiment 2, lever pressing behavior throughout self-administration, extinction, and spontaneous recovery were analyzed using a 2-way ANOVA with a Holm-Sidak multiple comparisons test to compare across treatment groups. Animals in Experiment 1 were eliminated from the experiment if they did not meet criteria of 10 correct lever presses within these two set-shifting testing sessions. Additionally, animals were eliminated from Experiment 2 if they did not meet self-administration criteria during the second phase of acquisition where ethanol concentration was at 10%.

Results

Two main experiments were completed to determine the effects of adolescent exposure to THC and ethanol on prefrontal cortex reliant behaviors including cognitive flexibility and drug seeking. Generally, animals across all experiments were exposed to THC vapor during mid-adolescence prior to behavioral testing across these two behavioral paradigms to widely assess behavioral responses to prefrontal cortex reliant tasks.

Effects of adolescent THC and ethanol exposure on cognitive flexibility

Experiment 1 was comprised of 40 male animals across the following treatment groups: Air exposed controls, THC exposed, CIE exposed, both THC + CIE exposed, and THC + CIE exposed animals that also received NAC (n = 8/group). Following the design outlined in **Figure 5-1**, animals were first exposed to THC vapor for a total of five days in which animals in the NAC treatment groups received injections directly after each exposure session. THC concentrations in the blood using this method of vapor administration has previously been established (Spencer et al. 2019) and, thus, was not obtained for these experiments.

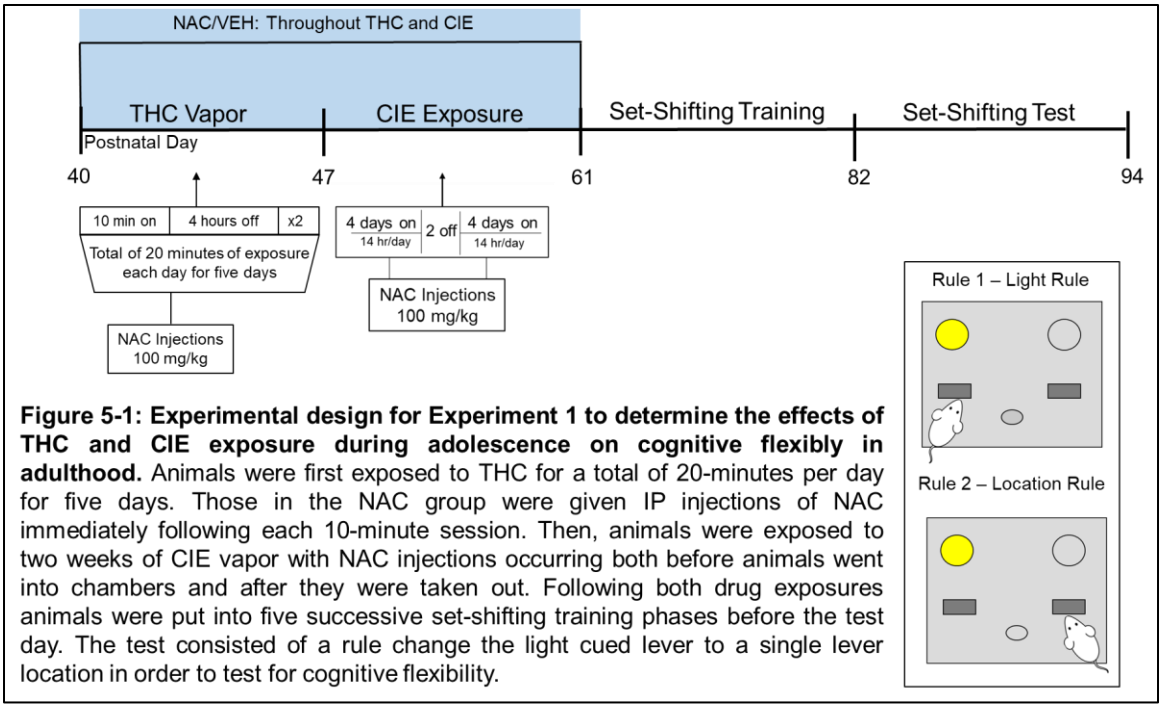

Figure 5-1: Experimental design for Experiment 1 to determine the effects of THC and CIE exposure during adolescence on cognitive flexibly in adulthood. Animals were first exposed to THC for a total of 20-minutes per day for five days. Those in the NAC group were given IP injections of NAC immediately following each 10-minute session. Then, animals were exposed to two weeks of CIE vapor with NAC injections occurring both before animals went into chambers and after they were taken out. Following both drug exposures animals were put into five successive set-shifting training phases before the test day. The test consisted of a rule change the light cued lever to a single lever location in order to test for cognitive flexibility.

CIE exposure commenced following the completion of THC exposure and behavioral intoxication ratings were taken following each night of exposure to confirm that animals were maintaining a moderate level of intoxication throughout the procedure (**Figure 5-2**). Animals were exposed to a total of eight nights of CIE vapor exposure with a goal of a behavioral intoxication rating of ~2-3 which corresponds to a blood ethanol concentration of ~250 mg% (Gass et al. 2016, Smiley et al. 2020).

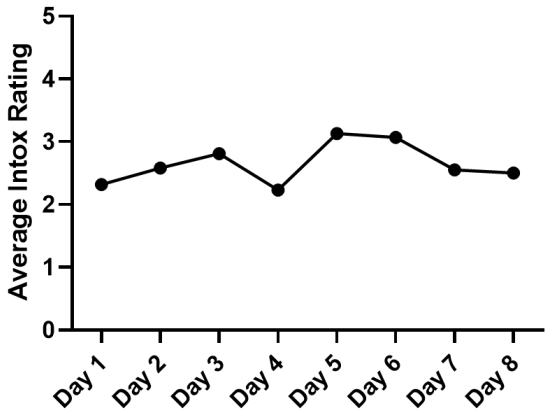

Figure 5-2: Average intoxication ratings from all animals during CIE for animals in Experiment 1. Animals were rated on a 5-point behavioral intoxication rating scale with a goal of moderate intoxication (2-3). Ratings are averaged across all animals each day (n = 24).

THC and CIE treated animals sought increased amounts of sucrose during set-shifting training

Set-shifting training commenced two days following the completion of CIE, and lever pressing for sucrose was monitored throughout all five training phases. During Phase1A animals were required to press either the right or left lever to receive a sucrose reward and trials continued daily until all animals met a criteria of ~50 lever presses within the 30-minute session. There was a significant effect of treatment on the number of infusions received and the number of well entries performed throughout Phase1A, with THC+CIE animals as well as animals exposed only to CIE exhibiting increases in sucrose seeking (**Figure 5-3A**) [$F(4, 70) = 15.0$, $p < 0.0001$]. Following Phase1A, animals were trained on retractable

levers in Phase1B where both levers were retracted following each lever press. Increases in sucrose seeking were observed in drug treated animals in this phase as well [F (4, 70) = 3.795, p = 0.0075], where THC+CIE and CIE treated animals performed more well entries than control groups while the number of infusions that were received remained similar between the groups (**Figure 5-3B**). This trend continued throughout Phase1C, where animals were trained to ten-second presentations of the levers. A similar effect was observed, where CIE exposed animals maintained an increased numbers of infusions as well as well entries compared to other treatment groups [F (4, 70) = 20.89, p < 0.0001] (**Figure 5-3C**).

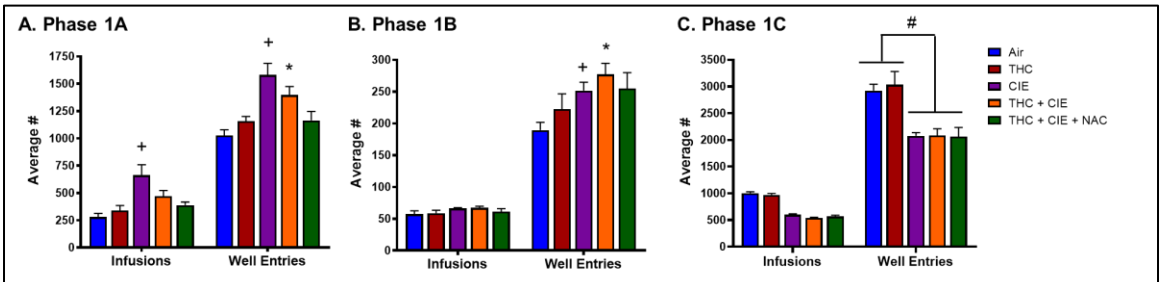

Figure 5-3: Sucrose seeking was measured during set-shifting training Phases 1A-1C. A) In Phase 1A of training, animals that were exposed to THC + CIE and CIE alone sought an increased number of reinforcers and performed significantly more well entries. **B)** This trend was continued in Phase1B where all animals received a similar number of reinforcers, but THC + CIE and CIE exposed animals performed more well entries. **C)** During Phase 1C, a similar effect was observed with CIE exposed animals showing increased sucrose seeking through a higher number of well entries performed across training (* p < 0.05 THC+CIE vs. Air, + p < 0.05 CIE vs. Air, # = p < 0.05 THC and Air vs. all other groups, n = 8/group).

These first three phases trained animals to lever press to receive sucrose in a manner that was specific to a ten-second availability followed by lever retraction. Next, animals were tested in a side-preference test to determine if they preferred either lever location. This test found that all animals exhibited a right lever preference (**Figure 5-4A**) and, thus, all subsequent testing used a left lever active program for the results not to be skewed by any innate lever preference. The final training phase of the set-shifting task was PhaseCue in which animals were trained to press either the right or left lever in response to an illuminated stimulus light that appeared above the lever. All animals had to press the indicated lever 8 times in sequence in a maximum of 30 trials to move on from this phase, and, while this criteria was the same between the groups and all animals received the same amount of reinforcers, those previously exposed to both THC+CIE performed more well entries for sucrose during this session [F (4, 70) = 4.786 p = 0.0018] (**Figure 5-4B**).

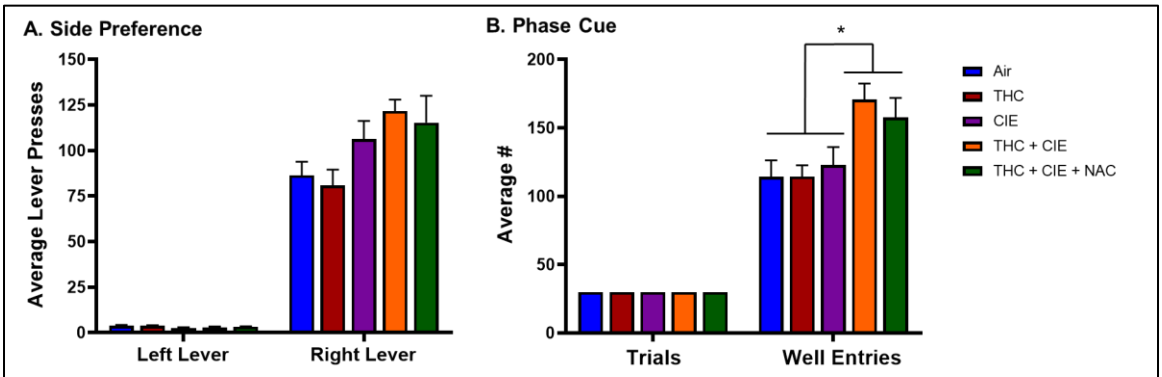

Figure 5-4: All animals exhibited a right lever preference and met criteria for phase cue before the test. A) Following the initial three phases of training, animals were tested in a single side preference session where all animals from all groups exhibited a clear right lever preference. **B)** By the last Phase Cue trial, all animals successfully met criteria of 30 trials, but during these trials animals exposed to THC + CIE performed significantly more well entries (* $p < 0.05$, n = 8/group).

Animals exposed to THC and CIE during adolescence display impairments in cognitive flexibility

Once these five training phases were completed, animals were tested on a between session set-shift to measure cognitive flexibility. During this test, animals were required to only press the left lever regardless of where the stimulus light was illuminated. The number of trials required for animals to learn this new rule and the number of perseverative and regressive errors that were performed when animals made an incorrect lever choice were recorded during this test. These studies found that THC+CIE exposed animals took an increased number of trials to meet criteria during the set-shifting test [$F(4, 29) = 3.615$, $p = 0.0165$], and that the increase was due to these animals performing more perseverative errors, or errors that occurred prior to successfully completing five correct lever presses ($p = 0.0135$), both of which indicate that THC+CIE exposed animals exhibit impairments in cognitive flexibility (**Figure 5-5B**). Additionally, this increase in both trials to criteria as well as perseverative errors was prevented by NAC treatment in THC+CIE exposed animals.

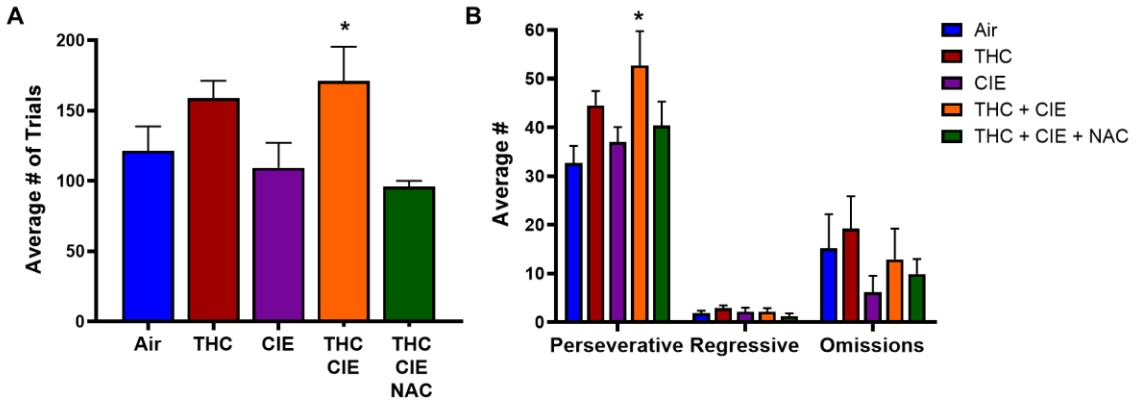

Figure 5-5: THC and CIE exposed animals took more trials to meet criteria during the set-shift due to increased perseverative errors. A) During the set-shifting test trial, animals exposed to THC + CIE and THC alone performed an increased number of trials in order to learn the new location rule, while those exposed to THC + CIE + NAC required a fewer number of trials to learn this rule. B) The increased number of trials required by the THC + CIE exposed animals was due to an increase in the number of perseverative errors performed when compared to the other treatment groups (* $p < 0.05$ THC + CIE vs. Air and THC + CIE + NAC, n = 8/group).

Effects of adolescent THC exposure on ethanol seeking in adulthood

Experiment 2 was completed using 48 male animals within the following treatment groups: Adolescents: THC, Air, THC+NAC, Adults: THC, Air, THC+NAC (n = 8/group). This experiment utilized the design outlined in **Figure 5-6** where animals were exposed to THC vapor for a total of five days and those in the NAC treatment groups received injections directly following each exposure session. Ethanol self-administration began following THC exposure and consisted of a total of 30 sessions, the first fifteen in which animals received 20% ethanol followed by fifteen subsequent sessions at 10% ethanol. Following successful acquisition of self-administration behavior, all animals were put through a week of extinction and

then a three-week break occurred before animals were tested for spontaneous recovery of ethanol seeking behavior.

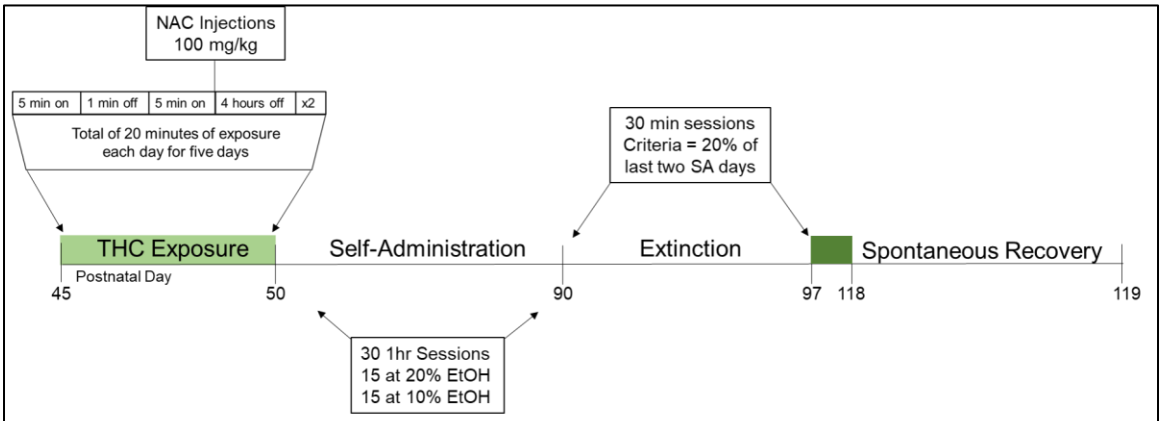

Figure 5-6: Outline of procedures for Experiment 2 to determine the effects of adolescent THC exposure on ethanol self-administration. Adolescent animals were first exposed to THC vapor for a total of 20 minutes per day for five days and those in the NAC treatment group received injections directly following each exposure. Then, animals were trained in ethanol self-administration paradigms followed by testing in extinction and spontaneous recovery sessions. For animals that were exposed to THC during adulthood, vapor exposure began on postnatal day 65, so the timeline is shifted twenty days forward for these animals.

Animals exhibit escalations in ethanol seeking during acquisition and extinction when exposed to THC during adolescence, but not adulthood

Following exposure to THC during either adolescence or adulthood, animals began training in self-administration paradigms. There was a significant effect of treatment [F (5, 19.67) = 2.909, p = 0.0398] on lever pressing behavior across all of self-administration, with animals exposed to THC during adolescence performing a significantly increased number of lever presses when compared to air exposed controls (**Figure 5-7A**). Notably, this effect was not present when THC

exposure occurred during adulthood. Similarly, this increase in ethanol seeking was also observed with regards to the number of reinforcers received throughout self-administration [F (5, 22.58) = 3.027, p = 0.0309], and was observed for adolescents but not adults (**Figure 5-7B**).

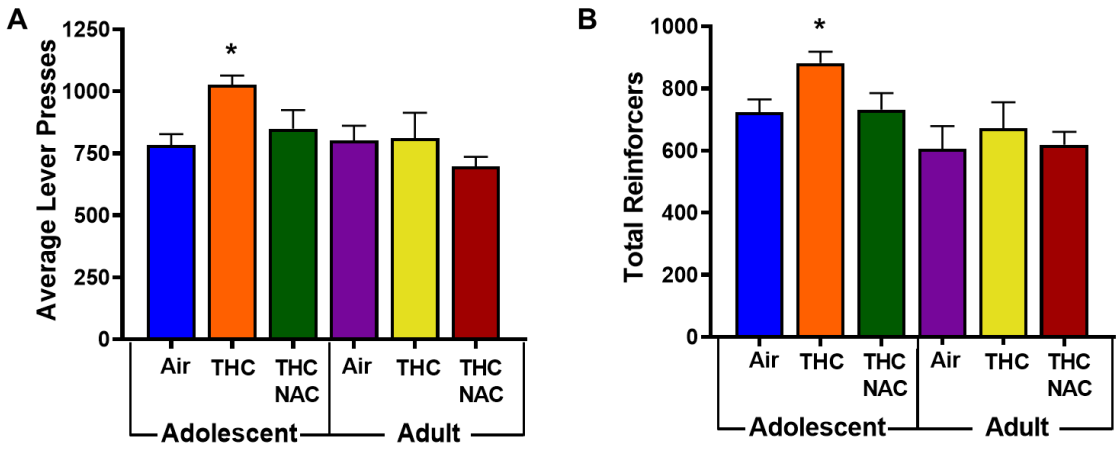

Figure 5-7: THC exposure increases ethanol seeking during self-administration for adolescents but not adults, and this effect is prevented with NAC treatment. A) When looking at total right lever presses performed during self-administration training, adolescent animals exposed to THC exhibited increased lever pressing for ethanol when compared to Air and THC animals that received NAC. This effect was not observed in adult animals exposed to THC. B) This trend was also true when looking at the total amount of reinforcers received throughout ethanol self-administration, where adolescent animals exposed to THC exhibited increased reinforcer delivery, while adult animals exposed to THC did not exhibit this effect (* $p < 0.05$ THC vs. Air and THC+NAC, n = 6-7/group).

A similar effect was observed during extinction training, where adolescent animals exposed to THC performed an increased number of lever presses during extinction when compared to air exposed animals [F (2, 102) = 4.943, p = 0.0089] (**Figure 5-8A)**, and this was not observed in animals exposed to THC during adulthood [F (2, 96) = 2.121, p = 0.1255] (**Figure 5-8B**).

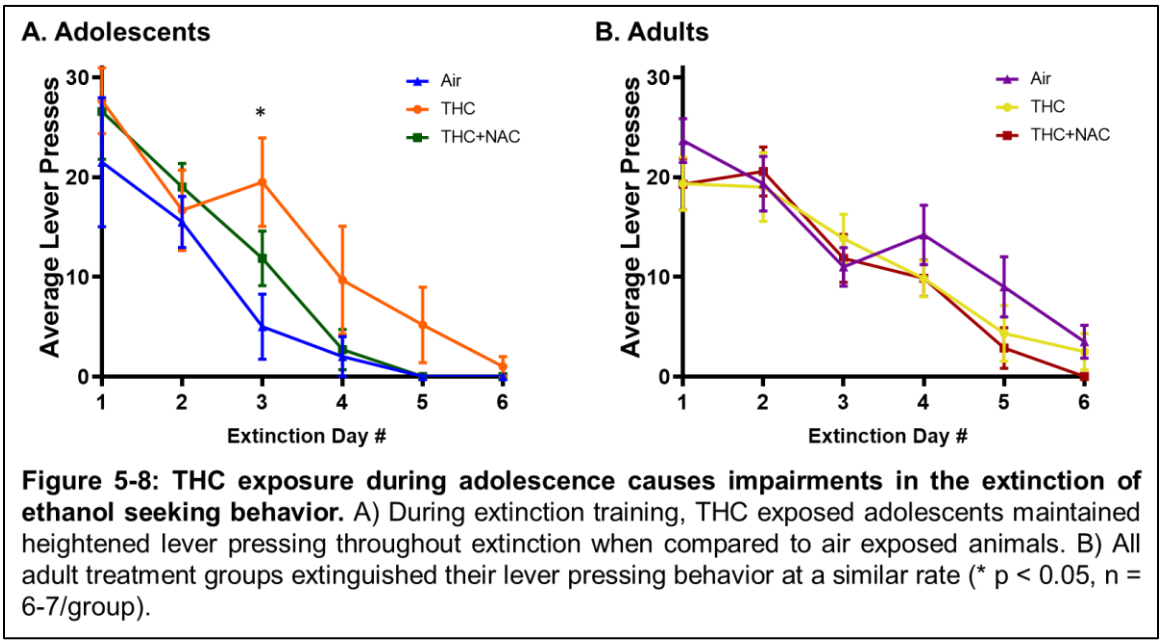

Figure 5-8: THC exposure during adolescence causes impairments in the extinction of ethanol seeking behavior. A) During extinction training, THC exposed adolescents maintained heightened lever pressing throughout extinction when compared to air exposed animals. B) All adult treatment groups extinguished their lever pressing behavior at a similar rate (* $p < 0.05$, n = 6-7/group).

This trend was carried over into spontaneous recovery testing where, while there were no effects of THC exposure on lever pressing during spontaneous recovery when compared to air exposed animals [F (5, 14.98) = 1.432, p = 0.2692], THC exposed adolescents performed more lever presses during this test when compared to THC exposed adults (p = 0.0364) (**Figure 5-9A**).

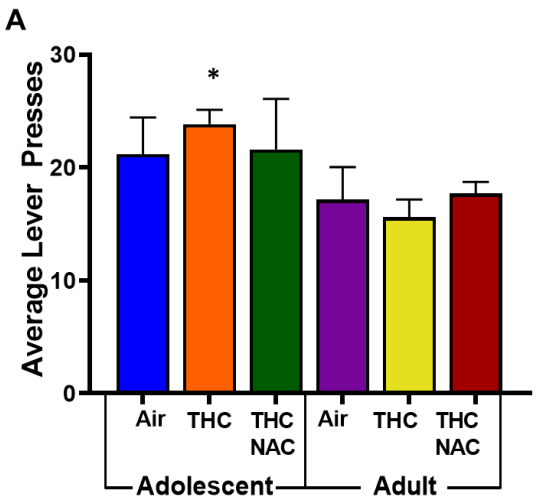

Figure 5-9: THC exposure does not alter the spontaneous recovery of ethanol seeking behavior. A) There were no differences in spontaneous recovery of ethanol seeking behavior between any of the treatment groups, but adolescent animals who were exposed to THC, performed more lever presses than THC exposed adults (* $p < 0.05$ THC Adolescent vs. THC Adult, n = 6-7/group).

Discussion

In summary, these experiments determined that exposure to THC and CIE during adolescence leads to impairments in cognitive flexibly shown by increases in trials to criteria as well as perseverative errors in a set-shifting test. Additionally, in Experiment 2, we observed that animals exposed to THC during adolescence increase ethanol seeking during self-administration, shown by increased lever pressing behavior, and that, in general, adolescent animals seek ethanol at an increased rate compared to adults. These experiment provide evidence that THC exposure during adolescence can have deleterious effects on prefrontal cortex

reliant cognitive tasks such as set-shifting and drug-seeking, and NAC is able to reverse these cognitive deficits.

While the data presented support the literature with regards to the effects of THC on cognition and drug-seeking behaviors, there are a number of limitations that need to be addressed. First, while adult groups were included for the analysis of ethanol seeking, no adults were tested in the experiments that involved cognitive flexibility testing. Although this is an important group that needs to be included to make conclusions regarding the specificity of this effect to the adolescent population, the specificity of these effects to the adolescent population is supported by previously published literature (Szkudlarek et al. 2019). Additionally, during CIE exposure, blood was not taken to test for blood ethanol concentration (BEC) to make sure that animals were appropriately intoxicated. From data collected across a variety of previous studies (Smiley et al. 2020; Trantham-Davidson et al. 2014; Gass et al. 2014a, Gass et al. 2017, Uys et al. 2016), we have determined that a behavioral rating of 2-3 will consistently be associated with a BEC of ~200-300 mg/dl and, thus, did not take samples from these animals. Further, these studies did not include a treatment group that received NAC in the absence of any other drug treatment. Preclinical studies completed using NAC to reduce neurotoxicity have found that NAC treated animals did not differ from controls, and an insult had to be present for NAC to impact oxidative injury and inflammation (Abdel-Wahab and Moussa 2019). This data is supported by our previous studies using NAC to prevent stress induced increases in drug relapse that found control animals treated

with NAC did not exhibit any behavioral differences (Garcia-Keller et al. 2020). Therefore, for these studies, we expect a NAC treated group to exhibit no differences in behavioral outcomes when compared to controls.

In conclusion, these studies found that adolescent exposure to THC and ethanol leads to deficits in prefrontal cortex reliant cognition, including set-shifting and drug seeking behaviors. These deficits could potentially be a result of impairments in glutamate functioning since NAC treatment led to reductions in ethanol seeking and increased cognitive flexibility in THC and CIE exposed animals. Further studies are needed to determine the exact mechanism of action that these drugs are following to exert these effects, but these studies supply valuable information regarding the behavioral effects of adolescent exposure to these drugs and a possible preventative treatment with NAC.

CHAPTER 6

EXPOSURE TO THC AND ETHANOL DURING ADOLESCENCE HEIGHTENS RESPONSES TO FEAR STIMULI DUE TO SIGNALING CHANGES IN THE PRELIMIBIC CORTEX

Background and Significance

Cannabis has become the most commonly used illicit drug that is increasingly being used for recreational purposes, with only ~10% of users citing use for exclusively medicinal reasons (National Academies of Sciences, 2017). As the rate of cannabis use has increased from 4.1% in 2002 to 9.5% in 2013 (Hasin et al. 2015) and almost all age groups have shown increases in past-month use within this time period (National Academies of Sciences, 2017). The population in

which heavy use and high intensity use is most prevalent is late adolescence, which is also the average age at which CUD is shown to develop (Hasin et al. 2016). Surveys of high school students reveal that ~50% of seniors have tried marijuana while 5% of them participate in daily use (Schweinsburg et al. 2008). While CUD itself is associated with adverse consequences, such as deficits in cognition and attention, this disorder is also highly comorbid with other substance use and psychiatric disorders that further add to these cognitive impairments. Clinically, CUD patients are observed to have up to a 10 times greater chance of developing alcohol use disorder (Hasin et al. 2015) and that ~80% of those with CUD also have AUD (Hasin et al. 2016). This comorbidity is also present for CUD and post-traumatic stress disorder, where CUD leads to a greater chance of developing PTSD and, conversely, PTSD leads to higher rates of cannabis abuse as well (Yarnell et al. 2015). This is especially true for the adolescent population, in which marijuana is the most commonly used illicit substance by adolescents with PTSD, and an adolescent with PTSD also has a two-fold increase in the likelihood of developing CUD (Bujarski et al. 2012). Exposure to trauma also increases alcohol use, especially when it occurs during adolescence, a period in which there are already high rates of binge drinking (Khoury et al. 2010). While the comorbidity between CUD with AUD and PTSD is extremely clinically relevant, there has been a lack of pre-clinical research into the underlying brain mechanisms that contribute to the maintenance and treatment of these co-morbid disorders. Therefore, in these experiments, we examined the impact of adolescent exposure to cannabis

and alcohol on responses to fear stimuli and sought to determine the underlying signaling changes responsible for these alterations.

Materials and Methods

Animals

Male Wistar rats were Postnatal Day 28 and ~100 grams on arrival and were individually house in standard polycarbonate cages. Animals were permitted one week to habituate to a 12/12 reverse light-dark cycle with lights off at 0900, allowing for behavioral testing to occur during the dark phase of the cycle. All experiments had prior approval by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and were completed within guidelines set forth by the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003). A total of 88 animals were used for this experiment and were broken up into the following treatment groups: Air, THC, CIE, THC+CIE, THC+CIE+NAC (n = 16/group). A subset of animals from each group (n = 8/group) received surgical implants to monitor brain activity during these behavioral paradigms (n = 8/group). A supplemental group was added that only received NAC treatment in the absence of all other drug manipulations and was only tested on the behavioral aspects of this experiment (n = 8). The general outline of the experimental design for these studies is outlined in **Figure 6-1**.

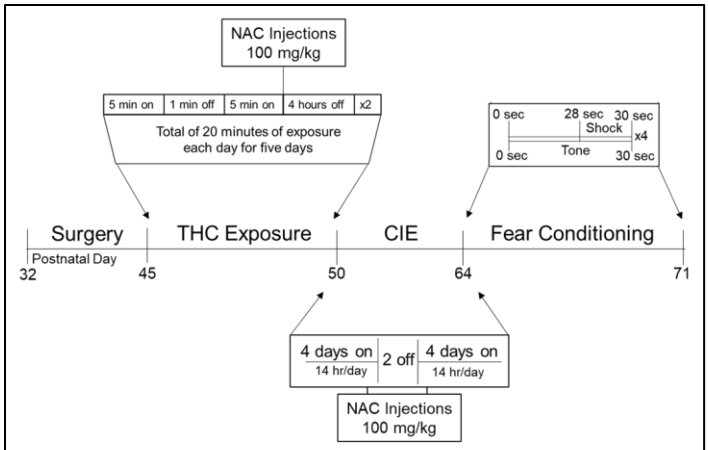

Figure 6-1: Experimental Design. Animals in the fiber photometry group were given surgery to inject a fluorescently signaling GCaMP virus into the prelimbic cortex. Following recovery, animals were exposed to THC vapor exposure for a total of five days and those in the NAC group were given injections directly after each exposure. Animals were then exposed to CIE for two weeks during which NAC injections were given prior to and following each exposure session. Following CIE, animals were put into fear conditioning paradigms in which they went into fear conditioning for three days in which they received four tone shock pairings per day. Fiber photometry recordings were obtained throughout all fear conditioning paradigms.

Surgical Procedures

For the subset of animals used to analyze brain signaling in response to fear conditioning, fiber photometry was used to measure calcium transients induced by neuronal signaling in real time during behavioral testing. Animals were injected with a virus containing genetically encoded calcium indicators to be used as a correlative measure of neural activity. Surgeries occurred between PD 35-36 and were followed by a one-week recovery period. Rats were anesthetized using vaporized isoflurane and oxygen (flow rate of 0.4 L/min, 5% for induction and 2.5-3.5% for maintenance) and were mounted in a stereotaxic apparatus once fully under (Kopf Instruments, Tujunga, CA). An adeno-associated virus encoding

GCaMP6f with a promoter for CaMKII (AAV1-CaMKII-GCaMP6f, AddGene, Watertown, MA) was injected into the prelimbic cortex (PrL) using a microsyringe (Hamilton Company, Reno, NV) at a volume of 300 nL and a rate of 1 nL/s. The PrL coordinates used in these surgeries were based on pilot experiments that used dye injections to optimize the PrL location in PD 35 animals (in mm from bregma and the skull surface, anterior/posterior +3.2, medial/lateral ± 0.6, and dorsal/ventral −2.8). There was a ten-minute period allowed for virus infusion following injection,. A handmade optical fiber probe (400 μm diameter patch cord in a 2. 5 mm ferrule, Thorlabs) was then implanted at the same coordinates. Two stainless steel screws were mounted in the skull and were covered in dental cement to keep the probe in place. The surgical incision was treated with 2% triple antibiotic ointment and 2% xylocaine directly after surgery and antibiotic ointment was applied as needed in the following days. Additionally, the following drugs at the listed doses were administered to each animal directly following surgery: ketorolac (body weight/1000), dexamethasone (body weight/1000), and saline (body weight/100) cefazolin (1/2 body weight/100). For post-operative care, ketorolac was used for pain management and delivered the day following surgery with cefazolin antibiotic administered for two days following surgery.

Adolescent THC Vapor Exposure

Once animals were allowed one week for surgical recovery, exposure to THC vapor occurred following the method established by Spencer et. al. (2018).

Starting at PD 40, each animal received a total of 20 minutes of vapor exposure per day for 5 days. Each THC exposure session was broken up into 5-minute administrations, with two in the morning and two in the afternoon following a three-hour break, to keep THC concentrations steady. Each exposure session used a vaporized solution consisting of THC at a concentration of 200 mg per mL of ethanol and CBD at 20 mg per mL of ethanol. These solutions were mixed at a 1:1:1 ratio with glycerol to obtain a final solution containing THC and CBD at a ratio of 10:1. Aliquots of 150 uL of this solution were vaporized using a Volcano (Storz and Bickel, Oakland, CA) and the resulting vapor was administered equally among the animals. If animals were assigned to the NAC treatment group, they received intraperitoneal (IP) injections of the drug immediately following both the morning as well as the afternoon session. Following the five total days of THC exposure, animals were permitted a two-day break before entering chronic intermittent ethanol exposure (CIE).

Chronic Intermittent Ethanol Exposure (CIE)

CIE commenced on PD 50 and was used to model binge-like alcohol consumption using repeated cycles of ethanol vapor exposure and abstinence. This paradigm consisted of four nights of vapor exposure from 1800-0800 (14 hours on/10 hours off) followed by a two-day break, and this cycle was completed a total of two times. A five-point behavioral scale was used to grade intoxication levels based on motor behavior. Rats were assigned the following scores based

on behaviors exhibited when taken out of the chambers in the mornings: 1: no signs of intoxication and no motor impairment, 2: slight intoxication and slight motor impairment, 3: moderate intoxication with obvious motor impairment but retains the ability to walk, 4: loss of righting reflex, highly intoxicated, 5: loss of righting reflex and eye blink reflex, extremely intoxicated (Nixon and Crews, 2002). An intoxication rating of 2-3 was the goal of each CIE session which corresponds to a blood ethanol concentration (BEC) of ~250 mg/dl. Animals in the NAC treatment group received injections two hours prior to CIE exposure and immediately after they came out of the chambers in the morning.

N-acetylcysteine (NAC) Preparation

NAC was administered at a dose of 100 mg/kg and was prepared on the day of injection. This dose was chosen to optimize the effects of NAC on the glutamate system based on previous experiments (Garcia-Keller et al. 2019). To achieve this concentration, 5 grams of NAC (Millipore Sigma) was added to 5 mL of 27 mg/mL NaOH in 0.9% saline. Since NAC is acidic, 200 mg/mL NaOH in saline was added in 50 uL increments until the solution reaches ~pH 7.5. On the day of injection animals were weighed and dosed accordingly and injections were delivered intraperitonially (IP).

Fear Conditioning Procedure

Two days following the end of CIE exposure, fear conditioning was used to determine differences in behavioral responses to fear stimuli and followed previously published methods (Cain et al. 2002, Holmes et al. 2012, Izquierdo et al. 2006, Sinclair et al. 2012, Wellman et al. 2007). This paradigm consisted of exposure to multiple tone/shock pairings to measure behavioral responses to the tone over time and between treatment groups. Each trial lasted approximately five minutes each and occurred once a day for three days total. Conditioning sessions consisted of an initial 120 second acclimation period followed by the presentation of four tones (80 dB, 3 kHz) separated by a 10 second inter-stimulus interval. Each tone was paired with a 0.75 mA shock that occurred during the final two seconds of the tone. Animals were presented with a total of 12 tone/shock pairings across three separate days. Trials were recorded and analyzed using AnyMaze software to determine freezing behavior (Stoelting Co. Wood Dale, IL).

Calcium Imaging

In the subset of animals previously implanted with fiber optic probes, calcium imaging was performed using a custom-built fiber photometry rig based on the design of the Deisseroth (Lerner et al. 2015) and Woodward labs (Braunscheidel et al. 2019). A LED driver (Thorlabs) provided both 405 nm and 490 nm illumination which were combined in a fluorescence mini-cube (Doric

Lenses). A custom made 400 μm diameter patch cord terminating in a ceramic sleeve was used to connect the mini-cube to the animal's fiber optic implant. Synapse software (TDT) was used to control a digital processor (TDT) that received input from a photodetector (Newport) that collected emission signals at both 405 nm and 490 nm. Integrated TTL signals were used to time-lock signals with the start of each fear conditioning session as well as the presentation of each tone throughout the session. Custom-written MatLab codes (MathWorks) were used to analyze these data. Signals from the 405 and 490 nm channels were subtracted from each other to calculate data as △F/F. Data for each testing day was then z-normalized to a combined baseline of all signaling across the whole session for that day.

Tail Immersion Test

Following the completion of all behavioral testing, animals in the surgical subset groups were exposed to a tail immersion test to test for differences in pain sensitivity between the groups. Prior to the test day, all animals were habituated to handling and immersion using room temperature water. Additionally, each animals' tail was marked 3 cm above the tip to standardize immersion length. On the day of the test, water was heated to 52°C and kept constant for each animal. The tail was dipped into the water to the 3 cm mark and animals were held into the water until their tail broke the surface. This was repeated for a total of three times per animal in succession and immersion time was averaged across the three tests

for each animal. This test was recorded and manually scored to determine average immersion time for each animal and averaged across treatment group.

Statistical Analysis

Behavioral experiments included 12-16 animals per treatment group while signaling data comprised 6-8 animals per group. The variation in sample size was a result of the following exclusion criteria. Animals were eliminated from behavioral experiments if they were not freezing over 50% of the time in response to the tone on Conditioning Day #3 and from signaling experiments if the fiber optic probe did not terminate above the virus in the prelimbic cortex. Data were analyzed using Prism version 8 (GraphPad software, La Jolla, CA) and a p value of less than 0.05 was considered statistically significant. Fear conditioning data were analyzed using a 2-way ANOVA with Holm-Sidak multiple comparisons as the post-hoc test used to determine differences between each treatment group. Signaling data was analyzed using a 1-way ANOVA with post-hoc testing completed using a Holm-Sidak multiple comparisons test.

Results

CIE exposure resulted in moderate levels of intoxication that were comparable between the groups

Behavioral intoxication ratings were complimented with blood ethanol values to assess the level of intoxication for all CIE exposed animals across the three ethanol exposed treatment groups. All animals achieved a moderate level of intoxication with behavioral ratings of between 2-3 (**Figure 6-2A**) and BEC levels of between 300-400 mg/dL (**Figure 6-2B**). Additionally, animals' weights were taken during each phase of the experiment to maintain health between the groups, and there were no differences in weight between treatment groups throughout the experiment (**Figure 6-2C**).

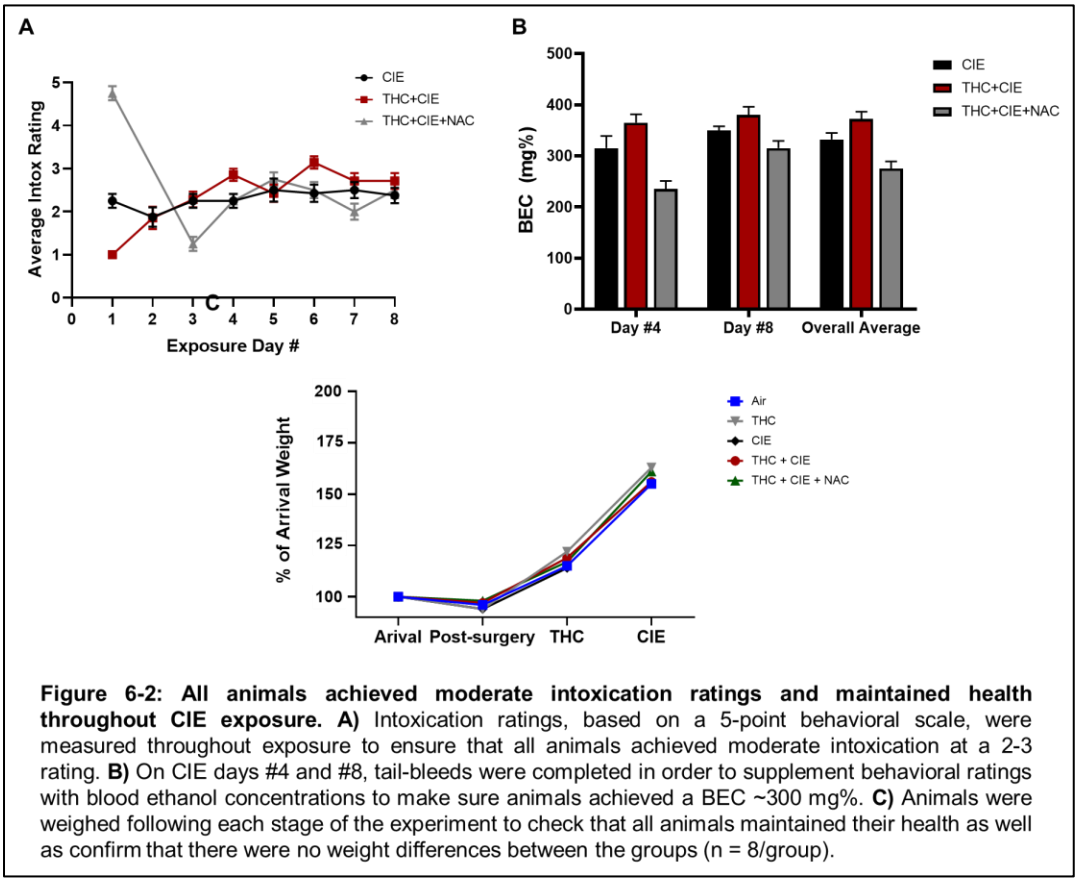

Figure 6-2: All animals achieved moderate intoxication ratings and maintained health throughout CIE exposure. A) Intoxication ratings, based on a 5-point behavioral scale, were measured throughout exposure to ensure that all animals achieved moderate intoxication at a 2-3 rating. **B)** On CIE days #4 and #8, tail-bleeds were completed in order to supplement behavioral ratings with blood ethanol concentrations to make sure animals achieved a BEC ~300 mg%. **C)** Animals were weighed following each stage of the experiment to check that all animals maintained their health as well as confirm that there were no weight differences between the groups (n = 8/group).

Adolescent exposure to THC and CIE increases behavioral responding to fear stimuli during conditioning

When conditioning behavior is examined as an average of percent freezing during the tones per each conditioning day, there was a significant effect of treatment [$F (5, 215) = 12.81$, $p < 0.0001$] such that THC+CIE and THC exposed animals exhibited an increased freezing response to the tones on Conditioning Day #2 when compared to all other treatment groups (**Figure 6-3A**). Additionally, this effect carried over to Conditioning Day #3, where drug treated groups exhibited heightened levels of freezing when compared to air treated controls as well as THC+CIE exposed animals that also received NAC treatment concurrent with each drug exposure (**Figure 6-3A**). During each conditioning day, animals received a total of four tone/shock pairings and, thus, freezing to each individual tone presentation were examined to determine when responses began to differentiate between the groups. There was a significant effect of treatment in individual tone responses [$F (5, 860) = 27.66$, $p < 0.0001$], with drug treated animals exhibiting a consistent increase in freezing when compared to control and NAC treated animals (**Figure 6-3B**). When comparing THC+CIE exposed animals vs. controls and NAC treated animals, the initiation of this effect was in response to tone presentation #6 on Conditioning Day #2 where these animals began exhibiting a significant increase in freezing to the tone (**Figure 6-3B**, $p = 0.0274$ vs. Air, $p = 0.0059$ vs. THC+CIE+NAC).

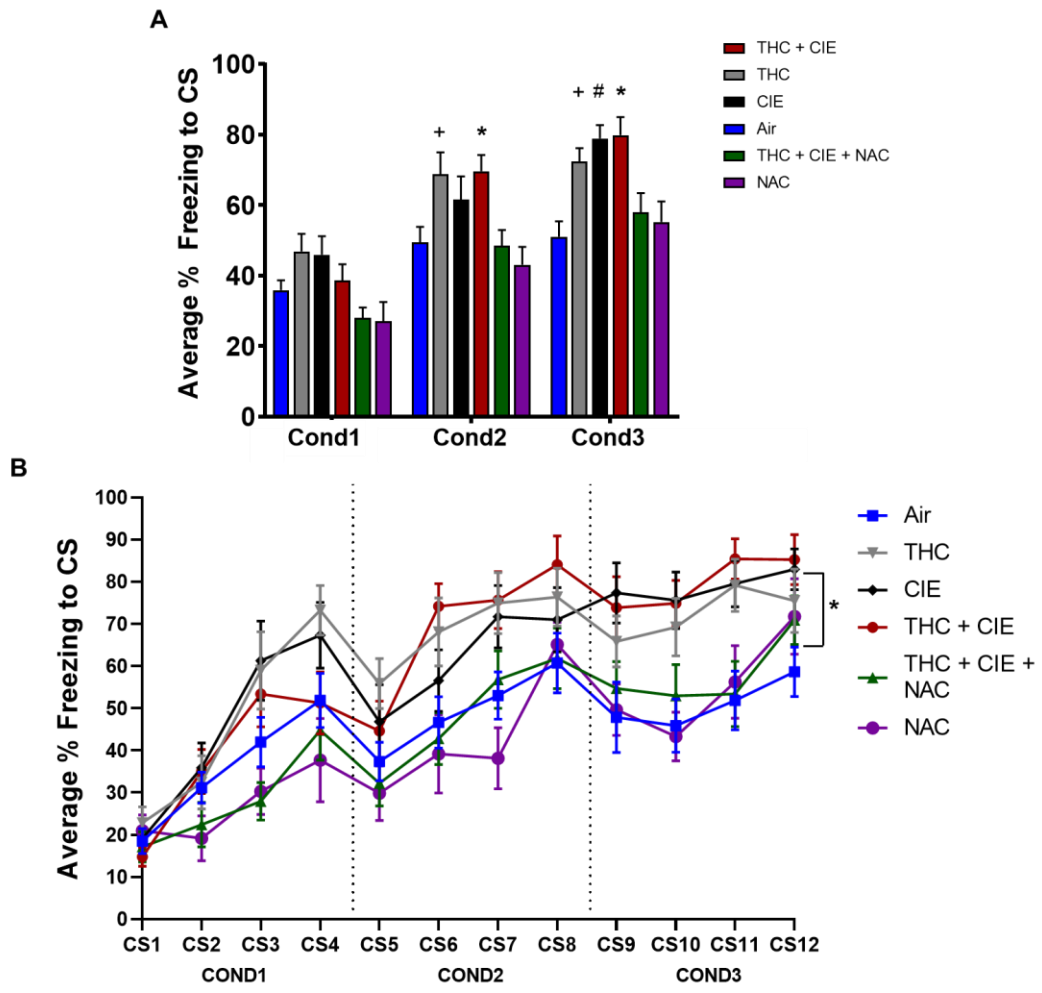

Figure 6-3: Exposure to THC, CIE, and a combination of both during adolescence leads to increased responses to fear stimuli that is prevented with NAC treatment. A) When looking at average freezing to all four tone presentations each day of conditioning, THC, CIE, and THC + CIE animals exhibit a heightened freezing response when compared to air exposed animals as well as THC + CIE animals that receive NAC. **B)** When looking at responses to individual tone presentations, there is a clear separation between the THC, CIE, and THC + CIE animals and the air controls and NAC treated groups (n = 12-16/group, *p < 0.05 THC+CIE vs. Air, NAC, and THC+CIE+NAC, +p < 0.05 THC vs. Air, NAC, and THC+CIE+NAC, #p < 0.05 CIE vs. Air, NAC, and THC+CIE+NAC).

Increased freezing during conditioning is not associated with alterations in pain tolerance

Following the completion of behavioral testing, all animals were exposed to a tail immersion test to ensure that behavioral responding during conditioning was not due to drug-related changes in pain sensitivity. There were no differences observed between any of the treatment groups in average immersion time during the tail immersion test designed to determine pain sensitivity (**Figure 6-4**) [F (4, 25) = 0.03744, p = 0.9971].

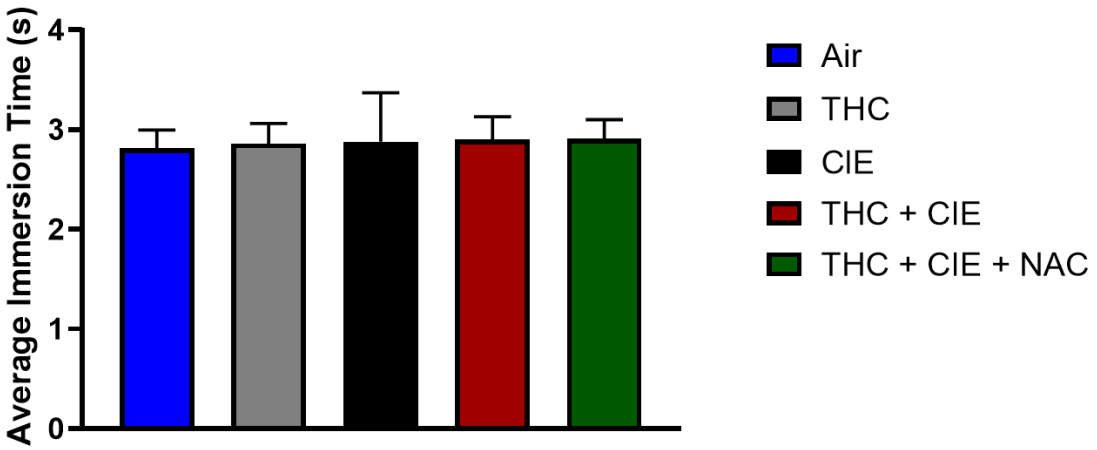

Figure 6-4: There were no differences between the groups with regards to pain sensitivity. There were no effects of treatment on average tail immersion time when exposed to a tail-immersion test, indicating no differences in pain sensitivity that could be responsible for the behavioral effects seen in response to shock exposures during conditioning (n = 6-8 animals/group).

THC and CIE exposure results in a freezing associated increase in prelimbic signaling in response to the shock during conditioning

In addition to the behavioral measures taken during fear conditioning, a subset of animals in each treatment group received fiber optic implants to measure signaling from the prelimbic cortex during each session. While signaling data was recorded throughout the session, the most variation in signaling was observed in response to each shock presentation and, therefore, this is the data focused on in this section. Each figure displays averaged signal from the prelimbic cortex across animals between each treatment group from the five seconds prior to each shock and five seconds following each shock, with the red mid-lines reflecting shock onset. On Conditioning Day #1, while most groups exhibit an increase in PrL activity following the shock, the magnitude of this difference differs between the groups (**Figure 6-5**).

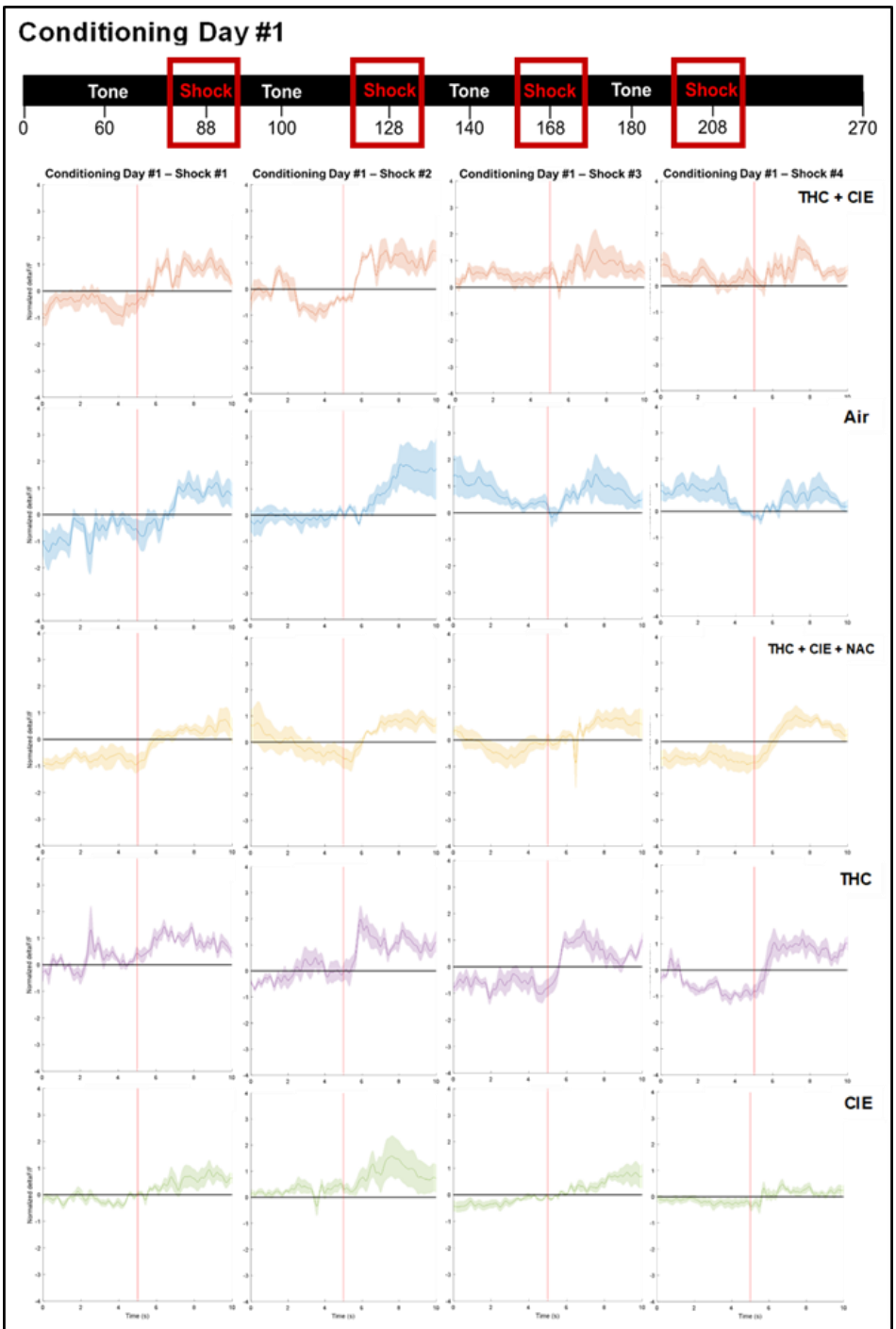

Figure 6-5: Average signaling recorded surrounding each shock presentation of Conditioning Day #1. When looking at the five seconds prior to and five seconds following each shock presentation, each group exhibits a slight increase in responding following the shock, but there are no significant differences between the groups (n = 6-8/group).

The magnitude of the PrL response to the shock is shown to differ between the groups when the difference in signal prior to vs. following the shock is quantified per each treatment group (**Figure 6-6**). In response to all four shock presentations on Conditioning Day #1, THC+CIE treated animals exhibit a heightened PrL response to the shock when compared to air exposed controls.

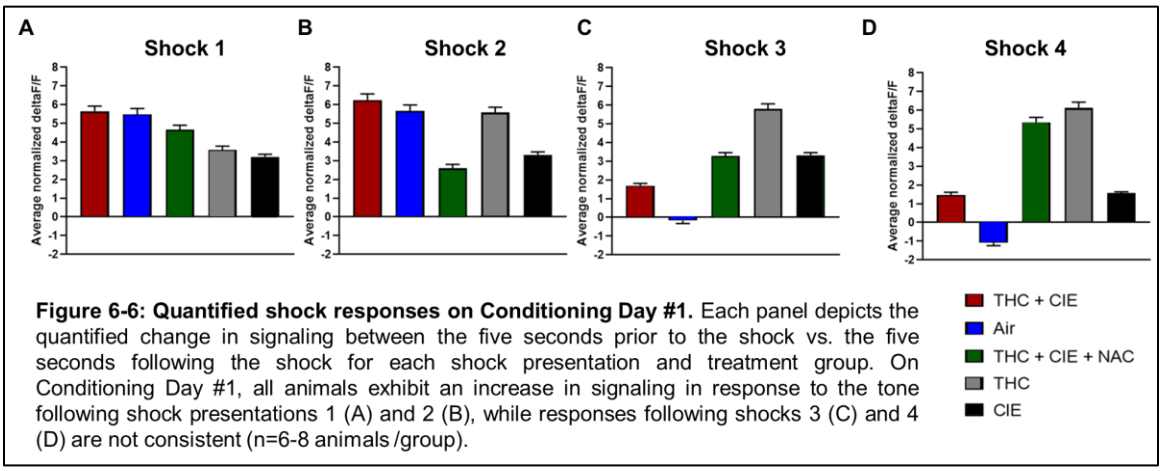

Figure 6-6: Quantified shock responses on Conditioning Day #1. Each panel depicts the quantified change in signaling between the five seconds prior to the shock vs. the five seconds following the shock for each shock presentation and treatment group. On Conditioning Day #1, all animals exhibit an increase in signaling in response to the tone following shock presentations 1 (A) and 2 (B), while responses following shocks 3 (C) and 4 (D) are not consistent (n=6-8 animals /group).

Further, signaling from Conditioning Day #3 reflects conditioned responding while shock responses on Conditioning Day #1 reflect responses during early phases of conditioning when animals have not yet formed a conditioned response. On Conditioning Day #3, THC+CIE exposed animals exhibit an increase in signal directly following each shock presentation (**Figure 6-7**).

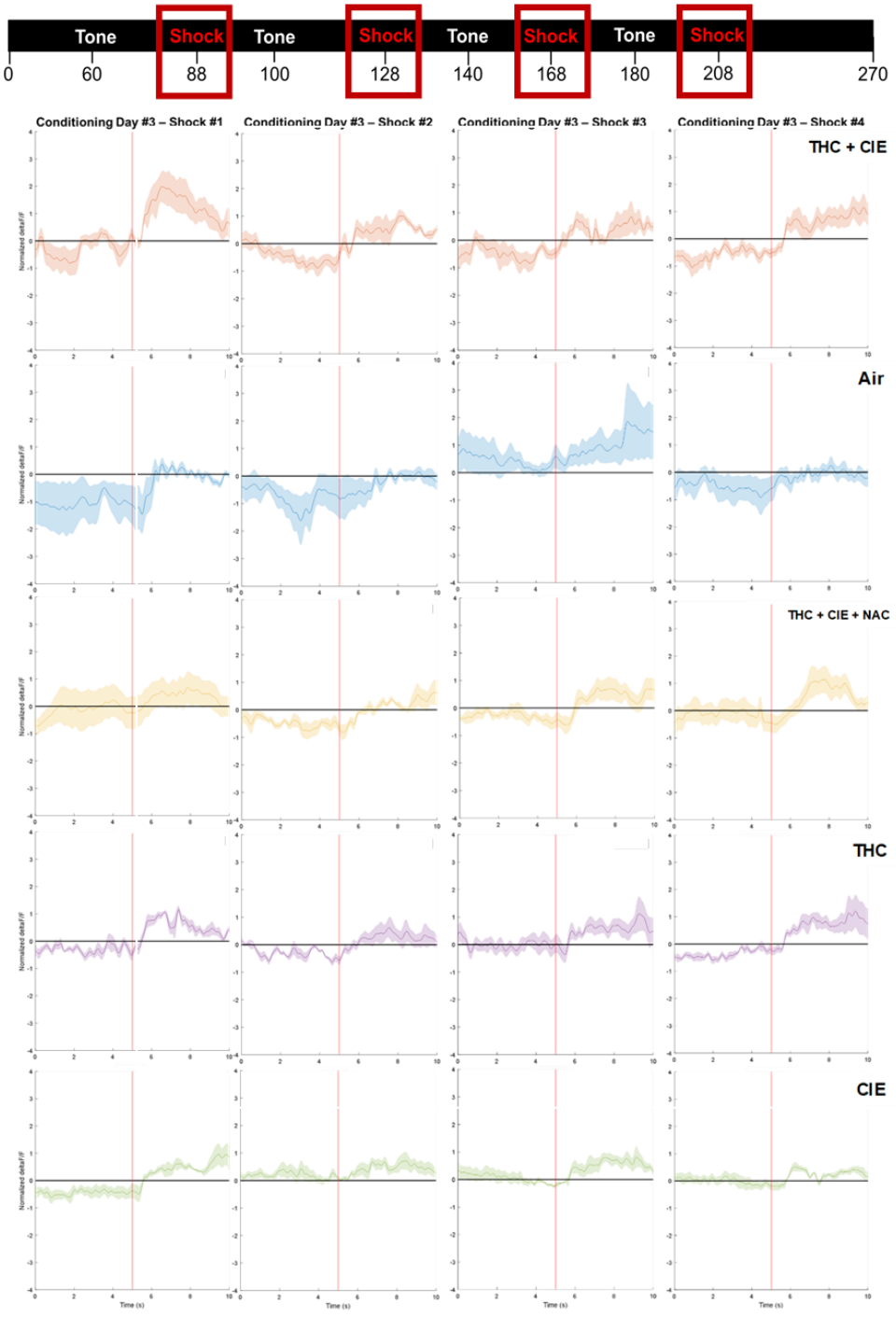

Figure 6-7: Average signaling in response to each shock presentation on Conditioning Day #3. When looking at prelimbic signaling surrounding each shock on Conditioning Day #3, THC + CIE exposed animals exhibit an increased response to the shock when compared to air exposed animals (n = 6-8 animals/group).

When quantifying signaling change in response to the shock on Conditioning Day #3, THC+CIE exposed animals exhibit a heightened response to each of the shocks when compared to air exposed animals as well as those exposed to THC+CIE treated with NAC. Additionally, the magnitude of response observed in the THC or CIE exposed animals is lower than when animals are exposed to both drugs (**Figure 6-8**).

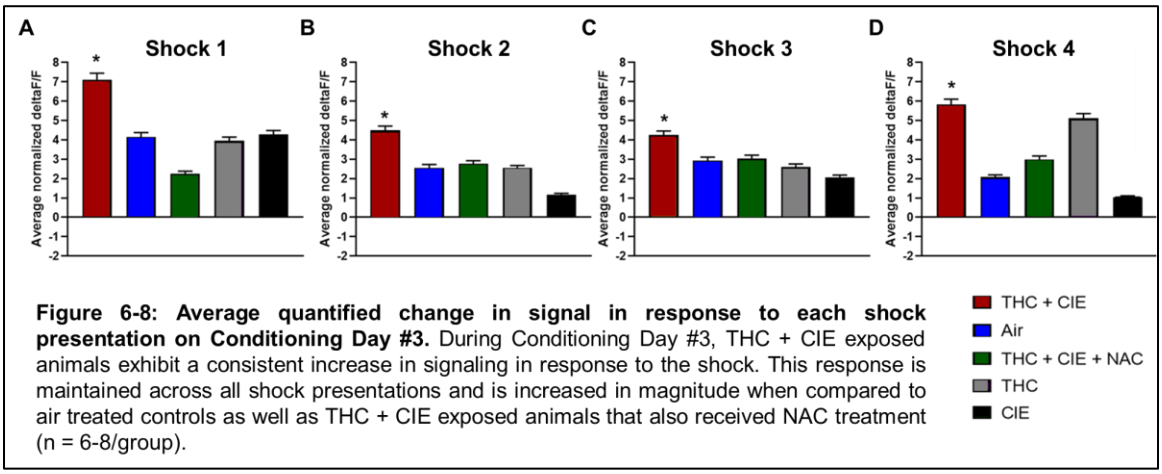

Figure 6-8: Average quantified change in signal in response to each shock presentation on Conditioning Day #3. During Conditioning Day #3, THC + CIE exposed animals exhibit a consistent increase in signaling in response to the shock. This response is maintained across all shock presentations and is increased in magnitude when compared to air treated controls as well as THC + CIE exposed animals that also received NAC treatment (n = 6-8/group).

This difference between treatment groups as well as the difference between responses on Conditioning Day #1 versus Day #3 are revealed when comparing the average response to all shocks across each day (**Figure 6-9**). While the pattern in shock responses on Conditioning Day #1 is not clear, by Conditioning Day #3, group differences in signaling reflect the differences in freezing behavior shown in **Figure 6-3**. There is a significant effect of treatment observed [$F(4, 70) = 15.02$, $p < 0.0001$], where THC+CIE exposed animals exhibit a heightened PrL response to the shocks on Conditioning Day #3 when compared to all other treatment groups

(**Figure 6-9**). Virus and probe location in the PrL are depicted in **Figure 6-10** to ensure that all recordings were from within the boundaries of the PrL.

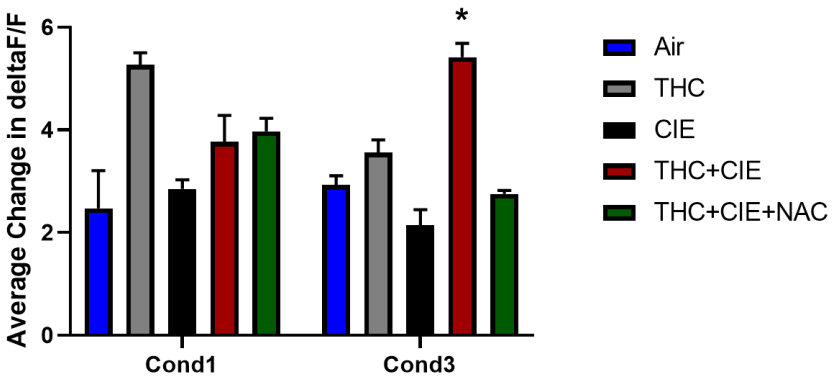

Figure 6-9: Change in prelimbic signal in response to the shock across Conditioning Day #1 and Day #3. When looking at an average of responses to all shock on each conditioning day, THC + CIE exposed animals exhibit a significant increase in prelimbic signal in response to the shocks on Conditioning Day #3 when compared to all other treatment groups (* $p < 0.05$ THC+CIE vs. all other groups, n = 6-8/group).

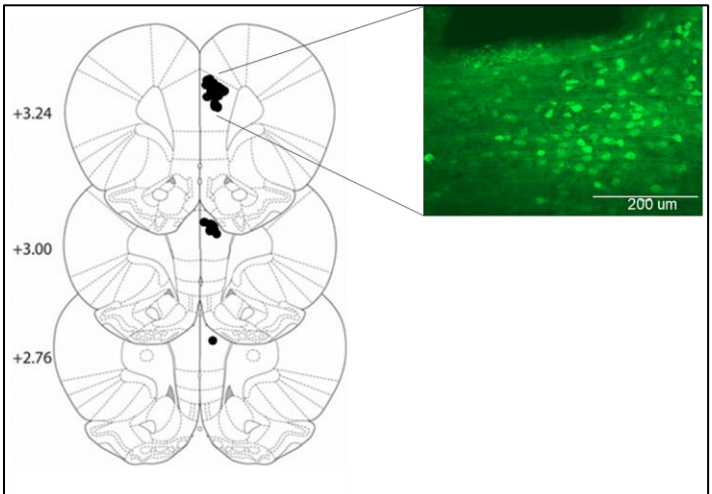

Figure 6-10: Histological verification of virus and probe placement. Following the completion of all behavioral testing, brains were extracted and sectioned in order to determine the location of the virus injections and placement of the fiber optic probe. Animals were eliminated if the fiber optic probe did not terminate directly above the virus within the prelimbic cortex (representative image inset).

Discussion

The results from these experiments indicate that THC and ethanol exposure during adolescence leads to increases in fear responses that are associated with a higher level of prelimbic signal in response to fearful stimuli. This effect was greater with combined drug exposure than either drug alone, and, when these animals were treated with NAC after each drug exposure, the behavioral and signaling alterations observed following adolescent THC and ethanol exposure were reversed. As such, these experiments identify the determinantal effects of adolescent THC and ethanol exposure on future behavioral and neuronal responses to fear stimuli. Further, these results support the hypothesis that alterations in prelimbic signaling may underlie the clinical relationship between adolescent drug exposure and development of PTSD.

These experiments examined pain sensitivity as an alternative explanation for the group differences observed with regards to freezing behavior, but there are a number of additional explanations that should be explored. First, exposure to these drugs could be causing a reduction in motor activity during fear conditioning paradigms that is recorded as increased freezing. While exposure to THC has been shown to cause short term decreases in motor activity observed in open field tests immediately following drug exposure, these are transient effects that are dependent on the drug being onboard and were not present 48 hours later (Bruijnzeel et al. 2016). Similarly, ethanol exposure has been shown to cause alterations in motor behavior, but these effects have been shown to dissipate

following the cessation of ethanol exposure (Ornelas et al. 2015). These motor effects that occur as a consequence of ethanol exposure on motor sedation are also less severe in adolescent animals when compared to adults (Acevedo et al. 2013, Little et al. 1996). As these experiments tested fear conditioning 18 days following the last THC exposure and four days after the last CIE session, we do not expect motor alterations as a result of these drugs to have impacted behavior during fear conditioning. While these experiments focused on alterations in prelimbic signaling, there are a number of fear related areas that could also be affected as a result of THC and ethanol exposure during adolescence. There are high levels of CB1 receptors in the amygdala, and cannabinoids are highly involved in signaling in this region especially in the basolateral amygdala (BLA) (Katona et al. 2001, Pistis et al. 2004, Phan et al. 2008). In general, adolescents exhibit stronger amygdala reactivity in response to fearful stimuli when compared to adults (Guyer et al. 2008). Additionally, it has been shown that adolescent cannabis users exhibit increased amygdala reactivity in response to fearful stimuli along with impaired discrimination between threat and neutral stimuli due to increases in prefrontal cortex activity (Spechler et al. 2015). Preclinical studies also support this effect, with animal models reporting inhibition of GABA transmission in the amygdala following exposure to CB1 agonists (Katona et al. 2001). Further, when animals were treated with THC and exposed to restraint stress, the effects on inhibitory transmission in the amygdala were enhanced (Patel et al. 2004). Therefore, while these experiments determined the effect of adolescent exposure to THC and ethanol on prelimbic cortex signaling during fear stimuli presentation,

future examinations of neuronal alterations following adolescent THC exposure should include analysis of downstream amygdala activity.

These experiments determined that adolescent exposure to THC and ethanol leads to increased behavioral and neuronal responding to fear stimuli during fear conditioning. Additionally, NAC treatment administered directly after each drug exposure session was able to prevent both the increased freezing exhibited during conditioning as well as the heightened prelimbic signaling recorded following the presentation of fear stimuli. These results are in accordance with the hypothesis that adolescent drug exposure can induce neuronal alterations that may cause an increased predisposition for developing disordered responses to fear stimuli that underlie PTSD. Furthermore, the use of glutamatergic modulators to normalize glutamate homeostasis in the prefrontal cortex could serve as a potential treatment to reverse the deleterious effects of adolescent drug exposure.

CHAPTER 7

DISCUSSION

The studies presented in this dissertation establish the detrimental effects of stress and drug exposure on multiple PFC-reliant behavioral tasks including drug seeking, cognitive flexibly, and fear-cue reactivity. Further, these experiments demonstrate that alterations in glutamatergic regulation in the prefrontal cortex are responsible for the drug and stress induced changes in responses to fear and drug cues. Finally, pharmacological modulators of glutamatergic function were used to reverse these stress and drug induced deficits.

N-acetylcysteine prevents stress induced alterations in alcohol seeking and cognitive flexibly

Restraint stress exposure followed by testing in self-administration paradigms revealed a stress induced increase in ethanol seeking during acquisition, extinction, and stress cue induced reinstatement testing. Additionally, treatment with N-acetylcysteine (NAC) delivered either prior to, following, or surrounding restraint prevented the stress induced increases in ethanol seeking observed throughout self-administration. Stress exposure was also found to cause impairments in cognitive flexibility that were prevented with NAC treatment as well.

Previous studies have examined the relationship between stress exposure and drug seeking across a wide variety of clinical studies as well as in preclinical models. For these experiments, restraint stress was used as our stress model and self-administration modeled ethanol seeking, but a variety of different techniques for stress and drug administration have been tested. Alternate models have shown increased drug seeking following stress exposure for drugs such as methamphetamine, heroin, cocaine, and ethanol (Goeders and Geurin 1994, Liu and Weiss 2002, Lewis et al. 2013, Pizzimenti et al. 2017, Shaham and Stewart 1994). In clinical studies, it has been shown that PTSD patients have an increased rate of substance use disorders when compared to the general population and are more likely to exhibit relapse events in response to stress associated cues (Roberts et al. 2015, Tipps et al. 2014, Shaham and Stewart 2000). Additionally, while those with PTSD and a co-morbid substance use disorder do not have

increased severity of substance use, they do exhibit a higher incidence of relapse compared to those with a substance use disorder alone (Tate et al. 2004, Najt et al. 2011, Burns et al. 2010). These clinical and preclinical studies support the results presented here, in which exposure to a stressor leads to increased ethanol seeking throughout self-administration paradigms as well as increases in ethanol seeking during fear cue-induced relapse testing. Additional results presented from these experiments include examinations of stress effects on cognitive flexibly. While these experiments determined that restraint stress exposure resulted in deficits in cognitive flexibility, further reports in the literature have achieved mixed results depending on the time scale of testing. In clinical studies, those exposed to an acute stressor exhibited impairments in cognitive flexibility (Shields et al. 2016), but this relationship has been shown to be more complicated in animal models with impairments, enhancements, and no effects on cognitive flexibility reported in rodent studies (Butts et al. 2013, Thai et al. 2013, George et al. 2015). Therefore, due to the previous reporting of both stress induced increases in relapse-like behavior and impairments in cognitive flexibility, the novelty of these experiments comes from the use of N-acetylcysteine (NAC) to prevent these effects.

The overlap in cognitive control of ethanol seeking and deficits in set-shifting is in the prefrontal cortex (PFC) and, thus, stress induced changes in PFC function need to be examined to determine the possible mechanism of action NAC is working through. The promotion of drug seeking behavior and strategy set-shifting have been shown to be reliant on the medial prefrontal cortex (mPFC) with

inactivation of the mPFC leading to increases in perseveration, similar to the results observed in these experiments (Birrell and Brown 2000, Floresco et al. 2008). Additionally, stress exposure has a variety of deleterious consequences on the structure and function of the mPFC (Arnsten 2009, Holmes and Wellman 2009). This region has been shown to be highly activated across a wide variety of stressors, leading to downstream increases glucocorticoid (GC) responses to stress (Ostrander et al. 2003, Herman et al. 2005, Singewald et al. 2003, Diorio et al. 1993). Further, exposure to stress and increases in GC activation are associated with morphological changes in these regions including dendritic remodeling (Liston et al. 2006, Sapolsky 2003, Cerqueira et al. 2005). These stress induced alterations in PFC structure have also been shown to be associated with modifications in glutamatergic functioning in this region (Popoli et al. 2011). The increase in GCs following exposure to physiological and environmental stressors is associated with increases in glutamate release in the PFC, and has been measured across multiple animal models including restraint stress, forced swim, foot-shock, and direct GC administration (Lowy et al. 1993, Moghaddam 1993, Musazzi et al. 2010). This increase in glutamate following stress is not only transient, since there are delayed and long-term effects on PFC glutamate function (Yuen et al. 2009, Yuen et al. 2011). Stress induced alterations in PFC glutamate are also associated with alterations in synaptic plasticity, including impairments in LTP in PFC to hippocampus signaling (Rocher et al. 2004, Mailliet et al. 2008). This is especially important for these experiments, as these signaling impairments

are associated with deficits in PFC-reliant behaviors including cognitive flexibly (Cerqueira et al. 2007).

The results from these experiments determined that N-acetylcysteine (NAC) had the ability to prevent fear-cue induced relapse and impairments in cognitive flexibility that occur following exposure to an acute stressor. These results are supported by clinical studies that used NAC in veterans with co-morbid PTSD and SUD and recorded reductions in both drug use and PTSD symptoms following this treatment (Back et al. 2016). Additionally, NAC may be affecting stress induced impairments in prefrontal glutamatergic functioning to impart these effects (Dean et al. 2011). NAC has been shown to have a restorative effect on deficient glutamatergic systems as a result of drug exposure across multiple addiction models (Knackstedt et al. 2009, Madayag et al. 2007). Further, NAC has been shown to have effects on oxidative stress and inflammation in the brain which could serve as additional candidates for the mechanism of action at work in these experiments (Dean et al. 2011). While NAC has widespread effects on brain function, the glutamatergic or inflammatory systems in the brain could be a potential mechanism of action for these experiments due to the overlapping effects of stress on these processes.

In summary, these experiments determined that acute stress exposure leads to increases in ethanol seeking during self-administration and stress-cue induced reinstatement and leads to impairments in cognitive flexibility, both of which are prevented with N-acetylcysteine treatment surrounding the stressor. NAC may

potentially be regulating glutamate homeostasis in the prefrontal cortex to impart these effects, but further studies are required to determine the exact mechanism of action. These results add to the established body of literature examining the detrimental effects of stress exposure on prefrontal cortex reliant cognition and provide further evidence to support the use of NAC in comorbid PTSD/AUD.

Chronic ethanol exposure leads to deficits in fear extinction learning through alterations in mGlu5 signaling in the infralimbic cortex

This set of studies modeled PTSD/AUD in rodents using fear conditioning followed by CIE and found that ethanol exposure leads to impairments in fear extinction learning in both sexes. Additionally, using CDPPB and microinjections, these results determined that the effects of ethanol on fear learning are dependent on mGlu5 signaling in the infralimbic subregion of the prefrontal cortex. Further, these studies establish multiple sex differences with regards to responses to fear stimuli during conditioning and recall.

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 functions 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 (Marek et al. 2018). 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.

These studies also established the ability of CDPPB, an mGlu5 positive allosteric modulator, to reverse CIE induced deficits in fear extinction learning. 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 (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.

In support of the sex differences observed with regards to behavioral responses during conditioning and recall, these fear-related 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 examining 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 examining auditory fear memories, it has been shown that females exhibit enhanced learned fear expression that is associated with 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.

In conclusion, these studies demonstrate the deleterious effects of chronic ethanol exposure on fear extinction learning and establish mGlu5 activity in the IfL as a potential mechanism of action through which ethanol induces these effects. CDPPB as a glutamatergic modulator was also shown to be effective at reducing the harmful effects of ethanol, and supports the use of glutamatergic modulation for the treatment of comorbid PTSD/AUD.

Optogenetic inhibition of the prelimbic cortex during fear memory reconsolidation facilitates extinction learning in ethanol exposed animals

These studies were completed to examine the effects of prefrontal cortex manipulation following fear memory recall on future responses to fear stimuli. By optogenetically inhibiting the PrL cortex during fear memory reconsolidation, we were able to enhance extinction learning in a model of PTSD/AUD. These effects were specific to the PrL cortex, as repetition of this experiment with inhibition of the IfL cortex did cause any effects on fear extinction learning. Further, these experiments confirmed these results in both sexes and also led to long term changes in responses to fear stimuli during spontaneous recovery testing.

The acquisition and maintenance of psychiatric disorders such as PTSD and AUD rely on the fundamental principles of learning and memory. Based on classical theories of learning and memory, the environment in which the patient experiences the traumatic event can become a conditioned stimulus (CS) and the fear response that the person has to the trauma environment and cues will then become a conditioned response (CR). Further experiencing of the environmental cues can reinforce the association between the cues (CS) and the fear response (CR) which leads to PTSD symptoms such as such as re-experiencing the trauma through flashbacks and nightmares in response to trauma associated cues (VanElazakker et al. 2013). These principles are often applied clinically for the treatment of PTSD and AUD through techniques such as exposure therapy. Repeated exposure to the trauma-associated environment (CS) in a safe location

can help decrease the fear response (CR) to these cues in a process known as extinction learning (Merlo et al. 2014). Subsequent cue presentation offers a unique period of time where intervention can be made to disrupt the association between the trauma associated cues and the fear response. However, when alcohol is used to self-medicate in response to the PTSD symptoms, there are deficits in extinction learning which render these therapies unsuccessful (Conklin and Tiffany 2002; Back et al. 2014). These results are replicated preclinically in studies that establish impairments in fear extinction learning following binge ethanol exposure (Smiley et al. 2020). Therefore, in situations where chronic alcohol exposure occurs following trauma exposure, supplemental therapeutics are needed to treat PTSD symptoms. The two clinical mechanisms for enhancing these processes include the enhancement of extinction learning and the blockade of fear memory reconsolidation (Giustino et al. 2016). Our previous study was able to treat the behavioral deficits that occur with co-morbid PTSD/AUD through the first strategy by using a pharmacological modulator of mGlu5 to enhance extinction (Smiley et al. 2020). The present study utilized the second mechanism by using optogenetics to block fear memory reconsolidation. By blocking the activity of the PrL during fear memory reconsolidation, we stopped the “fear promotion” input to the memory engram (Kitamura et al. 2017; Tonegawa et al. 2015; 2018). Therefore, when fear memory recall is induced through exposure to the fear associated cues and environment, PrL activity is reduced and, behaviorally, we record a reduced fear response. While invasive brain manipulation techniques are not practical for human use, there has been recent research into therapeutic

agents that result in the inhibition of fear memory reconsolidation. For example, propranolol has become a popular pharmaceutical to use in conjunction with behavioral therapies to affect more successful treatment outcomes for PTSD patients by blocking fear memory reconsolidation (Giustino et al. 2016; Brunet et al. 2014; Schwabe et al. 2012). The data presented here provide novel evidence that the manipulation of fear memory reconsolidation is a viable treatment strategy for extinction learning deficits that are introduced through exposure to chronic alcohol following stress exposure in a model of comorbid PTSD/AUD.

Previous studies have found conflicting results regarding the effects of ethanol exposure on fear learning, but it is important to examine each study separately with regards to the specific model used for both fear conditioning as well as ethanol exposure. When animals were conditioned using six tone/shock pairings followed by injections of 30% ethanol for five days, ethanol exposure was found to cause a strengthening of the fear memories that were established during conditioning (Quiñones-Laracuente et al. 2015). These animals exhibited an increased freezing response when compared to vehicle injected animals across extinction training and in a single extinction recall test the following day. However, these effects were only displayed when extinction training occurred directly following ethanol exposure, since when extinction testing occurred 10 days following ethanol injections there was no change in freezing during extinction (Quiñones-Laracuente et al. 2015). The main differences between this previously published data and the results presented here are in the fear conditioning (12 tone/shock pairings vs. 6) and

ethanol exposure (14 days vapor exposure to 100% ethanol vs. 5 days of injections of 30% ethanol). Supporting evidence comes from mouse studies that determined the ability of ethanol to cause changes in fear learning and the glutamatergic system. These experiments were completed using a similar CIE model that found that ethanol exposure resulted in increased levels of freezing during extinction and retrieval testing that was associated with increased dendritic arborization in the PrL (Holmes et al. 2012). Additionally, these studies showed that a shorter CIE paradigm was not able to induce these effects (Holmes et al. 2012). Thus, the more intensive paradigm used for these studies could lead to longer lasting changes in prefrontal glutamate signaling as well as behavioral responses to fear cues.

Taken together, these studies suggest that inhibition of the PrL, but not the IfL, during fear memory reconsolidation facilitates extinction learning and prevents increased freezing during memory reconsolidation in both male and female animals in a preclinical model of PTSD/AUD. These studies support the use of modulation of memory reconsolidation for the treatment of learning and memory deficits that occur as a result of exposure to stress and chronic alcohol and, further, identify specific time-points and brain regions that are necessary for this treatment effect.

Adolescent exposure to THC results in impairments in cognitive flexibly and increases in ethanol seeking in adulthood

Previous studies illustrate the long-term effects on cognition that occur as a result of chronic cannabis use during adolescence (Jacobus and Tapert 2014). Clinically, cannabis exposure during adolescence results in impairments in memory performance (Silva de Melo et al. 2005) that persist even after prolonged abstinence (Schwartz et al. 1989). Additionally, cognitive flexibility has been examined in preclinical models where set-shifting was tested following exposure to various models of prolonged cannabis exposure (Gomes et al. 2014, Szkudlarek et al. 2019). These studies found that, while adolescent exposure to cannabinoid agonists induces deficits in strategy set shifting (Gomes et al. 2014), adult exposure to THC does not result in alterations in this behavioral task (Szkudlarek et al. 2019). Further, clinical studies have determined the effects of alcohol, cannabis, and use of both substances on cognition and have found that concomitant use of alcohol and cannabis by adolescents leads to deficits in cognitive flexibility (Winward et al. 2014). Thus, the results reported from the present experiment are in accordance with the literature, and further these data by using a mix of THC/CBD at a concentration that is clinically relevant.

Additionally, prior studies have shown that cannabis exposure can impact drug use in the future (Chadwick et al. 2013). Longitudinal studies have shown that adolescent cannabis use will positively predict levels of alcohol use for the following year, and further studies determined that ~25% of illicit drug users have

had previous cannabis exposure (Newcomb and Bentler 1986). This effect has also been shown to be dependent on cannabis exposure occurring during adolescence, with the associations between later drug abuse predicted by prior cannabis abuse decreasing with the age at which cannabis abuse began (Fergusson et al. 2006). This effect has also been exhibited preclinically as well, where rodents exposed to THC during adolescence increase self-administration to drugs of abuse in adulthood (Ellgren et al. 2007). Additionally, it has been previously established that adolescents in general drink more than adults in various preclinical models of voluntary ethanol seeking (Bergstrom et al. 2006). The results from these experiment support the hypothesis that adolescent exposure to THC will increase ethanol seeking while adult exposure does not have the same effect.

An added aspect of these studies is examination of the effects of NAC on cognitive deficits induced by adolescent THC exposure. NAC treatment concurrent with THC and CIE exposure was able to prevent deficits in cognitive flexibility as well as have an effect on THC induced increases in ethanol seeking. While NAC has been shown to affect a wide variety of brain systems, its function as a neuroprotective agent could be a potential mechanism for action for these effects (Tardiolo et al. 2018). NAC has been shown to cross the blood-brain-barrier in animal studies and can affect antioxidant pathways as well as regulate glutamatergic signaling (Farr et al. 2003). Due to THC and ethanol's ability to impair glutamate homeostasis in the prefrontal cortex, the regulatory action of NAC

on this system may be responsible for the pro-cognitive effects of NAC observed in these studies (Gilbert et al. 1991).

In summary, these experiments revealed that adolescent exposure to THC and ethanol leads to impairments in cognitive flexibility. This deficit was associated with perseveration in set-shifting testing. Furthermore, exposure to THC during adolescence leads to increases in ethanol seeking during self-administration. Finally, NAC was effective at preventing drug induced alterations in cognitive flexibility, potentially due to its effects on prefrontal glutamate homeostasis. These data provide further evidence of the detrimental effects of adolescent THC exposure on prefrontal cortex reliant behaviors and establish a treatment effect of NAC.

THC and ethanol exposure during adolescence leads to heightened responding to fear stimuli through increases in prelimbic activity

To examine the effects of adolescent drug exposure on future response to fear stimuli, animals were exposed to vaporized THC and ethanol during mid-adolescence before being tested in fear conditioning paradigms. The ability of NAC to impact drug induced alterations in fear responding was examined along with in-vivo recordings from the PrL during the presentation of fear stimuli. These studies found that adolescent exposure to THC and ethanol results in heightened behavioral responding during fear conditioning along with an increase in PrL signaling in response to each shock presentation. Additionally, when NAC was

administered concurrently with each drug exposure, the effects of THC and ethanol on both behavioral freezing responses as well as PrL signaling alterations were prevented. These studies revealed the deleterious effects of adolescent exposure to THC and ethanol and provide evidence of impairments in PrL function as a contributing factor that may underlie the clinical relationship between adolescent drug exposure and future susceptibility to developing PTSD following trauma exposure.

These results add to the present literature examining the effects of THC and ethanol on stress responses. Cannabis is commonly used in an attempt to decrease anxiety, but THC is known to be anxiogenic, especially when it is taken at higher concentrations (Sharpe et al. 2020). This relationship has been demonstrated across a wide variety of preclinical tests of anxiety and in clinical examinations (Rock et al. 2017, Raymundi et al. 2020). More recently, the long-term effects of THC on anxiety disorders has become a topic of study. Clinical studies have examined this relationship by putting chronic cannabis users through a fear conditioning paradigm and found that, during conditioning, cannabis use was associated with increases in threat generalization to safety stimuli (Papini et al. 2017). This reduction in differentiation between safe and threatening stimuli was also associated with impairments in fear extinction (Papini et al. 2017). These effects have been further investigated preclinically in the adolescent population. Specifically, studies have shown that adolescent animals exposed to THC exhibit alterations in fear learning after being exposed to a stressor, but this effect was not

observed when THC exposure occurred during adulthood (Saravia et al. 2018). Additionally, adolescent exposure to chronic ethanol has been shown to have variable behavioral effects on fear conditioning depending on the ethanol exposure paradigm, fear conditioning protocol, and timing of exposure. Due to varying ethanol and fear exposure paradigms, these studies have found no effects on fear conditioning (Broadwater and Spear 2013), impairments in learning during conditioning (Stephens et al. 2005), or heightened responding (Moberg et al. 2017) following ethanol exposure. By using chronic intermittent ethanol exposure followed by exposure to 12 tone/shock pairings during conditioning, our results provide novel data regarding ethanol's ability to increase responding during fear conditioning.

The effects of THC and ethanol observed during the behavioral paradigms completed in these studies were associated with signaling alterations in the PrL subregion of the prefrontal cortex. Generally, activity in the PrL cortex has been shown to correlate with freezing behavior during conditioning paradigms (Burgos-Robles et al. 2009). Exposure to either THC or ethanol during adolescence has been previously shown to alter the development and morphology of this region. During adolescence, the prefrontal cortex is uniquely situated to be affected by drugs of abuse that may affect the ongoing developmental pruning (Pattwell et al. 2016). In rodent models, THC exposure has been shown to disrupt developmental processes in the PrL subregion of the prefrontal cortex by altering gene networks that are responsible for dendritic development (Miller et al. 2019). Specifically,

overactivity at cannabinoid 1 (CB1) receptors in the prefrontal cortex during adolescence can lead to dysregulation of glutamatergic signaling at inhibitory interneurons leading to impaired development of these cells and ultimately resulting in downregulated inhibitory control in this region (Caballero and Tseng 2012). These findings are paralleled by clinical data demonstrating that those with a history of adolescent cannabis exposure exhibit alterations in both functionality and volume of the prefrontal cortex (Medina et al. 2009, Orr et al. 2013). Similar impairments in prefrontal cortex development have been observed as a result of adolescent alcohol exposure (Squeglia et al. 2014). The prefrontal cortex is known to be especially susceptible to the deleterious effects of alcohol, and significant differences in both gray and white matter have been observed following adolescent alcohol use (De Bellis et al. 2005, Medina et al. 2008). Given the ongoing development of the PrL as well as the ability of THC and ethanol to affect this region, these alterations could underlie the behavioral and signaling alterations observed as a consequence of adolescent drug exposure in the present studies.

The overlap between THC and ethanol's effects is most pronounced in the prefrontal cortex and, therefore, NAC could be working in this area to prevent the increased reactivity to fear stimuli observed in these experiments. In clinical studies, NAC has been shown to regulate glutamate levels in the prefrontal cortex (McQueen et al. 2018) as well as functional connectivity of these regions (Mullier et al. 2019). Additionally, NAC has the ability to promote regulation of glutamate homeostasis in patients with substance abuse, including adolescents that exhibit

cannabis dependence (Gray et al. 2010). Therefore, given THC and ethanol's effects on prefrontal cortex glutamate homeostasis, NAC could be acting through this pathway to reduce increases in prelimbic signaling and behavioral responding during fear conditioning following THC and CIE exposure.

In summary, these experiments determined that adolescent exposure to THC and ethanol results in heightened behavioral responding during fear conditioning that is associated with increases in PrL signaling in response to fearful stimuli. These results support the hypothesis that drug exposure during adolescence can lead to neurobiological alterations that cause a predisposition to developing fear cue related disorders such as PTSD, and that normalization of glutamatergic functioning in the PFC could serve as a pharmacological method of preventing these deleterious effects.

Summary

The overall goal of this dissertation was to determine the effects of stress, ethanol, and THC exposure on behavioral and neuronal responses to future exposure to fear and drug cues. To this end, experiments were completed using multiple behavioral models of stress and drug exposure, including fear conditioning and restraint stress accompanied by ethanol self-administration, chronic intermittent ethanol, and exposure to vaporized THC. Surgical techniques including microinjections, optogenetics, and fiber photometry were also used to examine the activity of the prefrontal cortex during these behavioral tasks. Further,

the pharmaceutical compounds CDPPB and N-acetylcysteine were used to prevent and reverse both stress and drug induced behavioral and signaling deficits. In total, the studies completed within this dissertation establish multiple behavioral deficits that occur as a result of stress and drug exposure, determined the neuronal localization of these effects in the prefrontal cortex, and used readily available pharmacological compounds to treat these impairments. These data supply valuable information regarding the underlying circuitry involved in common psychiatric disorders such as post-traumatic stress disorder and substance use disorder and help to further the field regarding the use of glutamatergic modulation as a treatment strategy.

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