THE EFFECTS OF CHRONIC INTERMITTENT ETHANOL EXPOSURE ON FEAR CONDITIONING IN RATS: AN INVESTIGATION OF THE COMORBIDITY OF POSTTRAUMATIC STRESS DISORDER AND ALCOHOL USE DISORDER

BY

NATHAN P MCMULLEN

A Thesis Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

In Partial Fulfillment of the Requirements

For the Degree of

MASTERS IN BIOMEDICAL SCIENCES

(Concentration in Neuroscience)

May 2018

Winston-Salem, North Carolina

Approved By:

Jeffrey L. Weiner, PhD, Advisor

Kimberly F. Raab-Graham, PhD, Chair

Brian A. McCool, PhD
Dedications and Acknowledgments

I would like to thank Dr. Weiner for his support and guidance throughout my project as my advisor; he was instrumental in formulating this project and the content within this review/manuscript. I want to also thank my committee members Dr. Kimberly Raab-Graham and Dr. Brian McCool for their guidance during my project. I would like to express gratitude to Ann Chappell for her immense help in training me and troubleshooting our fear conditioning and CIE equipment; without her this project would not be possible. Likewise, I would like to thank Eugenia Carter for her help in training me and data analysis. I would also like to thank PhD student Sarah Ewin for her integral role in my project, providing advice and help in running my experiments and data analysis. I want to thank Dr. Antoine Almonte, a junior faculty member in our lab, for his valuable training. I would also like to thank Whitt Morgan, a fellow Master’s student for all his input and help running my experiments. I would lastly like to thank the rotating PhD students in our lab, Sarah Sizer and Alexandra Baldassaro for their help in my experiments. I would also like to thank my family for the continued support throughout my academic career, particularly my parents Michael and Dolores McMullen, without whom none of this would be possible.
# Table of Contents

I. Comorbidity of PTSD and AUD: a Review of potential neurobiological mechanisms (pg. 1-24)
   A. Post-traumatic Stress Disorder (pg. 1-3)
   B. Alcohol Use Disorder (pg. 3-6)
   C. Comorbidity between Post-traumatic Stress Disorder and Alcohol Use Disorder (pg. 7-8)
   D. Animal models of PTSD and AUD (pg. 8-12)
   E. Neurobiological substrates of Comorbid PTSD and AUD (pg. 12-23)
      a. HPA-axis (pg. 12-14)
      b. Basolateral Amygdala and PTSD/AUD (pg. 14-15)
      c. The Hippocampus and PTSD/AUD: a Bimodal System (pg. 15-23)
   F. Summary (pg. 23-24)

II. The effects of Chronic Intermittent Ethanol Exposure on Fear Conditioning in Rats: an Investigation of the comorbidity of PTSD and AUD (pg. 25-)
   A. Abstract (pg. 25-26)
   B. Significance Statement (pg. 26)
   C. Introduction (pg. 27-29)
   D. Materials and Methods (pg. 29-33)
   E. Results (pg. 33-40)
   F. Discussion (pg. 41-46)

III. Discussion (pg. 47-53)

IV. Curriculum Vitae (pg. 76-77)
List of Figures:

Figure 1: Symptoms used to diagnose Alcohol Use Disorder (pg. 6)

Figure 2: CIE exposure impairs fear acquisition on Day 1 of Fear conditioning (pg. 34)

Figure 3: CIE exposure impairs fear extinction (pg. 35)

Figure 4: CIE exposure does not affect retrieval of fear extinction on Day 3 of fear conditioning (pg. 35)

Figure 5: CIE exposure significantly increases spontaneous recovery of fear 10 days following retrieval (pg. 37)

Figure 6: CIE exposure significantly alters spontaneous recovery to cued fear 10 days following retrieval (pg. 37)

Figure 7: Spontaneous recovery of fear 20 days later is not significantly affected (pg. 37)

Figure 8: Effects of CIE and fear conditioning exposure on binge-like drinking behavior (pg. 39)

Figure 9: Effects of CIE and fear conditioning exposure on 24 hour drinking behavior (pg. 40)
Abbreviations

PTSD = Post Traumatic Stress Disorder

AUD = Alcohol Use Disorder

CIE = Chronic Intermittent Ethanol

DHC = dorsal hippocampus

VHC = Ventral Hippocampus

BLA = Basolateral Amygdala

LA = Lateral Amygdala

CeA = Central Amygdala

AsI = Adolescent Social Isolation model

mITC = Medial Intercalated Cells

PFC = Prefrontal Cortex

IL = Infralimbic Cortex

PL = Prelimbic Cortex
Chapter 1: Comorbidity of PTSD and AUD: a Review of potential neurobiological mechanisms

Post-traumatic Stress Disorder

With approximately 18% of the population afflicted, anxiety disorders are the most common mental illness in the United States (ADAA 2016). With a lifetime prevalence of approximately 7.8% (Ralevski et al. 2014), post-traumatic stress disorder (PTSD) is one of the most common of these disorders. Defined as a mental disorder stemming from exposure to an extremely stressful event or events (Yamamoto 2009), PTSD is diagnosed utilizing four clusters of symptoms as outlined in the DSM-V, these clusters being: re-experiencing, avoidance, negative alterations in cognition and mood and marked alterations in arousal and reactivity (USVA 2017; Allen et al. 2016; see figure 1). Specifically, following direct or indirect exposure to death, extreme injury or sexual assault, patients diagnosed with PTSD exhibit a subset of well-defined symptoms including: intrusive thoughts, flashbacks, nightmares, distress following trauma reminders, avoidance (of reminders) and hypervigilance/increased startle response (USVA 2017). For upwards of 40-50 years following the traumatic event, PTSD patients are unable to properly cope with the stress of the traumatic event and constantly re-experience the trauma following reminiscent stimuli (Wess and Flor 2007). Essentially, patients with PTSD have dysregulated fear learning/conditioning in that they are unable to extinguish the learned fear properly; the fear lingers in response to stimuli that are no longer associated with danger.

With such obstructive symptoms, there is a great deal of interest in studying PTSD. Surprisingly, much remains unknown about this devastating disorder and more
effective treatments are needed. To illustrate this, the FDA and the European Medicines Agency have only approved sertraline and paroxetine, two serotonin reuptake inhibitors (SSRIs), as medications for PTSD (Ralevski et al. 2014). Although they have been approved by these two agencies, these drugs (along with other SSRIs and SNRIs such as venlafaxine) have only been shown to modestly reduce PTSD symptoms, in numerous randomized controlled trials (Ralevski et al. 2014; Marshall et al. 2001; Kucukalic et al. 2008). Due to the plethora of symptoms, and limited efficacy of approved treatments, physicians often prescribe other medications “off-label” to alleviate subsets of symptoms. One such medication is prazosin, an alpha-1 adrenergic receptor antagonist typically prescribed for high blood pressure, has recently been prescribed to mitigate PTSD-related nightmares (Kung et al. 2012; Koola et. al 2014). Potentially by blocking the effects of adrenaline, prazosin has mostly been shown to alleviate overall PTSD symptoms as well as comorbid depressive symptoms (Kung et al. 2012; Koola et. al 2014); however, it is not a cure, only alleviating some of the symptoms (nightmares mostly) and is not explicitly recommended by the Department of Veterans Affairs (DVA and DoD 2018).

Due to the nature of PTSD there are high rates of comorbid depression in PTSD patients, resulting in physicians often prescribing other antidepressants including: mirtazapine (tetracyclic antidepressant), nefazodone, tricyclic antidepressants and monoamine oxidase inhibitors (Ralevski et al. 2014; Chung et al. 2004; Davis et al. 2004; Frank et al. 1988; Kosten et. al 1991) To mitigate other symptoms, antipsychotics, anticonvulsants and benzodiazepines are frequently used with varying efficacy. For example, a few studies have shown some efficacy for topiramate (an anticonvulsant) in reducing PTSD symptoms (Berlant 2006; Ralevski et al. 2014). Due to the complex nature of PTSD and
its comorbid disorders, these pharmacological interventions have proven to be insufficient, with much room for progress.

In addition to pharmacological treatments, there are a few psychological treatments that have shown some promise in ameliorating symptoms. These treatments include: prolonged exposure, cognitive processing therapy, brief eclectic psychotherapy, narrative exposure therapy and eye movement desensitization and reprocessing (EMDR) (Allen et al. 2016; Cusack et al. 2016). All of these treatments are exposure therapies aimed at recounting traumatic events to allow for emotional and cognitive processing and learning, in hopes of having the patients separate their past experiences from new ones. The hope is these treatments allow the patient to regain control of their responses to their environment, understanding what triggers their symptoms so they can mitigate the response. However, as with all psychological interventions, these programs are not perfect and have varying levels of efficacies between patients.

**Alcohol Use Disorder**

Similar to PTSD, alcohol use disorder is a devastating disease that afflicts some 29.1% of American adults in their lifetime that in addition to other alcohol related problems costs the American public upwards of $200 billion yearly (Zorumski et al. 2014; Grant et al. 2017). Defined by 11 symptoms outlined by the National Institute of Alcohol Abuse and alcoholism (see figure 1), AUD is a brain disease which results in the sufferer’s inability to control their alcohol consumption and cravings (NIAAA 2017). This often results in negative consequences in the sufferer’s life in regard to their personal relationships, career, health and standings with the law. With so many negative consequences associated with their drinking, why do AUD sufferers return to drinking?
One potential reason is a shift in the drinkers’ motivation to drink. When alcoholics (as well as normal drinkers) begin to drink, they drink for the rewarding effects of alcohol, i.e. mood enhancement, extraversion, euphoria, etc. However, as their use continues at dangerous levels, their craving to drink shifts from seeking pleasure to staving off the negative effects of alcohol. Instead of drinking for pleasure, AUD patients drink to subdue the negative side effects of alcohol, withdrawal in particular. This creates a downward spiral in their drinking behaviors, resulting in some of the symptoms outlined in figure 1. With this sort of drinking, it is easy to see the correlation between this disorder and anxiety as these drinkers are drinking to reduce the anxiety associated with alcohol withdrawal. Thus, it has been seen through numerous studies that there are high rates of comorbidity between AUD and anxiety-disorders. Moreover, anxiety and stress have been shown to increase vulnerability to developing AUD and other substance use disorders (Spanagel et al. 1995; Dube et al. 2003; Silberman et al. 2009; McCool et al. 2010) For instance, it has been shown that exposure to two or more adverse childhood events (ACE), as defined by the Substance Abuse and Mental Health Services Administration (SAMHSA), significantly increases the risk for alcohol dependence later in life (Pilowsky et al., 2009; SAMSHA 2017).

Currently, there are three medications approved by the FDA for AUD treatment including: naltrexone, acamprosate and disulfiram (Ralevski et al. 2014; NIAAA 2017). Despite their approval, each of the aforementioned drugs have only shown partial efficacy in mitigating symptoms. Each of these drugs works by helping to reduce the patients craving for alcohol (naltrexone and disulfiram) or by reducing the effects of withdrawal (Acamprosate) (NIAAA 2017). Although not directly approved for treatment,
numerous other drugs are prescribed to help patients including benzodiazepines, which have shown some efficacy in reducing withdrawal symptoms. Some other drugs have shown some preliminary promise for treatment, including two drugs previously mentioned for PTSD treatment: topiramate and prazosin. Both topiramate and prazosin have been shown in human and animal trials to reduce drinking, potentially via decreases in anxiety (Skelly and Weiner 2014; Johnson et al. 2007; Johnson et al. 2003 Simpson et al. 2008). With numerous treatments showing some promise, much more research must be done to study the mechanisms of action in hopes of finding even better treatments.
Figure 1. adapted from NIAAA. Symptoms used to diagnoses Alcohol Use Disorder. The severity of the condition is determined by the number of symptoms present.
Comorbidity between PTSD and Alcohol Use Disorder

Despite all of these promising treatments for PTSD and AUD, no completely effective treatment has been found for either. This is due to many reasons, one being the high rates of comorbidity with other disorders and each other. With a 30-59% comorbidity (Ralevski 2014), PTSD patients often suffer from AUD, with the prognosis being worse for both during co-occurrence. The exact link between these two disorders is uncertain. It is not known if AUD develops due to the stress related to PTSD (and the associated traumatic event) or if the comorbidity is due to some sort of proclivity/risk factor. AUD may develop as a coping mechanism for the PTSD symptoms, i.e. self-medication, or there may be alterations within the brain that may increase the risk for both disorders. These alterations may promote both resilience and vulnerability for these disorders. For this reason, it is imperative to study these two disorders in relation to each other, to discover this link in hopes of finding a more efficacious treatment for these comorbid conditions. Current treatments for each of these respective disorders have been shown to have mixed efficacy in comorbid patients. Treatments that may be effective in AUD or PTSD patients are often less effective when the patient exhibits both disorders. For instance, a review of six studies of PTSD/AUD comorbid treatment by Ralevski et al. found treatments to be fairly successful in decreasing drinking, but not in reducing PTSD symptoms (Ralevski et al. 2014). Moreover, some treatments for these disorders may not be safe for everyone, treatments have to be tailored to each individual. For instance, if patients suffer from liver damage (a common symptom of alcohol dependence) or chronic pain (co-occurrence in both PTSD and AUD), it is advisable to avoid naltrexone, a pharmacotherapy with promise of comorbid treatment. Despite some mixed results in
pharmacological treatments, there is some promise in psychopharmacological treatments in which psychosocial and pharmacological treatments are combined, such as naltrexone and prolonged exposure therapy. Moreover, some alternative treatments such as mindfulness meditation, acupuncture and yoga have shown to effective in reducing PTSD and AUD symptoms (Marlatt et al. 2004; Kearney et al. 2012; Kim et al. 2013; Wupperman et al. 2012; Descilo et al. 2004; see Ralevski for citations). Further research is critical to improve these treatments and to find more, as those listed above are not sufficient. By studying animal models for each and finding common grounds, it may be possible to find novel targets for the treatment of both. Moreover, by studying models which incorporate this comorbidity researchers may come closer to finding a more effective treatment.

Animal Models of PTSD and AUD

Much remains unknown about the neural substrates underlying the comorbidity between PTSD and AUD. One major impediment has been the difficulty in establishing valid animal models of either disorder, let alone one that recapitulates the symptoms of both. With regard to PTSD, it has been difficult to develop a reliable animal model since it is hard to objectively study affect in animals (Yamamoto et. al 2009). Moreover, for a multitude of reasons, it has been difficult to ethically produce PTSD-like symptoms in animals since inducing PTSD-like symptoms is inherently stressful and potentially harmful. However, some paradigms have begun to be utilized and appear to be able to engender many of the key symptom clusters associated with PTSD. One such paradigm, is the single prolonged stress model in which animals (usually rats) are introduced to an assortment of extremely stressful scenarios (including forced swims, restraint and loss of
consciousness) for a prolonged length of time to simulate the traumatic events associated with PTSD (Yamamoto et al. 2009). Following this model, animals exhibit the telltale signs of PTSD such as increased anxiety-like behavior, impaired fear extinction, abnormal HPA axis functioning and low cortisol levels. Another similar model is the predator-exposure model, in which animals are exposed to predators either directly or indirectly (using predator odors) while typically restrained. Like the aforementioned model, this model induces changes similar to PTSD such as enhanced startle response, heightened anxiety and dysregulated HPA axis (Zovkic and Sweatt 2013).

Models of AUD are a bit more agreed upon within the research community. One such model aims to produce an animal model of dependence-like behavior; this model being the chronic-intermittent ethanol exposure model. In this model, rats are subjected to 12 hours of ethanol vapor every other day for a set period of time (typically ten days). Following this paradigm, the rats exhibit significantly altered behavior, pharmacology and neurophysiology. These alterations include: increased anxiety-like behaviors, increased ethanol consumption, enhanced glutamatergic function, altered synaptic excitability/plasticity, altered endocannabinoid system expression, as well as altered HPA axis function (Maldinaddo-Devinnici et al. 2016; Griffin et al. 2015; McCool et al. 2011; Ewin unpublished). These alterations make this model an excellent means of studying both AUD as well as PTSD with it producing behavior reminiscent of both. Together with other treatments, CIE could be used to research comorbid alcohol dependence and PTSD symptoms.

Researchers have also begun to repurpose stress exposure paradigms to study stress-induced escalations of drinking as seen in the PTSD/AUD population. With PTSD
diagnoses typically preceding AUD, models have mainly been created to mirror this
development. Introducing the animals to a stressor at various time points, and observing
their resultant drinking behaviors, researchers may be able to find the neurobiological
underpinnings of this escalation. Despite challenges in inducing reliable prolonged
drinking effects, some laboratories have been successful in producing phenotypes
reminiscent of human behaviors. For instance, some laboratories have had success in
inducing escalated drinking following predator odor exposure. They found that exposure
to predator urine was sufficient to escalate rat self-administration of alcohol up to 3
weeks following exposure (Edwards et al. 2013; Manjoch et al. 2016). Therefore, these
studies would suggest that it is possible to mimic stress-induced ethanol consumption
escalations in animals. However, interestingly, this effect may only be certain types of
stress as a similar study in mice found that footshock, tail pinch, tail suspension and
restraint were not able to increase ethanol consumption like predator odor exposure
(Cozzoli et al. 2015); in fact, they found the opposite, exemplifying the complex nature
of this sort of behavior. The lack of ethanol consumption escalation in these other
paradigms may just be an effect of previous ethanol exposure, which has been shown to
mitigate stress-induced ethanol escalation in foot shock experiments (Meyer et al. 2013).
Despite these negative results, they were able to further validate the predator odor
exposure model as they found increases in corticosterone (CORT) levels following
exposure, reminiscent of the human condition (Cozzoli et al. 2015). Thus, it appears that
predator odor exposure is sufficient to induce escalated ethanol drinking similar to
PTSD/AUD. In the same way other labs have found that exposure to social defeat stress,
a classic depression animal model that relies on larger animals dominating others (Golden
et al. 2011), increases ethanol consumption during two-bottle choice drinking paradigms (Norman 2012; Molander et al. 2012; Logrip and Zorrilla 2012). Stress exposure has also been shown to increase the magnitude of effects seen in alcohol dependence models, with the combination of repeated forced swim tests and CIE increasing ethanol consumption above that of animals exposed to just one of these paradigms (Anderson et al. 2016; Lopez et al. 2016). The combined use of alcohol dependence and stress models thus may be useful in studying the comorbidity of PTSD and AUD.

Another such model that aims to mimic the comorbidity of AUD and anxiety disorders is the adolescent social isolation (AsI) model, which similarly induces long lasting behavioral deficits following stress exposure. With rats being such social creatures, the AsI model mimics early childhood stress by depriving the adolescent rats the ability to physically socialize, causing much stress. Previous research, has substantiated AsI as representative of anxiety disorders and comorbid alcohol abuse, with the socially isolated rats expressing: robust anxiety like behaviors, deficient fear extinction (characteristic of PTSD) and increased ethanol intake (Skelly et al. 2015). Via this paradigm, it may be possible to study the neural substrates that make certain populations of people more susceptible to both of these disorders, in particular those who undergo intense childhood stress. Studies have shown that early life stress significantly increases the likelihood of developing both PTSD and AUD later in life and is thus an important area of study (Enoch 2011).

These models, or a combination of them, could be utilized to study the comorbidity of PTSD and AUD in hopes of finding new targets of treatment. One means of studying these model’s validity would be Pavlovian fear conditioning where rats are
conditioned to associate a cue with an aversive stimulus, such as a footshock (Zovkic and Sweatt et al. 2016). Once they have learned to associate these two stimuli, the cue is presented without the aversive stimuli and the animal slowly learns to extinguish the fear memory. Those suffering from PTSD and AUD, or animal exhibiting PTSD/AUD-like symptoms, would have altered fear extinction, thus associating the two for longer periods than normal. Therefore, the use of fear conditioning in conjunction with one of the above models may prove as an important tool in the research of these disorders.

**Neurobiological Substrates of Comorbid PTSD and AUD**

**HPA axis**

One target that has received a great deal of research attention is the hypothalamo-pituitary-adrenocortical (HPA) axis, a system of organs and brain structures integral in the “stress response” (Smith and Vale 2006). The HPA axis describes the interactions of the hypothalamus, pituitary gland and the adrenal gland that allows for proper reactions to stressful stimuli. Following the presence of a stressful stimulus, hypothalamic neurons secrete corticotrophin-releasing hormones (CRH) into the pituitary gland (Herman and Cullinan, 1997). This hormone stimulates the release and production of adrenocorticotrophin-hormones (ACTH) which in turn stimulates the production and release of cortisol (in addition to adrenaline) from the adrenal glands. This steroid hormone is partially responsible for the body’s response to stressful and dangerous stimuli, the “fight or flight” response. To prepare the body to respond properly, high blood cortisol (a glucocorticoid) results in a few bodily changes including: increased blood glucose levels (to increase energy readability), increased blood pressure, decreased
pain sensitivity, increased blood calcium concentration and increased immune responses (Whitcomb 2011). In normal patients, there are negative feedback loops within this axis, with the release of cortisol inhibiting the production of both ACTH and CRF, keeping the levels of cortisol at proper levels (Levone et al. 2015; Zhu et al. 2014). This negative feedback loop occurs via interactions with mineralocorticoid and glucocorticoid receptors that are expressed throughout the brain, with high concentrations found in the: cerebral cortex, amygdala, hippocampus, olfactory cortex, locus coeruleus, hypothalamus, thalamus etc. (Ahima and Harlan 1990; Ahima et al. 1991; Morimoto et al. 1995).

Binding of cortisol to these receptors, particularly within the hippocampus, results in the dampening of the HPA axis, allowing modulation of the stress response.

When this system is overstimulated, such as in PTSD patients, this system appears to become dysregulated with the allostatic load of repeated and/or chronic stress overwhelming the system resulting in numerous alterations (Levone et al. 2015; McEwen and Stellar 1993; McEwen 2007). These changes are evident by decreased basal cortisol levels in PTSD patients (Yehuda et. al, 2009, Yamamoto 2009). It is believed that this arises due to hypersensitivity of the glucocorticoid receptors of the negative feedback system (Yehuda et. al 2010, Yamamoto 2009, Cohen et al. 2016) which results in a hypersensitive and reactive stress response. The exact mechanism by which this occurs, however, is uncertain as low cortisol levels and a hypersensitive feedback loop seem counter-intuitive for PTSD symptoms. Some hypothesize that these low levels are simply a factor of susceptibility, with those developing PTSD having congenitally low cortisol levels (Sherin and Nemeroff 2011). Others hypothesize that low basal cortisol may result in the disinhibition of autonomic and neuroendocrine responses to stress, resulting in the
increased stress seen in PTSD patients (Sherin and Nemeroff 2011). Although basal levels of cortisol are low, it does appear that PTSD patients exhibit increased cortisol levels and noradrenergic responses following stressors, potentially explaining the hyper-reactivity of these patients.

This hyperactivity appears to be centered in the hippocampus as increased glucocorticoids in this area, and not others such as the hypothalamus, induced HPA-axis hypersensitivity (Zhu et al. 2014). Moreover, the toxic effects of this enhanced glucocorticoid signaling occur mostly in the hippocampus with degradation of the hippocampus occurring in PTSD and stress disorder, as evident by decrease hippocampal volumes in PTSD patients (Woon et al. 2010; Bremner et al 2008). Therefore, it appears that the dysregulation of the HPA-axis, possibly via the hippocampus, is an important target for research in PTSD.

**Basolateral Amygdala and PTSD/AUD**

As mentioned previously, PTSD patients exhibit impaired fear learning as they are unable to correctly discern between dangerous and innocuous stimuli, due to previous trauma. Signals related to the trauma (such as loud noises) are incessantly associated with the danger of the trauma previously experienced, even if it occurred in the distant past. Therefore, researchers have begun to target brain regions implicated in fear conditioning, to find a source of dysregulation. One such structure is the basolateral amygdala, a main component of the limbic system, with roles in emotional behavior, emotions, memory and motivation. As such, it has become a target of study in PTSD and well as AUD, and in fact it appears to have roles in both. In animal models, the BLA has been implicated in
behaviors reminiscent of both disorders, with it being implicated in fear behaviors as well as ethanol consumption (Karkhanis et al. 2016; Dale et al. 2004).

Similar alterations have been found in human patients as well. The amygdala of patients suffering from PTSD is significantly altered in comparison to non-sufferers (Gilpin and Weiner 2016). For instance, PTSD patients have been shown to have significantly decreased amygdala volumes (Hanson et al. 2015, Matsuoka et al. 2003). Likewise, the classic biomarker of PTSD is a hyperactive amygdala which is associated with impaired fear extinction as well as impaired processing of dangerous stimuli (Etkin and Wagner 2007, Garfinkel et al. 2014). These same alterations, among others, are also found within sufferers of AUD, lending more credence to the linked mechanism behind these comorbid diseases (Gilpin and Weiner 2016). Currently, studies are underway to see if these alterations translate to impaired fear extinction in alcohol dependent humans.

The Hippocampus and PTSD/AUD: a Bimodal System

Since the amygdala appears to be hyperactive in individuals suffering from comorbid PTSD and AUD, research has begun to investigate the innervations and projections of the BLA in hopes of finding more pathological differences in these patients. One such projection of the BLA is the hippocampus, a reciprocal projection (Pitkanen et. al 2000) that has been shown to have roles in consolidation of fear-conditioning (Huff et al. 2016). This connection being mostly between the BLA and the ventral portion of the hippocampus, the “hot” hippocampus.

Despite this multitude of research, much is still unknown about each of these brain regions. Previously, there were two opinions held about the functions of the hippocampus, a cognitive function and an affective function (Fanselow and Dong 2010).
The common definition being of the cognitive function in which the hippocampus is the center of declarative memory. This claim being heavily substantiated by amnesia patients, such as the famous HM, who developed amnesia following hippocampal lesions and removal (Fanselow and Dong 2010). At the same time, other researchers have shown a more emotional aspect of the hippocampus, which had previously been thought to not be relevant with aspects fear not being affected by hippocampal lesions (Fanselow and Dong 2010). One study, performed by Kluver and Bucy, showed that removal of the medial temporal lobe (i.e. the hippocampus) induced significant behavioral and emotional disturbances in monkeys (Fanselow and Dong 2010). Likewise, a more recent study showed that selectively lesioning of one portion of the rat hippocampus (and not other portions) resulted in a decrease in unconditioned fear behavior on the elevated plus maze (Strange et al. 2014), a measure of anxiety-like behavior. One possible explanation for this result could be in the organization of the hippocampus, with different regions correlating to the cognitive and emotional functions. It has been put forth that this organization does occur, with the more cognitive processes occurring dorsally while the affective/anxiety processes occur ventrally (Fanselow and Dong 2010; Strange et al. 2014). In regard to humans and primates, this division appears to be along the anterior/posterior axis, with the anterior portion corresponding to the emotional ventral aspect in rats and posterior to dorsal (Satpute et al. 2012) It is not explicitly known, however, if these regions have distinct borders, and in fact it is believed there is an intermediate region with intermediate functions (Fanselow and Dong 2010). A gene expression experiment found evidence for nine distinct “demarcated” regions within the rat hippocampus, which could be subdivided into three distinct regions: ventral, dorsal
and intermediate (Strange et al. 2014). By performing lesions in specific regions of the hippocampus and studying the behavioral responses, the researchers found a more gradual separation of functions. For instance, it was found that spatial memory was mostly confined to dorsal 70% of the hippocampus (Strange et al. 2010); however, it was also found that large scale spatial memory is partially processed via the ventral hippocampus, exemplifying a more gradual separation of functions. Likewise, despite lesion studies finding only the ventral third being utilized in unconditioned fear behaviors, they found evidence for a more gradual distribution of emotional function. As evidence, they found in an anatomical study that reciprocal innervation between the ventral two-thirds of the hippocampus and the amygdala, suggesting that emotional function is spread over two-thirds of the hippocampus (Strange et al. 2010). Despite this gradual distribution of emotion, it still appears that emotional functions are centralized in the ventral hippocampus, with little to no BLA innervations to the most dorsal aspect of the hippocampus.

With such a pattern of innervation and function, researchers have therefore begun studying emotion, particularly anxiety, in the hippocampus specifically in the ventral aspect. As seen before, lesions to the ventral hippocampus results in reduced unconditioned fear behaviors, i.e. fear behavior in response to a threatening environment, pointing towards a role in ventral hippocampal anxiogenesis (Kjelstrup et al. 2002). Coinciding with the above finding of selective BLA innervations to the ventral hippocampus, researchers from the Picower Institute for Learning and Memory found evidence for the specific role of BLA to ventral hippocampus innervations in anxiety like behaviors. Utilizing an optogenetic approach in which BLA neurons were transfected...
with halorhodopsin (light dependent inactivating channel), the researchers found a decrease in anxiety-like behavior on the elevated plus maze when BLA neurons projecting to the ventral hippocampus were inhibited (Felix-Ortiz et al. 2013). When the neurons were inhibited, mice entered the open arm of the elevated plus maze significantly more, representing an anxiolytic effect of inactivating BLA to ventral hippocampus projections. Conversely, it was found that activating these same projections (with channelrhodopsin replacing halorhodopsin) produced an anxiogenic effect, with the mice entering the open arm less frequently (Felix-Ortiz et al. 2013). Glutamate receptor antagonists specifically injected to the ventral hippocampus further demonstrated that this projection was indeed responsible for changed behaviors as the anxiogenic effects of this circuit was attenuated by glutamate antagonism. Our lab recently substantiated these results as we found chemogenetic inhibition of synapses projecting from the BLA to the vHC decreases anxiety-like behaviors on the elevated plus maze, while also decreasing ethanol intake (Weiner Lab, unpublished). Therefore, there lies a projection between the BLA and the ventral hippocampus that modulates anxiety-like behaviors as well as ethanol drinking behaviors.

In fact, a monosynaptic connection between the posterior BLA and the ventral hippocampus was shown experimentally to regulate anxiety-like behaviors such as learned helplessness and hopefulness in mice and rats (Yang et al. 2016). Optogenetic inhibition and activation of this circuit was sufficient to disrupt these anxiety/fear-like behaviors, suggesting a direct role of this circuit in anxiety. This role appears to be synergistic, with both regions modulating potentially different mechanisms, as lesions of just the vHC or the BLA were not sufficient to create full anxiolytic effects in the
successive alleys test (a test of unconditioned anxiety), anxiety-like behaviors were still observed (McHugh et al. 2004). Although these regions work in concert to modulate anxiety, it appears that they also modulate separate independent mechanisms.

Similar results have been found in fear conditioning experiments in which the roles of the BLA and dorsal/ventral hippocampus were studied utilizing various methods including specific lesions. In general, it was found that both the amygdala and the hippocampus had roles in fear conditioning, however, they were slightly different and somewhat independent. To illustrate this, lesion studies performed by Phillips and Ledoux found that the hippocampus appears to have a role in the contextual processing of fear conditioning while the amygdala has roles in both the cue and the contextual learning, both crucial aspects of PTSD symptoms (Phillips and Ledoux 1992). Regarding the dorsal ventral axis of the hippocampus, it appears that these regions modulate different aspects of fear conditioning; the dorsal hippocampus appears to have roles in contextual fear, while the ventral hippocampus has a more general role.

As per its role in spatial memory, it is only logical that the dorsal hippocampus would have roles in contextual processing in fear and fear conditioning. In fact, the study above by Phillips and Ledoux was performed in the dorsal hippocampus, with lesions resulting in impaired contextual but not cued conditioning (Phillips and Ledoux 1992). Further lesion studies substantiated this claim with lesions of dorsal CA1 and CA3 regions resulting in deficits in the encoding and retrieval of contextual fear (Kim and Fanselow 1992; Ji and Maren 2005; Hunsaker and Kesner 2008). Therefore, the dorsal hippocampus has roles in contextual fear conditioning and may be influential in PTSD symptoms.
The studies of the ventral hippocampus in fear conditioning, however, are not as conclusive as the dorsal hippocampus. It appears that the vHC, has roles in not only contextual fear conditioning, but also in general fear and anxiety expression during fear conditioning. For instance, it was found that the ventral hippocampus plays roles in the aversive behavior associated with contextual fear-conditioning, i.e. conditioned place aversion, as lesioning this region produces an anti-aversive effect. When animals were trained to associate the aversive stimulation of the dorsal periaqueductal grey (dPAG) or foot shock with a certain context, the selective lesioning of the ventral hippocampus (not dorsal) significantly reduced defensive behaviors in the context, suggesting a role of the vHC in fear behaviors associated with contextual fear conditioning (Ballesteros et al. 2014). The ventral hippocampus also appears to have roles in the retrieval and retention of contextual fear conditioning with lesions of the vHC inhibiting these behaviors (Rogers et al. 2006; Hunsaker and Kesner 2008), similar to the dorsal hippocampus. Unlike the dorsal hippocampus, however, the ventral portion appears to also have roles in cued fear conditioning. For example, muscimol induced inactivation of the ventral (not dorsal) hippocampus as well as electrolytic lesions were sufficient to disrupt both contextual and auditory cued conditioning (Maren and Holt 2004). This is further validating in experiments which found selectively activating NMDA receptors in the ventral hippocampus blocked contextual and cued fear conditioning (Zhang, Bast and Feldon 2001); inactivation of NMDA receptors only blocked contextual conditioning. These studies suggest a role of the ventral hippocampus and its projections (i.e. BLA and possibly the Nucleus Accumbens) in contextual and cued fear conditioning. In fact, as seen in anxiety-like behaviors, the reciprocal innervations between the BLA and the
ventral hippocampus appear to modulate fear consolidation. Utilizing an altered contextual fear conditioning paradigm which isolates contextual and foot shock consolidation, Huff et al. found that optogenetic stimulation and inhibition of this circuit altered foot shock learning but not contextual consolidation (Huff et al. 2016). Activating this circuit enhanced the retention of the foot shock related fear memory (not context) while inactivation reduced retention. Thus, the role of the ventral hippocampus in fear learning appears multi-faceted, requiring future study especially regarding its projections to the BLA and other limbic regions as it may prove a target for PTSD.

Since chronic and/or severe stress may result in the formation of disorders such as PTSD electrophysiological experiments within the dorsal and ventral hippocampus further exemplify a role of the ventral hippocampus in stress as acute stress exposure resulted in alterations in synaptic functions. Typically, the ventral hippocampus expresses diminished LTP in comparison to the dorsal hippocampus, however, following acute stress the vHC expresses enhanced LTP while the dHC expresses diminished LTP (Maggio and Segal 2009). Thus, stress produces differential synaptic alterations in the dorsal and ventral hippocampus, suggesting an increased role of the ventral hippocampus in stress. These two effects were attenuated via antagonists to the glucocorticosterone and mineralocorticosterone receptors, further suggesting a role in stress.

Stress, has been shown to be similarly related to the ventral hippocampus, particularly in relation to 5-HT, a neurotransmitter shown to be involved in stress (Graeff et al. 1996; Sachs et al. 2015). In studying the effects of stress on 5-HT release from Raphe nuclei onto forebrain areas, researchers found that the ventral hippocampus appears to be involved in stress. Following a forced swim test, a behavioral test for stress-
like behavior, mice had decreased levels of 5-HT within their ventral hippocampus in comparison to controls (Adell et al. 1997). This could possibly show a mechanism by which stress is expressed within the ventral hippocampus, furthering its designated role in emotion. This is especially exemplified by the fact that the ventral hippocampus is highly responsive to stress, with stress related hormones potentially causing major alterations in synaptic outputs (to the HPA-axis for instance) as well as alterations in morphology, i.e. adult neurogenesis (Jacobson and Sapolsky 1991; Levone et al. 2014). It appears that following binding of stress hormones to corticoid receptors in the hippocampus, the hippocampus sends modulatory signals to the hypothalamus to regulate the HPA-axis (Jacobson and Sapolsky 1991). Moreover, stress has been shown to induce changes in hippocampus morphology with alterations in adult neurogenesis (Levone et al. 2015) as well as increased atrophy occurring following stress exposure (McEwen and Sapolsky 1995). This role appears to be particularly relevant in the more affective region of the hippocampus (anterior in humans and ventral in rodents) with alterations in receptors found mostly in this region in individuals with Major Depressive Disorder (MDD), a disorder that is highly comorbid with PTSD (Medina et al. 2013).

With the hippocampus, particularly the ventral hippocampus, being associated with anxiety and stress responses and behaviors, researchers have begun to study the effects of drugs and alcohol in the ventral hippocampus. One such study looked at 5-HT levels within the ventral hippocampus during amphetamine withdrawal in the context of anxiety behaviors. Withdrawal from drugs, including alcohol, is characterized by increased anxiety and stress phenotypes as well as a decreased 5-HT response in the ventral hippocampus (Tu et al. 2014). This blunted 5-HT response could partially be
response for the anxiety-like phenotypes following withdrawal. In fact, a study conducted on mice undergoing amphetamine withdrawal found that increasing 5-HT levels within the ventral hippocampus, via the serotonin reuptake inhibitor paroxetine, attenuated anxiety-like behavior (Tu et al. 2014). Conversely, the associated decreases in 5-HT during withdrawal increased anxiety-like behaviors, i.e. decreased open arm entries during elevated plus maze tests.

The ventral hippocampus therefore appears to have a significant role in emotion, anxiety-like behavior, fear conditioning and drug related behaviors, making it an ideal target for the study of comorbid PTSD and AUD. With roles in both drug/alcohol behavior as well as anxiety/stress behavior, the hippocampus (specifically the ventral hippocampus) may have a role in the comorbidity of these two debilitating disorders.

**Summary**

PTSD and AUD are two debilitating disorders that plague large, overlapping populations of people across the world. Unfortunately, patients diagnosed with PTSD are often diagnosed with comorbid AUD, resulting in worsened care outcomes for both clusters of symptoms. The neural substrates responsible for the development of these disorders are largely unknown. Whether the development of AUD stems from a desire to self-medicate and alleviate the negative affect associated with PTSD or from an underlying susceptibility is currently unknown and being studied. Without this knowledge, treatments have been shown to have limited efficacy in treating both. Some medications and therapies, such as prazosin, have shown some promise in treating symptoms of both, however, more targeted treatments must be found to properly treat both clusters of symptoms. With each patient expressing different symptoms, it is
imperative for treatment to be targeted for specific treatments. Before this can be done, more needs to be known about the pathogenesis of these two disorders, both separately and together. To this end, our lab and others have begun looking at animal models that may mimic symptoms of these disorders, to find regions of interest in hopes of finding alterations that could help explain these disorders. Although not an exhaustive list, such regions involved could be: the ventromedial prefrontal cortex (IL and PL cortices), the amygdala, the hippocampus (particularly the ventral aspect), the nucleus accumbens and the ventral tegmental area. In our research, we have begun to focus on the role of the BLA as well as the hippocampus, in the development of these disorders, utilizing animal models such as chronic intermittent ethanol exposure, adolescent social isolation and fear conditioning. If our lab and others can find regions that are responsible for the development of PTSD and AUD symptoms, more targeted treatments may be developed.
Chapter 2

The effects of chronic intermittent ethanol exposure on fear conditioning in rats: an investigation of the comorbidity of PTSD and AUD

Nathan McMullen, Ann Chappell, Sarah Ewin, Eugenia Carter, Whitt Morgan, Antoine Almonte, Jeff Weiner

Abstract

Numerous studies, in both animal models and humans, have found direct interconnections between anxiety disorders and alcohol use disorder (AUD). Post-traumatic stress disorder (PTSD) and AUD are two such disorders which express extremely high rates of comorbidity that results in a worsened prognosis for each. Despite the high rates of each of these disorders, little is known about the neural substrates responsible for the frequent co-occurrence of these disorders. In particular, it is unknown whether or not AUD develops as a means of self-medication of PTSD symptoms or if there is an underlying mechanism of vulnerability to both disorders. To this end, we aimed to perform and unbiased study of a possible AUD-PTSD comorbidity rat model, expanding on an experiment previously performed in mice. Similar to the mouse study, we tested the effect of chronic intermittent ethanol (CIE), a model of alcohol dependence, on fear conditioning, a model of fear learning that engages circuits that are dysregulated in PTSD, in male Long Evans rats. We further expanded this research by looking at the effects of this exposure on subsequent ethanol drinking behaviors, using a two-bottle choice paradigm. We hypothesized that we would validate previous findings by seeing alterations of fear extinction in CIE exposed rats, similar to symptoms of PTSD patients.
Moreover, we predicted that the exposure to both CIE and fear conditioning would result in increased long-term drinking in comparison to CIE or fear conditioning alone, as seen in PTSD/AUD comorbid patients. As predicted, dysregulation of fear extinction was observed. Unexpectedly, we also noted alterations in acquisition of fear and spontaneous recovery of fear. We also found that rats exposed to CIE (with and without fear conditioning) exhibited increased binge-like drinking in comparison to those exposed to air; those exposed to CIE and fear trended to drink more than cohorts exposed to just one of these conditions. Together, these results are consistent with the notion that these animal models may recapitulate salient features of comorbid PTSD and AUD and that they may prove useful in elucidating neural adaptations that promotes these disorders.

**Significance Statement**

There are few validated animal models of PTSD and AUD comorbidity, with each only highlighting some symptoms in each disorder. A previous study (Holmes et al. 2012) has shown an effect of chronic intermittent ethanol exposure on fear learning in inbred mice, with associated alterations in the mPFC. This current study aimed to replicate these findings in outbred rats, a model that may more closely reflect human genetic heterogeneity, to validate these results. Moreover, we aimed to explore associated drinking behaviors to further validate this approach to study the neural underpinnings of the comorbid condition.
Introduction

Although previously referred to by different names, such as shell shock, post-traumatic stress disorder (PTSD) is an anxiety disorder that has been seen in patients throughout the history of modern medicine. However, similar to other psychological disorders, it has only been recently that serious research has been done on this disorder. The DSM-V defines this anxiety disorder as a mental disorder with roots in an exposure to an extremely stressful event (e.g. violence, sexual assault, etc.), with defined clusters of symptoms including: re-experiencing symptoms, avoidance symptoms, negative alterations in cognition/mood and marked alterations in arousal and reactivity (USVA 2017; Allen et al. 2016). On top of these symptoms, patients often suffer from comorbid disorders such as major depressive disorder (MDD) and numerous substance use disorders. Alcohol use disorder (AUD), in particular, has a very high prevalence in the PTSD population, with a comorbidity ranging from 30-59% (Ralevski 2014) and even as high as 75% in some veteran populations (Jacobsen 2001). This comorbidity is often met with worsened treatment outcomes and relapses rates. The exact link between these disorders, however, is unknown. Researchers are uncertain whether or not AUD occurs via self-medication mechanisms or if there is a common underlying neuropathology or other risk factors associated with both disorders.

For this reason, our lab, and others, has begun looking into ways of modeling this comorbidity in animals. For instance, Holmes et al. (2012) combined a model of AUD (that also promotes some PTSD symptoms) with fear conditioning to study the effects of alcohol dependence on fear learning. These researchers found that mice exposed to the chronic intermittent ethanol paradigm (a model of alcohol dependence) expressed
dysfunctional fear extinction and retrieval. Similar to PTSD patients, these mice showed delayed extinction of fear conditioning as well as impaired fear extinction retrieval. In other words, the dependent mice expressed fear longer than those only exposed to fear conditioning. Similar deficits in fear extinction have been reported in studies with PTSD patients using very similar experimental procedures (Wicking et al. 2016; Zuj et al. 2016; Maeng and Milad 2017). Their study also reported that this impairment appeared to involve morphological and synaptic alterations within the medial prefrontal cortex (mPFC). Following CIE, these mice expressed remodeled prelimbic (PL) neurons which have been associated with fear expression during fear conditioning (Giustino and Maren 2015). Likewise, during extinction, these researchers found diminished NMDAR-mediated currents in infralimbic (IL) neurons during extinction. This being consistent with other studies illustrates that IL neurons are implicated in fear suppression (Quirk and Mueller 2008; Sotres-Bayon and Quirk 2010; Maren et al 2013). However, if this model is to be used to study symptoms associated with PTSD and AUD, it must also express worsened AUD symptoms, i.e. increased drinking (in comparison to control and to those simply dependent). Therefore, we aimed to replicate the above study in rats while also expanding it by including drinking behaviors to further mimic the human condition.

To achieve this expansion, we allowed rats to freely consume ethanol 3 days a week (intermittent two-bottle choice) for 4 weeks prior to and 4 weeks following exposure to CIE and fear conditioning. In the future, we aim to expand previous research by exploring the role of the basolateral amygdala (BLA), ventral hippocampus (vHC) and the dorsal hippocampus (dHC) in these potential alterations as these regions (and interconnecting circuitry) have also been implicated in fear and drinking behaviors
(Phillips and Ledoux 1992; Holt and Maren 1999; Bannerman et al. 2003; Rogers et al. 2006; Feliz-Ortiz et al. 2013; Yang et al. 2015). However, before conducting neurobiological studies, we first sought to conduct an unbiased study of CIE and fear conditioning as outlined above.

Based on prior findings, which found increased ethanol consumption following CIE (O’Dell et al. 2004; Gilpin et al. 2009; McCool and Chappell 2015; Morales et al. 2015; Lopez et al. 2013), we hypothesized that exposure to CIE would increase ethanol consumption and dysregulated fear extinction by prolonging fear responses to the conditioned stimulus. This prolonged fear expression following exposure to both paradigms was also hypothesized to further increase ethanol consumption in comparison to rats exposed to just fear conditioning or CIE.

Materials and Methods

Animals

All animals used in this study were male Long Evans rats purchased from Envigo, IN and arrived at approximately 8 weeks postnatal. Upon arrival, animals were singly housed in clear cages (25.4 cm x 45.7 cm) and given food ad libitum. Animals were maintained on a 12 hour light cycle, with lights on at 7:00am and lights off at 7:00pm. Rats were allowed to acclimate for 1 week before undergoing any paradigms. Rats were handled and weighed daily during this acclimation. Cages were changed weekly when rats were consuming ethanol while cages were changed daily during CIE exposure. Three cohorts of animals were used during this study. Two of the cohorts drank before and after CIE/fear exposure, while one group was only exposed to CIE and fear conditioning (no
drinking). Statistical analyses did not find any significant difference between ethanol drinking and naïve rats during behavioral tests (data not shown). Animal care procedures were carried out in accordance with NIH Guide for the Care and Use of Laboratory Animals and were approved by the Wake Forest University Animal Care and Use Committee.

**Intermittent two bottle choice**

Following 1 week of acclimation, rats were allowed to freely consume 20% EtOH three days a week. In their home cages, rats were provided the choice between water and 20% EtOH for 24hrs. Consumption was determined by weighing the bottles before and after rats drank. The amount of ethanol and water drank was determined following 30 minutes and 24hrs of exposure. Rats were allowed to drink for 4 weeks before undergoing their respective exposures. Rats were separated into four exposure groups (Control, CIE, air + fear conditioning, and CIE + fear conditioning) to ensure that pre-fear-conditioning drinking levels were consistent across groups. Rats were ranked based off their average amount of ethanol consumed during the first four weeks of the drinking regimen. The rats were then sorted in to the 4 groups from this list in a snake draft manner, with order of selection inverting to keep even levels of drinking (in other words, the 4th and 5th highest drinkers were in the same group, while the 1st and 8th are in another). The groups were assigned to an exposure condition using a random number generator. Following CIE and/or fear conditioning, rats again underwent this paradigm to determine if there were any changes in drinking from baseline. Drinking behavior was determined by the amount of ethanol consumed (g/kg) as well as percent preference for ethanol in comparison to water. Baseline drinking was determined by averaging the
amount of ethanol consumed 3 sessions directly prior to paradigm exposure. Percent of baseline, found by dividing amount consumed by each rat’s respective baseline, was used as a measure of altered drinking behavior.

**Chronic Intermittent Ethanol Exposure**

The CIE protocol employed was adapted from (Morales, McGinnis, and McCool 2015). Rats in the two drinking cohorts started in the CIE protocol following four weeks of ethanol consumption. One cohort of alcohol naïve rats started following 1 week of acclimation. Throughout the paradigm, animals were housed in home cages placed in sealed Plexiglas chambers (Triad Plastics, Winston-Salem NC) with 4-6 cages per chamber. Ethanol vapors were produced by bubbling 190 proof ethanol in a flask attached to the chambers. Beginning at 7am, ethanol vapors were pumped in to the chambers for 12 hours straight to attain blood ethanol levels (BECs) within the target 175-225 mg/dL range. To determine BECs, blood plasma was separated from tail blood samples and process using an alcohol dehydrogenase enzymatic kit (Caroline Liquid chemistries Corp, Brea, CA). Ethanol concentrations were determined using standards and a spectrophotometer (Molecular Devices Spectra Max). Health and weight checks occurred daily while the cages were being changed.

**Fear Conditioning**

Fear conditioning consisted of four sessions: acquisition (Day 1), extinction (Day 2), retrieval (Day 3) and spontaneous recovery (10-20 days after retrieval). Acquisition began 36 hours after the last exposure to ethanol vapors (36hr withdrawal). For all parts of the study, rats were placed in enclosed square Plexiglas chambers (41.5cm x 41.5 cm x
30cm) with electrifiable metal grid floors and a Fusion stimulus hub (Omnitech Electronics, Inc.) on top which was able to control delivery of auditory tones and foot shocks. Chambers were placed within Omnitech Superflex Sensors (Omnitech Electronics, Inc.), which utilize photo beams to track animal behaviors (freezing, locomotion, rearing etc.). This whole apparatus was then housed within a sound-attenuated chamber with a 7.5 watt overhead (white) light. Fusion 6.4 software developed by Omnitech Electronics Inc. was used to control stimulus hubs and analyze all data.

Freezing behavior, as defined by complete immobility outside of breathing for at least 2 seconds, was used as a measure of fear response to the context and the cue. Throughout experiments, all tones were delivered at 100dB and 1000 Hz, lasting 30 seconds. There was a 90 second delay between trials on each day. All foot shocks were delivered through the grid floor for 0.5 seconds with an intensity of 0.32mA. Overhead light was consistent throughout all trials and between cages (7.5W white lightbulbs).

On acquisition day, rats were placed in the activity box and allowed to acclimate for 5 minutes. They were then exposed to 5 pairings of the acoustic tone (CS) and the foot shock (US). Acoustic tones were played for 30 seconds, while foot shocks were paired with the tones for 0.5 seconds starting 29.5 seconds into the tone presentation. Acquisition was determined by measuring the rat’s freezing behavior in response to the tones. 24 hours after first exposure, rats were placed in the same context and allowed to acclimate again for 5 minutes. The same tone presented on day 1, was presented again 16 times (for 30 seconds) with a 90 second delay between tones to assess fear extinction. This test was repeated a day later to assess the retention of fear extinction. To assess spontaneous recovery of fear, rats were placed in the same context 10 or 20 days after last
exposure to context. One group of rats was also exposed to 20 presentations of the conditioned stimulus on spontaneous recovery day following 10 minutes of acclimation. Rats not undergoing fear conditioning were still placed in the same context with tones, but no shocks were presented. This protocol was adapted from (Curzon et al. 2009).

Statistical analyses

Data was graphed and analyzed using SigmaPlot (Systat Software Inc.) and outlier tests were run using Prism (GraphPad). Fear acquisition and extinction data were analyzed using two-way repeated measures ANOVAs followed by Neuman-Keuls posthoc tests. Retrieval and spontaneous recovery of fear were analyzed using one-tailed t-tests. All drinking data were analyzed using two-way ANOVAs. All tests used a significance levels of 0.05. Post-hoc analyses were performed on fear conditioning data due to a priori hypotheses.

Results:

Chronic Intermittent Ethanol Exposure impairs fear acquisition and extinction

To test the effects of ethanol vapor exposure on behaviors associated with PTSD, rats were exposed to ethanol vapors for 10-11 days and then exposed to fear conditioning 36 hours in to withdrawal. Throughout the exposure, rat BECs were relatively consistent and within tolerable range with an average of 184.02mg/dL±63.32. Surprisingly, exposure to ethanol vapors delayed acquisition of fear in comparison to the control group, with a significantly lower rate of freezing during the third shock-tone pairing (Figure 1); however, the groups acquired the same amount of fear to the tone overall. Two-way repeated measures ANOVA found no significant difference between the CIE and Air
group during extinction (n=12 rats per group, F=3.981, p=0.059), however, due to our a priori hypothesis that CIE rats would freeze to a higher extent, we went ahead and did a post hoc analysis using Student-Newman Keuls method. We found, as seen in mice, that ethanol exposed rats expressed dysregulated fear extinction, freezing to the tone at a higher rate for a prolonged period in comparison to controls (Figure 2). Ethanol exposed rats froze to a higher extent during the 5th-8th tone presentations, with a particular difference during the 7th tone (Figure 2). Retrieval of extinction was not significantly different between groups (Figure 3).

Figure 2. Percent freezing on day 1 of fear conditioning in rats exposed to either air or ethanol vapor. CIE treated rats express altered fear learning, taking longer to acquire the fear. There was a significant difference in freezing time during the third conditioning pairing using Student-Newman-Keuls method (p=.018 n=12 per group). No significant difference between ethanol naïve and ethanol drinking rats (data not shown)
Figure 3. Average Percent time freezing during conditioning in bins of 4 extinction tones. There is a significant difference between CIE treated and AIR treated rats during tones 5-8 (p=.0424; n=8 per group)

Figure 4. CIE exposure had no effect on fear extinction retrieval. Graph shows comparison of baseline freezing and retrieval freezing during first tone presentation on day 3. There is no statistical difference between groups in either baseline or retrieval levels of freezing (p=0.805 and 0.527 respectively; n=12 per group)


CIE exposure increases spontaneous recovery of fear

Although there was no alteration of fear retrieval on day 3 of fear learning, rats exposed to ethanol vapors expressed increased fear behavior 10 days after prior exposure to the context. When placed in the same context as before, rats exposed to ethanol vapors froze to a higher extent than those exposed to air (Figure 4). Moreover, CIE rats froze to a greater extent when presented with the conditioned stimulus (tone) during the spontaneous recovery (Figure 5). Two-way repeated measures ANOVA found no significant differences between groups (F=5.682, p=0.054), but due to a priori hypothesis, we continued to look at interaction effects and found a significant increase in the freezing response of ethanol exposed rats during the first presentation of the conditioned tone (Figure 5). When freezing behavior to all tones was collapsed, the CIE rats also expressed higher rates of freezing. There was no significant difference between freezing behaviors when rats were placed back in the context 20 days later (Figure 6).
Figure 5. CIE exposure significantly increases spontaneous recovery of fear 10 days after retrieval. Percent time freezing during 10 minutes or re-exposure to context, 10 days after extinction retrieval. CIE exposed rats expressed significantly higher spontaneous recovery of fear-like behavior than air exposed subjects (p=0.013, n=8 per group).

Figure 6. CIE Exposure significantly alters spontaneous recovery to cued fear 10 days after retrieval. Percent time freezing in response to the first tone, and all tones combined is shown. CIE exposed rats express higher rates of freezing during the first tone (p=0.027; n=4 per group) and in response to all tones (p=0.03621, n=4 per group, one-tailed T-test).
**Figure 7. CIE exposure does not affect spontaneous recovery of fear 20 days later.**

Percent freezing time in 5 minutes of re-exposure to the conditioning context was not different between groups (p=0.274, n=4 per group. 1-tailed T-test).

*CIE exposure alters binge-like ethanol drinking but not daily drinking behavior*

Following exposure to CIE and or fear conditioning (or neither), rats were allowed to freely drink ethanol in their home cages 3 days a week (intermittent two bottle choice). Drinking behavior was evaluated based upon the percent change in baseline drinking, baseline drinking being the average of the 3 drinking sessions directly prior to CIE exposure. The amount of ethanol consumed by each rat (g/kg) was divided by this baseline to determine percent of baseline drinking. This allows for the direct comparison between each rat’s drinking behaviors following CIE and fear conditioning exposure. There was a significant effect of CIE exposure on binge-like drinking (intake in the first 30 minutes of each ethanol drinking session), with CIE rats consuming more than air treated rats during the first 30 minutes (F=4.902, p=0.036). There was no significant
effect of fear exposure (F=1.357, p=.255) or interaction effect (F=0.0967, p=.758). There was also no significant effect of exposure to CIE (F=1.544, p=0.225), fear conditioning (F=.157, p=0.695) or interaction effect (F=.175, p=0.679) on 24 hour drinking behavior. There appears to be a trend towards the CIE and fear conditioning exposed rats having the highest levels of binge-like drinking in comparison to the control fear conditioning group (t=1.858, p=0.074) but not the CIE exposed group (t=1.086, p=0.287).

Figure 8. Effects of CIE and fear conditioning on binge-like drinking behavior. Average Percent of baseline drinking (g/kg) of each exposure group during the first 30 minutes. Two-way ANOVA found no significant effect of fear conditioning (F=0.669, p=0.421) or interaction effect CIE and fear conditioning exposure (F=0.528, p=0.473). There was significant increase in drinking by CIE exposed rats (F=4.902, p=0.036). There was a trend towards increased drinking in the CIE+FEAR (t=1.858, p=0.074) but not CIE group (t=1.086, p=0.287). There was not significant increase in drinking in fear conditioned rats (t=1.165. p=0.255).
**Figure 9.** Effects of CIE and fear conditioning exposure on 24 hour drinking behavior. There was also no significant effect exposure to CIE (F=1.373, p=0.252), fear conditioning (F=0.620) or both (F=0.162, p=0.691) on 24 hour drinking behaviors.
Discussion

As expected, we found that exposure to chronic intermittent ethanol vapors, a model of alcohol dependence, was sufficient to induce significant fear learning impairments including impaired acquisition and extinction. Following ethanol exposure rats had a delayed acquisition of fear conditioning, taking longer to associate the tone to the foot shock. This would suggest that CIE exposure results in neurological alterations in circuits and brain regions associated with fear acquisition/expression such as: the medial prefrontal cortex, the amygdala, and the hippocampus (Holmes et al. 2012; Tovote et al. 2015). The exposure to chronic ethanol may induce changes that alter synaptic function of neurons within these regions as well as neurons reciprocally projecting between these regions (Tovote et al. 2015; Maren and Holmes 2016). Although this was not observed in the paper we directly replicated, previous studies looking at chronic ethanol exposure (dietary not vapor exposure) found similar alterations in fear acquisition and expression (Ripley et al. 2003; Quinones-Laracuente et al. 2015). Despite these potential alterations, the CIE and air group acquired the same amount of fear to the tone overall, expressing fear levels similar to previous studies on fear conditioning.

Likewise, we found the CIE rats expressed impaired fear extinction, maintaining freezing behavior in response to the conditioned stimulus for longer than controls, as seen in previous experiments (Holmes et al. 2012). Since prior studies have found that fear extinction is not simply a model of forgetting but rather a new form of learning separate from acquisition (Gale et al. 2004; Myers and Davis 2007; Tovote et al. 2015), these results again suggest CIE-induced alterations of fear learning mechanisms that may be associated with impairments seen in PTSD and AUD. These impairments may be located
within distinct circuits in the same brain regions involved in fear acquisition. For instance, it appears that the infralimbic cortex of the mPFC is associated with fear extinction while the prelimbic cortex is involved in fear expression and acquisition (Milad and Quirk 2002; Farinelli 2006; Laurent and Westbrook 2009). These alterations appear to be long lasting as increased fear-like behavior was seen in CIE exposed rats during the spontaneous recovery tests 10 days after retrieval. Dependent-like rats expressed higher rates of freezing to both the context and the conditioned stimulus 12 days after acquisition of fear. These alterations do not seem life-long, as this effect was not seen 20 days after retrieval. This could mean that the effects were only transient, or that the paradigm was not stressful enough, i.e. stimulus intensity was too low. Therefore, further parametric-experiments should look at these long-term effects.

These fear learning deficits may arise due to changes in synaptic functioning, i.e. hyper- or hypo-excitability neurons. The original mouse CIE-fear conditioning study found such alterations in the mPFC, with overall decreases in evoked NMDAR-mediated currents following CIE as well as decreases in the GluN1 NMDA obligatory subunit (Holmes et al. 2012). Moreover, they found utilizing in vivo recordings, CIE diminished activity of infralimbic neurons in response to conditioned stimuli, suggestive of diminished fear extinction (Holmes et al. 2012). With decreased levels of NMDAR subunits, as well as NMDA activity, synaptic plasticity may be severely dampened in this region potentially contributing to the diminished fear learning behaviors observed. This may be particularly so regarding the infralimbic cortex. Decreasing the activity of infralimbic neurons in response to conditioned stimuli may result in the diminished extinction seen, potentially because of disinhibition of the amygdala. Typically, the
infralimbic cortex inhibits central amygdala (CEA) targets via medial intercalated cells (mITCs), a group of cells found between the BLA and CEA, to decrease fear expression (Royer et al. 1999; Asede et al. 2015; Bukalo et al. 2015). Moreover, IL neurons have also been shown to project to the BLA to specifically modulate fear extinction (Bloodgood et al. 2017). It is possible, that some of these deficits seen in our study and previous studies are due to decreased activity of these circuits, diminishing fear extinction. These same circuits could thus also be dysregulated in anxiety and stress disorders, such as PTSD and comorbid AUD.

Even though we observed altered fear-like behaviors in rats exposed to CIE, we did not observe robust alterations in ethanol drinking behaviors as reported by others (Gilpin et al. 2009; Lopez et al. 2012; Griffin 2014; McCool and Chappell 2015; Kimbrough et al. 2017) Contrary to our hypothesis we did not see an increase in daily ethanol intake in CIE exposed rats. Interestingly, CIE rats exposed to both fear conditioning and control conditions did significantly increase their intake during the first 30 minutes of drinking sessions. In this model, rats consume approximately 25% of their daily intake during the first 30 minutes of each ethanol session (Chappell et al. 2013). It does not appear that the lack of escalation is due to faults in our CIE protocol since most blood ethanol levels were within desired ranges with an average plasma concentration of 184.02mg/dL±63.32, with the exception of a few instances. In these instances, CIE exposure was prolonged a day to ensure desired BECs. It is possible that the escalation in ethanol intake did not occur due to the prolonged withdrawal period between CIE exposure and the next drinking session. The effects of withdrawal may have been mitigated by the start of drinking, preventing escalation. This however seems unlikely.
since previous experiments have shown that the escalations in drinking are long-lasting. The exact cause of the observed drinking behavior is unknown, but it may simply be due to small sample size or the fact that there was an extended period between final ethanol vapor exposure and first drinking session. Previous experiments found escalation in drinking when the animals were allowed to drink/administer ethanol directly following CIE exposure (Gilpin et al. 2009; Lopez et al. 2012; Griffin 2014; McCool and Chappell 2015; Kimbrough et al. 2017). Further studies may validate previous findings on the effects of CIE. Moreover, we did not observe any increases in drinking behavior of rats exposed to both CIE and fear conditioning in comparison to the control and CIE group. This may in part be due to the fact that we chose to use the minimal level of shock intensity that induced fear learning in this study. Further studies should be done using increased shock intensities to explore any possible escalations, mimicking the human condition more.

Given the findings that CIE impaired fear learning in our studies, the next logical step would be to begin to examine neurobiological adaptations in brain regions known to play a role in fear learning, i.e. the amygdala and the hippocampus, to see if alterations similar to those reported in the mPFC occur. Such alterations have been found in these regions following CIE exposure, with increases in excitability and diminishment of LTP found in both regions possibly because of upregulated NMDARs and increased AMPAR signaling (Läck et al. 2007; Christian et al. 2012; Roberto et al. 2002 Ewin et al unpublished). Hyper-excitability within these regions may result in increased expression of fear expression circuits over fear extinction circuits. For instance, increased activity of the reciprocal circuit between the basal amygdala and the prelimbic circuit may
overshadow the activity of infralimbic neurons projecting on the amygdala, the balance of which has already been shown to regulate the expression of fear and fear extinction (Senn et al. 2014; Tovote et al. 2015). Likewise, hyperexcitability and diminished LTP in the hippocampus may result in persistent expression of fear memories as well as persistent activation of the amygdala, a circuit that has been shown to directly modulate anxiety and fear expression/extinction (Felix-Ortiz et al. 2013; Sparta et al. 2014; Maren and Holmes 2016; Huff et al. 2016) Furthermore, this may diminish the hippocampus’ ability to inhibit behaviors to resolve conflict between two conflicting memories, such as the conflict between fear and extinction memory (Bannerman et al. 2014). The role of the hippocampus as a comparator, a role that has been shown to allow behavioral flexibility and memory updating (Vinogradova 2001; Hasselmo 2005; Bannerman et al. 2014; Numan 2015), may be thusly down regulated in AUD/PTSD patients. In fact, such impairments have been found by our lab in an animal model which expresses a phenotype reminiscent of alcohol addiction and stress disorder vulnerability. Following adolescent isolation, the ventral hippocampus expressed impaired synaptic plasticity as well as increased excitability, as seen in CIE experiments (Almonte et al. 2017). Therefore, impairments that are indicative of alcohol addiction vulnerability may also be an indicator of PTSD vulnerability.

In conclusion, although this study was only preliminary, the observed fear learning deficits may be suggestive of an underlying mechanism between the symptoms of PTSD and comorbid AUD, i.e. they develop due to a neurological susceptibility to both. However, future experiments should include alter fear conditioning paradigms, i.e. increased shock intensity and using differently aged/sex rats, to promote a fuller spectrum
of behaviors. In fact, a previous study saw that the age of chronic ethanol exposure had
significant effects on fear conditioning behaviors later in life; so much so that they saw
reversals in fear behaviors in different aged animals (Broadwater and Spear 2013). Based
off this study as well as previous studies, it appears that brain regions and circuits
involved in fear learning and expression are altered by exposure to CIE. A model of
alcohol dependence is sufficient to not only induce symptoms reminiscent of AUD
(anxiety-like behaviors, increased drinking etc.) but also fear learning deficits like those
seen in PTSD. In other words, symptoms associated with PTSD and AUD may have
similar underlying neurological deficits, in regions such as the: medial prefrontal cortex
(infralimbic and prelimbic cortex), hippocampus and amygdala (CeM and BLA).
Chapter 3: Discussion

As highlighted throughout this review and study, posttraumatic stress disorder and alcohol use disorder are two diseases that, despite high levels of prevalence and comorbidity, are still relatively poorly understood. Not much is known about the neurobiological underpinnings associated with the development of each of these disorders respectively. We do not specifically know the exact causes of all of the symptoms associated with these disorders. Even more elusive is the reason behind differences in susceptibility. With numerous people being exposed to trauma and alcohol on a daily basis across the world, it is not clear why only certain populations develop AUD and PTSD following exposure. For instance, why do only sub-populations of veterans develop PTSD when exposed to the same conditions as those who are relatively unaffected? Likewise, why are there populations, such as people exposed to childhood stress, that more readily develop AUD and PTSD? Why are women twice as likely to develop PTSD than men? These questions become even more complicated when the comorbidity of these two disorders is taken into account. With rates of co-occurrence ranging all the way up to 59% by some estimates (Ralevski 2014), patients diagnosed with PTSD are often diagnosed with AUD as well. This complicates research into the aforementioned questions, as well as creating a new question: what causes the development of these two disorders? Researchers are debating whether or not AUD development stems from a need to self-medicate in PTSD patients or from an underlying proclivity to develop both disorders. The PTSD patients may simply be looking for any sort of outlet to alleviate their negative affective symptoms, and alcohol (as well as other drugs of abuse) may develop into this outlet. Conversely, the neurobiological
mechanisms that result in PTSD development may also be those that aid in the development of AUD; these patients may be primed to become alcohol dependent, either congenitally or following trauma-exposure.

For this reason, researchers have begun to look at potential brain regions that have been implicated in both disorders such as: the medial prefrontal cortex, the hippocampus, the amygdala, the ventral tegmental area, nucleus accumbens and insula (Hughes and Shin 2011; Chattopadhyay et al. 2011; Gilpin and Weiner 2016). The hope is that by exploring these regions in animal models, the development of the disorders could be traced. However, few reliable animal models have been developed since it is hard to produce an animal model that leads to the expression of the full spectrum of symptoms of each of these disorders. Moreover, since these disorders are somewhat emotionally based, it is hard to test subjective measures in animals for obvious reasons. Some animal models have been developed and validated to promote some elements of this comorbidity, i.e. models of stress-induced drinking escalations such as social defeat stress, repeated forced swim tests, etc. (Norman 2012; Molander et al. 2012; Logrip and Zorrilla 2012; Meyer et al. 2013; Hwa 2016; Anderson et al. 2016; Lopez et al. 2016). However, there remains a need to develop animals models that more closely resemble the human comorbid condition.

This study aimed at further characterizing and validating one such research paradigm, previously studied by Holmes et al (2012). Both our lab and his found that exposure to chronic intermittent ethanol, a paradigm shown to induce alcohol-dependence like behaviors in animals, alters fear learning (specifically fear extinction) reminiscent of human patients who both express alcohol dependence and an inability to
extinguish intrusive thoughts and re-experiencing of traumatic events (PTSD/AUD). This finding has been now validated in both mice and rats, and has been shown to potentially involve the mPFC, with its projection to the BLA being implicated in extinction (Holmes et al. 2012; Bloodgood et al. 2018). The exposure to chronic ethanol vapors appears to be sufficient to induce neurobiological alterations that not only alter drinking behaviors as seen in other studies, but also fear related circuits. Although we were not able to completely replicate the increased levels of drinking following CIE exposure (we did see increases in binge-like drinking), we did see alterations in fear behavior following exposure; suggesting a possible mechanism that could contribute to the development of both disorders. There was an increase in binge-like drinking in rats exposed to CIE, with a trend toward further increases in CIE rats additionally exposed to fear conditioning; however, these escalations were not seen in total drinking behaviors. The lack of escalated drinking behavior in the fear exposed rats may simply be a result of our low shock intensity, as in our experiment we sought to use the lowest shock levels that induced significant fear learning. Although we saw freezing levels and acquisition levels similar to previous studies, increased shock intensity may elucidate stronger phenotypes more mimicking human PTSD and AUD. Future studies should be done using varying shock intensities to, perhaps, better mimic PTSD in humans. However, recent studies in human PTSD patients that meet criteria for alcohol dependence found that self-medication may be the more relevant factor in comorbidity development. They found that the amount the participants drank was directly related to their PTSD symptoms the previous day (Simpson et al. 2012; Simpson et al. 2014) suggesting self-medicating motives. In other words, when PTSD symptoms were the worst, the patients drank more
that day and the next days which would suggest the drinking escalation is dependent on negative affect, suggestive of self-medication. However, since this escalation persists more than one day, this self-medication may be unsuccessful; the lingering PTSD symptoms may be causing this prolonged drinking (Simpson et al. 2014), suggestive of a “mutual maintenance model” for both PTSD and AUD (i.e. underlying mechanisms). Therefore, the progression of these comorbid diseases may not be as simple as one hypothesis or another. It may be a combination of self-medication and an underlying mechanism, possibly with these alterations inducing the urge to self-medicate via drugs.

As mentioned before, this study was only a small exploration of the effects of CIE on fear learning and drinking behaviors, but significant and trending results were still found. Suggesting that further studies expanding upon this one may be useful. For instance, as in the human population, there may be individual rats that are more susceptible or vulnerable to these exposures, resulting in the limited results seen in this experiment. We may find in larger studies that there are two populations of susceptible and vulnerable groups in rats exposed to both CIE and fear conditioning. If this were the case, studying these vulnerable populations may help in targeting animal research that may one day aid in precise treatments. This idea of susceptible and vulnerable populations could be further explored using previous models of anxiety-disorder vulnerability, i.e. our adolescent social isolation model. In fact, previous research in our lab found that adolescent social isolation not only resulted in altered fear extinction but also increased ethanol consumption, in a fear conditioning paradigm that used higher levels of shock intensity (Skelly et al. 2015), suggesting the viability of such future changes.
Likewise, to further our knowledge of these disorders, more research must be done on the particular brain regions and circuits that contribute to the behavioral phenotypes promoted by CIE and fear conditioning, particularly the ventral hippocampus, BLA and PL/IL cortices. For example, electrophysiological experiments could be conducted on animals exposed to these paradigms to see alterations in the synaptic function of the BLA and the hippocampus, since the PL and IL has already been studied (Holmes et al. 2012). Since previous experiments by our own lab have already found alterations in synaptic plasticity and excitability in the ventral hippocampus in rats exposed to adolescent social isolation (a model of AUD vulnerability) and CIE (Almonte et al. 2017; Ewin et al. unpublished), it is probable that similar adaptations would be found, with possible synergistic changes in animals exposed to CIE and fear conditioning. To identify circuits that play a causal role in the AUD and PTSD-like behaviors promoted by these models, optogenetic and chemogenetic studies could be performed to modulate specific circuits (e.g. BLA→vHC) and test whether these manipulations directly influence fear learning and ethanol drinking. For example, we would predict that excitation of the BLA-vHC circuit might exacerbate deficits in fear learning and promote escalations in alcohol drinking whereas inhibition of this circuit might ameliorate fear learning deficits and attenuate alcohol intake.

Further experiments could also look at the downstream targets of the vmPFC, particularly its effects on the BLA. Since fear learning and extinction deficits may be a result of altered circuitry between the mPFC and the BLA via the medial intercalated cells, research should be done to explore the roles of each region in the fear learning. This could once again be achieved via optogenetic and chemogenetic approaches that could
either activate or inhibit these regions during various time points of learning, in rats either exposed to CIE or air.

Since one of the limitations of this study is the fact that the rats were only exposed to CIE prior to fear conditioning, further studies should also look at exposing animals to CIE at various time points throughout the paradigm to isolate the effects and determine the crucial period during which effects occur. Some periods of interest could be during adolescence, prior to fear extinction, prior to retrieval or after fear conditioning (if drinking alterations are of particular interest). As noted earlier, the paradigm could also be combined with other models such as the adolescent social isolation model, to see similarities between altered behaviors seen in each model as well as to further mimic the human condition.

Despite some promising results, conclusions from this study should be tempered for a number of reasons, some of which were outlined as limitations and future directions above. One such limitation is the possibility that altered fear expression may simply be due to altered pain related behaviors in rats in withdrawal as withdrawal from alcohol has been shown to induce hyperalgesia (Gatch 2009). This could explain the altered response of the CIE exposed rats to the shock during conditioning and thus their behavior during extinction. Likewise, since fear is a subjective experience, it cannot be directly stated that the behaviors seen in this study were a result of fear as experienced by humans, but rather fear-like behaviors that may be indicative of fear in humans. Lastly, since fear conditioning is not a model of PTSD but rather a test of a symptom indicative of PTSD, further studies should be done on models that more closely resemble PTSD manifestation in conjunction with AUD models. Moreover, these studies should also look to incorporate
pharmacotherapies that have been prescribed for the treatment of PTSD and AUD respectively, such as: topiramate, naltrexone, disulfiram, sertraline, paroxetine, fluoxetine, prazosin and others.

In conclusion, this study is the first to replicate in rats, previous findings in mice, that chronic intermittent ethanol vapor exposure (CIE) leads to altered fear learning, specifically fear extinction as well as fear acquisition. Although it is not a direct model of PTSD and AUD comorbidity, this paradigm could be combined with other models, like adolescent social isolation, to further expand our knowledge of potential mechanisms that induce susceptibility to PTSD and AUD. This study as well as previous studies lay groundwork for future explorations of the mechanisms underlying comorbid PTSD and AUD. With these mechanisms explored, future treatments may become more targeted and efficacious.
References


conditioning and unconditioned defensive behavior induced by electrical stimulation of the dorsal periaqueductal gray. *PLoS ONE, 9*(1).

https://doi.org/10.1371/journal.pone.0083342


https://doi.org/10.1101/172791


https://doi.org/10.1080/08897070903250084


ethanol drinking and ethanol concentrations in the nucleus accumbens.

*Psychopharmacology, 201*(4), 569–580. [https://doi.org/10.1007/s00213-008-1324-3](https://doi.org/10.1007/s00213-008-1324-3)


   *Science*, 256(5057), 675–677. [https://doi.org/10.1126/science.1585183](https://doi.org/10.1126/science.1585183)

   CA1 Hippocampal Neurons to the Medial Prefrontal Cortex and Basal Amygdala. 
   *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 

   Acupuncture for Posttraumatic Stress Disorder: A Systematic Review of Randomized 
   Controlled Trials and Prospective Clinical Trials. *Evidence-Based Complementary 
   and Alternative Medicine, 2013*, 1–12. [https://doi.org/10.1155/2013/615857](https://doi.org/10.1155/2013/615857)

   Access to Ethanol Drinking Facilitates the Transition to Excessive Drinking After 
   Chronic Intermittent Ethanol Vapor Exposure. *Alcoholism: Clinical and 
   Experimental Research, 41*(8), 1502–1509. [https://doi.org/10.1111/acer.13434](https://doi.org/10.1111/acer.13434)

   Moser, M.-B. (2002). Reduced fear expression after lesions of the ventral 
   hippocampus. *Proceedings of the National Academy of Sciences, 99*(16), 10825–10830. [https://doi.org/10.1073/pnas.152112399](https://doi.org/10.1073/pnas.152112399)

   [https://doi.org/10.1177/2045125313500982](https://doi.org/10.1177/2045125313500982)

   Pharmacotherapy for posttraumatic stress disorder using phenelzine or imipramine.
Journal of Nervous and Mental Disease, 179(6), 366–370.
https://doi.org/10.1097/00005053-199106000-00011


https://doi.org/10.1016/j.bbr.2016.03.009


https://doi.org/10.1038/npp.2015.180


https://doi.org/10.1038/nrn3492


https://doi.org/10.1176/appi.ajp.158.12.1982


https://doi.org/10.1016/S0074-7742(10)91007-6

https://doi.org/10.1152/physrev.00041.2006


https://doi.org/10.1037/0735-7044.118.1.63


129. Skelly, Mary J., & Weiner, J. L. (2014). Chronic treatment with prazosin or duloxetine lessens concurrent anxiety-like behavior and alcohol intake: Evidence of


https://doi.org/10.1371/journal.pone.0097689

https://doi.org/10.1016/j.alcohol.2013.09.045

Nathan McMullen
100 Warwick Road Haddonfield, NJ 08033 • 856-417-1280• nate0308@gmail.com

Education

Wake Forest University School of Medicine
Masters of Biomedical Sciences
- Concentration in Neuroscience
- GPA: 4.00/4.00

Wake Forest University
Bachelor of Science in Biology
- GPA: 3.82/4.00
- 8 time nomination to Dean’s List (Fall 2013-Spring 2017)
- Minor in Neuroscience and Chemistry

Clinical Experience

Wake Forest University
Shadowing
- Shadowed Roy Strowd, M.D. of the Neurology department during his rounds meeting with patients
- Observed Dr. Strowd in his consultation with neuro-oncology patients
- Gained insight into patient care as well as understanding patient charts and scans

Hospital of the University of Pennsylvania
Volunteer
- Worked as a volunteer aid in the cardiac intensive care unit 4 hours a week for 12 weeks
- Assisted nurses and nursing assistants in caring for patient’s basic needs
- Talked with patients and their families, making sure they had all they needed

Philadelphia Veterans Affairs Medical Center
Volunteer
- Worked as a volunteer on the Surgical Intensive Care Unit
- Integral in a project headed by Jamie Torres, aimed at bettering patient care and satisfaction

Professional Experience

Wake Forest University
Graduate Student
- Graduate student researcher in the lab of Jeff Weiner, PhD, researching alcohol addiction vulnerability
- Currently studying the comorbidity of PTSD and Alcohol Use Disorder
Thomas Jefferson University  
*Research Assistant*  
*June 2015-August 2015*

- Worked in the lab of Hui Zhang, PhD, researching Parkinson’s Disease for a 10 week internship
- Researched and presented recent developments in Parkinson’s research for the lab

Set in Stone Tile  
*Assistant*  
*Summer 2014*

- Aided proprietor in tiling and renovation of bathrooms and kitchens
- Prepared materials, such as tile and grout, for construction

University of Delaware Rehabilitation Institute  
*Research Assistant*  
*June 2013-August 2013*

- Worked in the lab of Thomas Buchanan, PhD, supporting researcher Stephen Suydam during a 7 week internship
- Contributed to data acquisition, calculation and interpretation
- Acknowledged in published paper on Echogenicity as a viable metric for evaluating tendons (Journal of Biomechanics 03/2014)

Kids Alley  
*Volunteer*  
*Spring 2012-Summer 2014*

- Tutored elementary aged students from impoverished backgrounds
- Cared for and watched students after school
- Private tutor, in math, for a girl struggling in mathematics (helped raise her grade from D to A)
- Volunteered 7 hours a week during the Spring of 2013

Honors and Awards

- *Dean’s List, Wake Forest University*  
  *Fall 2013-Spring 2017*
- *Phi Beta Kappa, Wake Forest University*  
  *Spring 2017*
- *Third Place Neuroscience Student Research Day, Wake Forest University*  
  *Fall 2017*

Activities

- *Treasurer Alpha Sigma Phi Beta Mu Chapter,*  
  *Fall 2014-Fall 2015*
- *Pledge Class President Alpha Sigma Phi Beta Mu Chapter,*  
  *Spring 2014*

Publications and Presentations


Computer and Language Skills

- *Computer:* Proficient in ImageJ, Microsoft Office, SigmaPlot, Clampfit and Clampex
- *Language:* Native English speaker and Proficient in German