Exploring the use of histone deacetylase inhibitors in treating rodent models of post-traumatic stress disorder and addiction

By:

Christie L. Pizzimenti

A DISSERTATION

Presented to the Department of Behavioral Neuroscience

Oregon Health & Science University School of Medicine

In partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

January 2018

TABLE OF CONTENTS

List of Figures and Tables

Chapter 2: **Persistent effects of acute stress on fear and drug-seeking in a novel model of the comorbidity between post-traumatic stress disorder and addiction**

Chapter 3: **The effects of histone deacetylase inhibitors during retrieval and extinction on stress-enhanced fear learning**

Chapter 4: The effects of a histone deacetylase-3 specific inhibitor on extinction in the comorbid PTSD-SUD model

List of Abbreviations

Abbreviation	Word or Phrase
ACTH	Adrenocorticotropin releasing hormone
BLA	Basolateral amygdala
CeA	Central amygdala
COC	Cocaine
CPP	Conditioned place preference
CORT	Corticosterone
DNA	Deoxyribonucleic acid
DEX	Dexamethasone
EtOH	Ethanol
FR	Fixed ratio
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HPA	Hypothalamic-pituitary-adrenal
IL	Infralimbic cortex
IP	Intraperitoneal
IM	Intramuscular
ISI	Inter-shock interval
IV	Intravenous
LTP	Long-term potentiation
METH	Methamphetamine
mPFC	Medial prefrontal cortex
NAcc	Nucleus accumbens
PL	Prelimbic cortex
PTSD	Post-traumatic stress disorder
NaB	Sodium butyrate
RM ANOVA	Repeated measures analysis of variance
SEFL	Stress-enhanced fear learning
SUD	Substance use disorder
SQ	Subcutaneous
TBI	Traumatic brain injury
TSA	Trichostatin A
VEH	Vehicle
VP	Ventral pallidum

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Christie Pizzimenti

has been approved

_____

K. Matthew Lattal, Mentor/Advisor

_____

Chris Cunningham, Oral Exam Committee Chair

_____

Barbara Sorg, Member

_____

Andrey Ryabinin, Member

_____

Matthew Ford, Member

Acknowledgments

"The people that you work with are just, when you get down to it, *your very best friends*."

- Michael Scott

I owe a huge debt of gratitude to so many people for helping me make it to this point. Thank you to my advisor Dr. K. Matthew Lattal and to my committee members Dr. Andrey Ryabinin, Dr. Christopher Cunningham, Dr. Barbara Sorg, and Dr. Matthew Ford for their support and guidance in preparing this manuscript. Thank you to Kris Thomason, Jeanne Sutter, Julie Rogers, Dr. Jackie Wirz, and Dr. Rachel Dresbeck (power women!) for the advice, opportunities, and support over so many years. I admire you ladies so profoundly.

A special thank you to my undergraduate-mentor-turned-life-mentor-turned-best-friend Dr. Maria Gomez. Honestly, without you, I wouldn't be here at all. You remain the single greatest teacher I have ever had the privilege of learning from. Your passion for neuroscience was infectious and set me on this path. My whole life would be different had you not been randomly assigned as my faculty mentor. I will look up to and adore you forever.

To the single greatest colleague anyone could ask for- Tommy Navis- you are a king among men. I quite literally couldn't have done this without you. You have been the greatest asset in my graduate school toolbox and I sincerely appreciate every single moment of working with you. Thank you for being my birthday twin. I am so excited to watch your career blossom; your success is inevitable my friend.

A gigantic thank you to my classmates Daicia Allen, Brian Mills, Nick Miner, Brett Dufour, and Vanessa Jimenez; I couldn't have asked for better people to stumble through this journey with. Thank you for being easily the sanest and most reasonable year of students this department has ever seen. Vanessa, thank you for being my person for so many years. Our sushi dates and rapid-fire seminar texts kept me sane over the past five years. Brian, honestly, I don't even know what to say. I can't imagine graduate school, or my life in general, without you. We were friends before we ever met, we just didn't know it yet. Thank you for being my brother from another mother, thank you for introducing me to Steph, Hamel, and Haley- thank you for all the memories. You remain the derpiest, silliest, strangest man-boy I've ever met. I love you very much. Please never change.

If I could shout it from the rooftops I would, THANK YOU THANK YOU THANK YOU to Amy Williams, Bene Ramirez, Sami Friedrich, Andre Walcott, Alfredo Zuniga, Eileen Torres, Monique Smith, and Gaby Alarcon for making graduate school more than just an educational experience; you guys have changed me for the better and I will never be the same for having known such wonderful people. Amy, one day when I grow up I want to be as good and kind as you are. I'll probably never get there but it's a great level to aspire to. I don't have any little sisters but if I did, I would want them to be just like you. Dreidel, thank you for being my person. There were so many times that talking to you about something was the only way it would get better. You both made me feel safe in ways I so desperately needed and I love you both so much for it. Bene, you remain the single most magnanimous person I've ever met. Your spirit, your heart, your

kindness- they are as big and bright as they come. Thank you all for saving me over and over and over again. I'll never be able to say it enough.

To the wolf pack- AROOOOO! Emma Schifsky, Moosa Ahmed, Brian Mills, Amy Williams, Bene Ramirez, Haley Todd, Stephanie Honeywoman, and Ryan Hamel-Spencer- what could I possibly say? What words are there to describe our tribe? Thank you from the bottom of my heart for helping make Portland more than just where I live, you made it my home. If ever we're apart just listen to the wind and you'll hear me ululating in the distance, just trying to make my way back to you.

To Stephanie Honeyman thank you for being such a prize of a human being. You are the kind of friend everyone deserves, but few ever get. I am so grateful to have found a life mate in you. To Froof, thank you for moving into the spare room in my house, bringing my nephew-dog Dexter, and exponentially improving my quality of life by 1000%. To Haley, thank you for our beautiful friendship that I wouldn't trade for anything in this world. I am so proud of our special, wonderful, organic connection. To Sami- thank you for being such a strong, powerful, brilliant badass lady. I am so proud of you and so excited to watch you soar. To Emma- your friendship feels like a cold glass of lemonade on a hot summer day; light, refreshing, and *just right* every single time. I could spend every single day laughing and talking with and admiring you and it would never get old.

To my best friend, my sister, the original love of my life and greatest person I've ever met- Ashley Barter- thank you for your unceasing love and support. Thank you for catching every 30 Rock reference, thank you for knowing me better than I know myself, thank you for traveling all the way to Portland to watch me defend this thesis, thank you

for being born in the first place so you could ensure I'd never truly be alone. I will never know or love anyone the way that I know and love you. If you were the only friend I had I'd still never feel lonely. I love you from the bottom of my heart.

To my partner Conor Kennedy- thank you for being the single greatest support system any person could ever ask for. I wouldn't have been able to do this without you. You have changed my life in so many wonderful ways, there's truly no acknowledgment section that could contain it. Thank you for being both a bear and a bunny. Thank you for always making me dinner. Thank you for your patience. Thank you for every single day we have shared together. Thank you for loving me and helping me become a better version of myself. I love you.

To my dog, Max, every single moment is improved by having and loving you. Thank you for being the fluffiest orange floof and the greatest dog of all time.

To the single strongest, most powerful, most generous, most awe-inspiring woman I've ever had the privilege of knowing and my very first best friend, Mom; I owe it all to you. To say that I am profoundly grateful for you would be such an understatement. Everything I have accomplished in life has been an extension of the unconditional love and support you have provided me every single day. Everything I achieved in graduate school would have been impossible had it not been for you acting as the greatest, loudest, one-woman cheering section the world has ever known.

Finally, to my beautiful grandmother who passed away while I was in graduate school, there are no words to describe how much you've impacted me. Everything I do is motivated by the desire to be the kind of person you would be proud of. I am so lucky to have had a grandmother as wonderful as you, someone who always lifted me up,

someone who was only ever proud of me, someone who saw in me only goodness. This thesis is dedicated to you and all you did in your lifetime to champion women. You will always be my hero.

Abstract

A large body of evidence shows that prolonged stress or even a single traumatic experience can lead to neurobiological and behavioral changes that are persistent. These changes have many deleterious long-term consequences and can lead to the development of post-traumatic stress disorder (PTSD). PTSD is associated with epigenetic changes that result in altered gene expression and subsequent protein synthesis. These epigenetic changes may account for the development of the disease itself in the form of exaggerated fear responses to neutral or mild stimuli and may render individuals more susceptible to the use and subsequent dependence on drugs of abuse. Substance use disorders (SUDs) similarly evoke persistent changes in gene expression that are mediated by epigenetic mechanisms. Because both PTSD and SUDs involve a memory component and an inability to extinguish unwanted behavioral responses, similar epigenetic changes may underlie long-term memory effects in both anxiety disorders and addiction. There is an emerging consensus that the best treatment strategies engage the circuits involved in behavioral inhibition, coupled with pharmacological manipulations designed to target those circuits, with the goal of creating lasting behavioral inhibition. An ultimate goal of research on these processes is to understand how appetitive and aversive memories interact, and what causes stressful experiences to induce relapse even after long periods of abstinence. This dissertation work focuses on this interaction, emphasizing how an epigenetic approach to memory suppression may be a particularly useful avenue to pursue in designing treatments for disorders that involve failures of inhibition, such as PTSD and substance abuse. Here, in Chapter 1, I review the literature on this comorbidity and describe

opportunities for novel behavioral approaches to address unanswered questions. In Chapter 2, I characterize a novel behavioral model of the comorbidity between PTSD and SUDs that demonstrates the persistent, long-term changes in fear responding and drug seeking that follow exposure to an acute stressor. In Chapter 3, I explore the use of epigenetic modulators to enhance memory inhibition in a rodent model of PTSD. In Chapter 4, I explore the ability of epigenetic modulators to enhance memory inhibition in the comorbid PTSD-SUD model described in Chapter 1. Finally, in Chapter 5, I synthesize these findings within the broader canon of existing literature and the implications of this research are discussed.

CHAPTER 1

General Introduction

Portions of Chapter 1 are adapted from the publication:

Pizzimenti CL & Lattal KM. (2015). Epigenetics and memory: causes, consequences and treatments for post-traumatic stress disorder and addiction. *Genes, Brain and Behavior, 14,* 73-84.

Addiction and relapse in PTSD

PTSD is classified as an axis I anxiety disorder that develops after an individual experiences a chronic or acute stressor, one that usually involves the threat of serious injury or death to oneself or to someone nearby (American Psychiatric Association, 2013). PTSD is accompanied by a constellation of symptoms including hyperarousal, avoidance, sleep disturbances and difficulty concentrating. The hallmark of PTSD is a re-experiencing of the traumatic event during which the individual relives the traumatic experience despite no longer being in danger.

One of the major challenges in developing effective treatments for PTSD is the high level of comorbidity between PTSD and substance use disorders (SUDs). Among people with lifetime PTSD, lifetime SUD is estimated at 21–43%, compared with 8–25% in those without PTSD (Jacobsen et al., 2001). Although this range is wide, the increased propensity for lifetime SUD in individuals with PTSD has been corroborated many times over (Keane & Wolfe, 1990; Kessler et al., 1995; Brown & Wolfe, 1994; Ouimette & Brown, 2003). Individuals with PTSD are more likely to develop SUDs (Kofoed et al., 1993) and once addicted, are more likely to relapse (Brown, Stout, & Mueller, 1999) than are unaffected individuals, even after long periods of abstinence (Bradizza et al., 2006). This increased vulnerability is especially troubling because those circuits and cellular signals that are involved in stress, fear, and memory are also involved in substance abuse (reviewed in Tipps et al., 2014).

Indeed, many have pointed out that addiction is a disorder of learning and memory, in which normal mechanisms in place to incorporate new information from the environment are hijacked by drugs of abuse (e.g., Hyman et al., 2006). Over time,

contexts evoke drug cravings, owing to the repeated intake of drugs in the presence of certain contextual cues (e.g., physical, social and temporal contexts). Drugs of abuse can also maintain anxious avoidance responses, preventing the extinction of anxiety in these situations, thus creating a vicious cycle in which problematic behaviors are maintained and even strengthened by both positive and negative reinforcement contingencies. Therefore, the treatments for addiction are difficult because the effects of drug abuse can persist even across contexts and long periods of abstinence.

PTSD also produces persistent changes that result in unwanted behavioral responses. Individuals who experience a stressor or series of stressors in a specific environment demonstrate inappropriate fear responses in otherwise innocuous environments in response to stimuli that remind the individual of the traumatic event. For example, a veteran may hear the crash of a wrecking ball tearing down a nearby building and be reminded of the sounds associated with his or her experience in a combat zone. Experiences like these can result in a re-experiencing of the memory associated with the original trauma, which leads to an exaggerated fear response. Therefore, both PTSD and addiction are examples of learning paradigms in which long-term memories related to discrete events persist over time and context. Thus, treatments of both PTSD and substance abuse require an understanding of the mechanisms that make memories long-lasting and protective against disruption.

A major goal of treatment interventions for PTSD and substance abuse is to weaken the ability of external and internal cues to evoke fear and/or drug seeking. A growing literature suggests that behavioral intervention paired with a pharmacological approach that targets the molecular mechanisms involved in fearful and drug-related

memory formation may lead to a weakening of the behavior evoked by those memories (e.g., Davis et al., 2006). It is abundantly clear that a key aspect of these treatments is a behavioral component that relies on principles of extinction.

Behavioral extinction as a therapeutic

At the level of behavior, treatment interventions for PTSD and substance abuse have focused on exposure therapy – presenting patients with cues associated with trauma or drug seeking, and allowing the responses evoked by those cues (anxiety or craving) to occur and extinguish as the expected outcome does not occur. This basic principle of exposure therapy is similar regardless of whether the treatment is directed toward behaviors that produce a positive outcome (attenuation of substance abuse) or remove a negative outcome (anxiety reduction), and there are numerous demonstrations of the power of extinction to change maladaptive behavior that occurs in a variety of disorders (e.g., Campbell, 2003).

Despite some success of exposure therapy based on extinction processes (e.g., Wachen et al., 2014), there are several challenges that can lead to poor clinical outcomes in the treatment of PTSD or substance abuse. First, extinction is known to suppress behavior (fear, avoidance, drug seeking) without necessarily altering the content of the original memory. Therefore, the original associative memory can still influence future behavior given the right circumstances (see Delamater & Westbrook, 2014). Second, the changes that occur during extinction may not be long lasting; extinguished behavior returns with changes in context, with reminders of the original association, and after the simple passage of time. Enhancements in extinction that

occur within a therapeutic setting may not subsequently persist outside of that treatment context. This is problematic, of course, because patients are likely to encounter cues associated with trauma or drug taking within their daily lives outside of the therapist's office. For example, an alcoholic may experience intense cravings when walking by the neighborhood bar or a nicotine addict may crave a cigarette with a morning cup of coffee. Third, exposure therapy involves the mental re-experiencing of anxiety or cravings and this type of experience may be aversive, which may decrease the likelihood that a patient will return to treatment sessions. A fourth challenge that is beginning to emerge in thinking about models for PTSD is that extremely stressful experiences have deleterious effects on the long-term efficacy of extinction (e.g., Knox et al., 2012; Rau & Fanselow, 2009). Thus, there is a need for interventions that promote extinction, make it occur more rapidly, and persist outside of the treatment context.

Testing treatments in models of PTSD and the comorbid substance use disorder condition

Traditional approaches to evaluating how aversive memories are formed and modulated involve fear conditioning. These approaches have been useful for delineating basic mechanisms of memory, but it is not clear that there is a direct relation between basic fear conditioning in the lab and PTSD in the clinic**.** Attempts to model the comorbid condition have evaluated drug-taking within the fear related environment, but this is not translatable, as individuals are highly unlikely to consume drugs in a trauma-related environment.

Other rodent models of PTSD have included exposure to predators (Adamec et al.,1997) or predator urine (Adamec & Shallow, 1993; Wallace & Rosen, 2000), single prolonged stress, which consists of animals being restrained for 2 h, run through a forced swim procedure, then exposed to ether until loss of consciousness (Khan & Liberzon, 2004), exposure to unsignaled footshock (Mikics et al., 2008; Rau & Fanselow, 2005), chronic variable stress, in which animals are exposed to a variety of stressors for several weeks (Willner,1997), and social defeat (Buwalda et al., 2005). Although these models of PTSD demonstrate behavioral differences between animals exposed to stressors relative to controls, an important feature of the disorder is not considered. One of the most prominent features of PTSD, and perhaps the most detrimental to everyday living is an enhanced fear response to mild or neutral stimuli that are not associated with trauma. Although exposure to some of these fear paradigms results in altered fear responses, these responses are often evaluated in the same environment in which the stressor was administered, or in close temporal proximity (immediately following exposure to the stressor). In individuals with PTSD, unwanted fear responses arise long-after the stressor has ended and in a variety of non-trauma related contexts.

In the case of modeling the comorbid PTSD-SUD condition, researchers often expose animals to stressors and then evaluate enhancements in drug-seeking behavior immediately after (Piazza et al., 1990; Quadros & Miczek, 2009). Although these models shed light on how exposure to trauma may contribute to an enhancement in short-term drug-taking behavior, they fail to capture the ability of a traumatic event to influence drug-seeking behavior long after the stressor has ended in non-trauma

associated contexts. In fact, it has been repeatedly reported that individuals that are comorbid for PTSD and SUDs do not differ from individuals with SUDs alone in substance use severity (Brown, Stout & Mueller, 1999; Eggleston et al., 2008), but are more likely to relapse (Tate et al., 2004; Kubiak, 2004; Burns et al., 2010; Najt et al., 2011), which highlights the importance of understanding how temporally and contextually distinct events may ultimately influence behavior.

Our current understanding of the role of behavioral extinction in the treatment of PTSD as well as PTSD-SUD comorbidity is quite limited; it remains unclear how behavioral extinction might affect fear and drug-taking responses in non-trauma related contexts. It is therefore critical that we evaluate the ability of behavioral extinction (with or without pharmacological enhancement) to attenuate exaggerated fear responses to mild stimuli/drug-seeking behavior when they do not occur in the same context.

Epigenetic mechanisms in PTSD and addiction

Given that one of the key challenges in using extinction as a therapeutic is that it may not always create lasting effects, there is a need to look for molecular mechanisms that may provide a long-term signature for extinction. As we understand more about persistent epigenetic changes that are associated with fear, PTSD, and addiction, it is becoming clear that targeting epigenetic processes may provide a long-term solution to the problem of creating persistent extinction (reviewed in Stafford & Lattal, 2011; Zovkic & Sweatt, 2012).

One reasons that epigenetic mechanisms are receiving so much attention in the fields of fear and drug seeking is the fact that they provide experience-dependent

positive and negative regulation of gene expression in the circuits that mediate memory. Broadly speaking, epigenetic processes provide molecular mechanisms through which environmental experience can have lasting effects on behavior. Because these mechanisms lead to lasting cellular changes, there is great focus on how they translate into lasting memories involving trauma and drugs of abuse. By enhancing the formation of memories where fear or drug conditioned stimuli no longer predict drug availability using agents that influence the epigenetic machinery, the extinction memory may persist outside the treatment context, preventing relapse in vulnerable populations.

Broadly, epigenetics refers to changes in chromatin structure that influence gene expression without affecting gene sequence (Kwapis & Wood, 2014; Levenson & Sweatt, 2005). Two primary mechanisms of epigenetic regulation are through direct deoxyribonucleic acid (DNA) methylation and histone modification, and these mechanisms allow for the integration and storage of information in species ranging from yeast and plants to humans (reviewed in Hitchcock & Lattal, 2014; Vanyushin, 2006). Chromatin refers to the complex of DNA and proteins, primarily histones, that reside in the nucleus of a cell. Chromatin and DNA interact as an octamer of histones known as a nucleosome (Luger et al., 1997). The default structure of chromatin is largely in a repressed state, with changes in either DNA methylation or histone modification on lysine residues along the N-terminal tail affecting the activity of a number of transcription factors and the transcriptional machinery itself, which in turns affects gene expression. There are many types of modifications that can occur on DNA or histones (see Strahl & Allis, 2000), but memory and addiction work has focused largely on histone acetylation.

Histone acetylation involves two key molecules, histone acetyl transferase (HAT) and histone deacetylase (HDAC). The addition of an acetyl group by HAT causes chromatin to relax, thus making the DNA available to the transcription machinery, which subsequently leads to the enhanced production of proteins that contribute to the formation of memories. Acetyl groups are removed by HDACs, which results in the tightening of chromatin that renders the DNA unavailable to transcription machinery. One mechanism through which memories may be enhanced is through the use of HDAC inhibitors, which relax chromatin, causing it to remain open and increasing transcription.

One of the early demonstrations of the importance of acute epigenetic mechanisms in learning and memory came from Levenson et al. (2004), who found that contextual fear conditioning induced acetylation of histone H3 in the CA1 region of the hippocampus and that injection of an HDAC inhibitor before a conditioning session enhanced the long-term fear memory. Many subsequent studies have demonstrated that HDAC inhibitors promote initial memories (e.g., Raybuck et al., 2013; Vecsey et al, 2007) and memories that form during extinction (Bredy & Barad, 2008; Gräff et al., 2014; Lattal et al., 2007; Marek et al., 2011; Stafford et al., 2012; Wei et al., 2012).

Epigenetic mechanisms have similarly been implicated in drug addiction. Both acute and chronic cocaine administration increase histone acetylation on H3 and H4 in the nucleus accumbens (NAcc), a critical brain region associated with reward (Kumar et al., 2005). Mice that lack class 1 HDAC1, but not HDAC2 or HDAC3, in the NAcc specifically show deficits in cocaine-induced locomotor sensitization (Kennedy et al., 2013). Knockout of HDAC1 in the striatum attenuates amphetamine-induced

desensitization of the c-fos gene, an immediate early response gene that is rapidly induced in the striatum following acute exposure to psychostimulants (Renthal et al., 2008). Epigenetic changes have been implicated in the transition from drug use to drug addiction, as well as in chronic stress, through the activity of HDAC5 (Renthal et al., 2007). Mice that received site-specific injections of the HDAC class I and II inhibitor suberoylanilide hydroxamic acid to the NAc during place conditioning show enhancements in cocaine-induced conditioned place preference (CPP; Renthal et al., 2007). This group also found that a single injection of cocaine in mice chronically exposed to cocaine induced HDAC5 expression, an effect not present following a single dose of cocaine in naïve mice. Conversely, overexpression of HDAC5 using HSV-mediated transgene expression attenuates cocaine-induced CPP (Renthal et al., 2007). Similarly, chronic, but not acute, social defeat downregulates Hdac5 mRNA in the NAc (Renthal et al., 2007). Epigenetic regulation has also been observed in experiments looking at non-psychostimulants, including heroin. Heroin induces acetylation of H3 in the NAc, and intra-accumbal injection of the HDACi trichostatin A (TSA) increases heroin-induced CPP, suggesting increased reward salience (Sheng et al., 2011).

Exposure to both trauma and drugs of abuse induce epigenetic changes that result in persistent behavioral changes, some of which may contribute to the formation of a drug addiction or a stress-related psychiatric disorder. It is therefore of great interest to find new therapeutic options to treat these disorders, and the use of agents that promote extinction as a promising new class of drugs.

Epigenetics and treatment of PTSD and addiction

Epigenetic changes that result from stressful situations, experienced either early in life or during adulthood, may predispose individuals to aberrant stress responses, as well as SUDs. For example, changes in methylation of both the genes for glucocorticoid receptors and brain derived neurotrophic factor have been associated with early-life trauma in human populations (McGowan et al., 2009; Roth et al., 2009). These changes may lay the groundwork for long-term dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which may account for increased rates of relapse to drugs of abuse in response to stressful stimuli. What is particularly promising for an epigenetic approach to PTSD is that these long-term changes can be reversed through environmental or pharmacological means. Injection with the HDAC inhibitor TSA can reverse changes in DNA methylation and HPA responsiveness to restraint stress (Weaver et al., 2004). This finding suggests that the changes that lead to sensitivity to PTSD can be reversed using epigenetic regulators in adulthood, and that this reversal can normalize HPA activity and behavioral responses to stress. Of course, these long-term epigenetic changes may result in developmental programming effects that set in motion a chain of events that lead to endpoints that are far removed from their initial triggers. For example, even if acetylation of lysine residues on histone tails can be reversed long after a stressful experience, the developmental program induced by that acetylation pattern may have already resulted in long-term cellular changes that now function independently of that initial pattern. Nonetheless, the long-term changes that can be induced in adulthood by drugs that target epigenetic mechanisms have exciting implications for potential treatment options for PTSD. Given the vicious circle that exists

between stress, PTSD and substance abuse, as well as many common underlying cellular and molecular mechanisms, drugs that can successfully treat one aspect of these disorders may simultaneously treat other aspects of those disorders. The power of an epigenetic approach that is informed by basic science on the neurobiology of stress and extinction has the potential to ultimately alter specific circuits that mediates memory.

Dissertation Studies

To summarize the literature and theories discussed above, the high rate of comorbidity between PTSD and SUDs creates a substantial barrier to treatment of both disorders, especially long-term abstinence from drug-use. Current models of PTSD-SUD comorbidity fail to capture the ability of a massive stressor to affect fear-related and drug-seeking behavior long after the stressor has ended and in non-fear associated contexts. Therefore, our understanding of how behavioral extinction influences these behaviors, and the utility of behavioral extinction as a potential treatment option, remains quite limited. Because both PTSD and SUDs, specifically relapse to drugs of abuse, involve a failure of extinction learning to persist across space and time there has been interest in developing therapeutic approaches that involve enhancing inhibitory learning. One such method that has shown promise in both drug- and fear-related paradigms is the use of epigenetic modulators paired with discrete learning events. This series of dissertation experiments investigate the role of behavioral extinction and epigenetic regulation in a novel model of the PTSD-SUD comorbidity in rats.

In the subsequent chapters of this dissertation I focus on the development of the novel comorbid condition in rodents (Chapter 2). First, I aim to replicate the basic finding that massive footshock in a distinct context results in exaggerated fear responses to mild stimuli in a different context. I then explore if this effect is maintained in environments that are associated with reward. To probe the ability of PTSD to affect long-term drug-seeking behavior I combine massive footshock and self-administration of intravenous (IV) methamphetamine (METH) in rats and massive footshock and CPP for cocaine (COC) in mice. I also investigate the role of the hypothalamic-pituitary-adrenal axis in this model.

Chapter 3 focuses on the role of behavioral extinction and epigenetic enhancement of extinction-related memories in the stress-enhanced fear learning (SEFL) paradigm. First, I examine the ability of behavioral extinction to influence fear responses in this model. Then, I investigate the ability of several epigenetic modulators to enhance extinction learning. Because fear conditioning paradigms are often conducted during the light phase of the light cycle, I investigate how circadian time of day may influence fear responses and account for differences observed between laboratories.

In Chapter 4 I investigate the ability of an HDAC 3 specific inhibitor to enhance extinction of drug seeking and subsequently attenuate reinstatement to drug-related cues in the comorbid PTSD-SUD model described in Chapter 1.

Chapter 5 summarizes the findings and provides a broader context for interpreting the results from these studies based on our current understanding of these principles. A detailed review of the implications of this body of work are discussed.

CHAPTER 2

Persistent effects of acute stress on fear and drug-seeking in a novel model of the comorbidity between post-traumatic stress disorder and addiction

Portions of Chapter 2 are adapted from the publication:

Pizzimenti CL, Navis TM, & Lattal KM. (2017). Persistent effects of acute stress on fear and drug-seeking in a novel model of the comorbidity between post-traumatic stress disorder and addiction. *Learning & Memory, 24(9),* 422-431.

Contributions: TM Navis collected behavioral data in the conditioned place preference and self-administration of ethanol experiments.

Abstract

Even following long periods of abstinence, individuals with anxiety disorders have high rates of relapse to drugs of abuse. Although many current models of relapse demonstrate effects of acute stress on drug seeking, most of these studies examine stressful experiences that occur in close temporal and physical proximity to the reinstatement test. Here, we assess the effects of a stressful experience in one context on fear and drug seeking in a different context. We adapt the stress-enhanced fear learning procedure to examine impacts on drug seeking long after the stressful experience occurred. We find massive footshock in a distinct environment produced an acute increase in corticosterone, long-term hyper-responsivity to a single shock in different contexts with extensive histories of drug-seeking behaviors, enhancements in cocaine-induced conditioned place preference in mice, and persistent enhancements in cue-induced reinstatement of drug-seeking behavior in rats. Together, these experiments demonstrate that an acute trauma causes persistent changes in responsivity to mild stressors and drug-seeking behavior in other contexts, which mirrors aspects of the comorbidity between PTSD and SUDs. These behavioral approaches provide novel procedures for investigating basic mechanisms underlying this comorbidity and they provide powerful tools for testing preclinical pharmacological and behavioral interventions.

Introduction

Compared with the general population, individuals diagnosed with PTSD have higher rates of SUDs (Back et al., 2000; McCauley et al., 2012; McFarlane, 1998; Ouimette et al., 1998; Roberts et al., 2015; Sonne et al., 2003; Stewart, 1996; Tipps et al., 2014) and are twice as likely to use METH than are individuals with trauma exposure that does not lead to PTSD (Smith et al., 2010). Individuals with PTSD are also more likely to relapse to drugs of abuse when cues associated with drug seeking are encountered, even long after periods of acute stress have ended (Bradizza et al., 2006), suggesting stressors that are temporally and contextually dissociated from drug seeking may induce long-term changes that contribute to an increased risk for relapse.

It has long been observed that stress is a potent inducer of reinstatement (an animal model of relapse) in rodents (e.g., Boutrel et al., 2005; Erb et al., 2001; Redila & Chavkin, 2008; Sanchez & Sorg, 2001; Shaham et al., 2000; Schindler et al., 2010). Although the ability of stress to induce reinstatement has been well established in the literature, most studies have focused on effects when the organism is tested in a state of acute stress within the drug-seeking context; few studies have evaluated the persistent effects of an acute stressor long after the stress has ended. Individuals with PTSD have traumatic experiences long before relapse and are unlikely to use drugs in the trauma-associated context; avoiding the location in which the trauma occurred is one of the DSM criteria for a diagnosis of PTSD (American Psychiatric Association, 2013). Stressors that occur within a distinct environment (e.g., social defeat; Quadros & Miczek, 2009) or are administered repeatedly (e.g., chronic tail pinch; Piazza et al., 1990) have been shown to increase acquisition of drug self-administration, but

comparatively little is known about how an acute stressor causes persistent changes in drug-seeking responses long after that stressful experience has ended. Developing a model of this persistence is key to understanding the PTSD-SUD comorbidity and to evaluating novel treatment interventions for both disorders.

There is evidence that an acute stressor (a battery of footshocks) associated with a specific environment can have lasting effects on fear responses to a mild stressor (a single footshock) in a different environment (Rau et al., 2005). This stress-enhanced fear learning (SEFL) persists across long intervals (Rau & Fanselow, 2009) and shows properties that differ from weaker forms of fear conditioning, such as resistance to extinction (Long & Fanselow, 2012) and N-methyl-D-aspartate receptor independence (Rau et al., 2005). An advantage of the SEFL approach is that it incorporates a fear conditioning procedure that has been characterized extensively at behavioral, cellular, and molecular levels (Kim & Jung, 2006; Maren et al., 2013), resulting in a stress procedure that has measureable memory and affective components (reviewed in Blouin et al., 2016). This procedure results in the same well-characterized behavioral response (conditioned freezing) both in the original stressful context and in novel contexts in which a single shock is encountered. Thus, the persistence of the stress response over time can be measured and manipulated in behaviorally tractable ways.

In the following experiments, we characterize the effects of a battery of footshocks in one context on exaggerated fear and drug-seeking responses in another context. It has previously been reported that massive footshock altered ethanol drinking patterns in a two-bottle choice paradigm only when footshock was administered before drinking patterns had been established (Meyer et al., 2013). Therefore, we sought to

investigate if this held true for intravenous self-administration of methamphetamine. To extend these findings we employed the same methods and included a 0 and 15 FS group, with no 1 or 4 FS group. Indeed, most papers investigating the SEFL effect following the initial description of this phenomenon in 2005 (Rau, DeCoa & Fanselow) have only included the 0 and 15 FS groups, with the intention of driving the effect to its highest degree (see Chapter 5 for a discussion on massed vs. spaced ISI).

First, we replicate the basic SEFL effect as it was originally reported. We then show that the basic SEFL effect occurs in contexts that have an extensive history of association with drug seeking. Further, we find that a battery of footshocks in one context causes persistent effects on cue-induced reinstatement and subsequent resistance to extinction of drug seeking in another context. Together, these findings show that a single acute trauma causes a hyper-response to a mild stressor and enhances cue-induced reinstatement long after that trauma. This model opens the doors to testing new treatment options for the comorbidity between PTSD and SUDs.

Methods and Materials

Animals

One hundred and twenty one male Long Evans rats (Charles River) that weighed 275-300 g (~9-11 weeks of age) at the start of the experiments were pair housed in a temperature (22 °C ± 1 °C) and humidity-controlled (70%) vivarium and were maintained on a 12/12 hr light/dark cycle (6:00 am/6:00 pm). Following surgery, animals were single housed, and three days prior to the initiation of self-administration training

animals were food restricted to ~90% free feeding body weight. Rats that did not receive jugular catheter surgery were not food restricted, but were single housed prior to the onset of behavioral testing.

Thirty-six adult, male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) weighing approximately 27 g at the start of the experiment were housed four to a cage on a 12 hr light/dark cycle (lights on at 6 AM). Mice were 11-16 weeks of age and were given *ad libitum* access to food and water. Mice were handled and weighed daily for five days prior to the start of the experiment. Housing conditions and treatment of these animals were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and conducted in accordance with the ethical guidelines of the National Institutes of Health.

Drugs

METH (Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline and administered IV as 0.06mg/kg/infusion over 5 sec. Cocaine hydrochloride (COC; Sigma) was dissolved in sterile saline and administered at a dose of 20 mg/kg via an intraperitoneal (IP) injection. DEX (Sigma) was dissolved in 0.01% propylene glycol injected subcutaneously (SQ) at a dose of 50 µg/kg.

Apparatus

In all experiments, drug seeking (self-administration or conditioned place preference) occurred in both a different room and chamber from massive footshock.

Rat Self-Administration and Fear Conditioning Apparatus (Experiments 1, 2, 3, 4, 5, and 7)

Context 1: METH self-administration (Experiments 2, 4, and 5) sessions were conducted in operant conditioning chambers (Med Associates, St. Albans, VT) (exterior dimensions: 31.8cm L x 25.4cm W x 34.3cm H) housed within sound attenuating cubicles (Med Associates, St. Albans, VT). All self-administration sessions lasted for 2 hours. The syringe pumps (Med Associates, St. Albans, VT) that delivered drug were located outside the sound attenuating chambers. Grid floors composed of 19 stainless steel rods (0.48 cm diameter with 1.6cm spacing between them) were connected to shocker/scramblers (Med Associates, St. Albans, VT) that delivered footshock. Each chamber was outfitted with two retractable levers, a stimulus light above each lever, and a houselight that was illuminated throughout the duration of every session. Between cohorts, 95% EtOH was used to clean these chambers.

These boxes were also used to deliver massive footshock in Experiment 1 (SEFL replication) and Experiment 3 (self-administration of EtOH) with the levers retracted and the houselight illuminated throughout the session.

Context 2: Operant conditioning chambers (exterior dimensions: 31.8cm L x 25.4 W x 26.7cm H, Med Associates, St. Albans, VT) housed within sound attenuating chambers (Med Associates, St. Albans, VT) were located in a different room from Context 1 chambers. These chambers had identical grid floors to Context 1. When used for the self-administration of EtOH these chambers were outfitted with two non-retractable levers with a stimulus light above each lever, a stainless steel cup between the levers into which EtOH was dispensed, and a houselight centered on the top panel.

These chambers were also used to deliver massive footshock in Experiment 1 (SEFL replication), Experiment 2 (freezing in METH context), Experiment 4 (footshock before self-administration of METH), Experiment 5 (footshock during self-administration of METH), and Experiment 6 (mouse CPP). For Experiment 6, the floors were exchanged with mouse-specific floors as described below. When used for these experiments the levers, stimulus lights, and stainless steel cup were removed so that only steel paneled walls and a houselight, which remained illuminated throughout the session, remained. Between cohorts, 0.5% bleach was used to clean the chambers.

Mouse Conditioned Place Preference and Fear Conditioning (Experiment 6)

Apparatus

CPP was performed using an unbiased procedure in unbiased apparatus (see Cunningham, et al. 2006) that consisted of 30x15x15cm clear acrylic walls divided in half by another clear acrylic wall, resulting in two 15x15x15cm compartments during acquisition. The bottom was supported by interchangeable half-floors composed of 2.3-mm stainless steel rods mounted 6.4 mm apart (GRID floor) or perforated stainless steel with 6.4-mm round holes on 9.5-mm staggered centers (HOLE floor). The CPP apparatus was located within a melamine sound-attenuating shell. A camera was mounted to the ceiling of the sound-attenuating chamber and mouse position in the apparatus was recorded by an automated tracking program (Noldus Ethovision, Leesburg, VA).

Fear conditioning occurred in a novel environment in a separate room from the CPP context using modified operant boxes (Context 2; Med Associates, St. Albans, VT)

housed within sound attenuating chambers. Floors consisted of 36 parallel stainless steel rods with a 0.327 cm diameter. The walls of the chamber were bare except for a houselight that remained illuminated for the duration of the session.

General Procedures

Jugular Catheter Surgery (Experiments 2, 4, and 5)

Catheter Assembly. Jugular catheters were made of 12 cm long silastic tubing (0.037 mm ID, 0.94 mm OD; Dow Corning, Midland, MI) with small beads of 100% silicone rubber sealant at 8.5 cm and 9 cm, respectively. One end of the catheter was inserted into the right jugular vein and run subcutaneously below the front right leg to exit the back between the shoulder blades. A stainless steel guide cannula (22 ga; Plastics One, Roanoke, VA) was inserted into an elastomer self-administration harness (Instech Laboratories, Plymouth Meeting, PA) and the jugular catheter was attached to the cannula within the harness.

Surgery. Anesthesia was induced with an intramuscular (IM) injection of ketamine/xylazine (85 mg/kg; 10 mg/kg), and was maintained throughout the duration of the surgery by vaporized isoflurane (1%). Catheters were implanted and connected to the harnesses as described above. Following surgery animals received a daily IV infusion of 0.1 ml 100 unit heparin and the antibiotic Timentin (238 mg/ml). On days when animals performed self-administration, an IV infusion of 0.1 ml 10 unit heparin was also administered prior to the session. Catheter patency was confirmed via 0.1 ml IV injection of 10 mg/ml sodium brevital before training began. Catheter patency was

not tested via injection of sodium brevital again, but catheter patency was monitored by the experimenter.

Self-Administration of Methamphetamine (Experiments 2, 4, and 5)

In all experiments, self-administration sessions (acquisition, extinction, and reinstatement) lasted for 2 hr. At the onset of each session a houselight was illuminated and two retractable levers were inserted into the chamber. One lever was designated the active lever, and upon completion of the fixed ratio (FR) requirement 88.5 µl of meth was administered IV over 5 sec (0.06 mg/kg/infusion), and the stimulus light above the active lever was concurrently illuminated for 5 sec. The inactive lever also had a stimulus light above it; however, pressing the inactive lever did not result in any programmed consequences. Levers were counterbalanced across animals. During extinction sessions, presses on either lever did not result in any programmed consequences. During cue-induced reinstatement sessions a press on the active lever resulted in the activation of the stimulus light above the active lever for 5 sec, but no drug was delivered. Animals received a single self-administration session per day.

Experiment 1: Replication of the SEFL Effect

Twenty-one rats received SEFL treatment following the methods of Rau et al. (2005). Animals received 0, 1, 4 (intershock interval (ISI) of 3-7 min), or 15 1 mA (ISI of 4-8 min), 1 sec footshocks in Context A; session durations were 93 min for the 0 and 15 shock groups, 3 min 44s for the 1 shock group, and 23 min for the 4 shock group. Twenty-four hr later, fear to Context A was assessed during an 8 min 32s nonreinforced session. Twenty-four hr later animals received a single 1 mA footshock in Context B

(delivered at 3 min 12 s of a 3 min 48 s session), and fear to Context B was assessed 24 hr later in an 8 min 32 s nonreinforced session. Contexts were counterbalanced between Contexts 1 and 2. Fear conditioning was measured by sampling freezing behavior, defined as the lack of movement except that which is required for breathing, every 8 s.

Experiment 2: SEFL Effect in a Context Associated with Methamphetamine

Rats were trained to lever press for METH in the self-administration context (METH; Context 1 in this experiment) during 9 FR1 sessions, followed by 19 FR5 sessions, followed by three 2-hr extinction sessions, in which responding on either lever had no programmed consequences. Twenty-four hr after the final extinction session, animals received 0 or 15 footshocks in the shock context (SHOCK; Context 2 in this experiment) over the course of 93 min. Twenty-four hr after footshock animals were returned to the METH context with levers retracted. After 5 min all animals received a single, 1-s 1 mA footshock, after which the levers were immediately extended. Reinstatement following the single footshock was assessed in extinction, and freezing behavior in the five min pre- and post-footshock was recorded. Thirteen rats were included in this analysis.

Experiment 3: SEFL Effect in a Context Associated with EtOH

Sixteen rats were trained to lever press for 10% EtOH using a sucrose fading procedure (adapted from Freedland et al., 2001). Rats were given *ad libitum* access to food and water while in the home-cage (animals were water restricted up to 20 hr prior to session

4 and 5 to promote acquisition). Sessions were 2 hr long and occurred every other day. Pressing the active lever caused a syringe pump to deliver 0.1 ml of liquid into a stainless steel cup over 1.66 s and activated a cue light above the lever for 1.66 s. Total rewards were limited to 200, equivalent to 20 ml of solution. Animals were trained to respond to sucrose alone (10%) for 8 sessions on an FR1 schedule (lever press requirements were set to FR1 for the duration of the experiment, with the exception of the 6 final sessions, which were FR5) in the EtOH context (Context 2 in this experiment). Ethanol was phased in according to the following schedule: 10% sucrose/2% EtOH for 3 sessions, 10% sucrose/5% EtOH for 3 sessions, 5% sucrose/5% EtOH for 3 sessions, 5% sucrose/10% EtOH for 3 sessions, 2% sucrose/10% EtOH for 3 sessions, 1% sucrose/10% EtOH for 3 sessions, 10% EtOH alone for 6 sessions, and 10% EtOH on an FR5 schedule for 6 sessions.

Seven days after the final FR5 session animals were given either 0 or 15 footshocks in the SHOCK context (Context 1 in this experiment), then were returned to the EtOH self-administration context (EtOH context; Context 2 in this experiment) and given a 2 hr 10% EtOH FR5 session. Animals were allowed to self-administer 10% EtOH on an FR5 schedule for 7 additional days, followed by four 2 hr extinction sessions. Twenty-four hr after the final extinction session animals were tested for cued reinstatement during which a press on the previously active lever resulted in the 1.66 s illumination of the cue light above the lever, but no drug delivery. Sixty-five days after massive footshock in the SHOCK context animals received a single, 1 mA footshock delivered 5 min into a 15 min session in the EtOH context.

Experiment 4: Massive Footshock During Acquisition of Methamphetamine Self-Administration in a Different Context

Rats were trained to respond for METH over 4 FR1 sessions, followed by 4 FR3 sessions, followed by 8 FR5 sessions in the METH context (Context 1 in this experiment). Prior to the 7th FR5 session (the 15th session of 30 total self-administration sessions), animals received either 0 or 15 footshocks in the SHOCK context (Context 2 in this experiment). Immediately following footshock animals were returned to the METH context and given an FR5 session. Following the 8th FR5 session the response requirement was increased to FR10 for 14 sessions, followed by 3 extinction sessions. Twenty-four hr after the last extinction session, animals were restrained (DecapiCones) within the METH context for 5 min, and were then allowed to lever press for 2 hr in extinction. Animals then received 4 additional extinction sessions, followed by a test of cue-induced reinstatement, followed by 6 additional extinction sessions. After an additional 5 days in the homecage, rats were returned to the operant chambers and retention was tested in extinction. Animals then received 13 additional extinction sessions, followed by a test of footshock-induced reinstatement (10s after placement into the chamber 10, 0.5mA shocks were delivered, variable ISI 10-70 sec, levers extended immediately following final shock and animals were allowed to lever press for the remaining 2 hr session). Eleven animals were included in this analysis.

Experiment 5: Massive Footshock Prior to Acquisition of Methamphetamine Self-Administration in a Different Context

Rats were exposed to 0 or 15 footshocks as described above in the SHOCK context (Context 2 in this experiment). Self-administration of METH began 24 hr later in the METH context (Context 1 in this experiment). Animals were trained to lever press for METH during 3 FR1 sessions, 2 FR3 sessions, and 10 FR5 sessions. Animals were then extinguished over 5 extinction sessions, followed by a cue-induced reinstatement test and one additional extinction session. Fifteen animals were included in this analysis.

Experiment 6: Massive Footshock in a Different Context Prior to Cocaine CPP tests.

CPP procedures followed Hitchcock et al. (2014). Mice were given a 5-min pretest in which they had access to both floors of the CPP chamber. Mice were then counter-balanced relative to floor preference to match time on the CS+ floor for the mice to be assigned to shock or no shock groups. Twenty-four hr following the pretest, all mice received an IP injection of cocaine (COC) and were placed on the CS+ floor (GRID or HOLE, counterbalanced). The following day, mice received an IP injection of saline alone and were placed on the CS- floor (HOLE or GRID, counterbalanced). Animals received 6 additional conditioning sessions over the next 6 days, with a single conditioning trial per day for a total of 4 CS+ and 4 CS- pairings. During conditioning, animals were restricted to one floor type and half the total area of the apparatus by

placing a clear Plexiglas divider between the two floor types. Conditioning sessions were 5 min in duration.

Three days following the final conditioning session mice were placed into the novel fear context described above. Mice in the shock group (n=16) received 15 intermittent, unsignaled footshocks (0.5 mA, 0.5 seconds, variable ISI 4-8 min) over a 93-min period. Control mice (n=20) were exposed to the context alone for an equivalent amount of time. Immediately after the fear conditioning session, mice were taken to the CPP context, given an IP injection of saline, and placed in the CPP chamber with both floors (GRID and HOLE) accessible for 15 min (Test 1). This test was repeated 24 hr later (Test 2).

Experiment 7: The Effect of Massive Shock on Corticosterone and Hypothalamic-Pituitary-Adrenal Axis Function

Blood samples were collected once prior to the onset of SEFL training. Rats then received either 0 (n=7) or 15 (n=7) footshocks as described above in Context A, and blood was drawn immediately following removal from the chamber. Blood samples were then collected once per week for 5 weeks to mirror the length of time between footshock and the cue-induced enhancements seen in Experiments 4 and 5. All blood samples were collected before 12:00 pm through the saphenous vein. Blood samples were mixed with 2 µl 0.5 MM EDTA, spun at 8,000 RPM for 15 min, and plasma was collected and stored at -80 °C until processing. CORT levels were determined using a radioimmunoassay kit (MP Biomedicals, Burlingame, CA; sensitivity of 7.7 ng/ml, intra-assay variance 8.35%).

DEX is a synthetic corticosteroid that induces negative feedback of the HPA axis, decreasing levels of cortisol (Yehuda et al., 1993). Five weeks following footshock rats received counterbalanced injections of DEX or Vehicle over two days and blood samples were collected 6 hr following administration. All blood draws were taken before 12:00 pm.

One week following DEX administration animals were returned to Context A and were tested for contextual freezing during an 8.5 min test. One week later animals received a single, 1 mA footshock in Context B and 24 hours later were tested for contextual freezing during an 8.5 min test. Contexts A and B were counterbalanced between Contexts 1 and 2.

Results

Experiment 1: Replication of the SEFL Effect

In Experiment 1 (overview shown in Figure 1A), we replicated the basic SEFL effect: rats that received 4 or 15 footshocks in Context A showed higher freezing during tests in Contexts A and B compared to rats receiving 0 or 1 footshock (Figure 1B; reliable main effects of group during Context A ($F_{(3,17)}$= 7.60, p = .002) and Context B tests ($F_{(3,17)}$= 3.97, p = .026)). There were no significant differences between the 0 and 1 shock groups (p > .257) or between the 4 and 15 shock groups (p > .41) in either test. However, there was a significant difference between 0 and 1 shock groups compared to the 4 shock (ps <.05) and the 15 shock group (ps < .05) in both tests.

A

	0,1,4, or 15 footshocks	Context A Test	1 footshock in Context B	Context B Test
Exp. Day	1	2	3	4
Context	A	A	B	B

B

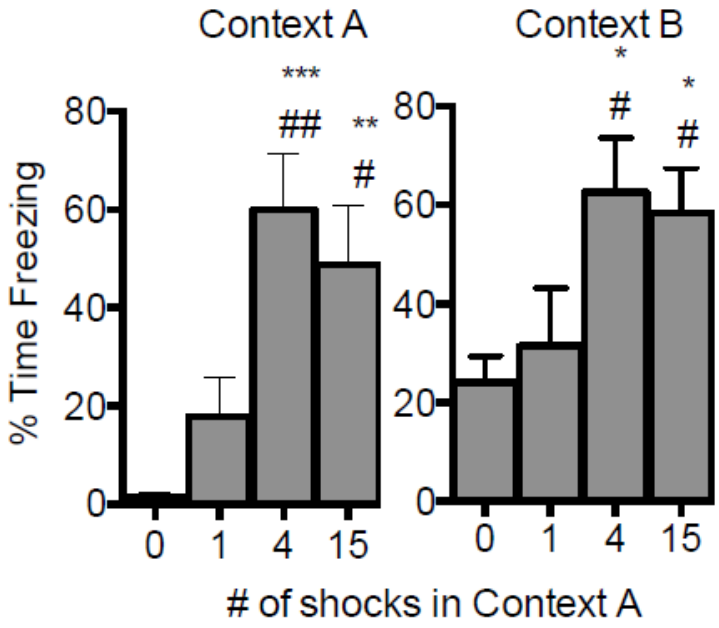


Figure 1. The stress-enhanced fear learning (SEFL) effect. (A) Overview of the design of Experiment 1. Rats received 0 (n=5), 1 (n=5), 4 (n=5), or 15 (n=6) shocks in Context A, followed by a single shock in Context B. Retention was tested in both contexts. (B) Animals that receive 4 or 15 shocks, but not 0 or 1 shocks in Context A demonstrate enhancements in freezing following a single shock in Context B. * p< .05, ** p < .01, *** p < .001 relative to 0 shocks, # p< .05, ## p < .01 relative to 1 shock. (FS: Footshock)

Experiment 2: SEFL Effect in a Context Associated with Methamphetamine

In Experiment 2 (overview shown in Figure 2A), we found that massive footshock in a different context (SHOCK) following extinction of self-administration caused a SEFL effect in the drug-seeking context (METH) but did not reinstate drug-seeking behavior. There were no reliable main effects of group or interactions involving group during acquisition, maintenance, or extinction (Figure 2B; see Table S1 in Supplemental Information). Animals averaged a total intake of 1.15 (± .09) mg/kg/session METH over the last three self-administration sessions. Animals that received massive footshock in the SHOCK context showed higher freezing in the 5 min following a single footshock in the METH context (Figure 2C; main effect of group *($F_{(1.11)}$ = 7.40, p <.01)*, time *($F_{(1.11)}$ = 31.7, p <.0001)*, and a significant interaction *($F_{(1.11)}$ = 8.8, p =.01)*); animals that had previously received massive footshock froze significantly more following exposure to a single shock than did exposure only controls ($t_{(11)}$= 2.87, p= .01).

The single footshock did not induce reinstatement of METH seeking in either group (Figure 2D). Analysis of the last day of extinction and the single footshock session revealed a decrease in lever pressing (main effect of session, ($F_{(1,11)}$= 17.27, p < .01)) and a significant main effect of lever ($F_{(1,11)}$= 7.63, p < .05) such that responding was higher on the previously active lever.

A

	Acquisition	Maintenance	Extinction	Massive Shock	Single Shock
Exp. Day	1-10	11-28	29-31	32	33
Context	METH	METH	METH	SHOCK	METH

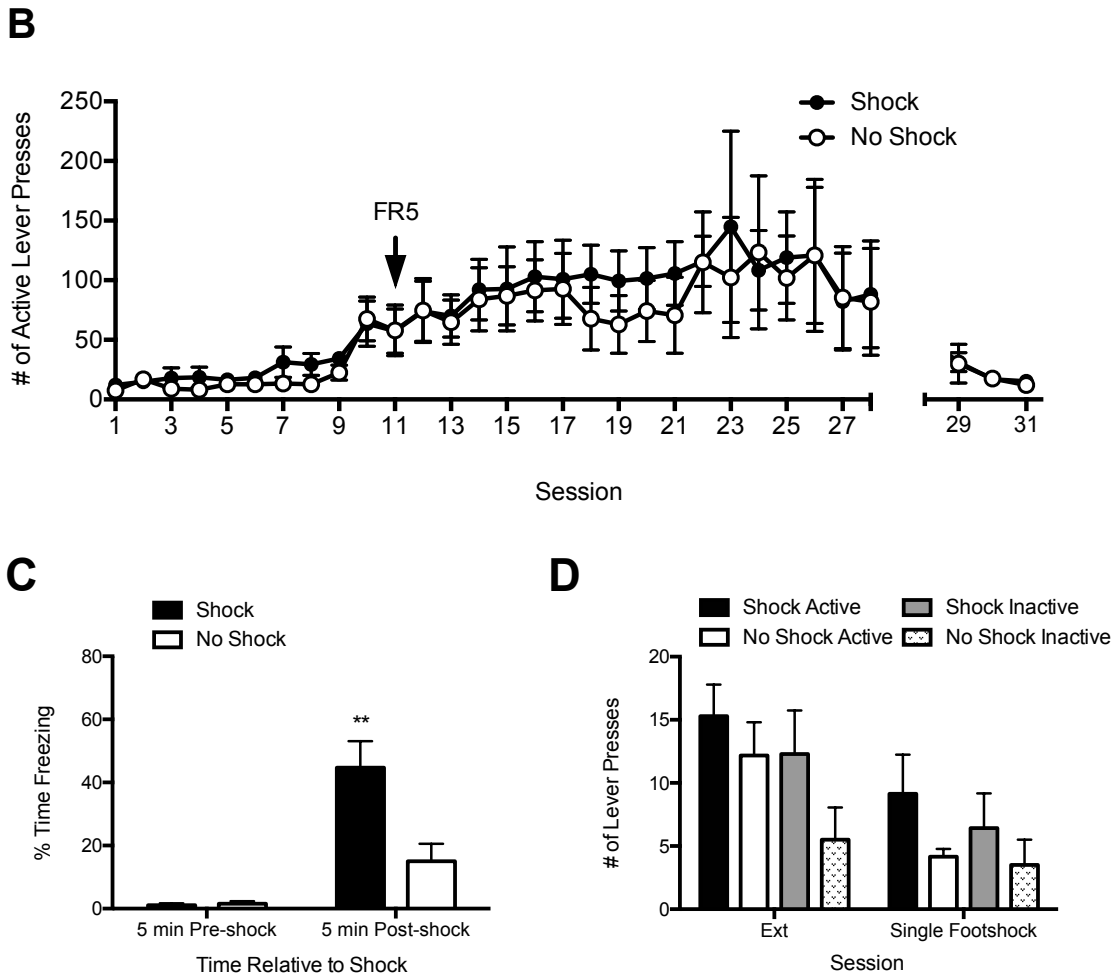


Figure 2. Effects of SEFL on freezing and reinstatement in a context associated with methamphetamine self-administration. (A) Overview of the design of Experiment 2. Rats acquired methamphetamine self-administration for 28 days, followed by 3 days of extinction, followed by either 15 shocks (n=7) or 0 shocks (n=6) in a different context (SHOCK), followed by a SEFL test in the methamphetamine context (METH). (B) Acquisition and extinction of responding for methamphetamine in groups that then received shock or no shock after extinction. (C) Freezing in the methamphetamine-associated context before and after the single shock. (D) Responding on active and inactive levers during the final extinction session (Session 31) and during the single shock reinstatement session. **p< .01

Experiment 3: SEFL Effect in a Context Associated with EtOH

In Experiment 3 (overview shown in Figure 3A), we found that massive footshock in a different context (SHOCK) caused an exaggerated long-term (60 days) fear response in the ethanol-seeking context (EtOH), even after that context had been associated with EtOH. There were no effects of shock on maintenance or extinction of ethanol-seeking (data not shown). Animals averaged an intake of .91 (± .06) g/kg/session EtOH over the last seven self-administration sessions (10% ethanol only, no sucrose). Analysis of freezing before and after the single shock in the EtOH context (Figure 3B) revealed a significant main effect of group *($F_{(1.14)}$ = 27.20, p <.0001)*, time *($F_{(1.14)}$ = 39.20, p <.0001)*, and a significant interaction *($F_{(1.14)}$ = 27.80, p <.0001)* such that animals with a history of footshock in Context A froze more than did exposure only controls ($t_{(13)}$=4.85, p < .0001).

As in Experiment 2, the SEFL effect did not induce reinstatement (Figure 3C). Analysis of the last day of extinction and the single footshock session revealed a significant main effect of session ($F_{(1,14)}$= 33.004, p < .0001) such that lever pressing decreased overall, lever x group ($F_{(1,14)}$= 13.72, p < .001), session x lever ($F_{(1,14)}$= 16.35, p < .001), but not session x group ($F_{(1,14)}$= .022, p =.884), nor lever x session x group interactions ($F_{(1,14)}$= 3.89, p > .05).

The high levels of freezing in Experiments 2 and 3 likely prevented any effect of the single shock on reinstatement to be observed. Consequently, in Experiment 4, we attempted to induce reinstatement with acute manipulations that should not result in a freezing response, such as brief restraint or exposure to drug-associated cues.

A

	Acquisition (sucrose fading)	Maintenance (10% EtOH FR5)	Massive Shock	Maintenance (10% EtOH FR5)	Extinction	Single Shock
Exp. Day	1-32	33-39	46	46-53	54-58	111
Context	EtOH	EtOH	SHOCK	EtOH	EtOH	EtOH

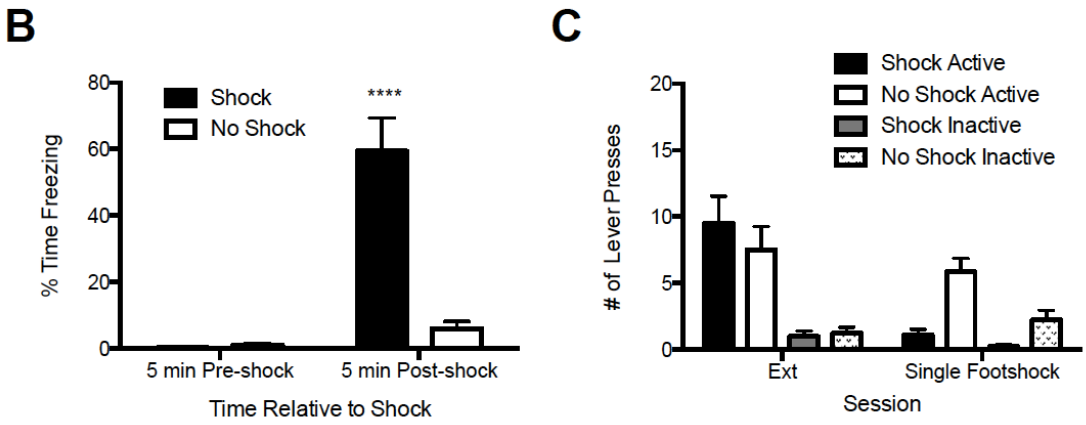


Figure 3. Effects of SEFL on freezing and reinstatement in a context associated with alcohol self-administration. (A) Overview of the design of Experiment 3. Rats were trained to respond for ethanol following a sucrose fading procedure and received 0 (n=8) or 15 shocks (n=8) in a different context during the maintenance phase. The SEFL test occurred in the ethanol-associated context after a long retention interval. (B) Freezing in the alcohol-associated context before and after the single shock. (C) Responding on active and inactive levers during the final extinction session and during the single shock reinstatement session. ****p<.0001

Experiment 4: Massive Footshock During Acquisition of METH Self-Administration in a Different Context Causes an Enhancement in Cue-Induced Reinstatement and a Resistance to Extinction

In Experiment 4 (overview shown in Figure 4A), there were no effects of shock on late acquisition, maintenance, or extinction of drug seeking (Figure 4B; no reliable main effects of group or interactions involving group; see Tables S2 and S3 in Supplemental Information), but there was a reliable increase in cue-induced reinstatement following extinction (Figure 4D), as well as a resistance to extinction following reinstatement (Figure 5). Animals averaged an intake of 1.1 (± .11) mg/kg/session METH over the last three sessions.

Restraint did not induce reinstatement in either group, but instead decreased active lever pressing (Figure 4C). Analysis of the last day of extinction and restraint-induced reinstatement revealed significant main effects of session ($F_{(1,9)}$= 7.96, p < .05) and lever ($F_{(1,9)}$= 12.78, p < .01) with no other reliable main effects or interactions (see Table S4 in Supplemental Information for statistics from reinstatement sessions).

Groups did not differ on the extinction trials that separated restraint-induced and cue-induced reinstatement (see Table S5 in Supplemental Information). Analysis of the cue-induced reinstatement session and the extinction session 24 hr prior revealed a significant session x lever x group interaction ($F_{(1,9)}$= 5.36, p = .04). The animals that had received footshock on Day 15 pressed significantly more on the active lever compared to exposure only controls during cue-induced reinstatement on Day 39 ($t_{(9)}$=2.4, p < .05; Figure 4D). The difference between groups during cue-induced reinstatement persisted through extinction that followed the cue test (Figure 5A; reliable

session x lever x group interaction ($F_{(5,45)}$= 2.46, p < .05) with higher active lever presses in the shock group).

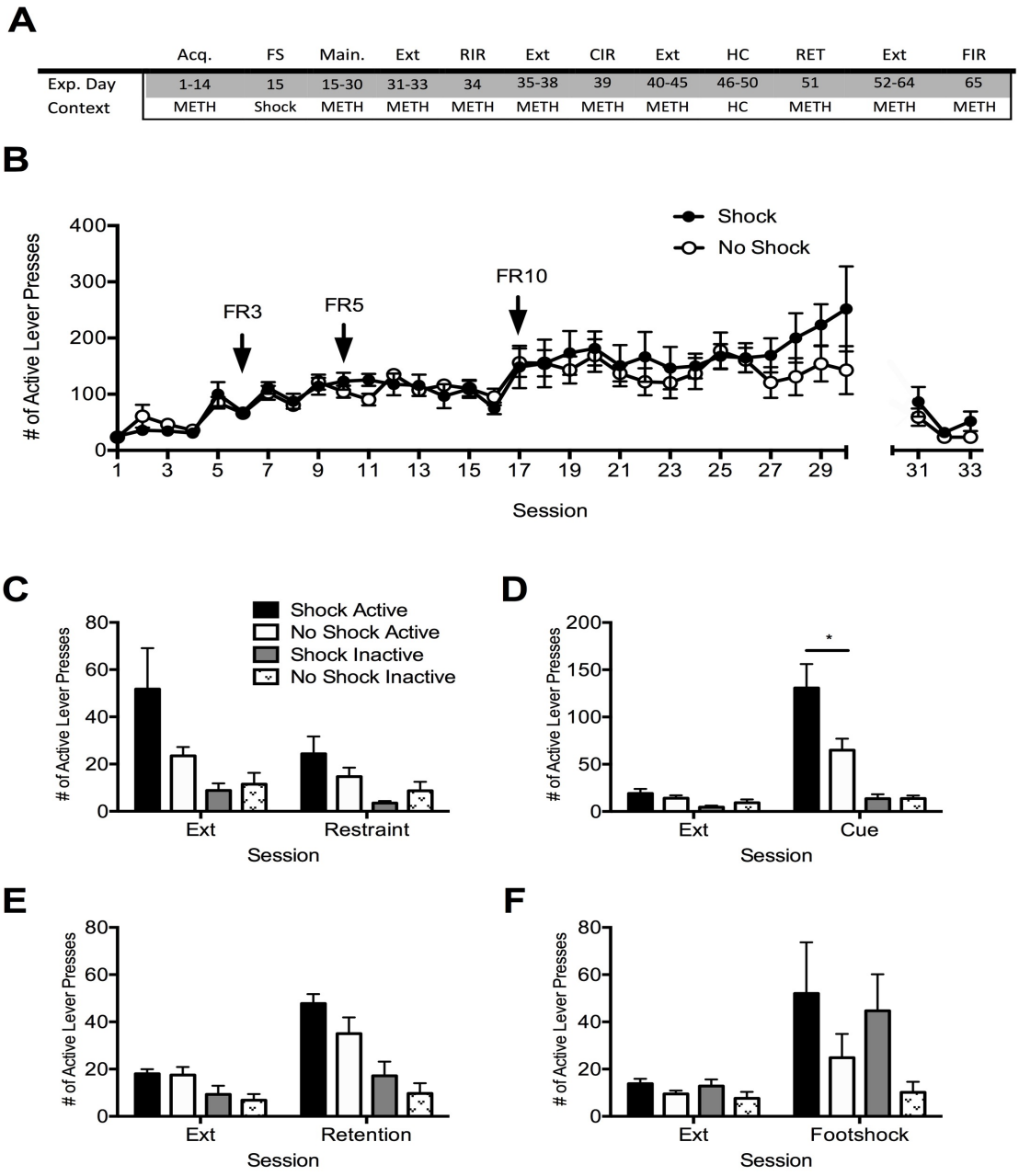


Figure 4. Effects of shock delivered during the course of acquisition of methamphetamine seeking on long-term tests of reinstatement. (A) Overview of the design of Experiment 4. Rats received 0 (n=5) or 15 shocks (n=6) in a different context during the maintenance phase. Following extinction, rats received several tests for reinstatement: restraint-induced reinstatement (RIR), cue-induced reinstatement (CIR), retention (RET), and footshock-induced reinstatement (FIR). Each reinstatement test was preceded by at least three additional extinction sessions. (B) Acquisition and extinction of methamphetamine self-administration. (C) Restraint-induced reinstatement. (D) Drug cue-induced reinstatement. (E) Spontaneous recovery retention test. (F) Footshock-induced reinstatement. *p<.05 (Acq: Acquisition; FS: Footshock; Main: Maintenance; Ext: Extinction; HC: Homecage)

Following extinction animals remained in the homecage for five days and were then tested for retention. Analysis of the retention test day and the last extinction session (six days prior) revealed a significant main effect of session ($F_{(1,9)}$= 29.14, p < .001), lever ($F_{(1,9)}$= 51.50, p < .001), and session x lever ($F_{(1,9)}$= 59.50, p < .001) such that both groups of animals significantly increased active lever pressing following a 5-day retention interval (Figure 4E).

The animals that received footshock did not extinguish as quickly as exposure only controls following the retention test (Figure 5B; significant main effect of session ($F_{(12,108)}$= 2.36, p < .01), lever ($F_{(1,9)}$= 11.7, p < .01), session x group ($F_{(12,108)}$= 2.44, p < .01), and session x lever interactions ($F_{(12,108)}$= 3.23, p < .01) during the 13 post-retention extinction sessions), as well as a trend toward a session x group x lever interaction ($F_{(12,108)}$= 2.5, p = .069). Analysis of the footshock-induced reinstatement session and the previous extinction session revealed a significant main effect of session ($F_{(1,9)}$=7.31, p= .02) and a significant effect of group ($F_{(1,9)}$=5.09, p= .05; Figure 4F) with no other significant main effects or interactions (Figure 4F).

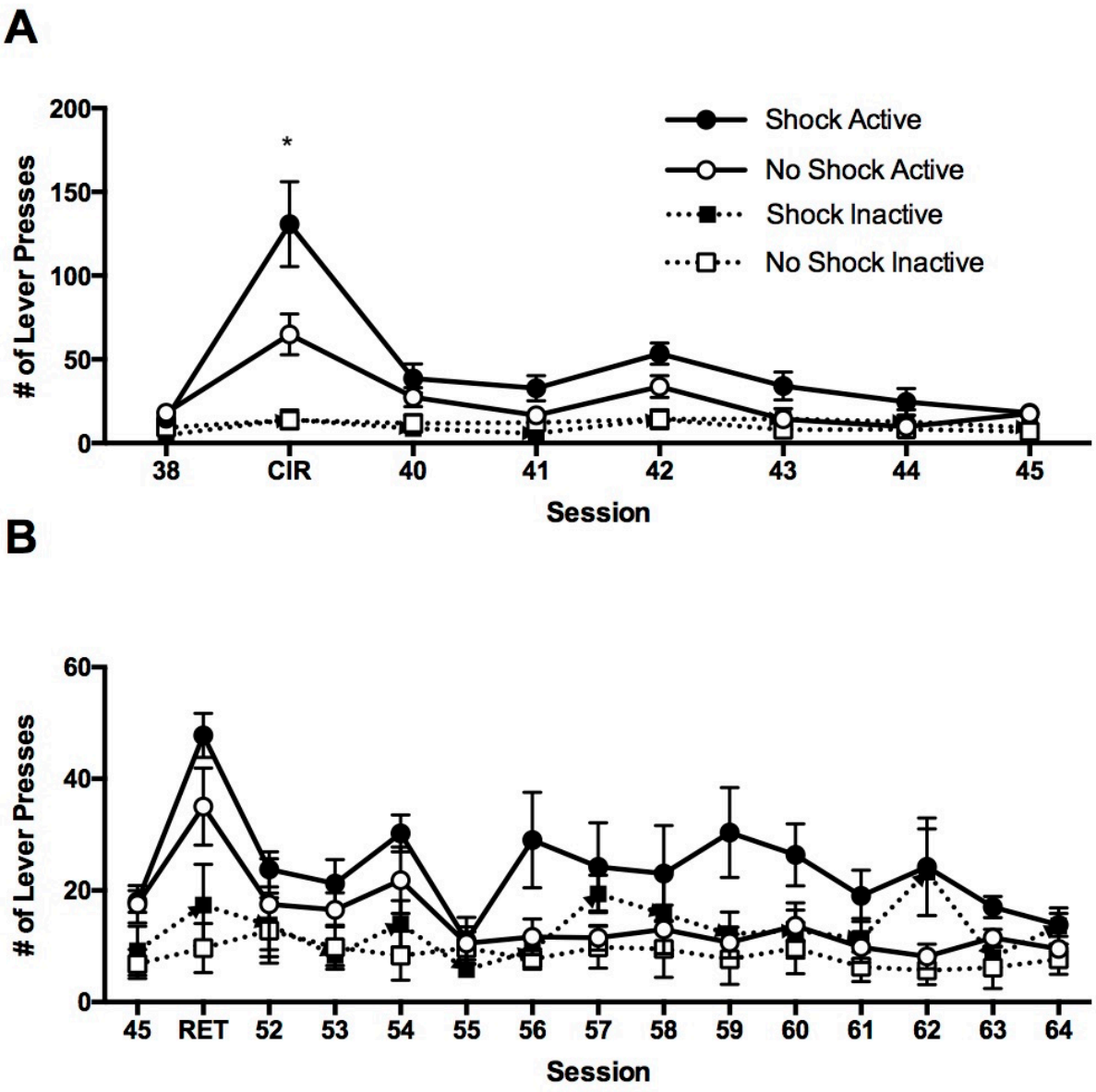


Figure 5. Persistent effects of shock on resistance to extinction of methamphetamine seeking following cue-induced reinstatement in Experiment 4. (A) Active lever pressing during the final extinction session before cue-induced reinstatement (CIR) and during six post-reinstatement extinction sessions. (B) Active lever pressing during the final extinction session before the retention test (RET; Session 51) and the 13 post-retention extinction sessions. *p< .05

Experiment 5: Massive Footshock Prior to Acquisition of Methamphetamine Seeking Enhances Cue-Induced Reinstatement and Slows Post-Reinstatement Extinction

In Experiment 5 (overview shown in Figure 6A), we found that exposure to the battery of footshocks prior to acquisition of METH self-administration increased cue-induced reinstatement after extinction 3 weeks later (Figure 6C). Massive footshock had no effect on acquisition, maintenance, or extinction of responding for METH (Figure 6B; no reliable main effects of group or interactions involving group; see Table S6 in Supplemental Information). Animals averaged an intake of 1.69 (± .12) mg/kg/session METH over the last three self-administration sessions. A repeated measures analysis of variance (RM ANOVA) conducted on the last day of extinction and cued reinstatement revealed a main effect of lever ($F_{(1,13)}$= 10.31, $p < .01$), session ($F_{(1,13)}$= 52.39 $p < .001$), lever x session ($F_{(1,13)}$= 16.54, $p < .001$), and lever x session x group ($F_{(1,13)}$= 4.99, $p < .05$); animals with a history of footshock pressed the active lever significantly more than exposure only controls ($t_{(13)}$= 1.83, $p < .05$; Figure 6C). In a final extinction session 24 hr following cued reinstatement, responding on the active ($p < .01$), but not the inactive (p =.14) lever was significantly higher in animals with a history of footshock relative to exposure only controls (Figure 6D).

A

	0 or 15 FS	Acquisition	Maintenance	Ext	CIR	Ext
Exp. Day	1	2-6	7-15	16-20	21	22
Context	SHOCK	METH	METH	METH	METH	METH

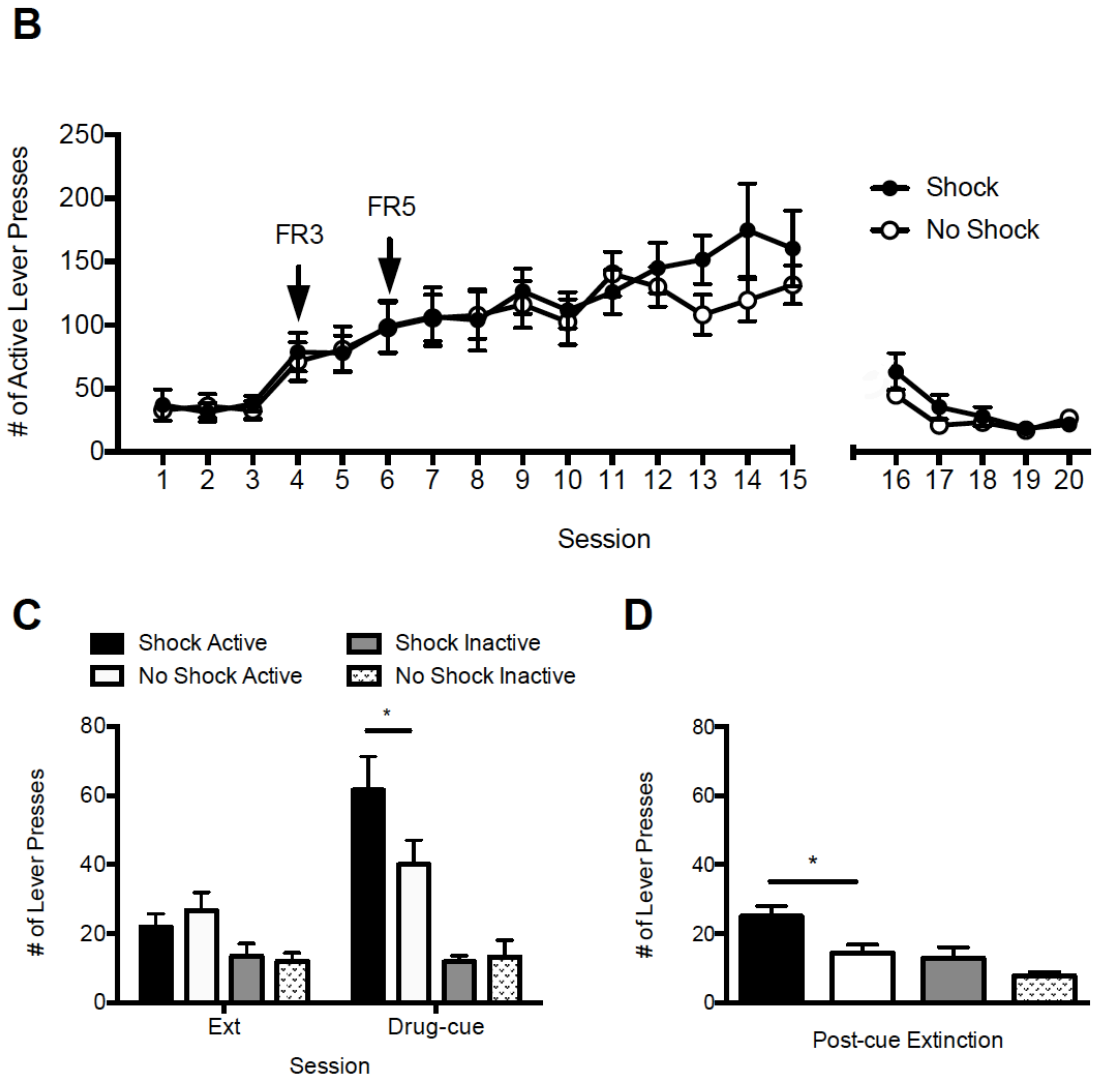


Figure 6. Effects of shock delivered prior to acquisition of methamphetamine seeking on acquisition, extinction, and cue-induced reinstatement. (A) Overview of the design of Experiment 5. Rats received 0 (n=8) or 15 shocks (n=7) in a different context (SHOCK) prior to acquisition of methamphetamine seeking (METH). (B) There were no effects of the battery of shocks on acquisition or extinction. (C) Rats with a history of shock showed greater cue-induced reinstatement following extinction. (D) This effect persisted to an extinction session the next day during which the cue was not presented. *p< .05 (FS: Footshock; Ext: Extinction; CIR: Cue-induced reinstatement)

Experiment 6: Massive Footshock in a Different Context Prior to Cocaine CPP

In Experiment 6 (overview shown in Figure 7A), we found that mice with a history of footshock showed enhanced expression of cocaine-induced CPP during preference tests conducted immediately and 24 hr after footshocks. As can be seen in Figure 7B, there were no differences between groups in activity during pretest or during the conditioning trials (CS+ or CS-). During pretest and conditioning, there was no reliable main effect of group ($F_{(1,38)}$= .005, p = .954) or group x session interaction ($F_{(1,38)}$=2.73, p = .10), but there was a main effect of session ($F_{(1,38)}$= 247.33, p < .0001*)*, with increased activity during cocaine conditioning trials (CS+). Following the shock, there again were no group differences in activity during the preference tests (ps>.05).

A RM ANOVA conducted on the pretest, Test 1, and Test 2 revealed that relative to exposure only controls, mice with a history of footshocks showed increased preference immediately (Test 1) and 24 hr later for a previously cocaine paired floor (Test 2; Figure 7C; reliable main effect of time ($F_{(1,34)}$= 105.27, p > .001), group ($F_{(1,34)}$= 5.57, p = .02), and a reliable time x group interaction ($F_{(1,34)}$= 4.57, p = .01)).

A

	Pretest	CS+ (coc)	CS- (saline)	CS+ (coc)	CS- (saline)	CS+ (coc)	CS- (saline)	CS+ (coc)	CS- (saline)	0 or 15 Footshocks	Test 1	Test 2
Exp. Day	1	2	3	4	5	6	7	8	9	10	10	11
Context	CPP	CPP	CPP	CPP	CPP	CPP	CPP	CPP	CPP	SHOCK	CPP	CPP

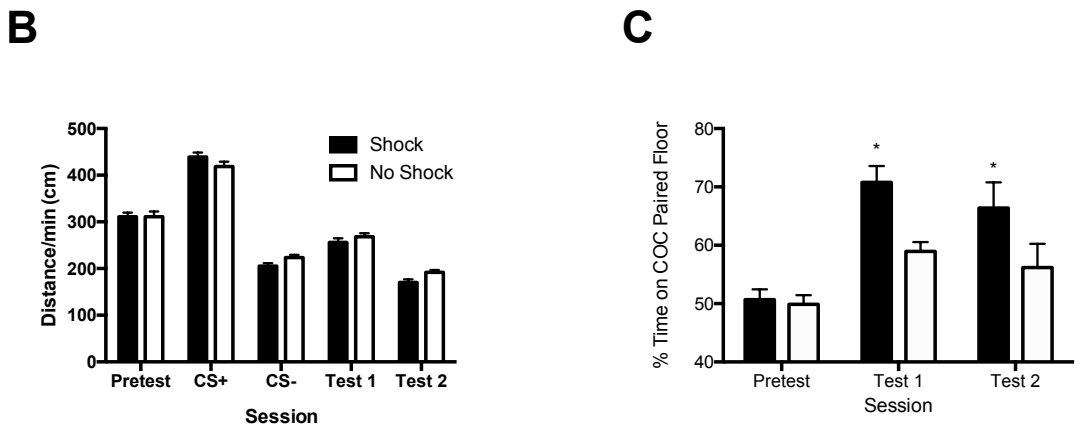


Figure 7. Effects of shock on expression of cocaine-induced CPP in mice. (A) Overview of the design of Experiment 6. Mice received pretest, CS+, and CS- conditioning trials over 5 days, followed by 0 (n=20) or 15 (n=16) shocks in a different context, followed by tests in the CPP context. (B) Activity during Pretest, conditioning trials (CS+ with cocaine; CS- with saline), and post-shock tests. (C) Relative to the No Shock controls, mice that were shocked showed increased preference for the CS+ (cocaine-paired) floor immediately (Test 1) and 24 hr after shock (Test 2). *$p<0.05$.

Experiment 7: The Effect of Massive Footshock on CORT and Hypothalamic-Pituitary-Adrenal Axis Function

In Experiment 7 (overview shown in Figure 8A), we found that animals that received 15 footshocks demonstrated significantly elevated levels of CORT relative to No Shock controls immediately following footshock (Figure 8). A two-tailed students t-test demonstrated that animals that received footshock had significantly elevated levels of CORT relative to exposure only controls (t=2.22$_{(12)}$, p < .05). A RM ANOVA on the pretest and 5 post-shock blood draws revealed no main of time, no main effect of group, and no significant interaction of time x group. A Mann-Whitney nonparametric t-test performed on CORT values following DEX administration revealed there was no difference between groups (Mann-Whitney U=14, asymptotic significance (2-tailed) p > .05). A Mann-Whitney nonparametric t-test performed on CORT following saline administration revealed there was also no difference between groups in response to saline (Mann-Whitney U=20.5, asymptotic significance (2-tailed) p > .05).

Forty-eight days following 0 or 15 footshocks in Context A animals with a history of shock demonstrated significantly elevated freezing when re-exposed to that context ($F_{(1,12)}$= 125.29, p <.001), as well as significantly elevated freezing in Context B the day after receiving a single footshock in that context ($F_{(1,12)}$= 8.87, p = .01; Figure 8D).

A

	0 or 15 FS	Blood Draw	Blood Draw	Blood Draw	Blood Draw	Blood Draw	Dex Test	Dex Test	Context A Test	1 FS	Context B Test
Expt. Day	1	7	14	21	28	35	39	40	49	59	60
Context	A	HC	HC	HC	HC	HC	HC	HC	A	B	B

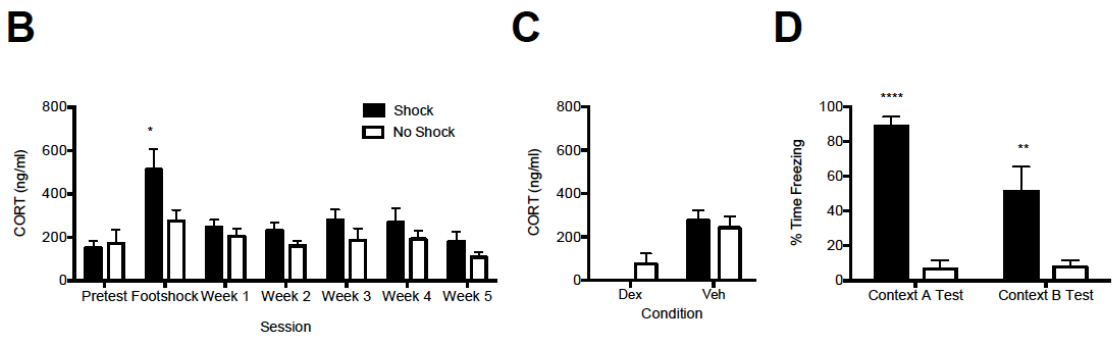


Figure 8. Effects of shock on CORT levels. **A)** Overview of the design of Experiment 7. Rats received 0 (n=7) or 15 (n=7) footshocks (FS), followed by blood draws on the day of shock and weekly thereafter in the homecage (HC). SEFL was tested on Days 49-60. **B)** Massive shock increased corticosterone (CORT) relative to exposure only controls, but this increase returned to near baseline levels during subsequent weeks. **C)** The synthetic corticosteroid dexamethasone (Dex; 50 µg/kg, SQ) induced potent negative feedback in both groups of animals. **D)** The SEFL effect occurred during a test 60 d after the initial battery of shock (Context B Test. * p <.05, **p<.01, ****p<.0001, FS: Footshock; HC: Homecage)

Discussion

These experiments show a consistent and long-lasting effect of exposure to a bout of massive footshocks in one context on fear and drug seeking in another context. This effect occurred when the shock occurred prior to or during acquisition of METH self-administration in rats and after acquisition of cocaine-induced CPP in mice. Our findings also extend the basic SEFL effect to show that it persists up to 60 days after the initial battery of shocks and that it can be revealed in a drug-seeking context even after 30 (METH) or 60 (EtOH) daily 2 hr sessions, suggesting that an extensive history of drug associations with a context does not prevent that context from revealing a SEFL effect. Together, these results suggest that this combination of massive fear conditioning and cue-induced reinstatement of drug seeking provides a strong preclinical model of the comorbidity between PTSD and substance use disorders.

It is notable that when the SEFL effect on freezing was observed, there were no effects of the single shock on reinstatement of extinguished responding. The single shock used in our SEFL procedure resulted in a strong freezing response, which was particularly true in Experiment 3 in which the single footshock completely suppressed lever pressing in the group that previously received the battery of footshocks. That likely occurred because the freezing response prevented the animals from engaging in the instrumental drug-seeking response and is consistent with many studies showing an inability of animals to perform instrumental actions in a state of high fear (e.g., Bouton & Bolles, 1980). It is also important to note that although in Experiment 1 four footshocks in Context A were sufficient to produce a robust SEFL effect, we decided to move forward with 15 footshocks in subsequent experiments for several reasons. In our

laboratory we have repeatedly found that freezing behavior in rats following four footshocks is rapidly extinguished and does not produce a lasting fear response (unpublished observations). In addition, all experiments investigating the SEFL effect (including the original report that demonstrated it persists up to 90 days) have included a 0 and 15 footshock group with no 1 or 4 footshock group. Therefore, we sought to make meaningful comparisons to the current literature by using the same experimental groups. Indeed, in Meyer et al. (2013), the report that found effects of massive footshock on ethanol drinking and initiated this series of experiments, only 0 and 15 footshock groups were used. Because refining the procedures used in animal studies is such an important endeavor, future studies investigating this effect may seek to replace the 15 footshock group with 4 footshocks.

In Experiment 4, we aimed to reveal an effect of the massive battery of footshocks on the reinstatement of drug-seeking behavior that was not confounded by differences in a freezing response during the reinstatement session. We therefore evaluated several tests that may result in a reinstatement or return of drug seeking. We found that regardless of shock history, brief restraint did not reinstate drug seeking. Previous literature has demonstrated mixed effects of restraint to induce reinstatement of drug seeking (Sanchez et al., 2003; Shalev et al., 2000). However, allowing time to pass between extinction and a subsequent test did cause spontaneous recovery of drug seeking in both groups. Animals demonstrated footshock-induced reinstatement, but this was not specific to the active lever, which is consistent with findings that shocks evoke general activity both during and soon after their presentation (Fanselow, 1982). Although other studies have documented shock-induced reinstatement, the specificity of

this effect to the drug-seeking lever is not always clear (see McFarland, 2004; Shaham et al., 1998; Lê et al., 1999; Liu & Weiss, 2002).

Our most consistent finding was that massive footshocks outside of the drug-seeking contexts resulted in an increase in cue-induced reinstatement or expression of cue-associated CPP (Experiments 4-6), even though the shocks did not immediately alter acquisition, maintenance, or extinction of drug-seeking behaviors. In general, effects on drug seeking that have been revealed during acquisition involve repeated stressors (e.g., Goeders & Guerin, 1994; Lewis et al., 2013). Shaham and Stewart (1994) found that intermittent footshock prior to several daily self-administration sessions increased the progressive ratio breakpoint for heroin. Other work has shown that stressors administered outside of the drug-associated context, including chronic tail pinch (Piazza et al., 1990), social defeat (Tidey & Miczek, 1997), or exposure to a hot plate or repeated footshocks (Ramsey & Van Ree, 1993) increase acquisition of self-administration. A more recent report in a procedure similar to ours found that repeated exposures to predator odor over 5 days had no effects on acquisition or extinction of METH seeking, but resulted in enhanced cue-induced reinstatement (Ferland et al., 2016). Thus, there is precedent for observing an effect that is specific to cue-induced reinstatement, but it remains possible that our specific parameters for acquisition or extinction were not sensitive enough to reveal differences as a function of history of shock. Exploring different response requirements or session durations would be useful for future characterizations of these effects.

The novelty of our findings is that a single, acute stressor delivered outside of the self-administration context resulted in persistent effects on drug seeking in a different

context. The effects were similar when the acute stressor occurred prior to acquisition or during maintained responding for METH. A study by Meyer et al. (2013) found that the same acute, massive shock stressor used here causes persistent changes in alcohol consumption, but only in those animals that had not established drinking patterns prior to shock. Determining how this shock stressor interacts with drug taking, drug seeking, and previous drug history will be important next steps in evaluating this model of comorbidity.

In humans, it has been repeatedly reported that individuals that are comorbid for PTSD and SUDs do not differ from individuals with SUDs alone in substance use severity (Brown, Stout & Mueller, 1999; Eggleston et al 2008), but are more likely to relapse (Tate et al., 2004; Kubiak, 2004; Burns et al., 2010; Najt, Fusar-Poli, & Brambilla, 2011). This finding is corroborated by the results in our experiments revealing no differences in acquisition or maintenance between previously shocked or unshocked rats, but shocked rats showed greater reinstatement after successful extinction. The specificity to cue-induced reinstatement makes this model a potentially powerful tool to model the comorbid condition.

One particularly interesting finding from our experiments was that even though Shock and No Shock groups did not differ at any point prior to cue-induced reinstatement, the differences that occurred during the reinstatement session persisted during subsequent drug- and cue-free tests. In Experiment 4, shocked animals continued to show elevated responding over spontaneous recovery tests and additional extinction sessions up to 25 days after the battery of footshocks. These findings suggest that there may be an interaction between a past experience of stress and exposure to a

cue previously associated with drugs that causes a persistent resistance to extinction of drug seeking.

Our finding that a battery of shocks before or during acquisition may confer an increased vulnerability to reinstatement in response to cues previously paired with drug is also consistent with human studies of addiction and PTSD. A meta-analysis of cue-induced reactivity found that the effect size for self-reported cravings in addicts following exposure to drug-related cues was large across a wide range of drugs, arguing in favor of the importance of a model of heightened cue-induced reactivity (Carter & Tiffany, 1999). It has also been shown that PTSD symptom severity correlates with self-reports of cue-elicited craving in comorbid individuals (Saladin et al., 2003). The relationship between a history of trauma and substance use may explain in part why individuals with anxiety disorders have an increased vulnerability to relapse, even following long periods of abstinence, especially in response to previously drug-paired cues (Bradizza et al., 2006). Our experiments demonstrate in rodents that this heightened cue-induced reactivity persists long-term and interferes with the extinction of drug-seeking behaviors.

Massive footshock produced significantly elevated levels of CORT relative to exposure only controls immediately following footshock, but these levels normalized quickly and remained at baseline for five weeks post footshock. A five-week range was selected to investigate if CORT levels were elevated during tests of reinstatement in Experiments 4 and 5, thus driving the enhancements in cued responding. In addition, a DEX challenge revealed that a single bout of massive footshock did not produce an alteration to the HPA axis's ability to provide reliable negative feedback. Our findings fall in agreement with human literature that individuals with PTSD do not exhibit chronically

elevated levels of cortisol (e.g., Meewisse et al., 2007; Yehuda & Seckl, 2011). Because we did not sample blood at shorter time points (30, 60, 90 min) following footshock it is unclear when exactly CORT is normalized within this study. However, we were primarily interested in investigating long-term changes in CORT that may be responsible for our observed enhancements in cued-responding. Because CORT levels over time remain the same between groups and there is no difference between groups in responses to dexamethasone it is unlikely that chronic elevations in CORT or impaired sensitivity to negative feedback are responsible for this effect. It should be noted, however, that these tests do not entirely rule out the potential for HPA involvement in these effects. Dysregulation of the HPA at the level of the pituitary or adrenal response to corticotropin releasing factor or adrenocorticotropic hormone (ACTH), respectively, may still influence behavioral responses or the development of PTSD-like symptoms. It has been well established that traumatic brain injuries (TBI) pose a substantial risk to pituitary function (reviewed in Aimaretti and Ghigo, 2005). Because TBI sustained from explosive blasts remains one of the most common injuries for combat veterans (Military Health System, 2011) dysregulation of the pituitary and subsequent abnormalities in target-organ hormone levels (Wilkindson et al., 2012) may contribute to PTSD symptomology. It has also been shown that individuals with PTSD demonstrate greater release of ACTH in response to metyrapone, indicating increased activity of the pituitary (Yehuda et al., 1996). Therefore, there remains the possibility that dysregulation of the HPA axis (in the form of alterations to pituitary function) may contribute to the expression of PTSD-like symptoms in general, as well as the behavioral enhancements in drug-seeking observed in the self-administration experiments. In addition, differences in

glucocorticoid receptor density were not assessed in these studies. Previous work has shown that individuals with PTSD express significantly elevated levels of glucocorticoid receptors on lymphocytes, even when urinary levels of cortisol were similar to controls (Yehuda et al., 1993). Future studies should continue to probe the role of the HPA in the development of PTSD, especially in cases where individuals may be comorbid for a TBI. It is also worth noting that the fear assessment to the massive footshock-associated context occurred nearly 7 weeks following footshock, suggesting the massive footshock protocol used in these studies produces persistent alterations in fear behavior, consistent with previous reports (e.g., Rau & Fanselow, 2009).

Our findings reflect a novel, interactive model of fear conditioning and drug seeking that demonstrates the ability of stress during a single session in a specific context to lead to persistent changes in drug seeking in another context. These changes include reinstatement to drug-related cues and a resistance to extinction following reinstatement. Clinical studies have shown that trauma-focused treatments are significantly more effective at improving SUDs in comorbid individuals (Hien et al., 2010), suggesting that our preclinical approach could be used in situations that may better model the clinical condition (Hariri & Holmes, 2015). Because this model involves measurable behavioral responses in the stress-associated context (freezing) and the drug-associated context (drug seeking), it can serve as a tool for understanding the relation between learned fears and substance abuse, as well as the potential to test novel therapeutic agents designed to weaken fear and attenuate reinstatement.

Supplemental Information

Table S1.

Experiment	Session	Effect	*F* value	DF	*p* value
Experiment 2	FR1	group	< 1.0	1,11	> .05
		session	2.7	8,88	< .01
		lever	7.8	1,11	> .05
		session x group	< 1.0	8,88	> .05
		session x lever	1.8	8,88	0.08
		lever x group	1.3	1,11	> .05
		session x lever x group	< 1.0	8,88	> .05
	FR5	group	< 1.0	1,11	> .05
		session	1.3	18,198	> .05
		lever	10.9	1,11	< .01
		session x group	< 1.0	18,198	> .05
		session x lever	1.5	18,198	0.07
		lever x group	< 1.0	1,11	> .05
		session x lever x group	0.1	18,198	> .05
	Extinction	group	< 1.0	1,11	> .05
		session	3.3	2,22	.052
		lever	8.1	1,11	< .05
		session x group	< 1.0	2,22	> .05
		session x lever	4.4	2,22	< .05
		lever x group	0.1	1,11	> .05
		session x lever x group	< 1.0	2,22	> .05

Table S2.

Experiment	Session	Effect	*F* value	DF	*p* value
Experiment 4	FR1	group	3.8	3,27	> .05
		session	1.7	3,27	> .05
		lever	8.8	1,9	.01
		session x group	< 1.0	3,27	> .05
		session x lever	1.6	3,27	> .05
		lever x group	< 1.0	1,9	> .05
		session x lever x group	0.9	3,27	> .05
	FR3	group	< 1.0	1,9	> .05
		session	4.9	3,27	< .01
		lever	36	1,9	< .001
		session x group	< 1.0	3,27	> .05
		session x lever	2.9	3,27	0.051
		lever x group	1.0	1,9	> .05
		session x lever x group	3.0	3,27	> .05
	FR5 (pre-shock)	group	1.5	1,9	> .05
		session	1.2	5,45	> .05
		lever	28.2	1,45	<.001
		session x group	2.1	5,45	0.08
		session x lever	< 1.0	5,45	> .05
		lever x group	1.4	1,9	> .05
		session x lever x group	< 1.0	5,45	> .05

Table S3.

Experiment	Session	Effect	*F* value	DF	*p* value
Experiment 4	FR5 (post-shock)	group	2.9	1,9	> .05
		session	15.9	1,9	< .01
		lever	15.7	1,9	< .01
		session x group	2.4	1,9	> .05
		session x lever	11.3	1,9	< .01
		lever x group	< 1.0	1,9	> .05
		session x lever x group	1.6	1,9	> .05
	FR10	group	< 1.0	1,9	> .05
		session	1.3	13,117	> .05
		lever	22.0	1,9	< .01
		session x group	1.0	13,117	> .05
		session x lever	< 1.0	13,117	> .05
		lever x group	< 1.0	1,9	> .05
		session x lever x group	< 1.0	13,117	> .05
	Extinction	group	1.4	1,9	> .05
		session	8.5	2,18	< .01
		lever	23.1	1,9	< .01
		session x group	< 1.0	2,18	> .05
		session x lever	5.8	2,18	< .05
		lever x group	2.6	2,9	> .05
		session x lever x group	< 1.0	2,18	> .05

Table S4. Tests of Reinstatement. Session in these analyses has two levels (extinction prior o to reinstatement test and the reinstatement test itself)

Experiment	Reinstatement Test	Effect	*F* value	DF	*p* value
Experiment 4	Restraint	group	1.6	1,9	> .05
		session	7.9	1,9	< .05
		lever	12.7	1,9	<. 01
		session x group	1.8	1,9	> .05
		session x lever	4.0	1,9	> .05
		lever x group	4.0	1,9	> .05
		session x lever x group	1.2	1,9	> .05
	Cue-Induced	group	3.4	1,9	0.09
		session	51.3	1,9	< .0001
		lever	51.4	1,9	< .0001
		session x group	7.1	1,9	< .05
		session x lever	37.6	1,9	<.0001
		lever x group	8.2	1,9	< .05
		session x lever x group	5.3	1,9	< .05
	Retention	group	1.1	1,9	> .05
		session	29.1	1,9	< .001
		lever	51.5	1,9	< .001
		session x group	2.6	1,9	> .05
		session x lever	59.5	1,9	< .001
		lever x group	< 1.0	1,9	> .05
		session x lever x group	2.1	1,9	> .05
	Footshock	group	5.0	1,9	0.05
		session	7.3	1,9	< .05
		lever	< 1.0	1,9	> .05
		session x group	2.7	1,9	> .05
		session x lever	< 1.0	1,9	> .05
		lever x group	< 1.0	1,9	> .05
		session x lever x group	< 1.0	1,9	> .05

Table S5.

Experiment	Session	Effect	*F* value	DF	*p* value
Experiment 4	Extinction	group	1.4	1,9	> .05
		session	8.5	2,18	< .01
		lever	23.1	1,9	< .01
		session x group	< 1.0	2,18	> .05
		session x lever	5.8	2,18	< .05
		lever x group	2.6	1,9	> .05
		session x lever x group	< 1.0	2,18	> .05
	Extinction After Restraint	group	< 1.0	1,9	> .05
		session	6.0	3,27	< .001
		lever	20.1	1,9	< .01
		session x group	1.6	3,27	> .05
		session x lever	1.4	3,27	> .05
		lever x group	4.1	1,9	> .05
		session x lever x group	< 1.0	3,27	>.05
	Extinction After Cued Reinstatement	group	2.2	1,9	> .05
		session	5.8	5,45	< .001
		lever	66.3	1,9	< .001
		session x group	< 1.0	5,45	> .05
		session x lever	9.8	5,45	< .001
		lever x group	10.7	1,9	< .01
		session x lever x group	2.4	5,45	< .05
	Extinction After Retention	group	2.7	1,9	> .05
		session	2.3	12,108	< .01
		lever	11.7	1,9	< .01
		session x group	2.4	12,108	< .01
		session x lever	3.2	12,108	< .01
		lever x group	1.3	1,9	> .05
		session x lever x group	2.5	12,108	.069

Table S6.

Experiment	Session	Effect	*F* value	DF	*p* value
Experiment 5	FR1	group	< 1.0	1,13	> .05
		session	< 1.0	2,26	> .05
		lever	20.7	1,13	.001
		session x group	< 1.0	2,26	> .05
		session x lever	< 1.0	2,26	> .05
		lever x group	< 1.0	1,13	> .05
		session x lever x group	1.2	2,26	> .05
	FR3	group	< 1.0	1,13	> .05
		session	2.7	1,13	> .05
		lever	31.5	1, 13	< .001
		session x group	< 1.0	1,13	> .05
		session x lever	< 1.0	1,13	> .05
		lever x group	< 1.0	1,13	> .05
		session x lever x group	< 1.0	1,13	> .05
	FR5	group	< 1.0	1,13	> .05
		session	6.0	9,117	< .001
		lever	34.2	1,13	< .001
		session x group	1.2	9,117	> .05
		session x lever	< 1.0	9,117	> .05
		lever x group	< 1.0	1,13	> .05
		session x lever x group	< 1.0	9,117	> .05
	Extinction	group	< 1.0	1,13	> .05
		session	9.3	4,52	< .001
		lever	49.4	1,13	< .001
		session x group	1.0	4,52	> .05
		session x lever	10.7	4,52	< .001
		lever x group	2.1	1,13	> .05
		session x lever x group	1.5	4,52	> .05

CHAPTER 3

The effects of histone deacetylase inhibitors during retrieval and extinction on stress-enhanced fear learning

Contributions: TM Navis assisted in the collection of behavioral data in the experiment involving massive behavioral extinction.

Abstract

A component of treatments for PTSD is the re-experiencing of the traumatic memory in a safe setting. One of the challenges in modeling this type of exposure therapy is that many of the rodent models of PTSD involve exposure to multiple stressors that lack a tractable behavioral measure of memory. Therefore, little is known about how extinction learning in a given context might affect fear responses in other contexts. Here, we evaluate extinction following SEFL. SEFL is a highly translatable model of PTSD in which animals that receive a massive bout of footshock in one context demonstrate exaggerated fear responses to a single footshock in a different context. Using this model we can investigate how extinction training following massive footshock in a distinct context modifies behavioral responses in non-trauma associated contexts. A failure of extinction learning to persist long-term is a particularly challenging aspect in the treatment of PTSD, but promoting learning through the use of epigenetic modulators may hold promise for enhancing extinction-related learning. Specifically, histone deacetylase inhibitors (HDACi) have been used to enhance extinction of both fear and drug-related memories, but their success in the literature is mixed, with some reports demonstrating null or detrimental findings. Here, we investigate the ability of two HDACi to enhance extinction following SEFL. We find that the both the pan-HDACi sodium butyrate (NaB and the HDAC3 specific inhibitor RGFP 966 fail to enhance the extinction of fear and do not attenuate the SEFL effect. We also find that behavioral extinction in the trauma-associated context is capable of attenuating the exaggerated fear responses observed in neutral contexts.

Introduction

Many treatments for PTSD rely on retrieval of memories, but many common laboratory approaches to PTSD do not have easily measurable memory components (e.g., chronic variable stress, single prolonged stress). SEFL is a powerful model of PTSD that takes advantage of well characterized behavior and neurobiological mechanisms involved in fear learning and leads to lasting changes in responsivity to mild shocks and to other challenges (Pizzimenti, Navis, & Lattal, 2017; Rau et al., 2005). Briefly, in this procedure animals that receive massive footshock in a specific context demonstrate significantly elevated freezing in response to a single footshock delivered in a different context. This model captures the ability of a traumatic experience to modify behavior in non-trauma associated contexts. In addition, this phenomenon has been shown to persist up to 90 days (Rau & Fanselow, 2009) and even in contexts that have long been associated with reward (Pizzimenti, Navis, & Lattal, 2017).

Exposure therapy is a key component of many treatments for PTSD. Much of what is known about the neurobiology of exposure comes from studies of extinction in the rodent lab. These studies have found that extinction, the loss of a behavioral response that was once evoked by a conditioned stimulus, does not reflect the loss of the original associative learning that produced that response (Bouton et al., 2006; Berman & Dudai, 2001, but instead reflects the development of new learning. Several behavioral phenomena (e.g., spontaneous recovery, reinstatement, rapid reacquisition) demonstrate that under certain conditions, the original learning may be recovered and expressed (reviewed in Bouton, 2002). This is likely due to several mitigating factors, particularly that extinction learning appears to be especially dependent on the context in

which the learning occurred (Vlachos et al., 2011; Maren et al., 2013). Although extinction has been examined after many different conditioning procedures, little is known about extinction in conditions that lead to a SEFL response.

Recent research has focused on ways to enhance extinction pharmacologically. One particularly promising approach is targeting epigenetic mechanisms to create lasting changes in gene expression that may be needed to inhibit salient memories. One mechanism that has been extensively characterized is promoting histone acetylation through the systemic administration of HDACi which have been shown to promote extinction learning in both fear and drug-related learning paradigms (Whittle & Singewald, 2014). Our chromosomes are comprised of DNA and related proteins wrapped tightly around histones, a material that is collectively known as chromatin. When acetyl groups are removed from the lysine tails on histones by a class of enzymes known as HDACs the chromatin structure closes, causing the histones to be packaged so tightly that genes are no longer accessible to the cellular machinery, attenuating protein synthesis (reviewed in Chen, Zhao, & Zhao, 2015). HDACi force the chromatin to remain in an open, permissive state by preventing HDACs from removing acetyl groups from histones, thus prolonging protein synthesis (reviewed in Abel & Zukin, 2008). When HDACi are paired with a learning event the chromatin structure remains open and permissive for a longer period, thus increasing the amount of protein synthesis related to that learning event.

Our current understanding of what is known about the enhancement of extinction responding using HDACi largely comes from studies using relatively mild conditioning, which may not accurately capture the etiology of PTSD, nor the role of histone

acetylation and HDACs in fear learning. Indeed, Federman et al (2009) found that H3 acetylation occurred only following strong fear training, suggesting that the involvement of acetylation in fear conditioning is dependent on the degree of conditioning. Therefore, using this model we can investigate the ability of behavioral extinction to attenuate the exaggerated fear response to a neutral context in a strong model of PTSD.

It has previously been shown that HDACi can promote the extinction of fear learning (Stafford et al., 2012) and the extinction of drug-seeking behavior (Malvaez et al., 2010). However, the ability of HDACi to promote extinction learning remains mixed in the literature, with some demonstrations that HDACi have null or deleterious effects on extinction. Specifically, Bowers et al. (2015) found that the Class I HDACi RGFP 963 enhanced extinction of cued fear, but RGFP 966, a strong inhibitor of HDAC3, did not significantly enhance consolidation of cued fear extinction. Others have found that knockout of HDAC1 does not affect cued or contextual fear learning or extinction (Morris et al., 2013) or that knockout of HDAC1 prevents extinction of contextual fear (Bahari-Javan et al., 2012).

In this paper, we first probe the ability of moderate (Experiment 1) and relatively strong (Experiment 2) behavioral extinction to attenuate the SEFL effect. There is evidence that animals trained during the light cycle acquire fear faster, extinguish fear more slowly, and demonstrate greater levels of contextual fear when tested (Albrecht & Stork, 2017; Chaudhury & Colwell, 2002) when compared with dark trained animals. Therefore, in Experiment 3 we also investigate the role of the light cycle in influencing behavioral responses to the extinction of fear in this model.

We also investigate the ability of two HDACi to promote the extinction of fear learning following massive footshock. In Experiment 4, we use NaB, a pan-HDACi that inhibits the activity of all classes of HDACs. Because HDAC3 is the most highly expressed Class I HDAC in the brain and has previously been shown to be a critical regulator of learning and memory (McQuown et al., 2011) we use the HDAC3 specific inhibitor RGFP 966 with combination with brief (Experiment 5) and extended (Experiment 6) extinction. Together, these experiments shed light on the role of behavioral extinction in augmenting fear responses to mild or neutral stimuli following exposure to a massive bout of footshock.

Methods and Materials

Animals

Two-hundred and two male, Long Evans rats (Charles River) that weighed 275-300 g (~9-11 weeks of age) at the start of the experiments were single housed in a temperature (22 °C ± 1 °C) and humidity-controlled (70%) vivarium and were maintained on a 12/12 hr dark/light cycle (lights off 6:00 am/lights on 6:00 pm). Experiments were performed during the dark phase of the light cycle, with the exception of Experiment 11 which was partially conducted during the light phase. Animals were given access to food and water *ad libitum* throughout the duration of the experiment. Housing conditions and treatment of these animals were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and conducted in accordance with the ethical guidelines of the National Institutes of Health.

Drugs

NaB (Sigma) was dissolved in sterile water and administered at a dose of 1.2 g/kg (Malvaez et al., 2010) via an IP injection. RGFP 966 (Abcam) was dissolved in 75% PEG 400 and 25% 6.25mM sodium acetate and administered at a dose of 10 mg/kg (Malvaez et al., 2013) via an SQ injection.

Apparatus

Context A: Conditioning chambers (exterior dimensions: 31.8cm L x 25.4 W x 26.7cm H, Med Associates, St. Albans, VT) were housed within sound attenuating chambers (Med Associates, St. Albans, VT). Chambers were outfitted with a houselight centered on the top panel that was illuminated throughout the duration of each session. Grid floors composed of 19 stainless steel rods (0.48 cm diameter with 1.6cm spacing between them) were connected to shocker/scramblers (Med Associates, St. Albans, VT) that delivered footshock. During conditioning sessions red light was used to illuminate this room. Between cohorts 0.5% bleach was used to clean the chambers.

Context B: Conditioning chambers (exterior dimensions: 31.8cm L x 25.4cm W x 34.3cm H, Med Associates, St. Albans, VT) housed within sound attenuating cubicles (Med Associates, St. Albans, VT) were located in a different room than Context A. In addition to a houselight that was illuminated throughout the duration of each session, these chambers were outfitted with two retracted operant levers with a stimulus light above each lever (these stimulus lights were never illuminated during these experiments). Grid floors were identical to those used in Context A. During conditioning

sessions white light was used to illuminate this room, and 95% ethanol was used between cohorts to clean the chambers.

General Procedures

Stress-Enhanced Fear Learning

This procedure was adapted from the paradigm originally developed by Rau, DeCola, & Fanselow in 2005. Animals are exposed to 0 or 15 footshocks in Context A (variable ISI, 4-8 min, 1 mA, 1 sec) over the course of 93 min. Twenty-four hours later animals are reexposed to Context A during a 12 min test during which no shocks are presented (in Experiments 1 and 2 an 8.5 min test was used). The following day all animals, regardless of their treatment in Context A receive a single, 1 mA footshock in Context B following a 3 min and 12 second delay. All animals remain in Context B for an additional 30 seconds following footshock before being removed. Twenty-four hours later animals are tested for fear behavior in Context B during a 12 min test during which no shocks are delivered (in Experiments 1 and 2 an 8.5 min test was used).

Freezing

Freezing behavior is interpreted as an index of fear, where higher levels of freezing indicate higher levels of fear. Freezing behavior, defined as the lack of movement except that which is required for breathing, was sampled every 8 s.

Experiment 1: Moderate Behavioral Extinction Does Not Attenuate the SEFL Effect

All animals received 15 footshocks (variable ISI, 4-8 min, 1 mA, 1 sec) over 93 min in Context A (contexts were counterbalanced across groups). The following day animals received either 1 min (N=8), 6 min (N=8), or 30 min (N=8) of extinction in Context A or 0 min of extinction but were handled in the homecage (N=8). Twenty-four hours all animals received a single footshock in Context B, followed by an 8.5 min Context B test the following day. Twenty-four hours later animals were re-exposed to Context A during an 8.5 min test.

Experiment 2: Massive Behavioral Extinction Attenuates the SEFL Effect

Animals received (*N*=23) 15 footshocks (variable ISI, 4-8 min, 1 mA, 1 sec) or 0 footshocks (*N*=24) over 93 min in Context A. The following day half of the animals that received 15 footshocks (*N*=11) and half of the animals that received 0 footshocks (*N*=12) returned to Context A and were given 30 min of extinction, which was repeated once per day for two additional days resulting in 3, once daily 30 min extinction sessions. Twenty-four hours later all animals received a single 1 mA, 1 sec footshock in Context B; the following day fear was assessed during an 8 min 26 second Context Test. The following day animals were re-exposed to Context A.

Experiment 3: SEFL Training During the Light Cycle Promotes Acquisition of Fear

All animals received 15 footshocks (variable ISI, 4-8 min, 1 mA, 1 sec) over 93 min in Context A; half of the animals (*N*=16) received footshock beginning 2 hours into the

dark cycle, half (*N*=16) received footshock 2 hours into the light cycle. Animals that were conditioned during the dark cycle received all subsequent behavioral training/tests in the dark cycle, and all animals that were conditioned during the light cycle received all subsequent behavioral training/tests in the light cycle at the same time of day as initial conditioning. Twenty-four hours following conditioning animals were returned to Context A and were given 30 min of extinction, which was repeated once per day for two additional days resulting in 3, once daily 30 min extinction sessions. The next day animals received a single 1 mA, 1 sec footshock in Context B; the following day fear was assessed during a 12 min test. Twenty four hours later all animals were returned to Context A and fear was assessed during a 12 min test.

Experiment 4: Sodium Butyrate Prevents Extinction

All animals received 15 footshocks (variable ISI, 4-8 min, 1 mA, 1 sec) over 93 min in Context A. The following day animals either received 3 (n=16) or 24 (n=15) min of extinction in Context A, or were handled in the homecage (n=16). Half of the animals from each extinction group received NaB immediately following extinction, and half received saline. Twenty-four hours later all animals were tested for contextual fear during a 12 min test in Context A. The next day all animals received a single 1 mA, 1 sec footshock in Context B; twenty-four hours later contextual fear was assessed during a 12 min test.

Experiment 5: The HDAC3 Specific Inhibitor RGFP 966 Does Not Enhance Moderate Extinction

Animals received 15 footshocks in Context A (counterbalanced across contexts). The following day all animals received 6 min of extinction in Context A. Animals received an injection of vehicle (N=10) or RGFP 966 (N=10; 10 mg/kg, SQ) 10 min prior to extinction. Twenty-four hours later all animals received a single, 1 mA footshock in Context B. The following day animals received a 8.5 min Context B test, followed by a 12 min Context A test 24 hr later.

Experiment 6: The HDAC3 Specific Inhibitor RGFP 966 Does Not Enhance Extended Extinction

Animals received 15 footshocks in Context A (counterbalanced across contexts). The following day all animals received 30 min of extinction in Context A. Animals received an injection of vehicle (N=12) or RGFP 966 (N=12; 10 mg/kg, SQ) 10 min prior to extinction. Twenty-four hours later all animals received a single, 1 mA footshock in Context B. The following day animals returned to Context B for a 12 min test. Twenty-four hours later animals were returned to Context B for a 2nd 12 min test, followed by a 3rd 12 min test in Context B the following day. Twenty-four hours later animals were exposed to Context A during a 12 min test. The next day all animals received a single footshock in Context A, followed by a 2nd 12 min Context A test 24 hr later.

Results

Experiment 1: Moderate Behavioral Extinction Does Not Attenuate the SEFL Effect

There was a main effect of time ($F_{(3,81)}$=86.79, p < .001) but no main effect of group nor a group x time interaction (p < .05) during acquisition of fear (see Figure 9A). Freezing levels during extinction are shown in Figure 9B.

There was a main effect of time ($F_{(1,27)}$=60.83, p < .001) following a single footshock in Context B such that freezing increased in all groups following footshock, but there was no main effect of group nor a group x time interaction (p > .05; Figure 9C). A one way ANOVA revealed there were no differences between groups during an 8.5 min Context B Test ($F_{(3,30)}$= 1.56, p = .22; see Figure 9D). However, a simple t-test comparing the 0 min and 30 min extinction groups revealed a significant (p < .05) difference in levels of freezing. There were also no differences between groups during an 8.5 min Context A Test ($F_{(3,30)}$= 2.06, p = .12; see Figure 9E).

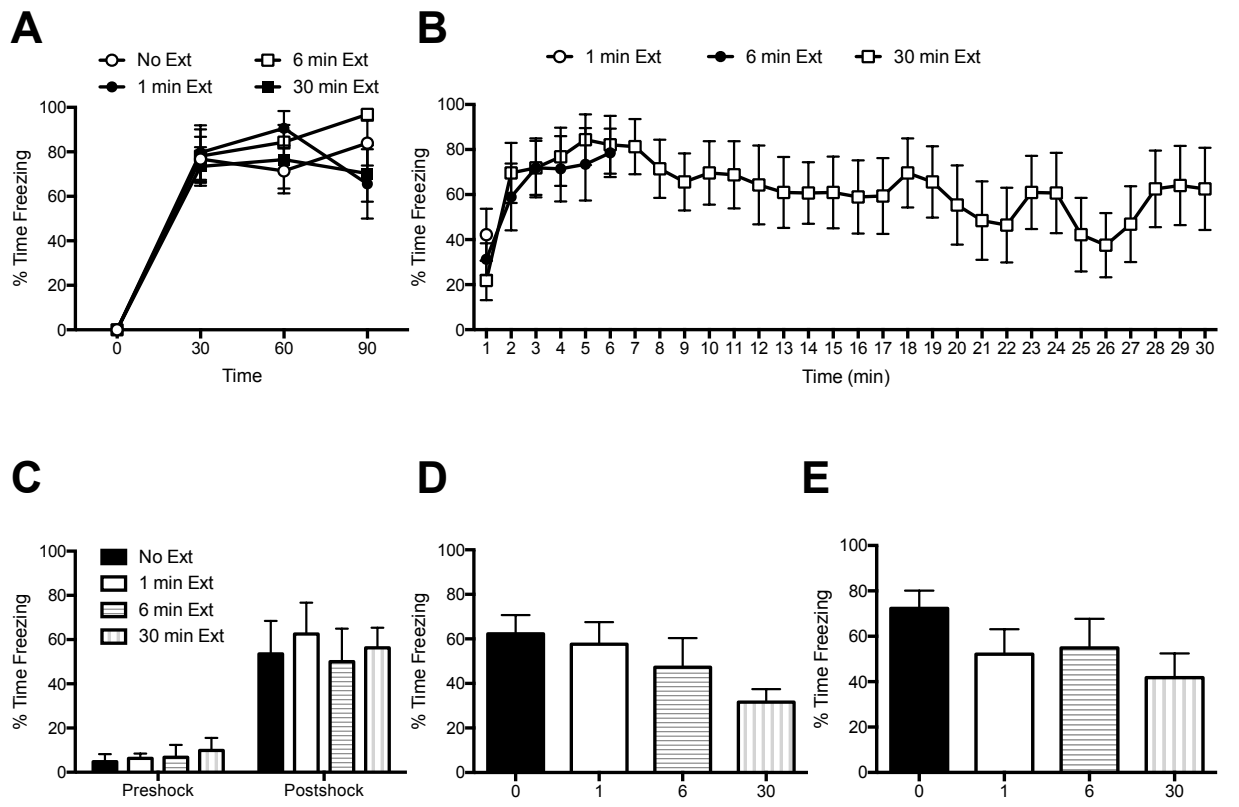


Figure 9. Moderate Behavioral Extinction Does Not Attenuate the SEFL Effect. **A)** There were no significant differences between groups during the acquisition of fear in Context A ($p > .05$). **B)** Percent time freezing during 1 min (n=8), 6 min (n=8), or 30 min (n=8) extinction session in Context A in 1 min bins. **C)** Prior to footshock in Context B, all animals, regardless of extinction training in Context A, demonstrated low levels of freezing. Following a single footshock in Context B, all animals demonstrated significantly elevated levels of freezing ($p < .01$). **D)** There were no significant differences between groups during an 8.5 min Context B test. **E)** There were no significant differences between groups during an 8.5 min Context A test.

Experiment 2: Massive Behavioral Extinction Attenuates the SEFL Effect

There was a main effect of time ($F_{(9,189)}$= 4.61, p < .001), a main effect of group ($F_{(1, 21)}$= 22.07, p < .001), and a significant time x group interaction ($F_{(9,189)}$=6.29, p < .001) on the first day of extinction such that animals that received 15 footshocks the day prior demonstrated significantly elevated freezing during the first 7 of 10 time bins (p < .05; see Figure 10B). There were no differences between groups on extinction day 2, nor on extinction day 3 (p > .05; see Figure 10B).

There was a significant main effect of group following a single foot shock in Context B ($F_{(3,43)}$=15.1, p < .001), a significant main effect of time ($F_{(1,43)}$=41.12, p < .001), with freezing increasing in general following a single footshock, and a significant time x group interaction ($F_{(3,43)}$=7.78, p < .001). Animals that received 15 footshocks and no extinction in Context A demonstrated significantly elevated freezing relative to animals that had received 15 footshocks and 3 x 30 min extinction in Context A (p < .001), with both groups demonstrating significantly elevated freezing relative to the 0 footshocks groups (p < .001); there was no difference between 0 footshocks groups (p > .05; see Figure 10C).

During a Context B Test there was a significant main effect of time ($F_{(8,344)}$= 6.62, p < .001, group ($F_{(3,43)}$=11.88, p < .001), and a significant group x time interaction ($F_{(24,344)}$= 2.70, p < .001). There was no difference in freezing between animals that received 15 footshocks and 3 x 30 min extinction in Context A and animals that had received 0 footshocks (regardless of extinction condition; p > .05). Animals that received 15 footshocks and 3 x 30 min extinction in Context A demonstrated significantly lower

levels of freezing relative to animals that received 15 footshocks with no extinction (p < .05; see Figure 10D).

During a Context A Test there was a main effect of time ($F_{(8,24)}$=4.29, p < .001), a main effect of group ($F_{(3,43)}$=58.58, p <.001), and a significant time x group interaction ($F_{(24,344)}$=2.84, p < .001). There was no difference between the 15 footshocks + extinction, 0 footshocks + extinction, and the 0 footshocks + no extinction groups (p > .05), but the 15 footshocks + no extinction animals demonstrated significantly elevated freezing during the Context A Test relative to all other groups (p < .001; see Figure 10E).

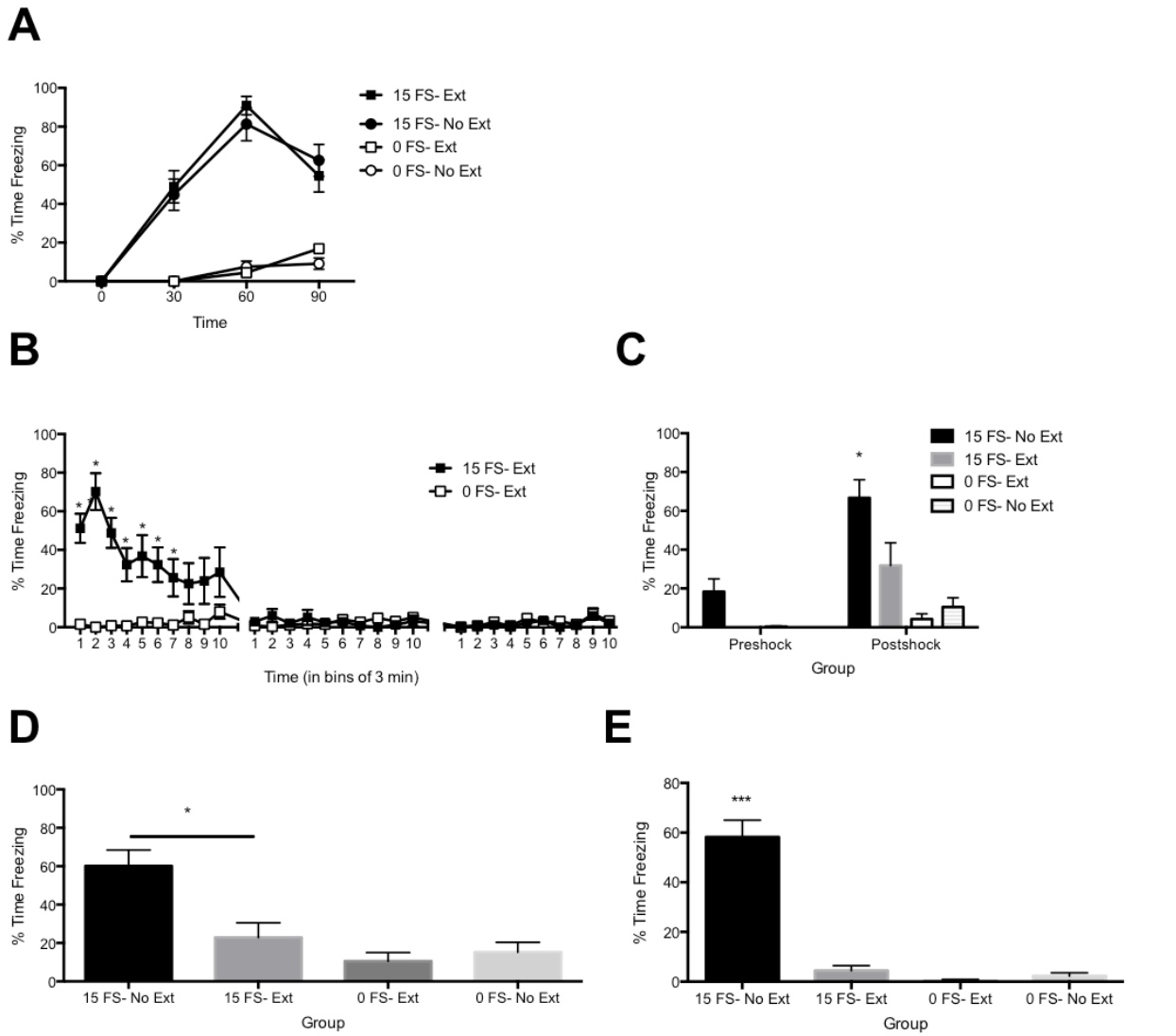


Figure 10. Massive Behavioral Extinction Attenuates the SEFL Effect. A) Animals receiving 15 footshocks (n=23) in Context A demonstrated significantly elevated freezing relative to exposure only controls (n=24; $p < .05$). **B)** On Day 2 animals with a history of footshock in Context A (n=11) demonstrated significantly elevated freezing relative to exposure only controls (n=12) during the first 21 min of a 30 min extinction session in Context A ($p < .05$). There were no differences between groups at any time during the second (Day 3) and third (Day 4) 30 min extinction session ($p > .05$). **C)** Prior to a single footshock in Context B, all animals demonstrated low levels of fear. Following a single footshock animals with a history of footshock in Context A and no extinction demonstrated significantly elevated levels of freezing relative to all other groups ($p < .05$). **D)** During a Context B test animals that had received 15 footshocks in Context A and no extinction demonstrated significantly elevated levels of freezing ($p < .05$) relative to all other groups. Animals that received 15 footshocks and extinction in Context A demonstrated levels of fear that were consistent with animals without a history of footshock. **E)** During a Context A test animals that had received 15 footshocks and no extinction demonstrated significantly elevated levels of freezing ($p < .05$) relative to all other groups. * $p < .05$, *** $p < .001$

Experiment 3: SEFL Training During the Light Phase Promotes Acquisition of Fear

On experimental day 1 (15 footshocks in Context A) animals trained during the light phase demonstrated significantly elevated freezing during the first 30 min of the paradigm as evidenced by a significant main effect of time ($F_{(29, 870)}$= 50.168, p < .0001) and a significant time x group interaction ($F_{(29,870)}$=1.975, p=.002). There was no main effect of group ($F_{(1,30)}$= 1.96, p=1.72; see Figures 11A and 11B). There were no significant differences between groups during any day of extinction training (see Figure 11C).

Following a single footshock in Context B there was a significant main effect of time (p < .001) such that all animals increased freezing following footshock, and a trend towards a significant interaction of time x group (p = .06; see Figure 11D).

A Context B Test revealed a main effect of group ($F_{(1,30)}$= 3.86, p = . 05), with animals trained during the light phase demonstrating significantly elevated levels of freezing, and a main effect of time ($F_{(11,330)}$=6.155, p < .0001), but no interaction of time x group ($F_{(11,330)}$= .860, p= .580; see Figure 11E).

A Context A Test revealed no main effect of time ($F_{(11,330)}$= .864, p= .57), no main effect of group ($F_{(1, 30)}$= 1.1, p =.295), and no interaction of group x time ($F_{(11,330)}$ = 1.02, p = .42).

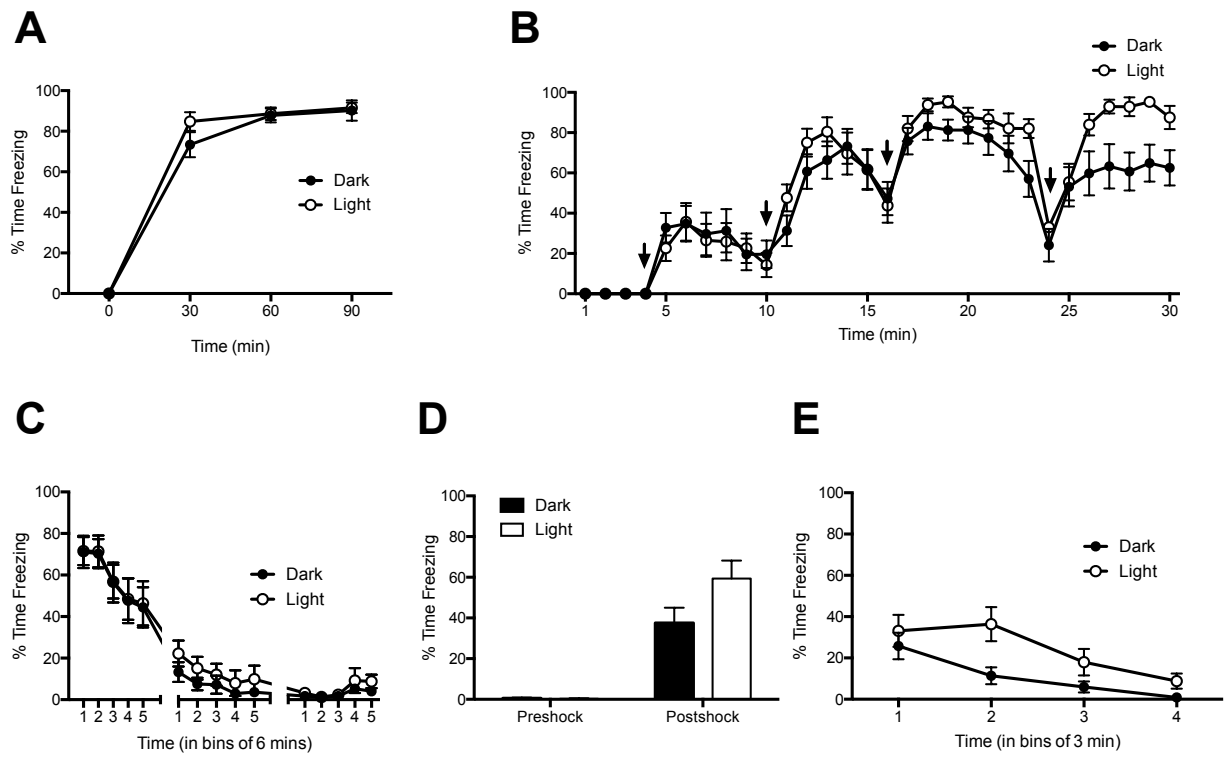


Figure 11. SEFL Training During the Light Phase Promotes Acquisition of Fear. A) Sampled freezing (in bins of 3 min) at 0, 30, 60, and 90 minutes during a 93 min footshock conditioning session. **B)** Animals fear conditioned during the light phase (n=16) demonstrate significantly elevated levels of freezing relative to animals conditioned during the dark phase (n=16) during the first 30 min of a 93 min footshock paradigm (p <.05). **C)** There were no differences in freezing levels between groups on any day of extinction (p > .05). **D)** Prior to delivery of a single footshock in Context B animals in both groups demonstrated low levels of fear; following a single footshock in Context B both groups animals demonstrated significantly elevated levels of freezing. **E)** Animals that were trained and tested during the light cycle demonstrated significantly elevated levels of freezing during a Context B test (p < .05). ↓ indicates a footshock was delivered

Experiment 4: Sodium Butyrate Prevents Extinction Consolidation

There was a main effect of time ($F_{(3,123)}$=101.41, p < .0001) , but no main effect of group ($F_{(1,41)}$=.045, p =.99) nor a group x time interaction ($F_{(15,123)}$=.11, p =1) during acquisition of fear in Context A (see Figure 12A). Levels of freezing did not differ between groups receiving the same length of extinction *(*p > .05; see Figure 12B).

A mixed factorial 2 x 3 RM ANOVA performed on freezing behavior during a 12 min Context A Test revealed a main effect of time ($F_{(3,123)}$= 9.88, p < .001), a main effect of length of time extinguished ($F_{(2,41)}$=4.00, p = .02), a trend toward a significant interaction of time x length of time extinguished ($F_{(6,123)}$=1.8, p =.09), a significant time x drug treatment interaction ($F_{(3,123)}$= 1.17, p = .05), and a significant time x length of time extinguished x drug treatment interaction ($F_{(6,123)}$=3.23, p < .01). All other main effects and interactions were not significant (p > .05; see Figure 12C).

All animals showed a significant increase in freezing following a single footshock in Context B (main effect of time; $F_{(1,41)}$= 107.31, p < .001). There was a significant interaction of drug treatment and extinction time ($F_{(2,41)}$= 3.74 p = .03), and a trend toward an interaction of time x length of time extinguished ($F_{(2,41)}$=2.84, p = .07). All other main effects and interactions were not significant (p > .05; see Figure 12D).

A Context B Test revealed a significant main effect of time ($F_{(3,123)}$= 9.77, p < .001) such that, in general, freezing decreased over time, and a significant interaction of drug treatment x time extinguished ($F_{(2,41)}$= 3.15, p = .05; see Figure 12E). All other main effects and interactions were not significant (p > .05).

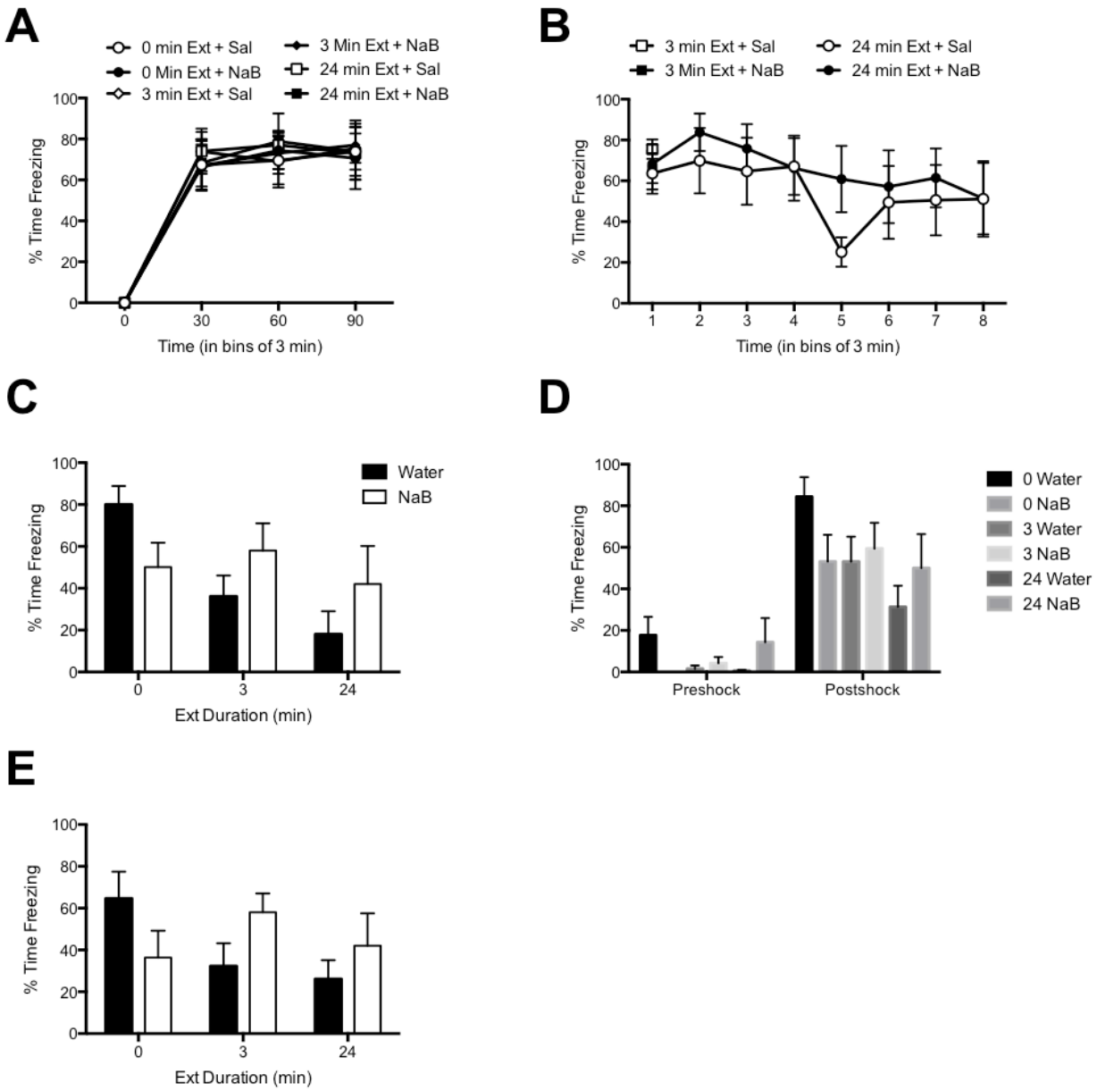


Figure 12. Sodium Butyrate Prevents Extinction. A) There were no differences between groups during acquisition of fear in Context A ($p > .05$). **B)** Animals received 3 (n=16) or 24 min (n=15) min extinction in Context A (displayed in 3 min bins). **C)** During a Context A test there was a significant time x group x length of time extinguished interaction such that NaB promoted fear in animals that received extinction training. **D)** Prior to the onset of footshock in Context B all animals demonstrated low levels of fear; following a single footshock all animals demonstrated significantly elevated levels of fear relative to the preshock period. **E)** During a Context B test there was a significant interaction of drug treatment x length of time extinguished. (Ext: Extinction)

Experiment 5: The HDAC3 Specific Inhibitor RGFP 966 Does Not Enhance Brief (6 min) Extinction

There was a main effect of time ($F_{(3,54)}$= 102.83, p < .001) but no main effect of group, nor a group x time interaction during acquisition (p > .05; see Figure 13A). There was a main effect of time during extinction ($F_{(1,18)}$=25.02, p < .001) such that freezing increased in both groups over time, but no main effect of group nor a group x time interaction (p > .05 see Figure 13B).

Following a single footshock in Context B there was a main effect of time such that all animals increased freezing ($F_{(1,18)}$= 11.34, p < .001), but there was no main effect of group nor a group x time interaction (p > .05; see Figure 13C).

There was a main effect of time such that freezing decreased over time ($F_{(8,144)}$= 4.72, p < .001) during a Context B Test, but no main effect of group nor a group x time interaction (p > .05; see Figure 13D). There was a main effect of time ($F_{(8,144)}$=4.72 ,' p < .001) during a Context A Test, but no main effect of group nor a group x time interaction (p > .05; see Figure 13E).

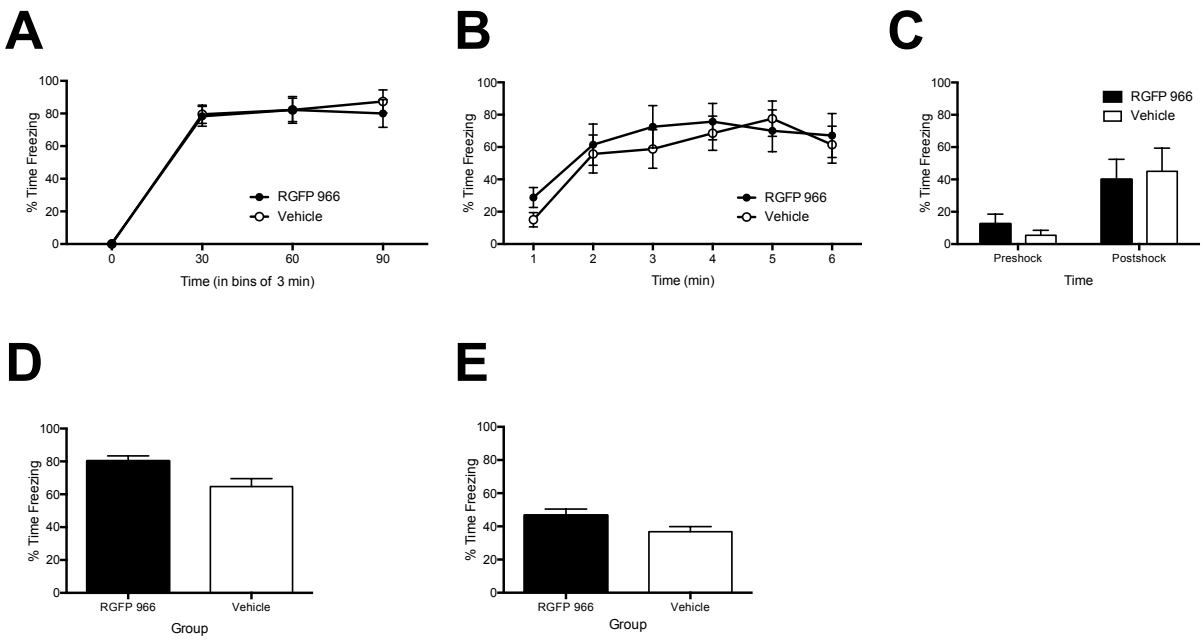


Figure 13. RGFP 966 Does not Enhance Brief Extinction. A) There was no difference between groups during the acquisition of fear in Context A ($p > .05$). **B)** Groups did not differ during a 6 min ext session in Context A ($p < .05$). **C)** Following a single footshock in Context B both vehicle (n=10) and RGFP 966 (n=10) treated animals demonstrated significantly elevated levels of freezing ($p < .05$), although there was no difference between groups ($p > .05$). **D)** There was no difference between groups during a Context B Test ($p > .05$). **E)** There was no difference between groups during a Context A test ($p > .05$).

Experiment 6: The HDAC3 Specific Inhibitor RGFP 966 Does Not Enhance Extended Extinction

There was a main effect of time ($F_{(3,66)}$= 112.29, p < .001) , but no main effect of group nor a group x time interaction during acquisition (p > .05; see Figure 14A). There was a main effect of time ($F_{(9,198)}$= 7.10, p < .001), but no main effect of group nor a group x time interaction during extinction (p > .05; see Figure 14B).

There was a main effect of time ($F_{(1,22)}$= 84.40, p < .001) such that both groups increased freezing following a single footshock in Context B, but there was no main effect of group, nor a group x time interaction (p > .05; see Figure 14C).

There was a main effect of time ($F_{(11,242)}$= 17.12, p < .001) such that freezing decreased over time, but no main effect of group, nor a group by time interaction during Context B Test 1 (p > .05). There was a main effect of time ($F_{(11,242)}$= 2.87, p = .01) such that freezing decreased over time, but no main effect of group, nor a group by time interaction (p > .05) during Context B Test 2. There was no main effect of time or group, nor a group by time interaction (p > .05) during Context B Test 3 (see Figure 14D).

There was no main effect of time or group (p > .05), but there was a trend toward a time x group interaction ($F_{(11,242)}$= 1.65, p = .08) during Context A Test 1 (see Figure 14E). There was a main effect of time ($F_{(1,22)}$= 28.71, p < .001) such that both groups increased freezing following a single footshock in Context A, but there was no main effect of group, nor a group x time interaction (p > .05; see Figure 14F). There was no main effect of group (p > .05), but there was a main effect of time ($F_{(11,242)}$= 3.54, p <

.001) and a significant time x group interaction ($F_{(11,242)}$= 1.81, p = .05) during Context A Test 2 (see Figure 14G). Posthoc analysis did not reveal significance (p > .05).

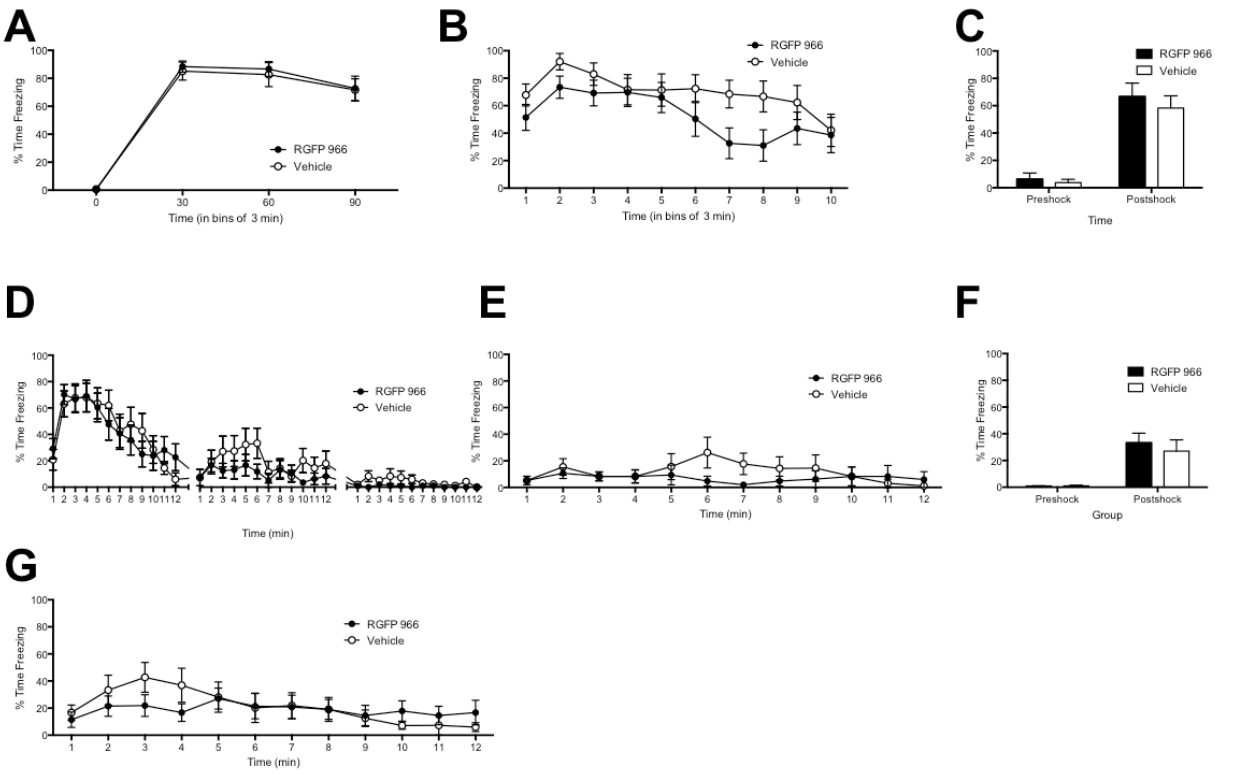


Figure 14. RGFP 966 Does not Enhance Extended Extinction. A) All animals received 15 footshocks in Context A. **B)** There was no significant difference in levels of freezing between groups during a 30 min extinction session in Context A. **C)** Both vehicle (n=12) and RGFP 966 treated (n=12) animals demonstrated low levels of freezing prior to the onset of footshock in Context B ($p > .05$); following footshock all animals demonstrated a significant enhancement in freezing ($p < .05$). **D)** There was no difference in levels of freezing between groups during any 12 min test in Context B (Context B Test 1, Context B Test 2, and Context B Test 3 shown in 1 min bins). **E)** There was no difference between groups in levels of freezing during a 12 min Context A Test. Both groups showed minimal levels of freezing throughout the session. **F)** All animals, regardless of drug treatment, demonstrated extremely low levels of freezing prior to the onset of footshock in Context A. Following footshock, all animals significantly elevated freezing levels, but there were no differences between groups. **G)** There was a significant interaction of time x group ($p < .05$) such that the vehicle treated animals demonstrated elevated levels of fear early on, but these levels drop over the course of the session.

Discussion

These experiments demonstrate that SEFL produces long-lasting alterations in fear responses to mild stimuli across a number of conditions. In general, the most robust finding from these experiments is that the length of behavioral extinction is the most powerful predictor of attenuated fear responses following massive footshock. In Experiment 1 we probed the ability of behavioral extinction to attenuate the SEFL effect by testing different lengths of extinction (1, 6, or 30 min) in Context A and found that none attenuated the SEFL effect in Context B. However, when the data was analyzed using a simple t-test to compare the 0 min extinction group with the 30 min extinction group (rather than a RM ANOVA on all four groups) we did find that 30 min extinction attenuated the SEFL effect. This suggested that longer lengths of behavioral extinction may in fact confer a protective effect in this model.

In Experiment 2 we therefore investigated if over-extinction was capable of attenuating the SEFL response and found that massive behavioral extinction attenuated the SEFL effect entirely. This finding is at odds with previously published data that found that five rounds of behavioral extinction in animals with a history of massive footshock was still incapable of attenuating the SEFL effect (Rau, De Cola, & Fanselow, 2005). Both experiments used the same strain of rat and identical behavioral procedures, but the animals used in this report were housed in a reverse light/dark facility; the animals used in Rau's experiments were not. Previous work has demonstrated that animals that receive fear conditioning during the light phase demonstrate significantly elevated levels of freezing relative to animals conditioned during the dark phase, even when animals are housed in constant darkness, and show greater levels of contextual freezing

following conditioning (Chaundry & Cowell, 2002). We therefore hypothesized that differences in behavioral training and testing as they related to the light cycle might account for the difference between experiments. In Experiment 3 we sought to address this by performing identical experiments during different phases of the light cycle.

As has been previously shown, animals that received footshock during the light phase demonstrated significantly elevated freezing during the first third of the acquisition paradigm. While this difference was not maintained during the entire footshock period it is likely that both groups reached a ceiling following multiple inescapable footshocks that washed out any potential group differences. Although there were no group differences during extinction we found that animals that were trained and tested during the light phase demonstrated significantly elevated levels of freezing during a Context B test. However, the difference between groups in this experiment is less dramatic than the difference between our data and Rau's, suggesting that additional factors may contribute to the persistence of the SEFL effect. Other potential mitigating factors may include the type of bedding, cage material, or differences in personnel. These data contribute to the growing evidence that circadian time influences behavioral responses in fear related paradigms (reviewed in Albrecht & Stork, 2017). It is important to note that the time of day is a confound in these experiments. Animals trained and tested during the dark phase of the light cycle were run beginning at 8 am, while animals trained and tested during the light phase of the light cycle were run beginning at 8 pm. Therefore, animals differed on both phase of light cycle and time of day. It is unclear if differences in behavior were driven by differences in learning that occurred as a result of the light cycle or were simply artifacts of performance differences

influenced by the light cycle. Because animals were not tested under common conditions, inferences about what drives behavioral differences should be made cautiously. Nonetheless, these data corroborate previous findings that demonstrate enhanced fear learning during the light phase of the light/dark cycle (Chaudhury & Colwell, 2002).

Because massive, but not moderate, behavioral extinction was capable of attenuating the SEFL effect, we next investigated if pharmacological intervention with an HDACi could promote the extinction of fear in this model. HDACi have previously been shown to promote the extinction of fear behavior (Stafford et al., 2012) and drug-seeking behavior (Malvaez et al., 2010; Malvaez et al., 2013). In Experiments 4 and 5 we investigated the ability of the pan-HDAC inhibitor NaB (Experiment 4) and the Class I HDACi RGFP 966 (Experiment 5) to enhance extinction and attenuate fear responses. In general, NaB seemed to promote fear, regardless of the length of extinction; rats that were given either 3 or 24 min extinction demonstrated an ordinal trend towards greater fear when NaB was administered prior to extinction. RGFP 966 appeared to have no effect on extinction learning (see Figure 13 & 14).

Others have found little to no effect of HDACi on memory extinction (Guan et al., 2009; Morris et al., 2013) or that there must be a significant reduction of fear during an extinction session (within session extinction) in order for HDACi to influence behavior (Whittle et al., 2013). A careful review of the mixed results on these findings reveals that different learning models recruit different classes of HDACs that are paradigm specific (Fischer et al., 2010) and acetylation patterns that are region-, task-, and age-specific (Graff & Tsai, 2013). In addition, the specific activity of many HDACi remain elusive.

For example, both RGFP 963 and RGFP 966 are Class I HDACi. However, Bowers et al. (2015) demonstrated that when administered to mice at a dose of 10mg/kg, RGFP 963 reached brain levels of capable of inhibiting HDAC1, HDAC2, and HDAC3; RGFP 966 administered at the same dose (10mg/kg, the same as the dose used in this paper) only reached levels capable of inhibiting only HDAC3, while minimally inhibiting HDAC1 and HDAC2. Therefore, it is clear that future studies should pay careful attention to the role of specific HDACs in the form of learning being studied, as well as the ability of a given HDACi to influence the activity of those HDACs.

The exact mechanism through which HDACi promote the extinction of fear remains poorly understood. Despite their emerging use as therapeutic agents, it has been shown that many genes are sheltered from global histone acetylation. Microarray expression analysis revealed altered transcript levels in only ~9% of genes in HL60 cells that were treated with different HDACi (vaproic acid, TSA, & suberoylanilide hydroxamic acid; Halsall et al., 2012). Interestingly, these HDACi produced similar levels of up- and down-regulation and rarely produced acetylation at the site of gene promoters. Therefore, although HDACi have been shown to promote acetylation, including at the site of gene promoters (reviewed in Struhl, 1998), it is unclear if it is through their effect on acetylation that HDACi influence memory. It is also important to note that in these studies we were unable to confirm that HDACi administration affected acetylation levels, as animals could not be euthanized following administration. Often, the molecular effects of HDACi are confirmed through immunohistochemistry using antibodies for acetylated lysine residues on histones (e.g., Lys14 on H3, Stafford et al., 2012). In the

studies described herein, we could not confirm the acetylation effects because animals continued to be tested for several days following injection of HDACi.

Taken together these experiments demonstrate that SEFL is a powerful model of PTSD that is resistant to mild behavioral extinction, but the stress-induced enhancement in fear may be augmented through massive behavioral extinction. Although HDACi failed to promote extinction in this model, future studies should systematically investigate the specific HDACs involved in this learning pattern and the brain regions that demonstrate hyperacetylation. Understanding the specific involvement of HDACs will allow investigators to select the most appropriate HDACi and subsequently refine the use the HDACi as therapeutic options.

CHAPTER 4

The effect of a histone decetylase-3 specific inhibitor on extinction in the comorbid PTSD-SUD model

Contributions: TM Navis and E Kim assisted in the collection of behavioral data.

Abstract

Relapse to drugs of abuse remains one of the most persistent challenges in maintaining long-term abstinence. This challenge is especially true in individuals who are comorbid for PTSD, as this disorder increases the likelihood of relapse, especially to drug-related cues. Current strategies for treating SUDs include forms of behavioral extinction therapy during which individuals learn to inhibit drug-seeking responses elicited by previously drug-salient cues. The problem with this approach, however, is that extinction learning is especially context dependent and when individuals encounter cues in their home environment this extinction learning often fails and individuals relapse. One strategy to promote extinction learning across time and contexts has been the use of HDACi, which have previously been shown to promote extinction learning in other drug-related learning paradigms. HDACi modify chromatin structure and allow for increased protein synthesis; when paired with a learning event like behavioral extinction HDACi may promote better long-term extinction-memory retention and prevent relapse. In this paper, we investigate the ability of a novel HDACi, KDAC 0008, to attenuate cue-induced reinstatement in a rodent model of the comorbid PTSD-SUD condition in which rodents are trained to self-administer IV METH following massive footshock in a distinct environment or exposure only to that context. We find that, similar to previous reports, footshock in a distinct context produces long-term enhancements in drug-seeking in the form of significantly enhanced responding to previously drug-paired cues, but KDAC 0008 failed to attenuate this enhancement and did not prevent reinstatement in exposure only controls.

Introduction

PTSD and SUDs are highly comorbid. Approximately half of the individuals seeking treatment for SUDs meet the diagnostic criteria for PTSD (Brady et al., 2004), an estimate over 5 times greater than the average lifetime prevalence of SUDs in the United States (Kessler et al., 2005). Treatment outcomes for comorbid individuals seeking treatment for their SUD are poorer than those without PTSD, with individuals reporting greater amounts of craving for drugs of abuse (Drapkin et al., 2011; Saladin et al., 2003; Simpson et al., 2012) and higher rates of relapse (Brady et al., 2004).

The high co-occurrence of PTSD and SUDs suggest they share a common etiology, although the exact nature of the relationship between the two disorders remains unclear. It is, however, clear that regardless of which disorder develops first, PTSD and SUDs influence one another. Comorbid individuals tend to suffer from more severe PTSD symptoms (McCauley et al., 2012) and an improvement in PTSD symptom severity is associated with a decrease in substance use (Back et al., 2006). Indeed, one study found during a 6-month post-treatment follow up that individuals whose PTSD symptoms had remitted reported significantly less substance use than individuals whose symptoms persisted (Ouimette et al., 1998). One prospective study monitored individuals with PTSD and concurrent alcohol/cocaine dependence or substance dependence alone for 28 days following last substance use. They found that over the 28 day period PTSD-related symptoms significantly declined, regardless of withdrawal substance, even though none of the patients were seeking treatment for PTSD at that time (Coffey et al., 2007). These results suggest that treatments that

target maintaining abstinence from drugs of abuse may also improve symptoms of PTSD in comorbid individuals.

One common barrier to prolonged abstinence from drugs of abuse is relapse. Individuals seeking treatment for SUDs may develop skills to resist the urge to use drugs in controlled environments, like a rehabilitation center, but these strategies often fail when individuals return home. Current strategies to improve long-term treatment outcomes have focused on strengthening the inhibitory learning that prevents drug seeking in response to salient cues. One promising avenue has been through the use HDACi. HDACi prevent the de-acetylation of lysine residues on histones that causes the chromatin structure to close, which attenuates protein synthesis. Therefore, HDACi may promote learning by forcing the chromatin structure to remain in an open, permissive state, and when paired with a discrete learning event, increase the synthesis of proteins related to that learning. HDACi have previously been shown to promote extinction learning in various drug-related learning paradigms in rodents. Mice treated with the pan-HDAC inhibitor sodium butyrate (NaB) extinguish a cocaine-induced conditioned place preference more quickly and to a greater degree than do vehicle-treated animals (Malvaez et al., 2010; Malvaez et al., 2013). NaB also enhances extinction of a conditioned place preference for morphine (Wang et al., 2010).

In this paper, we examine the ability of HDACi to enhance extinction of drug seeking in a newly developed rodent model of the comorbid PTSD-SUD condition. It has recently been shown that animals that experience massive footshock prior to the onset of self-administration of METH demonstrate a significant increase in reinstatement to previously drug-paired cues (Pizzimenti et al., 2017). Using this model

we can test the ability of new therapeutic options to attenuate this enhancement. Here, we employ the use of the novel HDACi KDAC 0008 to promote extinction. KDAC 0008 is highly selective for HDAC3, the most highly expressed Class I HDAC in the brain, is rapidly detectable in the brain following systemic administration, and levels remain high for over 3 hours following injection. Because levels of KDAC 008 remain stable over a long period of time we are able to affect a larger window of consolidation as it occurs over time. Here we replicate the basic finding that animals with a history of footshock in a distinct environment demonstrate a significant enhancement in cue-induced responding following extinction. We also find that animals that received KDAC 0008 paired with extinction did not significantly differ from vehicle treated animals during a test of cue-induced reinstatement.

Methods and Materials

Animals

Twenty male Long Evans rats (Charles River) were pair housed in a temperature (22 °C ± 1 °C) and humidity-controlled (70%) vivarium and were maintained on a 12/12 hr light/dark cycle (6:00 am/6:00 pm). Following surgery, animals were single housed, and three days prior to the initiation of self-administration training animals were food restricted to ~90% free feeding body weight (average 380 grams). Housing conditions and treatment of these animals were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and conducted in accordance with the ethical guidelines of the National Institutes of Health.

Drugs

METH (Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline and administered IV as 0.06mg/kg/infusion over 5 sec. KDAC 00 (KDAc Therapeutics, Cambridge, MA) was dissolved in 45% PEG 400, 45% saline, and 10% DMSO and was administered at a dose of 10 mg/kg IP.

Apparatus

Massive Footshock (Context A)

Massive footshock took place in operant conditioning chambers (Med Associates, St. Albans, VT; 31.8cm L x 25.4 W x 26.7cm H) housed within sound attenuating cubicles (Med Associates, ST Albans, VT). These chambers were different dimensions than the conditioning chambers used for self administration of METH and were located in a different room. In each chamber there was only a houselight on the front center panel; no other cues, levers, or stimuli of any kind were present. Grid floors composed of 19 stainless steel rods (0.48 cm diameter with 1.6cm spacing between them) were connected to shocker/scramblers (Med Associates, St. Albans, VT) that delivered footshock. Between cohorts, 0.5% bleach was used to clean the chambers.

Self-administration of IV Methamphetamine (Context B)

METH self-administration sessions were conducted in operant conditioning chambers (Med Associates, St. Albans, VT; 30.5 cm x 24.1 cm x 29.2 cm) housed within sound attenuating cubicles (Med Associates, St. Albans, VT). The syringe pumps (Med

Associates, St. Albans, VT) that delivered drug were located outside the sound attenuating chambers. Each chamber was outfitted with two retractable levers, a stimulus light above each lever, and a houselight that was illuminated throughout the duration of every session. Between cohorts, 95% EtOH was used to clean these chambers.

General Procedures

Jugular Catheter Surgery

Catheter Assembly. Jugular catheters were made of 12 cm long silastic tubing (0.037 mm ID, 0.94 mm OD; Dow Corning, Midland, MI) with small beads of 100% silicone rubber sealant at 8.5 cm and 9 cm, respectively. One end of the catheter was inserted into the right jugular vein and run subcutaneously below the front right leg to exit the back between the shoulder blades. A stainless steel guide cannula (22 ga; Plastics One, Roanoke, VA) was inserted into an elastomer self-administration harness (Instech Laboratories, Plymouth Meeting, PA) and the jugular catheter was attached to the cannula within the harness.

Surgery. Anesthesia was induced with an intramuscular (IM) injection of ketamine/xylazine (85 mg/kg; 10 mg/kg), and was maintained throughout the duration of the surgery by vaporized isoflurane (1%). Catheters were implanted and connected to the harnesses as described above. Following surgery animals received a daily IV infusion of 0.1 ml 100 unit heparin and the antibiotic Timentin (238 mg/ml). On days when animals performed self-administration, an IV infusion of 0.1 ml 10 unit heparin was also administered prior to the session.

Self-Administration of Methamphetamine

At the onset of each session a houselight was illuminated and two retractable levers were inserted into the chamber. One lever was designated the active lever, and upon completion of the FR requirement 88.5 µl of METH was administered IV over 5 sec (0.06 mg/kg/infusion), and the stimulus light above the active lever was concurrently illuminated for 5 sec. The inactive lever also had a stimulus light above it; however, pressing the inactive lever did not result in any programmed consequences. Levers were counterbalanced across animals. During extinction sessions, presses on either lever did not result in any programmed consequences. During cue-induced reinstatement sessions a press on the active lever resulted in the activation of the stimulus light above the active lever for 5 sec, but no drug was delivered.

Experiment 1: The effects of KDAC 00 on extinction in the comorbid PTSD-SUD model

Twenty rats received either 15 footshocks (1 mA, ISI 4-8 min, *N*=10) or 0 footshocks (*N*=10) over a 93 minute session on Day 1 in Context A. Twenty-four hours later all animals began IV self-administration of METH in Context B. All animals received 4 hr of FR1 training for two consecutive days. Following extended access, animals were moved to 2 hr FR1 training followed by FR3, then FR5 training according to each animal's performance (all FR3 and FR5 sessions were 2 hr). Therefore, all animals did not receive the same number of FR1, FR3, and FR5 sessions, nor the same total number of self-administration sessions. All animals completed a minimum of 3 FR5 sessions before moving on to extinction. All animals received 3 extinction sessions. Thirty

minutes prior to the first extinction session animals received either an IP injection of vehicle or KDAC 00 (10mg/kg) resulting in the following groups: No Shock + Veh (*N*=5), No Shock + KDAC 00 (*N*=5), Shock + Veh (*N*=5), and Shock + KDAC 00 (*N*=5). All animals subsequently completed two additional extinction sessions once per day for a total of 3 extinction sessions (all extinction sessions lated 2 hr). The day after the third extinction session all animals underwent cue-induced reinstatement during which a press on the previously active lever resulted in activation of the stimulus-light above the lever for 5 sec, but no drug was delivered (total session length of 2 hr). Twenty-four hours later all animals completed a 2 hr extinction session.

Results

Freezing During SEFL

During fear conditioning there was a main effect of time ($F_{(3,48)}$=19.58, $p < .001$), a main effect of shock ($F_{(1,16)}$=45.22, $p < .001$), and a main effect of shock x time ($F_{(3,48)}$=8.9, $p < .001$), such that animals that received footshock significantly increased their freezing behavior relative to exposure only controls over time (see Figure 15A). All other main effects and interactions were not significant (see Table S7).

Self-Administration and Cued Reinstatement

There were no differences between groups during the final 3 FR5 IV METH self-administration sessions that preceded reinstatement (see Figure 15B; Table S8). Animals averaged an intake of 1.43 (± .04) mg/kg/session METH over the final three

FR5 sessions. During cued reinstatement there was a main effect of session ($F_{(1,16)}$= 14.72, $p < .01$) and a main effect of lever ($F_{(1,16)}$=23.42, $p < .01$) such that all animals elevated responding on the previously active lever in the presence of drug-paired cues. Animals with a history of footshock demonstrated significantly elevated responding during cued reinstatement as evidenced by a significant session x shock interaction ($F_{(1,16)}$=4.77, p =.04); this elevated responding was specific to the active lever (lever x shock; ($F_{(1,16)}$=5.38, p =.03); see Figure15C). All other main effects and interactions were not significant (see Table S9).

During the final extinction session following reinstatement there was a main effect of lever ($F_{(1,16)}$=14.26, $p < .01$) such that all animals pressed more on the active lever. All other main effects and interactions were not significant (see Supplementary Table S10).

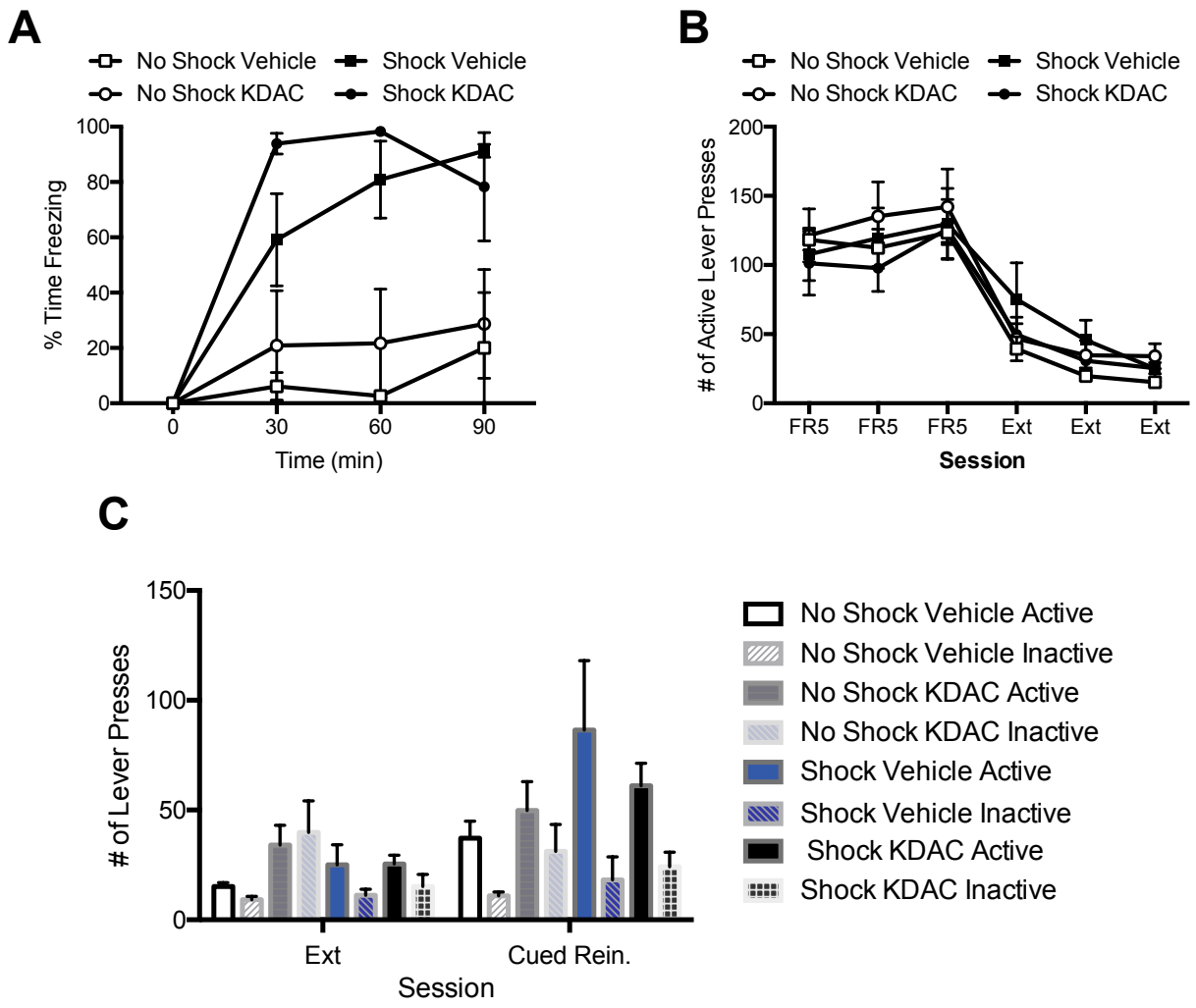


Figure 15. Effects of KDAC 0008 During Extinction of METH Self Administration. A) Animals that received 15 footshocks (n=10) demonstrated significantly elevated freezing relative to animals that received exposure to that context only (n=10). There were no significant differences in freezing between groups that both received shock or both received exposure only. **B)** Active lever pressing during the final 3 FR5 sessions and 3 extinction sessions that preceded cue-induced reinstatement did not differ between groups ($p > .05$). **C)** All animals reinstated to drug-paired cues, however animals that received footshock, regardless of drug condition, demonstrated significantly elevated levels of responding relative to animals that received exposure only. There was no main effect of drug treatment (n=5/group).

Discussion

In this experiment we replicate the basic finding that animals with a history of footshock in a distinct context demonstrate significant enhancements in cue-induced reinstatement. When paired with extinction, the HDAC3 specific inhibitor KDAC 0008 failed to attenuate reinstatement to cues in animals with and without a history of footshock.

The group that demonstrated the greatest degree of reinstatement to cues was Shock + Vehicle, followed by Shock + KDAC 0008. Although we did not find a statistically significant difference between groups, the groups follow a promising ordinal trend. Because the N was low (N=5 for all groups) it may be that this study was underpowered to reveal significant differences between groups. What is extremely promising, however, is the replication of the enhancement in cued-reinstatement in animals with a history of footshock in a distinct environment. This finding is remarkable because it demonstrates the robustness of this effect, even with small experimental groups.

Although some groups have had success demonstrating the effects of HDACi on memory enhancement, the literature remains mixed on these effects. While the HDACi RGFP 966 has been shown to enhance CPP for cocaine (Malvaez et al., 2010; Malvaez et al., 2013), it has also been shown to have no effect on consolidation of cued fear extinction (Bowers et al, 2015). It has also been shown that HDACi attenuates CPP for nicotine without affecting nicotine aversion (Pastor et al., 2011) and has also been shown to decrease responding for intracerebral infusions of ethanol (Jeanblanc et al.,

2015). Taken together, these results suggest that HDACi mediated enhancements in memory are inconsistent and may be affected by drug type.

HDACi have been shown to enhance extinction in experiments modeling drug seeking with conditioned place preference paradigms, but we failed to observe an extinction enhancement using an operant self-administration procedure. One group found that NaB administered immediately before extinction of nicotine self-administration did not enhance extinction, even when the drug was administered prior to each extinction session (≥6 extinction sessions; Castino et al., 2015), although they did find an attenuation of nicotine-primed reinstatement. In this experiment, as in the one described here, the cue light was not present during extinction, which produced relatively low levels of responding during the sessions. To increase responding during extinction this group ran a second experiment with the cue light present during extinction. When the conditions changed and the cue light was available during extinction they observed a significant extinction enhancement in NaB treated animals, strongly suggesting that if levels of behavioral responding during extinction are not high enough a difference between groups will be impossible to detect. Therefore, it is possible that in our case that, because cue light was not available and levels of responding during extinction were relatively, a significant difference between groups was washed out.

It is also worth noting that this group administered NaB prior to each extinction session, while we only injected animals only prior to the first extinction session. It is possible that repeated injection of HDACi with extinction is necessary to distinguish differences between groups. They also found the greatest difference between groups

was on the day following treatment, suggesting that the most salient time for pharmacological enhancement of memory is likely following initial learning.

A separate study found that rats treated with one of two HDACi (NaB and MS-275) during operant self-administration of ethanol significantly reduced their intake of ethanol; however, this was only true in dependent animals (Simone-O'Brien et al., 2015). Similarly, alterations in histone acetylation were observed in dependent, but not non-dependent animals, and these alterations were normalized by HDACi treatment. Therefore, HDACi may interact with a history of drug-taking and subsequently may only be effective in treating animals that have undergone specific changes in gene expression. The animals in this study were not dependent on METH which may have also contributed to a lack of an observed effect.

Additionally, inhibition of a given class of HDACs may alter the levels of other classes of HDACs. For example, treatment with MS-275, a selective inhibitor of Class I HDACS, leads to a reduction of HDAC8 expression in the nucleus accumbens (NAcc) (Covington et al., 2011). Because KDAC 0008 is a novel compound it is still unclear what actions, if any, it may have on other classes of HDACs in the brain. Finally, it should be noted that KDAC 0008 is a relatively untested HDACi and to the best of our knowledge has not yet been shown to result in significant memory enhancements. The current study was a first attempt to probe the memory enhancing effects of this drug, and the null findings here do not preclude the potential therapeutic value of this compound in the future.

Taken together, the literature suggests that HDACi-mediated memory enhancements are extremely sensitive to the specific parameters employed in each

learning paradigm. Drug of abuse, cues, dependence, even the HDACi chosen may influence behavioral outcomes as well as the activity of other classes of HDACs in the brain. Indeed, the effects of HDACs on METH-induced locomotor sensitization have been shown to be especially variable (Godino et al., 2015), with behavioral outcomes varying depending on experimental parameters (Kalda et al., 2007; Coccurello et al., 2007; Shen et al., 2008; Harkness et al 2013; Li et al., 2005). METH itself induces changes in HDAC levels (Cadet et al., 2013) across the brain, further obfuscating the role of HDACi in drug-extinction related memory enhancements. Future studies should aim to systematically approach the use of these compounds in learning paradigms to shed light on the circumstances that lead to the greatest treatment outcomes.

Supplementary Information

Table S7.

Session	Effect	F value	DF	*p* value
Footshock	time	19.58	3,48	< .001
	shock	45.22	1,16	<.001
	drug treatment	1.87	1,17	0.19
	Time x shock	8.9	3,48	<.001
	Time x drug treatment	1.3	3,48	0.26
	time x shock x drug treatment	0.55	3,48	0.64
	shock x drug treatment	0.03	1,18	0.95

Table S8.

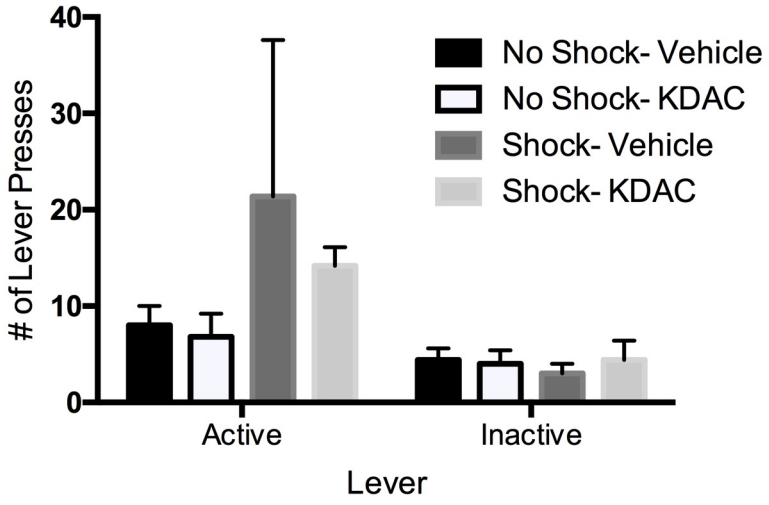
Session	Effect	F value	DF	*p* value
Last 3 FR5 Sessions	session	2.48	2,32	0.1
	shock	2.45	1,16	0.13
	lever	1.59	1,16	0.22
	drug treatment	0.68	1,16	0.42
	session x shock	0.59	2,32	0.55
	session x drug treatment	0.31	2,32	0.73
	session x lever	0.51	2,32	0.6
	lever x shock	1.8	1,16	0.19
	lever x drug treatment	0.77	1,16	0.39
	lever x shock x drug treatment	0.88	1,16	0.36
	session x lever x shock	1.45	2,32	0.24
	session x lever x drug treatment	0.42	2,32	0.86
	session x drug treatment x shock	0.01	2,32	0.981
	session x lever x drug treatment x shock	0.42	2,32	0.65
	shock x drug treatment	1.58	1,16	0.22

Table S9.

Session	Effect	F value	DF	*p* value
Cued Reinstatement	session	14.72	1,16	0.001
	shock	0.3	1,16	0.58
	lever	23.42	1,16	< .001
	drug treatment	0.89	1,16	0.35
	session x shock	4.77	1, 16	0.04
	session x drug treatment	1.15	1,16	0.29
	session x lever	29.49	1,16	< .001
	lever x shock	5.38	1,16	0.03
	lever x drug treatment	2.31	1,16	0.14
	lever x shock x drug treatment	0.17	1,16	0.67
	session x lever x shock	2.47	1,16	0.13
	session x lever x drug treatment	0.17	1,16	0.67
	session x lever x shock x drug treatment	1.91	1,16	0.18
	session x drug treatment x shock	0.03	1,16	0.85
	shock x drug treatment	1.87	1,16	0.19

Table S10.

Session	Effect	F value	DF	*p* value
Extinction After Cued Reinstatement	shock	1.57	1,16	0.22
	lever	14.26	1,16	<.01
	Drug treatment	0.5	1,16	0.48
	lever x shock	3.04	1,16	0.1
	lever x drug treatment	0.63	1,16	0.43
	lever x shock x drug treatment	0	1,16	0.98
	shock x drug treatment	5.66	1,16	0.03



Supplementary Figure 1. Cued reinstatement during the first 15 minutes of session.

Table S11.

	Effect	DF	F	*p*
First 15 Mins of Cued Reinstatement (KDAC 0008)	time	2,32	11.25	< .0001
	shock	1,16	1.15	0.299
	injection	1,16	0.161	0.693
	lever	1,16	5.12	0.03
	time x shock	2,32	1.33	0.27
	time x injection	2,32	0.75	0.47
	time x lever	2,32	0.2	0.81
	lever x shock	1,16	2.03	0.17
	lever x injection	1,16	0.37	0.54
	time x shock x injection	2,32	4.28	0.02
	lever x shock x injection	1,16	0.26	0.61
	time x lever x shock	2,32	2.26	0.12
	time x lever x injection	2,32	0.03	0.96
	time x lever x shock x injection	2,32	0.72	0.49

CHAPTER 5

Discussion

Portions of Chapter 5 are adapted from the publication:

Pizzimenti CL & Lattal KM. (2015). Epigenetics and memory: causes, consequences and treatments for post-traumatic stress disorder and addiction. *Genes, Brain and Behavior, 14,* 73-84.

Summary of findings

Chapter 1 discussed how both stress and exposure to drugs of abuse induce` persistent behavioral changes, some of which may contribute to the formation of a drug addiction or a stress-related psychiatric disorder. Converging evidence suggests that similar behavioral, neurobiological and molecular mechanisms control the extinction of learned fear and drug-seeking responses. This may, in part, account for the fact that individuals with PTSD have a significantly elevated risk of developing a substance use disorder and have high rates of relapse to drugs of abuse, even after long periods of abstinence. At the behavioral level, a major challenge in treatments is that extinguished behavior is often not persistent, returning with changes in context, the passage of time or exposure to mild stressors. A common goal of treatments is therefore to weaken the ability of drug-related cues to induce relapse. With the discovery of epigenetic mechanisms that create persistent molecular signals, recent work on extinction has focused on how modulating these epigenetic targets can create lasting extinction of fear or drug-seeking behavior. In this dissertation, I targeted these mechanisms in combination with behavioral therapy to investigate their treatment potential in a novel, comorbid model.

Chapter 2 focused on the development of the novel model for the comorbid condition in rodents. First, I replicated the basic finding that rats with a history of massive footshock in a distinct environment demonstrate exaggerated fear responses to mild footshock in a novel environment. I then showed that this effect persists even when the mild footshock is administered in a context that has long been associated with reward. Most significantly, I found massive footshock in a distinct environment

administered before or during self-administration of IV METH results in significantly elevated levels of responding to cues following extinction, as well as significantly elevated responding following reinstatement. I also showed that mice with a history of massive footshock demonstrate a significant preference for a cocaine paired floor in a test of conditioned place preference. I found that although massive footshock acutely elevates levels of CORT, this difference does not persist long-term and animals respond normally to dexamethasone, suggesting differences in drug-seeking behavior are not likely to be driven by chronically elevated CORT or alterations in glucocorticoid feedback.

Chapter 3 investigated the ability of behavioral extinction as well as behavioral extinction paired with epigenetic modulators to attenuate exaggerated fear responses to mild stimuli following exposure to massive footshock. I first showed that moderate levels of behavioral extinction are not sufficient to attenuate the SEFL effect. I then showed that massive behavioral extinction (animals demonstrated no fear for two consecutive days) was sufficient to attenuate this effect, suggesting treatments that turn a “weak” conditioning session into a “strong” conditioning session may hold promise for promoting extinction. Because previous research has demonstrated the profound influence of circadian rhythms and time of day on fear learning, I investigated if the light/dark cycle influences SEFL outcomes. I found that animals trained during the light phase demonstrate significantly greater levels of freezing during fear acquisition and a significantly enhanced SEFL effect (greater freezing during a Context B test). I then probed the ability of two HDACi, NaB and RGFP 966, to enhance extinction memory within the SEFL paradigm. Despite previous reports, I found that NaB actually increased

freezing levels, regardless of the length of time the animal was extinguished, suggesting NaB was actually promoting fear. Although others have demonstrated success with NaB, it is a pan-HDACi and therefore does not target the processes specific to learning and memory. Because HDAC3 has previously been shown to be an important regulator of learning and memory (McQuown et al., 2011) I also used RGFP 966, an HDAC3 specific inhibitor, in this paradigm. I found that following both brief (6 min) and extended (30 min) extinction, RGFP 966 does not produce a significant enhancement in extinction memory.

In Chapter 4 I investigated the ability of a novel HDAC3 specific inhibitor (KDAC 0008) to enhance extinction of drug seeking in a rodent model of comorbid PTSD-SUD. Animals received massive footshock in a distinct environment and were then allowed to acquire and extinguish self-administration of METH in a different environment. I replicated the basic finding that animals with a history of footshock demonstrate significant enhancements in cue-induced responding, and that this enhancement is specific to the previously active lever. I also did not find evidence that administration of KDAC 0008 30 min prior to the initial extinction session attenuated cued-reinstatement in animals that did not receive shock. I similarly did not find evidence that KDAC 0008 attenuated the enhancement observed in animals with a history of shock.

Stress-enhanced fear learning as a model of PTSD

SEFL is a powerful model of PTSD that allows researchers to directly model the ability of a contextually specific stressor to produce long-term alterations in fear responses in non-trauma associated contexts. Because the stressor (massive

footshock) is administered in a specific context, manipulations that are specific to that context can be tested to investigate how they influence fear responses in other contexts. This is an advantage over other models of PTSD that lack a specific trauma-related contextual component (e.g, chronic variable stress, single prolonged stress, maternal separation). The disadvantage of these models is that there is no specific context in which the stressful experience occurs, therefore extinguishing that context and investigating the influence on behavior is not possible. SEFL also produces a fear response (freezing) that is well characterized, easily measured, and tractable.

A diagnosis of PTSD requires that symptoms, which may include a heightened startle reaction, persist for at least one month (American Psychological Association, 2013). The SEFL effect has previously been shown to persist up to 90 days following the initial battery of footshocks in Context A (Rau & Fanselow, 2009). In this dissertation, I also demonstrated the persistence of this effect over time (60 days; Chapter 1: Experiment 3), even in a context that has long been associated with reward (Chapter 1: Experiment 2 & 3). Therefore, this model accurately captures the persistence of exaggerated fear responses over long periods of time, even in contexts that previously failed to elicit a fear response, consistent with the diagnostic criteria in humans.

An interesting feature of SEFL is the long ISI (4-8 min). It has been previously shown that learning is more robust when trials are spaced apart by longer intervals rather than massed closely together in time (Rescorla, 1988; Lattal, 1999). The ISI used in the SEFL paradigm is generally longer than most fear conditioning paradigms and may contribute to the exaggerated fear response observed in response to a single

footshock.

Models of the PTSD-SUD comorbidity

Often, when researchers seek to investigate the PTSD-SUD comorbidity stressors are paired with drug taking both contextually and temporally. Take, for example, footshock-induced reinstatement, arguably the most well characterized example of stress induced reinstatement (it should be noted that other stressors besides footshock that are administered in a similar temporal/contextual fashion have also been shown to induce reinstatement of drug-seeking; reviewed in Mantsch et al., 2016). In these experiments animals are trained to self-administer a drug of abuse in Context A; subsequently this behavior is extinguished in Context A. Following extinction animals are exposed to multiple footshocks delivered in rapid succession in Context A and are then allowed to lever press in the absence of drugs. This model has been shown to induce reinstatement of drug-seeking for a wide class of drugs of abuse including heroin, cocaine, methamphetamine, ethanol, and nicotine (Buczek et al., 1999; Erb, Shaham, & Stewart, 1996; Le et al., 1998; Shaham & Stewart, 1995; Shephard et al., 2004). and footshock stress has also been shown to reinstate a CPP for cocaine (Sanchez & Sorg, 2001; Wang et al., 2010). Interestingly, this phenomenon seems only to persist when the footshock is administered within the drug-taking environment; when animals receive footshock in a novel context it failed to produce reinstatement (Shalev et al., 2000). While these studies have demonstrated the ability of stress itself to induce drug-seeking behavior and have shed light on the putative circuits that promote this behavior, they fail to capture the essence of the PTSD-SUD

comorbidity. Although the exact nature of the relationship between PTSD and SUDs remains unclear, the majority of research supports the primacy of PTSD prior to the onset of SUD development (McCauley et al., 2012), as PTSD has been shown to precede SUDs in both retrospective (Kessler et al., 1995; Mellman et al., 1992) and prospective studies (Chilcoat & Breslau, 1998). Therefore, models where stress induces reinstatement after drug-seeking patterns have already been established may not accurately capture the etiology of this comorbidity.

In addition, these models fail to demonstrate the ability of exposure to trauma to augment long-term behavioral responses. Indeed, in these preparations animals experience an acute stressor and immediately respond with drug-seeking behaviors. As described in Chapter 1, individuals with PTSD are unlikely to use drugs of abuse in the trauma related context, and avoidance of the trauma associated environment is one of the diagnostic criteria for a diagnosis of PTSD (American Psychiatry Association, 2013). Therefore, although these studies have been helpful in mapping the circuitry related to acute, stress-induced reinstatement of drug-seeking behavior, they do not adequately describe the human condition in which an individual with PTSD relapses to drugs of abuse long after their trauma has ended in a non-trauma related location. The studies described in this dissertation are therefore the first of their kind. Here, I have demonstrated the ability of acute exposure to footshock to augment drug-seeking responses long after the footshock has ended in a non-footshock associated environment in response to drug-paired cues. This paradigm is more translatable because the animals never drug seek in the footshock associated environment and I

observe the same increased propensity to reinstate (“relapse”) in response to drug-paired cues that is noted in humans.

What is perhaps even more promising is that this effect was observed in both CPP and self-administration paradigms, in both rats and mice, and in response to both cocaine and methamphetamine. These findings suggest that, like the classic footshock-induced reinstatement preparation, this model persists across a variety of species, classes of drugs, and learning paradigms. This novel paradigm is therefore an exciting addition to the canon as it allows for the investigation of novel therapeutic options to attenuate this enhancement and prevent relapse in comorbid individuals. From a treatment perspective, understanding the circuits that are engaged during the extinction of fear and drug seeking is critical in order to develop treatments that target those circuits and subsequently promote extinction learning.

Common extinction circuits in fear and substance abuse

Models of treatment for fear related disorders and drug seeking have involved brain regions that mediate, stress, fear and addiction. By better understanding the circuits that promote this behavior, researchers can develop treatment strategies that target these circuits to attenuate unwanted responses. One avenue that has received attention is the enhancement of extinction of fear and drug seeking behavior. This work has revealed that common circuits mediate the acquisition of pro-fearful responses and drug seeking, much like common circuits mediate the extinction of these same behaviors.

Early work with lesions and temporary inactivation demonstrated a role for the

medial prefrontal cortex (mPFC) in the expression and extinction of fear (e.g., Morgan et al., 1993). Since then, many studies have extended these findings to other preparations, including extinction of drug seeking in conditioned place preference (CPP; e.g., Groblewski et al., 2012) and self-administration paradigms with drugs of abuse (LaLumiere et al., 2010). There is also increasing evidence that the mPFC may become dysregulated with prolonged stress (Knox et al., 2010; 2012). The specific circuits regulating extinction within the mPFC and between the mPFC and other structures are still being identified and may differ as a function of strain and species (e.g., Camp et al., 2012; Chang & Maren, 2010; MacPherson et al., 2013). Nonetheless, some general properties are emerging.

Subregions of the mPFC are involved in regulating both the expression and extinction of fear. Although there is clear overlap in function, it is generally thought that the infralimbic (IL) region is involved in the development and consolidation of extinction, whereas the prelimbic (PL) region is involved in the expression and contextual modulation of fear (Laurent & Westbrook, 2009; Sharpe & Killcross, 2015). Effects on fear modulation are due to excitatory glutamatergic projections from the mPFC to the amygdala (Krettek & Price 1977; Paré & Smith, 1994). The more dorsally located PL mPFC sends projections to the basal amygdala, which then synapses onto the central amygdala (CeA), which promotes fear behavior through projections to the periaqueductal gray area (e.g., McNally et al., 2011). Projections from the IL target GABAergic cells found in the CeA and intercalated cells between the basolateral and CeA (reviewed in Pape & Pare, 2010). Many studies using a variety of manipulations have demonstrated a role for these structures in expression and consolidation of fear

and fear extinction (e.g., Moustafa et al., 2013).

Overlapping circuits also appear to regulate extinction of drug-seeking behavior (see McNally, 2014). Divergent projections from the mPFC regulate the increase in glutamate that leads to locomotor sensitization, as well as drug seeking and the cessation of drug seeking in a similar manner to that of fear expression. The IL, which attenuates fear expression by inhibiting the CeA through the GABAergic-intercalated cell projections, sends an excitatory projection to the medial portion of the NAc shell, which in turn sends an inhibitory GABAergic projection to the medial ventral pallidum (VP) which inhibits the motor output necessary for drug-seeking behavior (McFarland et al., 2004; Peters et al., 2009). The PL, which promotes the expression of fear through a glutamatergic projection to CeA, sends an excitatory projection to the dorsal NAc core, which putatively sends enkephalins to VP (Peters et al., 2009). These enkephalins may bind to mu opioid receptors on GABAergic inhibitory interneurons in the VP, thus disinhibiting the VP and promoting the locomotor behavior necessary for drug seeking (Peters et al., 2009). Therefore, the IL promotes the attenuation of drug-seeking behavior, just as it attenuates the expression of fear, and the PL promotes drug-seeking behavior, just as it promotes the expression of fear.

Additional similarities may underlie the converging circuitry that promotes both fear expression and drug seeking. For example, the basolateral amygdala (BLA), an integral structure for the expression of fear, is critically involved in the initial consolidation and subsequent modulation of memory associated with different rewards, such as food, ethanol and cocaine (Fuchs et al., 2006). Similarly, a large literature implicates the hippocampus in the contextual modulation of extinction in both fear and

substance abuse paradigms (reviewed in Maren et al., 2013).

The question that remains is how acute activity in these circuits leads to changes in the brain that persist over time and that produce long-term changes in behavior. How these changes occur is a particularly important question to consider in the context of PTSD and addiction because many different experiences can disinhibit the circuit, resulting in relapse. Therefore, pairing behavioral extinction treatments with drugs that target receptors that are involved in modulating fear and drug seeking and extinction of these behaviors may hold promise for promoting better treatment outcomes for both disorders.

Putative circuits underlying the PTSD-SUD comorbidity

How footshock stress alters the neural circuitry and subsequently increases both freezing responses to subsequent stressors as well as the degree of behavioral reinstatement in these studies remains unclear. One potential mechanism may through the effects of footshock stress on the BLA. Disruption of the GABAergic interneurons in this brain region results in hyperexcitability of these neurons and has been shown to be involved in disorders characterized by high levels of fear and anxiety (reviewed in Prager et al., 2016). Manzanares et al. (2005) investigated the role of the BLA in exaggerated fear responses to footshock twenty-four hours after acute restraint. They found that pretreatment with both midazolam and bicuculine methiodide attenuated the exaggerated freezing response to footshock observed in animals who had previously experienced restraint. They went on to show that local field potentials recorded in slices obtained from drug treated animals demonstrated multispike responses, indicative of

GABA disinhibition, and that high-frequency stimulation resulted in LTP (long-term potentiation) in the BLA that was absent in control treated animals. This suggests that a loss of GABAergic inhibition in the BLA during stress may lead to hyperexcitability and subsequent LTP that underlies long term changes in behavior. Indeed, disruption of only 3% of GABAergic interneurons in the BLA through a targeted lesion results in an increase in fear and anxiety like behavior in rats (Truitt et al., 2007). In contrast, animals that express higher levels of BLA GABA interneurons are resistant to conditioned fear stimuli (Cunningham et al., 2009).

The BLA is also critically involved in cue-triggered motivated behaviors. Bilateral lesions of the BLA have been shown to disrupt cue-induced reinstatement of drug seeking as well as acquisition of cocaine self-administration on a second-order schedule of reinforcement (Meil & See, 1997; Whitelaw et al., 1996). Additionally, it has been shown that the degree of behavioral reinstatement to drug-conditioned cues correlates with c-FOS expression in the BLA in rats (Kufahl et al., 2009). Therefore, I hypothesize that in the experiments described in this dissertation that footshock disrupts GABAergic signaling in the BLA. In the case of exaggerated responses to fear (the SEFL effect), it seems likely that dysregulation of GABAergic signaling in the BLA alone promotes this behavioral response. Indeed, it has been shown that following SEFL three GABAA subunits (*Gabrb2, Gabrb3,* and *Gabra4*) are downregulated relative to control animals, and that pretreatment with etomidate, an allosteric modulator selective for *β*2 or *β*3 subunit-containing GABAA receptors attenuates the SEFL response (Ponomarev et al., 2010).

In the case of the comorbid model it may be that enhanced glutamatergic

signaling from the amygdala to the NAcc promotes an increase in drug-seeking behavior. An alternative interpretation may be that shock-induced LTP in the BLA alone promotes enhanced responding to cues, as the BLA is required for cued responding. LTP in the BLA may also explain why massive footshock produced significant enhancements in responding to drug-related cues, but not in response to spontaneous recovery or footshock-induced reinstatement (Chapter 2: Experiment 4).

Previous work has shown a significant decrease in the surface expression of GABAA receptors (Chhatwal et al., 2005) and that the GABAA receptor clustering protein gephyrin is significantly downregulated (Ressler et al., 2002) in the BLA following the acquisition of fear. However, mRNA and protein levels of gephyrin significantly increased following extinction training (Chhatwal et al., 2005), suggesting behavioral extinction may attenuate fear responses by normalizing GABAA receptor expression and subsequent GABAergic signaling in the BLA. Therefore, treatments that promote behavioral extinction may counteract the effect of fear learning on the BLA and subsequently lead to better long-term memory retention.

HDAC inhibition and learning and memory

One way to promote extinction learning (and the putative underlying GABAergic signaling in the BLA) is to pharmacologically promote the function of cellular and molecular pathways involved in memory. The thinking behind this approach is that potentiating these signals at the time of extinction will strengthen the memory that forms during extinction, resulting in a persistent form of extinction that transfers to situations outside of the treatment context (see Bukalo et al., 2014; Fitzgerald et al., 2014; Lattal & Wood, 2013).

Recent studies have moved from targeting cellular receptor-level processes to processes that may lead to a longer lasting molecular signature, potentially resulting in a longer lasting signature at the level of memory. These studies have targeted some of the final steps of transcriptional regulation necessary for memory formation – the expression of genes and the proteins they code during the formation of long-term memories. Understanding transcriptional regulation and gene expression may ultimately be the best path toward developing treatments for PTSD and addiction because both of these disorders are associated with long-term epigenetic changes that contribute to the persistence of the problems at the level of behavior. Drugs that target some of these processes, when paired with behavioral extinction, may be especially useful as treatment interventions.

The role of histone acetylation in memory formation was first explored by Levenson et al. (2004) who found that H3 acetylation (on K14) was significantly elevated in hippocampal area CA1 1 hr (but not 24 hr) following contextual fear conditioning, while H4 remained unchanged. In contrast, H4 acetylation was significantly increased following latent inhibition, with little change in H3. This early work demonstrates that different learning paradigms are accompanied by distinct epigenetic changes in the brain. The nuanced role of specific HDACs in learning have been further confirmed by reports that focal deletion of *Hdac3* from area CA1 of the hippocampus enhanced object location memory in mice, but did not influence object recognition (Morris et al., 2013). Both object recognition and location memory were unaffected by knockout HDAC1 and HDAC2 (Morris et al., 2013). Another study found that HDAC2, but not HDAC1, regulated associative and spatial memory (Guan et al., 2009) and

selective knockout of HDAC2 led to robust extinction of a conditioned fear response and a conditioned taste aversion (Morris et al., 2013). The critical involvement of HDAC2 in learning and memory suggests HDACi that target HDAC2, rather than HDAC3, may hold greater promise as cognitive enhancers in future studies. Therefore, the lack of extinction enhancements observed in Chapter 3 may not necessarily reflect a failure of HDACi in general, but rather a failure to select the most appropriate HDACi based on HDAC involvement in contextual fear conditioning.

Because HDACs often, but not always, act at the sites of gene promotion there remains the possibility that systemic administration induces changes in gene transcription or in brain regions that ultimately wash out any observable effect on behavior. For example, both chronically depressed patients and mice exposed to a chronic social-defeat stress paradigm display lower levels of HDAC2 in the NAcc (Covington et al., 2011), suggesting downregulation of a given HDAC may not be desirable in all brain regions. It is therefore a possibility that systemic administration of HDACi induced epigenetic changes in brain regions that counteracted the effect of HDACi on memory enhancement, or actually worked to promote fear by influencing gene transcription in brain regions that promote the acquisition of fear. As the involvement of specific HDACs in distinct learning paradigms becomes clear, it may be advantageous to perform site specific administration of HDACi to restrict activity to discrete brain regions to better understand their influence on gene transcription and ultimately behavior.

The role of HDAC 3 in extinction enhancements

Despite emerging evidence demonstrating the involvement of HDACs in the regulation of learning and memory, the specific ability of HDACi (paired with behavioral extinction) to promote extinction learning long-term remains unclear. In Chapter 5 I investigated the ability of the HDAC 3 specific inhibitor KDAC 0008 to enhance extinction in the novel rodent model of PTSD-SUD characterized in Chapter 1.

I observed a directional effect in which animals that had received footshock and KDAC 0008 during extinction reinstated less to a previously drug-paired cue than animals that received footshock and vehicle during extinction. (Although this difference was not statistically significant, the sample size for this experiment was small (N=5/group). The lack of statistical significance might indicate that this study was underpowered and that the simple addition of animals would reduce the standard error and subsequently reveal a statistically significant effect.) Although not significant, these data are in contrast with the data from Experiments 5 and 6 in Chapter 3 where I found no effect whatsoever of RGFP 966, also an HDAC 3 selective inhibitor, on extinction.

There are potentially several reasons this directional effect was observed following administration of an HDAC 3 selective inhibitor in Chapter 4, but not in Chapter 3. First, KDAC 0008 is more selective for HDAC 3 than RGFP 966 at the doses used in these studies. Therefore, RGFP 966 may not have revealed any extinction effects due to a reduced specificity to act on HDAC 3. Second, and perhaps more likely, is the differing involvement of HDAC 3 in fear and drug-related learning paradigms. Previous reports that have shown the effectiveness of RGFP 966 to enhance extinction have come from studies investigating the enhancement of extinction in drug-seeking

paradigms (Malvaez et al., 2010; Malvaez et al., 2013). In 2010 Malvaez et al. found that systemic administration of RGFP 966 promoted extinction of a CPP for cocaine in mice and significantly increased acetylation in the NAcc. However, to date, no studies have demonstrated the ability of RGFP 966 specifically to enhance fear extinction, although this effect has been shown using the pan HDACi NaB (Stafford et al., 2012). Indeed, similar to the results in Chapter 3: Experiments 5 & 6, Bowers et al. (2015) found that RGFP 966 administered systemically at a dose of 10mg/kg did not enhance cued fear extinction. These results suggest that HDAC 3 may be more involved in the extinction of drug-seeking than in the extinction of fear responses.

It has also been shown that systemic pretreatment with the pan-HDACi NaB facilitates morphine CPP extinction (Wang et al., 2010). Intra-BLA infusions of the pan-HDACi TSA also enhance extinction of morphine CPP in rats (Wang et al., 2015). However, NaB impaired the extinction of a cocaine-induced CPP (Itzhak, Liddie, & Anderson, 2013). Even a pan-HDACi, which affect all classes of HDACs have different effects when they are administered in the same learning paradigm (CPP) that use different classes of drugs (opiate vs. amphetamine). Therefore, a third explanation for the difference in findings from Chapter 3 and Chapter 4 may relate to the differing involvement of HDACs based on the class of drugs being studied; HDAC 3 may be involved in drug-seeking for cocaine, but not METH.

It should also be noted that in Chapter 4 I again demonstrated the ability of massive footshock in a distinct environment to produce significant enhancements in cue-induced reinstatement. This is remarkable because the sample size was low for these groups and error was large, however despite these challenges differences

between groups persisted. This underscores the validity of this effect in this model and highlights the importance of understanding the specific neurobiological changes that occur at the time of footshock that promote long-term drug-seeking behavior.

Potential Limitations

There are several limitations to the studies in this dissertation. First, these studies were conducted exclusively in males; therefore, these results may not generalize to females. This is important because it has been shown that females develop PTSD at twice the rate of males, despite greater trauma exposure in males (Adamson et al., 2008; Breslau et al., 1998). In rodents, sex differences have been observed in hippocampal-LTP (Maren, De Oca, & Fanselow, 1994), the learning and processing of Pavlovian fear (reviewed in Dalla and Shors, 2009), and extinction and recall of conditioned fear (Baren et al., 2009), with females demonstrating greater fear learning and impaired fear extinction. However, these differences are not always consistent. Future studies should include females to better understand the role of sex differences in these effects.

Second, these studies may not generalize to other ages, strains, or species. Adult, male, Long-Evans were selected for these studies because these biological variables are the most well characterized. Indeed, the original SEFL report was conducted using adult, male, Long-Evans rats. Therefore, I focused on known variables in the initial characterization of this model. Future iterations of these studies may manipulate biological variables and find disparate findings that may be interesting to probe.

Third, it is important to note that these results may not generalize to other classes of drugs. The effects of massive footshock on drug-seeking behavior were observed in experiments using METH and COC. Future experiments should include other classes of drugs (e.g., opiates) to better understand the generalizability of these behaviors.

Fourth, the potential for neurotoxicity in these studies was not assessed. However, the likelihood that the average intake of METH in these studies produced neurotoxic effects is low. One study administered 50 mg/kg METH to rats either in a single dose on a single day, or as a 10 mg/kg injection once per day for 5 days. Only rats that received a single injection of 50 mg/kg METH demonstrated elevated levels of apoptosis (Tokunaga et al., 2008). In general, when seeking to model the neurotoxic effects of METH using similar acute administration paradigms, METH is administered at a dose that far exceeds levels that were achieved in these studies, in both daily and overall intake (for a review, see Kobeissey et al., 2011). In a study of METH self-administration, signs of neurotoxicity were only observed in animals that achieved intake levels of over 10 mg/kg/session following 15 hr sessions (Krasnova et al., 2010). Still, the specific effects of daily low dose METH intake over an extended period of time (at least several weeks in the self-administration experiments described here) remains unclear. In addition, previous work has shown that chronic stress enhances excitotoxicity in the rodent striatum (Tata & Yamamoto, 2008). Although animals in these studies were not chronically stressed, it remains unclear how a single stressful experience might augment the neurotoxic and excitotoxic effects of METH. Future studies should investigate signs of neurotoxicity in rodents more closely following self-

administration to better understand how chronically administered, low-doses of drug may influence behavior.

Fifth, the sessions used in these experiments were not extended (typically 6 hr or more). However, the self-administered doses of methamphetamine are consistent with many published studies, both in terms of daily intake and overall intake (Ujike et al., 1989; Harrod et al., 2001; Vinklerova et al., 2002; Stefanski et al., 2004; Carson et al., 2010). In general, I would not regard the self-administered doses in this paper as low or insufficient to model substance use disorders. In one study that increased the dose to .1 mg/kg/infusion rats responded ~70 times over a 6hr session for methamphetamine; this level of responding is far lower than the level seen in my studies, even though my sessions were only 2 hr (Shepard et al., 2004). Because my primary interest was in drug-seeking behavior and the reinstatement of this behavior, using a dose that promoted lever pressing without intoxicating the animals to the point of attenuating their response rates was important. It is true that these models may not accurately model bingeing behavior specifically, but the daily doses administered in this experiment regularly induce reinstatement for methamphetamine (e.g., Carson et al., 2010), are higher than the dose required to develop a METH-induced CPP (Zakharova et al., 2009), and produce stereotypy and other signs of methamphetamine intoxication (Hadamitzky et al., 2012).

Sixth, the dose of self-administered ethanol is not high. However, this dissertation does not make claims regarding drug-seeking in the experiment investigating the effects of footshock on ethanol self-administration. The conclusions from that experiment were that 1) a single footshock administered in the drug-

associated environment produced the SEFL effect and 2) that like the original report, the effect persisted over time. The ethanol experiment in this dissertation used a sucrose fading procedure. This may pose potential problems for the interpretation of self-administration behavior, as it's unclear if after the sucrose is fully faded out if animals are responding for ethanol, or because of the prior association with sucrose. In general, responding decreased when sucrose was eliminated, making investigating drug-seeking behaviors difficult to study. In other self-administration experiments, the FR requirement was increased to increase behavior. In the absence of robust behavior, this proved to be a poor procedure for addressing the question I was most interested in. In light of this, we switched to self-administration of METH, which does not require baiting or encouragement of any kind to initiate responding.

Seventh, although self-administration paradigms model aspects of SUDs, no model can capture all facets due to the complex nature of these disorders. These self-administration studies investigate the effects of a single acute stressor in a distinct context on drug seeking in a different context. Specifically, these studies model the enhancements in relapse seen in individuals with PTSD. Across species, self-administration is a widely accepted model of SUDs (Koob & Le Moal, 1997; Platt, Carey, & Spealman, 2012; Lynch et al., 2010). The DSM 5 no longer refers to substance abuse or dependence, rather it refers to substance use disorders that are defined as mild, moderate, or severe. These levels are defined by the number of diagnostic criteria met by an individual (American Psychological Association, 2013). It is difficult to interpret how many of these diagnostic criteria these animals would qualify for, but the severity of SUDs encompasses a wide range and is not exclusive to daily,

binge level intake. Overall intake in these models does not reach binge levels, and physical dependence or symptoms of withdrawal during forced abstinence were not specifically assessed. Additionally, animals do not suffer negative consequences as a result of their drug use. Therefore, self-administration, like all rodent models of disease, does not completely capture the human condition. However, these studies demonstrate how stressful experiences alter long-term drug-seeking behavior, which is particularly relevant in the development of behavioral and pharmacological approaches to reducing relapse to drugs of abuse in humans.

Eighth, in the studies investigating the use of HDACi only one dose of drug was used. Subsequently, it remains unclear if the lack of effects resulted from an insufficient dose to see an effect. The doses of HDACi used in this study were selected based on published research both from our laboratory and the laboratory of our collaborators that demonstrated positive effects of these compounds at these particular doses. Therefore, although only one dose was tested, the doses had previously been shown to be effective in similar paradigms and to alter histone acetylation. Dose-dependence curves do not generally exist for HDACi, as the effects of their administration at low and high doses have been shown to be species and task specific (Graff & Tsai, 2013). Nonetheless, future studies should include several doses of HDACi to more thoroughly probe their therapeutic utility in these paradigms.

Finally, HDACi may have unwanted side effects that may limit their effectiveness as therapeutic agents. That being said, HDACi are currently used as therapeutics for a number of diseases, primarily cancer. In clinical trials of Romidepsin, an HDACi approved for cancer treatment in 2009, the most common side effects reported following

a year of treatment included fatigue, vomiting, ECT T-wave changes, and diarrhea (Wagner et al., 2010). Understanding the adverse side effects of HDACi are complicated by the numerous symptoms associated with the diseases HDACi are currently approved to treat (e.g., fatigue and vomiting are often reported in patients undergoing treatment for cancer). As a cognitive enhancer, unwanted effects may arise if HDACi are paired with experiences that promote fearful or drug-seeking behavior. Subsequently, the use of HDACi in the treatment of these disorders must be carefully characterized in future studies.

Future directions

There is ample evidence that common neural circuits mediate the acquisition and extinction of fear and drug seeking behavior (Brady & Sinha, 2005, Peters et al, 2009). The basolateral amygdala (BLA) is involved in both cue-induced reinstatement (Dwyer & Kilcross, 2006; Feltenstein & See, 2007) and the acquisition and consolidation of fear learning (Fanselow & Kim, 1994; Helmstetter & Bellgowan, 1994) which makes it a likely candidate as the locus of this interaction. Indeed, stress-induced enhancements in synaptic plasticity in the BLA have been hypothesized to increase the likelihood of drug cue-induced relapse in comorbid individuals (Herringa et al., 2004; Belujon & Grace, 2011). Based on previous reports that inactivation of the BLA during stress prevents the induction of synaptic plasticity and remodeling (Manzanares et al., 2005) and synaptic plasticity in the BLA underlies conditioned reinforcement (Kufahl et al., 2009), it is possible that inactivation of the BLA during footshock will prevent stress-induced enhancements in reinstatement to drug-paired cues. Testing this hypothesis is outside

the scope of this thesis, but understanding the specific neural circuitry that underlies stress-induced enhancements in cued-responding is an essential component to understanding how these disorders influence one another and developing effective treatment strategies.

It is also important to continue characterizing the role of specific HDACs in distinct learning paradigms, as well as the effectiveness of different HDACi to act on those HDACs at specific doses and pre- or post-treatment times. This refinement will help researchers make informed choices when designing experiments, as null effects may not necessarily reflect a failure of HDACi in general to enhance memory, but rather an inappropriate HDACi choice for those conditions.

Final comments

Converging evidence suggests that similar behavioral, neurobiological and epigenetic mechanisms control the extinction of learned fear and drug-seeking responses. With the discovery of epigenetic mechanisms that create persistent molecular signals, recent work on extinction has focused on how modulating these epigenetic targets can create lasting extinction of fear or drug-seeking behavior. The experiments described here establish a powerful novel model of the PTSD-SUD comorbidity in rats, demonstrating for the first time the ability of an acute stressor to produce long-term alterations in drug-seeking behavior. This model allows researchers to investigate the common mechanisms that underlie both of these disorders in a highly translatable model of relapse. Although these studies failed to reveal an effect of extinction enhancement, there remains great promise that with further characterization

of the role of specific classes of HDACs in learning that HDACi in combination with behavioral extinction may promote positive treatment outcomes for both PTSD and SUDs.

References

Abel T & Zukin RS. (2008). Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. *Current Opinion in Pharmacology, 8(1),* 57-64.

Adamec RE & Shallow T. (1993). Lasting effects on rodent anxiety of a single exposure to a cat. *Physiol Behav*, *54*, 101–109.

Adamec RE, Shallow T, & Budgell J. (1997). Blockade of CCK(B) but not CCK(A) receptors before and after the stress of predator exposure prevents lasting increases in anxiety-like behavior: implications for anxiety associated with post-traumatic stress disorder. *Behav Neurosci, 111,* 435–449.

Aimaretti G & Ghigo E. (2005). Traumatic brain injury and hypopituitarism. *Sci. World. J., 5,* 777-781.

Albrecht A & Stork O. (2017). Circadian rhythms in fear conditioning: an overview of behavioral, brain system, and molecular interactions. *Neural Plast.*

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual of Mental Disorders* (5th ed.). Arlington, VA: American Psychiatric Publishing.

Baran SE, Armstron CE, Niren DC, Hanna JJ, Conrad CD. (2009). Chronic stress and sex differences on the recall of fear conditioning and extinction. *Neurobiology of Learning and Memory, 91(3),* 323-332.

Back SE, Dansky BS, Coffey SF, Saladin ME, Sonne SC, & Brady KT. (2000). Cocaine dependence with and without post-traumatic stress disorder: A comparison of substance use, trauma history, and psychiatric comorbidity. *American Journal on Addictions*, *9*, 51–62.

Back SE, Brady KT, Jaanimagi U, & Jackson JL. (2006). Cocaine dependence and PTSD: a pilot study of symptom interplay and treatment preferences. Addict Behav, 31, 351–4.

Bahari-Javan S, Maddalena A, Kerimoglu C, Wittnam J, Held T, Bahr M, Burkhardt S, Delalle I, Kugler S, Fischer A, & Sananbenesi F. (2012). HDAC1 regulates fear extinction in mice. *J Neurosci, 32(15),* 5062-5073.

Berman DE & Dudai Y. (2001). Memory extinction, learning anew, and learning the new: Dissociations in the molecular machinery of learning in cortex. *Science, 291,* 2417-2419.

Belujon, P. & Grace, A.A. (2011). Hippocampus, amygdala and stress: interacting systems that affect susceptibility to addiction. *Annals of the New York Academy of Sciences, 1216(1),* 114-121.

Blouin, A. M., Sillivan, S. E., Joseph, N. F., & Miller, C. A. (2016). The potential of epigenetics in stress-enhanced fear learning models of PTSD. *Learning & Memory*, *23*, 576-586.

Bouton ME. (2002). Context, ambiguity, and unlearning: sources of relapse after behavioral extinction. *Biol Psychiatry, 52(10),* 976-988.

Bouton ME & Bolles RC. (1980). Conditioned fear assessed by freezing and by the suppression of three different baselines. *Animal Learning & Behavior*, *8*, 429-434.

Bouton ME, Garcia-Gutierrez A, Zilski J, & Moody EW. Extinction in multiple contexts does not necessarily make extinction less vulnerable to relapse. *Behav Res Ther, 44(7),* 983-994.

Boutrel B, Kenny PJ, Specio, SE, Martin-Fardon R, Markou A, Koob GF, & de Lecea L. (2005). Role for hypocretin in mediating stress-induced reinstatement of cocaine-seeking behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 19168-19173.

Bowers ME, Xia B, Carriero S, & Ressler KJ. (2015). The Class I HDAC inhibitor RGFP963 enhances consolidation of cued fear extinction. *Learn Mem, 22(4)*, 225-231.

Bradizza CM, Stasiewicz PR, & Paas ND. (2006). Relapse to alcohol and drug use among individuals diagnosed with co-occurring mental health and substance use disorders: a review. *Clinical Psychology Review*, *26*(2), 162–178.

Bredy, T.W. & Barad, M. (2008) The histone deacetylase inhibitor valproic acid enhances acquisition, extinction, and reconsolidation of conditioned fear. *Learn Mem* 15, 39–45.

Brady KT, Back S, & Coffey SF. (2004). Substance abuse and post-traumatic stress disorder. *Curr Dir Psychol Sci, 13*, 206–209.

Brady, K.T. & Sinha, R. (2005). Co-occurring mental and substance use disorders: the neurobiological effects of stress. *American Journal of Psychiatry, 162(8),* 1483-1493.

Brown PJ & Wolfe J. (1994). *Substance abuse and post-traumatic stress disorder comorbidity. Drug and Alcohol Dependence, 35(1),* 51-59.

Brown PJ, Stout RL, & Mueller T. (1999). Substance use disorder and post-traumatic stress disorder comorbidity: Addiction and psychiatric treatment rates. *Psychology of Addictive Behaviors, 13,* 115-122.

Buczek Y, Le AD, Wang A, Stewart J, & Shaham Y. (1999). Stress reinstates nicotine seeking but not sucrose solution seeking in rats. *Psychopharmacology, 144*, 183–188.

Bukalo, O., Pinard, C., & Holmes, A. (2014). Mechanisms to medicines: elucidating neural and molecular substrates of fear extinction to identify novel treatments for anxiety disorders. *British Journal of Pharmacology*, *171*(20), 4690-4718.

Burns MN, Lehman KA, Milby JB, Wallace D, & Schumacher JE. (2010). Do PTSD symptoms and course predict continued substance use for homeless individuals in contingency management for cocaine dependence? *Behaviour Research and Therapy, 48,* 588-598.

Buwalda, B., Kole, M.H.P., Veenema, A.H., Huininga, M., de Boer, S.F., Korte, S.M. & Koolhaas, J.M. (2005) Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning. *Neurosci Biobehav Rev* 29, 83–97.

Cadet JL, Jayanthi S, McCoy MT, Ladenheim B, Saint-Preux F, Lehrmann E, De S, Becker KG, & Brannock C. (2013). Genome-wide profiling identifies a subset of methamphetamine (METH)-induced genes associated with METH0induced increased H4K5Ac binding in the rat striatum. *BMC Genomics, 14,* 545.

Camp, M. C., MacPherson, K. P., Lederle, L., Graybeal, C., Gaburro, S., DeBrouse, L. M., Ihne, J.L., Bravo, J.A., O'Connor, R.M., Ciocchi, S., Wellman, C.L., Luthi, A., Cryan, J.F., Singewald, N., & Holmes, A. (2012). Genetic strain differences in learned fear inhibition associated with variation in neuroendocrine, autonomic, and amygdala dendritic phenotypes. *Neuropsychopharmacology*, *37*(6), 1534-1547.

Campbell, J.M. (2003.) Efficacy of behavioral interventions for reducng problem behavior in persons with autism: a quantitative synthesis of single-subject research. *Res Dev Disabil, 24,* 120–138.

Carson DS, Cornish JL, Guastella AJ, Hunt GE, & McGregor IS. (2010). Oxytocin decreases methamphetamine self-administration, methamphetamine hyperactivity, and relapse to methamphetamine-seeking behavior in rats. *Neuropharmacology, 58(1),* 38-43.

Carter BL & Tiffany ST. (1999). Meta-analysis of cue-reactivity in addiction research. *Addiction*, *94*, 327–340.

Castino MR, Cornish JL, & Clemens KJ. (2015). Inhibition of histone deacetylases facilitates extinction and attenuates reinstatement of nicotine self-administration in rats. *PLoS One*.

Chang, C. & Maren, S. (2010) Strain difference in the effect of infralimbic cortex lesions on fear extinction in rats. *Behavioral Neuroscience, 124*(3), 391-397.

Chaudhury D & Colwell CS. (2002). Circadian modulation of learning and memory in fear-conditioned mice. *Behav Brain Res, 133(1),* 95-108.

Chen HP, Zhao YT, & Zhao TC. (2015). Histone deacetylases and mechanisms of regulation of gene expression. *Crit Rev Oncog, 20(1-2),* 35-47.

Chhatwal JP, Myers KM, Ressler KJ, & Davis M. (2005). Regulation of gephyrin and GABAA receptor binding within the amygdala after fear acquisition. *J Neurosci, 25(2),* 502-506.

Chilcoat HD &Breslau N. (1998). Posttraumatic stress disorder and drug disorders: testing causal pathways. *Arch Gen Psychiatry,* 55, 913–7.

Coccurello R, Caprioli A, Ghirardi O, Virmani A. (2007). Valproate and acetyl-L-carnitine prevent methamphetamine-induced behavioral sensitization in mice. *Ann NY Acad Sci, 1122*, 260-75.

Coffey SF, Schumacher JA, Brady KT, & Cotton BD. (2007). Changes in PTSD symptomatology during acute and protracted alcohol and cocaine abstinence. *Drug Alcohol Depend., 87,* 241–8.

Covington HE, 3rd, Vialou VF, LaPlant Q, Ohnishi YN, Nestler EJ. (2011). Hippocampal-dependent antidepressant-like activity of histone deacetylase inhibition. *Neurosci Lett, 493*, 122–126.

Cunningham CL, Gremel CM, & Groblewski PA. (2006). Drug-induced conditioned place preference and aversion in mice. *Nature Protocols, 1,* 1662.1670.

Cunningham MG, Connor CM, Carlezon WA, & Meloni E. (2009). Amygdalar GABAergic-rich neural grafts attenuate anxiety-like behavior in rats. *Behavioral Brain Research, 204(1),* 146-153.

Dalla C & Shors TJ. Sex differences in learning processes of classical and operant conditioning. *Physiology & Behavior, 97(2),* 229-238.

Davis M, Myers KM, Chhatwal J, & Ressler KJ. (2006). Pharmacological treatments that facilitate extinction of fear: relevance to psychotherapy. *NeuroRx, 3*, 82–96.

Delamater AR & Westbrook RF. (2014). Psychological and neural mechanisms of experimental extinction: a selective review. *Neurobiol Learn Mem, 108,* 38–51.

Drapkin ML, Yusko D, Yasinski C, Oslin D, Hembree EA, & Foa EB. (2011). Baseline functioning among individuals with post-traumatic stress disorder and alcohol dependence. *J Subst Abuse Treat*, 41, 186–92.

Dwyer, D.M. & Killcross, S. (2006). Lesions of the basolateral amygdala disrupt conditioning based on the retrieved representations of motivationally significant events. *Journal of Neuroscience, 26(32),* 8305-8309.

Eggleston AM, Calhoun PS, Svikis DS, Tuten M, Chisolm MS, & Jones HE. (2009). Suicidality, aggression, and other treatment considerations among pregnant, substance-dependent women with post-traumatic stress disorder. *Comparative Psychiatry, 5,* 415-423.

Erb S, Shaham Y, & Stewart J. (1996). Stress reinstates cocaine-seeking behavior after prolonged extinction and drug-free periods. Psychopharmacology 128: 408–412.

Erb S, Salmaso N, Rodaros D, & Stewart J. (2001). A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. *Psychopharmacology, 158*, 360-365.

Fanselow MS. (1982). The postshock activity burst. *Animal Learning & Behavior, 10(4),* 448-454.

Fanselow, M.S. & Kim, J.J. (1994). Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. *Behavioral Neuroscience, 108(1),* 210-212.

Federman N, Fustinana MS, & Romano A. (2009). Histone acetylation is recruited in consolidation as a molecular feature of stronger memories*. Learning Mem, 16(10),* 600-606.

Feltenstein, M.W. & See, R.W. (2007). NMDA receptor blockade in the basolateral amygdala disrupts consolidation of stimulus-reward memory and extinction learning during reinstatement of cocaine-seeking in an animal model of relapse. *Neurobiology of Learning and Memory, 88(4),* 435-444*.*

Ferland CL, Reichel CM, & McGinty JF. (2016). Effects of oxytocin on methamphetamine-seeking exacerbated by predator odor pre-exposure in rats. *Psychopharmacology*, *233*, 1015-1024.

Fischer A, Sananbenesi F, Mungenast A, & Tsai LH. (2010). Targeting the correct HDAC(s) to treat cognitive disorders. *Trends Pharmacol Sci, 31(12),* 605-617.

Fitzgerald, P. J., Seemann, J. R., & Maren, S. (2014). Can fear extinction be enhanced? A review of pharmacological and behavioral findings. *Brain Research Bulletin*, *105*, 46-60.

Freedland CS, Sharpe AL, Samson HH, & Porrino LJ. (2001). Effects of SR141716A on ethanol and sucrose self-administration. *Alcoholism: Clinical and Experimental Research, 25(2),* 277-282.

Fuchs, R. A., Feltenstein, M. W., & See, R. E. (2006). The role of the basolateral amygdala in stimulus–reward memory and extinction memory consolidation and in subsequent conditioned cued reinstatement of cocaine seeking. *European Journal of Neuroscience*, *23*(10), 2809–2813.

Godino A, Jayanthi S, & Cadet JL. (2015). Epigenetic landscape of amphetamine and methamphetamine addiction in rodents. *Epigenetics, 10(7),* 574-580.

Goeders NE & Guerin GF. (1994). Non-contingent electric footshock facilitates the acquisition of intravenous cocaine self-administration in rats. *Psychopharmacology, 114,* 63-70.

Gräff, J., Joseph, N.F., Horn, M.E., Samiei, A., Meng, J., Seo, J., Rei, D., Bero, A.W., Phan, T.X., Wagner, F., Holson, E., Xu, J., Sun, J., Neve, R.L., Mach, R.H., Haggarty, S.J. & Tsai, L.H. (2014) Epigenetic priming of memory updating during reconsolidation to attenuate remote fear memories. *Cell, 156*, 261–276.

Gräff J & Tsai LH. (2013). The potential of HDAC inhibitors as cognitive enhancers. *Annu Rev Pharmacol Toxicol, 53,* 311-330.

Groblewski, P.A., Ryabinin, A.E., & Cunningham, C.L. (2012) Activation and role of the medial prefrontal cortex (mPFC) in extinction of ethanol-induced associative learning in mice. *Neurobiology of Learning and Memory, 97*(1), 37-46.

Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, Nieland TJF, Zhou Y, Wang X, Mazitschek R, Bradner JE, DePinho RA, Jaenisch R, & Tsai LH. (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature, 459,* 55-60.

Hadamitzky M, McCunney S, Markou A, Kuczenski R. (2013). Development of stereotyped behaviors during prolonged escalation of methamphetamine self-administration in rats. *Psychopharmacology, 223(3),* 259-269.

Halsall J, Gupta V, O'Neill LP, Turner BM, & Nightingale KP. Genes are often sheltered from the global histone hyperacetylation induced by HDAC inhibition. *PLoS One.*

Hariri AR & Holmes A. (2015). Finding translation in stress research. *Nature Neuroscience*, *18*, 1347-1352.

Harkness JH, Hitzemann RJ, Edmunds S, Phillips TJ. (2013). Effects of sodium butyrate on methamphetamine-sensitized locomotor activity. *Behav Brain Res, 239,* 139-147.

Harrod SB, Dwoskin LP, Crooks PA, Klebaur JE, & Bardo MT. (2001). Lobeline attenuates d-Methamphetamine self-administration in rats. *Journal of Pharmacology and Experimental Therapeutics, 298(1),* 172-179.

Helmstetter, F.J., Bellgowan, P.S. (1994). Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. *Behavioral Neuroscience, 108(5)*, 1005-1009.

Herringa, R.J., Nanda, S.A., Hsu, D.T., Roseboom, P.H., & Kalin, N.H. (2004). The effects of acute stress on the regulation of central and basolateral amygdala CRF-binding protein gene expression. *Molecular Brain Research, 131,* 17-25.

Hien DA, Jiang H, Campbell AN, Hu MC, Miele GM, Cohen LR, Brigham GS, Capstick C, Kulaga A, Robinson J, & Suarez-Morales L. (2010). Do treatment improvements in PTSD severity affect substance use outcomes? A secondary analysis from a randomized clinical trial in NIDA's Clinical Trials Network. *American Journal of Psychiatry*, *167,* 95-101.

Hitchcock, L.N. & Lattal, K.M. (2014) Histone-mediated epigenetics in addiction. In Lubin, F.D. & Akbarian, S. (eds), *Progress in Molecular Biology and Translational Science*. Epigenetics and Neuroplasticity: Evidence and Debate. Vol. 128. Academic Press, Burlington, MA, pp. 51–87.

Hitchcock LN, Cunningham CL, & Lattal KM. (2014). Cue configuration effects in acquisition and extinction of a cocaine-induced place preference. *Behavioral Neuroscience*, *128*, 217-227.

Hyman, S. E., Malenka, R. C., & Nestler, E. J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. *Annual Review of Neuroscience*, *29*(1), 565–598.

Inslicht SS, Metzler TJ, Garcia NM, Pineles SL, Milad MR, Orr SP, Marmar CR, & Neylan TC. (2013). Sex differences in fear conditioning in posttraumatic stress disorder. *Journal of Psychiatric Research, 47(1),* 64-71.

Itzhak Y, Liddie S, & Anderson KL. (2013). Sodium butyrate-induced histone acetylation strengthens the expression of cocaine-associated contextual memory. *Neurobiol Learn Mem, 102(34)*, 34-42.

Jacobsen, L. K., Southwick, S. M., & Kosten, T. R. (2001). Substance use disorders in patients with post-traumatic stress disorder: a review of the literature. *The American Journal of Psychiatry*, *158*(8), 1184–1190.

Jeanblanc J, Lemoine S, Jeanblanc V, Alaux-Cantin S, & Naassila M. (2015). The class I-specific HDAC inhibitor MS-275 decreases motivation to consume alcohol and

relapse in heavy drinking rats. *Int J Neuropsychopharmaol, 18(9).*

Kalda A, Heidmets L-T, Shen H-Y, Zharkovsky A, Chen J-F. (2007). Histone deacetylase inhibitors modulates the induction and expression of amphetamine-induced behavioral sensitization partially through an associated learning of the environment in mice. *Behav Brain Res, 181,* 76-84.

Keane TM & Wolfe J. (1990). Comorbidity in post-traumatic stress disorder: An analysis of community and clinical studies. *Journal of Applied Social Psychology, 20(21), 1776-1788.*

Kennedy,P.J.,Feng,J.,Robison,A.J.,Maze,I.,Badimon,A.,Mouzon, E., Chaudhury, D., Damez-Werno, D.M., Haggarty, S.J., Han, M.H., Bassel-Duby, R., Olson, E.N., & Nestler, E.J. (2013) Class I HDAC inhibition blocks cocaine-induced plasticity by targeted changes in histone methylation. *Nat Neurosci* 16, 434–440.

Kessler RC, Sonnega A, Bromet E, Hughes M, & Nelson CB. (1995). Post-traumatic stress disorder in the national comorbidity survey. *Arch Gen Psychiatry, 52,* 1048–60

Khan, S. & Liberzon, I. (2004) Topiramate attenuates exaggerated acoustic startle in an animal model of PTSD. *Psychopharmacology (Berl)* 172, 225–229.

Knox, D., Perrine, S. A., George, S. A., Galloway, M. P., & Liberzon, I. (2010). Single prolonged stress decreases glutamate, glutamine, and creatine concentrations in the rat medial prefrontal cortex. *Neuroscience letters*, *480*(1), 16-20.

Knox, D., Nault, T., Henderson, C., & Liberzon, I. (2012). Glucocorticoid receptors and extinction retention deficits in the single prolonged stress model.*Neuroscience*, *223*, 163-173.

Kobeissy FH, Mitzelfelt JD, Fishman I, Morgan D, Gaskins R, Zhang Z, Gold MS, & Wang KK. (2011). Methods in drug abuse models: Comparison of different models of methamphetamine paradigms. *Psychiatric Disorders,* 269-278.

Kofoed, L., Friedman, M. J., & Peck, R. (1993). Alcoholism and Drug Abuse in patients with PTSD. *Psychiatric Quarterly*, *64*(2), 151–171.

Koob GF & Le Moal M. (1997). Drug abuse: Hedonic homeostatic dysregulation. *Science, 278(5335),* 52-58.

Kim JJ & Jung MW. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neuroscience & Biobehavioral Reviews*, *30*, 188-202.

Krasnova IN, Justinova Z, Landenheim B, Jayanthi S, McCoy MT, Barnes C, Warner JE, Goldberg SR, & Cadet JL. (2010). Methamphetamine self-administration is

associated with persistent biochemical alterations in striatal and cortical dopaminergic terminals in the rat. *PlOS One*.

Krettek, J. E., & Price, J. L. (1977). Projections from the amygdaloid complex and adjacent olfactory structures to the entorhinal cortex and to the subiculum in the rat and cat. *The Journal of Comparative Neurology*, *172*(4), 723–752.

Kufahl PR, Zavala AR, Singh A, Thiel KJ, Dickey ED, Joyce JN, Neisewander JL. (2009). c-Fos expression associated with reinstatement of cocaine-seeking behavior by response-contingent conditioned cues. *Synapse, 63(10),* 823-835.

Kubiak SP. (2016). The effects of PTSD on treatment adherence, drug relapse, and criminal recidivism in a sample of incarcerated men and women. *Research on Social Work Practice, 14,* 424-433.

Kumar, A., Choi, K.-H., Renthal, W., Tsankova, N.M., Theobald, D.E.H., Truong, H.-T., Russo, S.J., Laplant, Q., Sasaki, T.S., Whistler, K.N., Neve, R.L., Self, D.W. & Nestler, E.J. (2005) Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in stria- tum. *Neuron* 48, 303–314.

Kwapis, J.L. & Wood, M.A. (2014) Epigenetic mechanisms in fear conditioning: implications for treating post-traumatic stress disorder. *Trends Neurosci* 37, 706–720.

LaLumiere, R. T., Niehoff, K. E., & Kalivas, P. W. (2010). The infralimbic cortex regulates the consolidation of extinction after cocaine self-administration. *Learning & Memory*, *17*(4), 168–175.

Lattal KM. (1999). Trial and intertrial durations in Pavlovian conditioning: issues of learning and performance. *J Exp Psychol Anim Behav Process, 25(4),* 433-450.

Lattal, K.M., Barrett, R. & Wood, M.A. (2007) Systemic or intrahip- pocampal delivery of histone deacetylase inhibitors facilitates fear extinction. *Behav Neurosci* 121, 1125–1131.

Lattal, K. M., & Wood, M. A. (2013). Epigenetics and persistent memory: implications for reconsolidation and silent extinction beyond the zero. *Nature Neuroscience*, *16*(2), 124–129.

Laurent, V. & Westbrook, R.F. (2009) Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. *Learn Mem, 16*, 520–529.

Lê AD, Poulos CX, Harding S, Watchus J, Juzytsch W & Shaham Y. (1999). Effects of naltrexone and fluoxetine on alcohol self-administration and reinstatement of alcohol seeking induced by priming injections of alcohol and exposure to stress. *Neuropsychopharmacology, 21,* 435-444.

Lê AD, Quan B, Juzytch W, Fletcher PJ, Joharchi N, & Shaham Y. (1998). Reinstatement of alcohol-seeking by priming injections of alcohol and exposure to stress in rats. *Psychopharmacology, 135*, 169–174.

Levenson, J.M., O'Riordan, K.J., Brown, K.D., Trinh, M.A., Molfese, D.L. & Sweatt, J.D. (2004). Regulation of histone acetylation during memory formation in the hippocampus. *J Biol Chem* 279, 40545 – 40559.

Levenson, J.M. & Sweatt, J.D. (2005) Epigenetic mechanisms in memory formation. *Nat Rev Neurosci* 6, 108–118.

Lewis CR, Staudinger K, Scheck L, & Olive MF. (2013). The effects of maternal separation on adult methamphetamine self-administration, extinction, reinstatement, and MeCP2 immunoreactivity in the nucleus accumbens. *Frontiers in Psychiatry.*

Li J-X, Han R, Deng Y-P, Chen S-Q, Liang J-H. (2005). Different effects of valproate on methamphetamine- and cocaine-induced behavioral sensitization in mice. *Behav Brain Res,161,* 125-32.

Liu X & Weiss F. (2002). Addictive effect of stress and drug cues on reinstatement of ethanol seeking: Exacerbation by history of dependence and role of concurrent activation of corticotropin-releasing factor and opioid mechanisms. *Journal of Neuroscience, 22,* 7856-7861.

Long VA, & Fanselow MS. (2012). Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. *Stress*, *15*, 627-636.

Luger, K., Mäder, A.W., Richmond, R.K., Sargent, D.F. & Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 389, 251–260.

Lynch WJ, Nicholson KL, Dance ME, Morgan RW, & Foley PL. (2010). Animal models of substance abuse and addiction: Implications for science, animal welfare, and society. *Comp Med, 60(3),* 177-188.

MacPherson, K., Whittle, N., Camp, M., Gunduz-Cinar, O., Singewald, N., & Holmes, A. (2013). Temporal factors in the extinction of fear in inbred mouse strains differing in extinction efficacy. *Biology of mood & anxiety disorders*, *3*(1), 13.

Marek, R., Coelho, C.M., Sullivan, R.K.P., Baker-Andresen, D., Li, X., Ratnu, V., Dudley, K.J., Meyers, D., Mukherjee, C., Cole, P.A., Sah, P. & Bredy, T.W. (2011) Paradoxical enhancement of fear extinction memory and synaptic plasticity by inhibition of the histone acetyltransferase p300. *J Neurosci* 31, 7486–7491.

Maren S, Phan KL, & Liberzon I. (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. *Nature Reviews Neuroscience*, *14*, 417-428.

Maren S, De Oca B, & Fanselow MS. (1994). Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in ras: positive correlation between LTP and contextual learning. *Brain Research, 661(1-2),* 25-34.

Malvaez M, McQuown SC, Rogge GA, Astarabadi M, Jacques V, Carreiro S, Rusche JR, & Wood MA. (2013). HDAC3-selective inhibitor enhances extinction of cocaine-seeking behavior in a persistent manner. *Proceedings of the National Academy of Science*, *110(7)*, 2647-2652.

Malvaez M, Sanchis-Segura C, Vo D, Lattal KM, & Wood MA. (2010). Modulation of chromatin modification facilitates extinction of cocaine-induced place preference . *Biol Psychiatry*, *67(1),* 36-43.

Mantsch JR, Baker DA, Funk D, Le AD, & Shaham Y. (2016). Stress-induced reinstatement of drug seeking: 20 years of progress. *Neuropsychopharmacology, 41(1),* 335-356.

Manzanares, P.A.R., Isoardi, N.A., Carrer, H.F., & Molina, V.A. (2005) Previous stress facilitates fear memory, attenuates GABAergic inhibition, and increase synaptic plasticity in the rat basolateral amygdala. *Journal of Neuroscience, 25(38), 8725-8734.*

McCauley JL, Killeen T, Gros DF, Brady KT & Back SE. (2012). Post-traumatic stress disorder and co-occurring substance use disorders: Advances in assessment and treatment. *Clinical Psychology*, *19*, 283-304.

McFarland K, Davidge SB, Lapish CC & Kalivas, PW. (2004). Limbic and motor circuitry underlying footshock-induced reinstatement of cocaine-seeking behavior. *The Journal of neuroscience*, *24*, 1551-1560.

McFarland, K., Davidge, S.B., Lapish, C.C., & Kalivas, P.W. (2004). Limbic and Motor Circuitry Underlying Footshock-Induced Reinstatement of Cocaine-Seeking Behavior. *The Journal of Neuroscience*, *24*(7), 1551–1560.

McFarlane AC. (1998). Epidemiological evidence about the relationship between PTSD and alcohol abuse: the nature of the association. *Addictive Behaviors*, 23, 813–825.

McGowan, P.O., Sasaki, A., D'Alessio, A.C., Dymov, S., Labonté, B., Szyf, M., Turecki G. & Meaney, M.J. (2009) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 12, 342–348.

McNally, G. P., Johansen, J. P., & Blair, H. T. (2011). Placing prediction into the fear circuit. *Trends in neurosciences*, *34*(6), 283-292.

McNally, G. P. (2014). Extinction of drug seeking: neural circuits and approaches to augmentation. *Neuropharmacology*, *76*, 528-532.

McQuown SC, Barrett RM, Matheos DP, Post RJ, Rogge GA, Alenghat T, Mullican SE, Jones S, Rusche JR, Lazar MA, & Wood MA. (2011). HDAC3 is a critical regulator of long-term memory formation. *J Neurosci, 31(2),* 764-774.

Meewisse ML, Reitsma JB, De Vrie GJ, Gersons BP, & Olff M. (2007). Cortisol and post-traumatic stress disorder in adults. *The British Journal of Psychiatry*, *191*, 387-392.

Meil WM & See RE. (1997). Lesions of the basolateral amygdala abolish the ability of drug associated cues to reinstate responding during withdrawal from self-administered cocaine. *Behav Brain Res, 87(2),* 139-148.

Mellman TA, Randolph CA, Brawman-Mintzer O, Flores LP, & Milanes FJ. (1992). Phenomenology and course of psychiatric disorders associated with combat related posttraumatic stress disorder, *Am J Psychiatry, 149,* 1568-1574.

Meyer ME, Long V, Fanselow MS, & Spigelman I. (2013). Stress increases voluntary alcohol intake, but does not alter established drinking habits in a rat model of post-traumatic stress disorder. *Alcoholism: Clinical and Experimental Research*, *37*, 566-574.

Mikics, E., Tóth, M., Varjú, P., Gereben, B., Liposits, Z., Ashaber, M., Halasz, J., Barna, I., Farkas, I. & Haller, J. (2008) Lasting changes in social behavior and amygdala function following traumatic expe- rience induced by a single series of foot-shocks. *Psychoneuroen- docrinology* 33, 1198–1210.

Morgan, M.A., Romanski, L.M. & LeDoux, J.E. (1993) Extinction of emotional learning: contribution of medial prefrontal cortex. *Neurosci Lett, 163*, 109–113.

Morris MJ, Mahgoub M, Na ES, Pranav H, & Monteggia LM. (2013). Loss of histone deacetylase 2 improves working memory and accelerates extinction learning. *J Neurosci*, *33(15)*, 6401-6411.

Moustafa, A. A., Gilbertson, M. W., Orr, S. P., Herzallah, M. M., Servatius, R. J., & Myers, C. E. (2013). A model of amygdala–hippocampal–prefrontal interaction in fear conditioning and extinction in animals. *Brain and cognition*,*81*(1), 29-43.

Najt P, Fusar-Poli P, & Brambilla P. (2011). Co-occuring mental and substance abuse disorders: a review on the potential predictors and clinical outcomes. *Psychiatry Research, 186,* 159-164.

Ouimette PC, Brown PJ, & Najavits LM. (1998). Course and treatment of patients with both substance use and post-traumatic stress disorders. *Addictive Behaviors, 23*, 785–795.

Ouimette PC & Brown PJ. (2003). Trauma and substance abuse: Causes, consequences, and treatment of comordbid disorders. American Psychological Association.

Pape, H. C., & Pare, D. (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiological reviews*, *90*(2), 419-463.

Paré, D., & Smith, Y. (1994). GABAergic projection from the intercalated cell masses of the amygdala to the basal forebrain in cats. *The Journal of Comparative Neurology*, *344*(1), 33–49.

Pastor V, Host L, & Bernabeu R. (2011) Histone deacetylase inhibition decreases preference without affecting aversion for nicotine. *J Neurochem*, *116(4),* 636-645.

Peters, J., Kalivas, P. W., & Quirk, G. J. (2009). Extinction circuits for fear and addiction overlap in prefrontal cortex. *Learning & Memory*, *16*(5), 279–288.

Piazza PV, Deminiere JM, le Moal M, & Simon H. (1990). Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration. *Brain Research*, *514*, 22–26.

Pizzimenti, CL & Lattal, KM. (2015). Epigenetics and memory: causes, consequences and treatments for post-traumatic stress disorder and addiction. *Genes, Brain and Behavior, 14,* 73-84.

Pizzimenti CL, Navis TM, & Lattal KM. (2017). Persistent effects of acute stress on fear and drug-seeking in a novel model of the comorbidity between post-traumatic stress disorder and addiction. *Learning & Memory, 24(9),* 422-431.

Platt DM, Carey G, & Spealman RD. (2012). Models of neurological disease (substance abuse): Self-administration in monkeys. *Current Protocols in Pharmacology*.

Ponomarev I, Rau V, Eger EI, Harris RA, & Fanselow MS. (2010). Amygdala transcriptome and cellular mechanisms underlying stress-enhanced fear learning in a rat model of posttraumatic stress disorder. *Neuropsychopharmacology, 35(6),* 1402-1411.

Prager EM, Bergstrom HC, Wynn GH, & Braga MF. (2016). The basolateral amygdala gamma-aminobutyric acidergic system in health and disease. *J Neurosci Res,* 94, 548–567.

Quadros IMH & Miczek KA. (2009). Two modes of intense cocaine bingeing: increased persistence after social defeat stress and increased rate of intake due to extended access conditions in rats. *Psychopharmacology*, *206,* 109–120.

Ramsey NF & Van Ree JM. (1993). Emotional but not physical stress enhances intravenous cocaine self-administration in drug-naïve rats. *Brain Research, 608,* 216-222.

Rau V, DeCola JP, & Fanselow MS. (2005). Stress-induced enhancement of fear learning: an animal model of post-traumatic stress disorder. *Neuroscience and Biobehavioral Reviews*, *29*, 1207–1223.

Rau V & Fanselow MS. (2009). Exposure to a stressor produces a long lasting enhancement of fear learning in rats. *Stress (Amsterdam, Netherlands)*, *12*, 125–133.

Raybuck, J.D., McCleery, E.J., Cunningham, C.L., Wood, M.A. & Lat- tal, K.M. (2013) The histone deacetylase inhibitor sodium butyrate modulates acquisition and extinction of cocaine-induced conditioned place preference. *Pharmacol Biochem Behav* 106, 109 – 116.

Redila VA & Chavkin C. (2008). Stress-induced reinstatement of cocaine seeking is mediated by the kappa opioid system. *Psychopharmacology*, *200*, 59-70.

Renthal, W., Maze, I., Krishnan, V., Covington, H.E., Xiao, G., Kumar, A., Russo, S.J., Graham, A., Tsankova, N., Kippin, T.E., Kerstetter, K.A., Neve, R.L., Haggarty, S.J., McKinsey, T.A., Bassel-Duby, R., Olson, E.N. & Nestler, E.J. (2007) Histone deacetylase 5 epigenet- ically controls behavioral adaptations to chronic emotional stimuli. *Neuron* 56, 517–529.

Renthal, W., Carle, T.L., Maze, I., Covington, H.E., Truong, H.-T., Alibhai, I., Kumar, A., Montgomery, R.L., Olson, E.N. & Nestler, E.J. (2008) Delta FosB mediates epigenetic desensitization of the c-fos gene after chronic amphetamine exposure. *J Neurosci* 28, 7344 – 7349.

Rescorla RA. (1988). Behavioral studies of Pavlovian conditioning. *Annu Rev Neurosci, 11,* 329-352.

Ressler KJ, Paschall GY, Zhou XL, & Davis M. (2002). Regulation of synaptic plasticity genes during consolidation of fear learning. *J Neurosci, 22,* 7892–7902.

Roberts NP, Roberts PA, Jones N, & Bisson JI. (2015). Psychological interventions for post-traumatic stress disorder and comorbid substance use disorder: A systemic review and meta-analysis. *Clinical Psychological Review, 38,* 25-38.

Roth, T.L., Lubin, F.D., Funk, A.J. & Sweatt, J.D. (2009) Lasting epigenetic influence of early-life adversity on the BDNF gene. *Biol Psychiatry* 65, 760–769.

Saladin ME, Drobes DJ, Coffey SF, Dansky BS, Brady KT, & Kilpatrick DG. (2003). PTSD symptom severity as a predictor of cue-elicited drug craving in victims of violent crime. *Addictive Behaviors*, *28*, 1611-1629.

Sanchez, C. J., & Sorg, B. A. (2001). Conditioned fear stimuli reinstate cocaine-induced conditioned place preference. *Brain research*, *908*, 86-92.

Sanchez CJ, Bailie TM, Wu W, Li N, & Sorg BA. (2003). Manipulation of dopamine d1-like receptor activation in the rat medial prefrontal cortex alters stress- and cocaine-induced reinstatement of conditioned place preference behavior. *Neuroscience, 119,* 497–505.

Schindler AG, Li S, & Chavkin C. (2010). Behavioral stress may increase the rewarding valence of cocaine-associated cues through a dynorphin/κ-opioid receptor-mediated mechanism without affecting associative learning or memory retrieval mechanisms. *Neuropsychopharmacology, 35*, 1932-1942.

Shaham Y & Stewart J. (1995). Stress reinstates heroin-seeking in drug-free animals: an effect mimicking heroin, not withdrawal. *Psychopharmacology, 119,* 334–341.

Shaham Y, Erb S, Leung S, Buczek Y, & Stewart J. (1998). CP-154,526, a selective non-peptide antagonist of the corticotropin-releasing factor$_1$ receptor attenuates stress-induced relapse to drug seeking in cocaine- and heroin-trained rats. *Psychopharmacology, 137,* 184-190.

Shaham Y, Erb S & Stewart J. (2000). Stress-induced relapse to heroin and cocaine seeking in rats: a review. *Brain Research Reviews*, *33*, 13–33.

Shaham Y & Stewart J. (1994). Exposure to mild stress enhances the reinforcing efficacy of intravenous heroin self-administration in rats. *Psychopharmacoloy, 114,* 523-527.

Shalev, U., Highfield, D., Yap, J., & Shaham, Y. (2000). Stress and relapse to drug seeking in rats: studies on the generality of the effect. *Psychopharmacology, 150,* 337-346.

Sharpe, M.J. & Killcross, S. (2015) The prelimbic cortex uses higher-order cues to modulate both the acquisition and expression of conditioned fear. *Front Syst Neurosci, 8,* 235.

Shepard JD, Bossert JM, Liu SY, Shaham Y (2004). The anxiogenic drug yohimbine reinstates methamphetamine seeking in a rat model of drug relapse. *Biol Psychiatry, 55,* 1082–1089.

Shen H-Y, Kalda A, Yu L, Ferrara J, Zhu J, Chen J-F. (2008). Additive effects of histone deacetylase inhibitors and amphetamine on histone H4 acetylation, cAMP

responsive element binding protein phosphorylation and DeltaFosB expression in the striatum and locomotor sensitization in mice. *Neuroscience,157*, 644-655.

Sheng, J., Lv, Z.G., Wang, L., Zhou, Y. & Hui, B. (2011) Histone H3 phosphoacetylation is critical for heroin-induced place preference. *Neuroreport* 22, 575–580.

Simon-O'Brien E, Alaux-Cantin S, Warnault V, Buttolo R, Naassila M, & Vilpoux C. (2015). The histone deactylase inhibitor sodium butyrate decreases excessive ethanol intake in dependent animals. *Addict Biol, 20(4),* 676-689.

Simpson TL, Stappenbeck CA, Varra AA, Moore SA, & Kaysen D. (2012). Symptoms of post-traumatic stress predict craving among alcohol treatment seekers: results of a daily monitoring study. *Psychol Addict Behav.*

Smith RC, Blumenthal H, Badour C, & Feldner MT. (2010). An investigation of relations between crystal methamphetamine use and post-traumatic stress disorder. *Addictive behaviors*, *35*, 625-627.

Sonne SC, Back SE, Zuniga CD, Randall CL & Brady KT. (2003). Gender differences in individuals with Comorbid Alcohol Dependence and Post-traumatic Stress Disorder, *The American Journal on Addictions, 12:* 412–423.

Stewart SH. (1996): Alcohol abuse in individuals exposed to trauma: a critical review. *Psychological bulletin*, *120*, 83-112.

Stafford, J.M. & Lattal, K.M. (2011) Is an epigenetic switch the key to persistent extinction?, *Neurobiol Learn Mem* 96, 35–40.

Stafford, J.M., Raybuck, J.D., Ryabinin, A.E. & Lattal, K.M. (2012) Increasing histone acetylation in the hippocampus-infralimbic network enhances fear extinction. *Biol Psychiatry* 72, 25–33.

Stefanski R, Justinova Z, Hayashi T, Takebayashi M, Goldberg SR, & Tsung-Ping S. (2004). $Sigma_1$ receptor upregulation after chronic methamphetamine self-administration in rats: a study with yoked controls. *Psychopharmacology, 175,* 68-75.

Strahl, B.D. & Allis, C.D. (2000) The language of covalent histone modifications. *Nature* 403, 41–45.

Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes & Development, 12,* 599-606.

Tata DA & Yamamoto BK. (2008). Chronic stress enhances methamphetamine-induced extracellular glutamate and excitotoxicity in the rat striatum. *Synapse, 62(5),* 325-336.

Tate SR, Brown SA, Unrod M, & Ramo DE. (2004). Context of relapse for substance-dependent adults with and without comorbid psychiatric disorders. *Addictive Behaviors, 29,* 1707-1724.

Tidey JW & Miczek KA. (1997). Acquisition of cocaine self-administration after social stress: role of accumbens dopamine. *Psychopharmacology, 130,* 203-212.

Tipps, M. E., Raybuck, J. D., & Lattal, K. M. (2014). Substance abuse, memory, and post-traumatic stress disorder. *Neurobiology of learning and memory*, *112*, 87-100.

Tokunaga I, Ishigami A, Kubo S, Gotohda T, & Ktamura O. (2008). The perioxidative DNA damage and apoptosis in methamphetamine-treated rat brain. *J Med Invest, 55(3-4)*, 241-245.

Truitt WA, Sajdyk tJ, Dietrich AD, Oberlin B, McDougle CJ, & Shekhar A. (2007). From anxiety to autism: spectrum of abnormal social behaviors modeled by progressive disruption of inhibitory neuronal function in the basolateral amygdala in Wistar rats. *Psychopharmacology, 191(1)*, 107-118.

Ujike H, Onoue T, Akiyama K, Hamamura T, & Otsuki S. Effects of D-1 and D-2 dopamine antaognists on development of methamphetamine-induced behavioral sensitization. *Psychopharmacology, 98(1),* 89-92.

Vanyushin, B.F. (2006) DNA methylation and epigenetics. *Russ J Genet* 42, 985–997.

Vlachos I, Herry C, Luthi A, Aertsen A, & Kumar A. (2011). Context-dependent encoding of fear and extinction memories in a large-scale network model of the basal amygdala. *PLoS Comput Biol, 7(3).*

Vecsey, C.G., Hawk, J.D., Lattal, K.M., Stein, J.M., Fabian, S.A., Attner, M.A., Cabrera, S.M., McDonough, C.B., Brindle, P.K., Abel, T. & Wood, M.A. (2007) Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent tran- scriptional activation. *J Neurosci* 27, 6128–6140.

Vinklerova J, Novakova J, & Sulcova A. (2002). Inhibition of methamphetamine self-administration in rats by cannabinoid receptor antagonist AM 251. *Journal of Psychopharmacology, 16(2),* 139-143.

Wachen, JS, Jimenez S, Smith K, & Resick PA. (2014) Long-term functional outcomes of women receiving cognitive processing therapy and prolonged exposure. *Psychol Trauma: Theory Res Pract Policy,16*, 58–65.

Wagner JM, Hackanson B, Lubbert M, & Manfred J. (2010). Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. *Clin Epigenetics, 1(1-3),* 117-136.

Wang R, Zhang Y, Qing H, Liu M, & Yang P. (2010). The extinction of morphine-induced conditioned place preference by histone deactylase inhibition. *Neurosci Lett., 483(2)*, 137-142.

Wallace KJ & Rosen JB. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav Neurosci, 114,* 912–922.

Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M. & Meaney, M.J. (2004) Epigenetic programming by maternal behavior. *Nat Neurosci* 7, 847 – 854.

Wei, W., Coelho, C.M., Li, X., Marek, R., Yan, S., Anderson, S., Mey- ers, D., Mukherjee, C., Sbardella, G., Milite, C., Rotili, D., Mai, A., Cole, P.A., Sah, P., Kobor, M.S. & Bredy, T.W. (2012) p300/CBP- associated factor selectively regulates the extinction of conditioned fear. *J Neurosci* 32, 11930–11941.

Whitelaw RB, Markou A, Robbins TW, & Everitt BJ. (1996). Excitotoxic lesions of the basolateral amygdala impair the acquisition of cocaine-seeking behavior under a second-order schedule of reinforcement. *Psychopharmacology, 127(3),* 213-224.

Whittle N., Schmuckermair C., Gunduz Cinar O., Hauschild M., Ferraguti F., Holmes A., & Singewald N. (2013). Deep brain stimulation, histone deacetylase inhibitors and glutamatergic drugs rescue resistance to fear extinction in a genetic mouse model. *Neuropharmacology, 64*, 414–423.

Wilkinson CW, Pagulayan KF, Petrie EC, Mayer CL, Colsaurdo EA, Shofer JB, Hart KL, Hoff D, Tarabochia MA, & Peskind ER. (2012). High prevalence of chronic pituitary and target-organ hormone abnormalities after blast-related mild traumatic brain injury. *Front. Neurol.*

Willner P. (1997). Chronic mild stress (CMS) revisited: consistency and behavior-neurobiological concordance in the effects of CMS. *Neuropsychobiology, 52*, 90-110.

Yehuda R, Boisoneau D, Mason JW, & Giller EL. (1993). Glucocorticoid receptor number and cortisol excretion in mood, anxiety, and psychotic disorders. *Biological Psychiatry, 34(1-2),* 18-25.

Yehuda R, Levengood RA, Schmeidler J, Wilson S, Guo LS, & Gerber D. (1996). Increased pituitary activation following metyrapone administration in post-traumatic stress disorder. *Psychoneuroendocrinology, 21(1),* 1-16.

Yehuda R & Seckl J. (2011). Minireview: stress-related psychiatric disorders with low cortisol levels: a metabolic hypothesis. *Endocrinology*, *152*, 4496-4503.

Zakharova E, Leoni G, Kichko I, & Izenwasser S. (2009). Differential effects of methamphetamine and cocaine on conditioned place preference and locomotor activity in adult and adolescent male rats. *Behavioral Brain Research, 1(2),* 45-50.

Zovkic, I.B. & Sweatt, J.D. (2012) Epigenetic mechanisms in learned fear: implications for PTSD. *Neuropsychopharmacology* 38, 77–93.