Involvement of the dorsal hippocampus and histone deacetylase 3

# in reward-seeking behaviors

A Dissertation Presented to the

Department of Behavioral Neuroscience, School of Medicine

Oregon Health & Science University

In Partial Fulfillment of the Requirements for

Doctorate of Behavioral Neuroscience

Leah Hitchcock

August 2017

#### OREGON HEALTH & SCIENCE UNIVERSITY SCHOOL OF MEDICINE – GRADUATE STUDIES Guidelines and Regulations for Completion of Master's and Ph.D. Degrees

School of Medicine

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# **CERTIFICATE OF APPROVAL**

This is to certify that the PhD dissertation of

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# List of Abbreviations

Abbreviation	Word or Phrase
A1	acquisition in a one-compartment apparatus
A2a	acquisition in a two-compartment apparatus, alternating sides
A2c/A2	acquisition in a two-compartment apparatus, consistent sides
AAV	adeno-associated virus
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMY	amygdala
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BLA	basolateral amygdala
cAMP	cyclic adenosine monophosphate
COC	cocaine
CON	control/empty vector expression (AAVHDAC3-CON)
CPP	conditioned place preference
CRE	cAMP response element
CREB	cAMP responsive element binding protein
CS (+ or -)	conditioned stimulus (positive or negative)
CTX	context
DH	dorsal hippocampus
DNA	deoxyribonucleic acid
E1	extinction in a one-compartment apparatus
E2a	extinction in a two-compartment apparatus in alternating sides
E2c/E2	extinction in a two-compartment apparatus in a consistent side
EC	entorhinal cortex
G (+ or -)	grid floor (conditioned with CS+ or CS-)
GABAa	gamma-aminobutyric acid (ionotropic) receptor
GSK3B	Glycogen synthase kinase 3 beta
H (+ or -)	hole floor (conditioned with CS+ or CS-)
H4K8	histone 4 lysine 8
HDAC	histone deacetylase
HDAC3	histone deacetylase 3
HDACi	histone deacetylase inhibitor
IHC	immunohistochemistry
IL	infralimbic
MUS	muscimol
NA	nucleus accumbens
NaB	sodium butyrate
NCoR	nuclear receptor co-repressor

# List of Abbreviations (continued)

Abbreviation	Word or Phrase
NMDA	N-methyl-D-aspartate
PBS	phosphate buffered saline
PFC	prefrontal cortex
PKA	protein kinase A
PM	point mutation overexpression (AAVHDAC3-Y298H-v5)
RGFP966	HDAC3 inhibitor (Repligen)
RSC	retrosplenial cortex
SA	self-administration
TF	transcription factor
v5	virus type 5 epitope tag derived from simian parainfluenza
VEH	vehicle
VH	ventral hippocampus
VPA	valproic acid
VTA	ventral tegmental area
WT	wildtype overexpression (AAVHDAC3-WT-v5)

#### Acknowledgments

Funding for this research was provided by the National Institutes on Drug Abuse (NIDA) in the National Institutes of Health, grants P50DA018165, R01DA025922, R01DA025922S1 (K.M.L.), and T32DA007262 (OHSU, trainee L.N.H.), the Department of Defense grant W81XWH-12-2-0048 (K.M.L.), and the American Psychological Association grant APA1006636 (L.N.H.).

I would additionally like to thank the people who supported me through this research. Many thanks to my mentor Dr. Matt Lattal and the rest of my dissertation committee (Drs. Chris Cunningham, Andrey Ryabinin, Aaron Janowsky, and Matthew Ford) for your exemplary instruction, leadership, and compassion throughout my training at OHSU. Thank you to Drs. Karlie Intlekofer, Brian Tracy, and the Navises for their extraordinary donation of time and help over the years, in and out of the lab.

I would like to acknowledge the ever-expanding support (understatement!) of my father Eric Hitchcock, my partner Kyle Donovan, and my friend and fellow graduate Melanie Pina. They kept me strong and laughing through the years, even when I doubted myself.

Thank you to my mother Susan Hitchcock, who was not able to see this work in its final stage. Ahead of the curve, she was the first one to smile and call me Dr. Hitchcock (and mean it), and the one who helped me understand the true value of my training during her final stage. If she were still with me, she would tell me to "Pop the Champagne Already!" I would like to dedicate my degree to her.

#### Preface

All of the work presented hereafter was conducted in K. Matthew Lattal's Laboratory at Oregon Health & Science University, Portland, Oregon. All experiments were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Correspondence concerning this dissertation may be addressed to hitchcol@ohsu.edu.

Portions of Chapters 1 (General Introduction) and 6 (General Discussion) have been published by Hitchcock & Lattal, 2014. I was the lead author, responsible for all concept formation, figure production, and manuscript composition. Dr. Lattal was the supervising author and was involved throughout the project in concept formation, manuscript edits, and composition.

A version of Chapter 2 has been published in *Behavioral Neuroscience* (Hitchcock, Cunningham, & Lattal, 2014). I was the lead investigator, responsible for concept formation, data collection and analysis, as well as manuscript composition. Dr. Cunningham was involved in the early stages of concept formation and contributed to manuscript edits. Dr. Lattal was the supervisory author on this project and was involved throughout the project in concept formation and manuscript edits.

A version of Chapter 3 is in preparation for submission. I was the only investigator for Chapter 3, responsible for concept formation, data collection, and analysis, as well as manuscript composition. Dr. Lattal was the supervising author and was involved throughout the project in concept formation and manuscript edits.

A version of Chapter 4 is in preparation for submission. I was the lead investigator for this work, responsible for all major areas of concept formation, data collection and analysis, as well as manuscript composition. Dr. Jon Raybuck was involved in the early stages of concept formation (including preliminary data collection), and for portions of project design and data collection. Dr. Antony Abraham (Experiment 1) helped collect extinction data and Rapheal Williams (Experiment 3) helped maintain catheter patency when I was unavailable. Repligen Corporation provided RGFP966. Dr. Lattal was the supervising author and was involved throughout the project in concept formation and manuscript edits.

I was the lead investigator for the project in Chapter 5, responsible for concept formation, data collection, analysis, and manuscript composition. On the final day of behavioral experiments, Tommy Navis assisted me in euthanizing rats and removing and flash freezing brains for immunohistochemistry (IHC). Dr. Wood (University of California-Irvine) was involved in the concept formation, provided technical expertise for IHC analysis, and provided the AAV plasmid services (cloning, DNA amplification, and sequence verification) for vector production by the University of Pennsylvania (UPenn) Vector Core. Dr. Lattal was the supervisory author on this project and was involved throughout the project in concept formation and manuscript edits.

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#### Abstract

Substance use disorder is a chronic, often relapsing disease that can include a loss of behavioral inhibition and compulsive drug-seeking. Acquisition of a reward seeking behavior often begins when cues are paired with rewards. These cues are thought to influence subsequent extinction (animal model of exposure-based therapy) and relapse-like behavior, both in humans and animals. My work found that acquisition and extinction of cocaine-induced conditioned place preference (CPP) were sensitive to the configuration of the apparatus (Chapter 2) and that the dorsal hippocampus (DH) regulated expression of CPP after acquisition and extinction (Chapter 3).

At the molecular level, the epigenetic enzyme histone deacetylase 3 (HDAC3), is a negative regulator of cocaine-associated learning and spatial memory. I determined that inhibition of HDAC3 activity enhanced this hippocampus-based extinction after operant self-administration. Extended extinction did not eliminate contextual or cue-induced reinstatement, but the systemic injection of RGFP966 (HDAC3 inhibitor) caused persistent extinction and weakened context and cue-induced reinstatement (Chapter 4).

The generality of these findings was examined by assessing overexpression of a wildtype or point mutant version of HDAC3 in the dorsal hippocampus during acquisition and extinction of responding for natural rewards (food pellets). In this experiment, the HDAC3 point mutant led to faster acquisition and faster extinction, similar to prior effects with the systemic HDAC3 inhibitor (Chapter 5). Together, this dissertation presents novel findings on interactions between the context, dorsal hippocampus, and HDAC3 regulating acquisition and extinction of Pavlovian (Chapters 2-3) or instrumental rewardseeking behaviors (Chapters 4-5). Further research on these factors and associated brain activity may be used to design novel prevention and treatment for problematic and context-dependent reward behavior.



Portions of Chapter 1 are adapted from the publication:

Hitchcock, L. N., & Lattal, K. M. (2014). *Histone-Mediated Epigenetics in Addiction. Epigenetics and Neuroplasticity – Evidence and Debate* (1st ed., Vol. 128).

#### **General Introduction**

Adapting to a constantly changing environment requires the ability to acquire new behaviors and change old ones in response to environmental contingencies. For this reason, much of our behavior is controlled by its consequences, with response probabilities in given situations changing as a function of the history of reinforcement in those situations. These experiences are learned and remembered through several well-defined neural circuits that are involved in acquisition, consolidation, retrieval, and expression of memories. In addiction, the reinforcing properties of the abused substance can be so powerful that the plasticity mechanisms involved in normal learning and memory are usurped, often resulting in repetitive and persistent behaviors that are resistant to change, even in the face of strong negative consequences. It is thought that such habitual changes in behavior are due to shifts in the underlying circuitry expressing dominance over behavior. One key to understanding addiction is to understand the basic mechanisms of learning and memory and how these may be altered by abused substances (e.g., Hyman et al., 2006). A great deal is now known about the ways in which memories are formed, from binding of neurotransmitters at the receptor level, to the activation of transcriptional machinery needed for the synthesis of new proteins that solidify long-term memories.

A growing literature indicates that long-term memories form at a molecular level as a consequence of changes in gene expression induced by activitydependent histone modifications (Levenson, O'Riordan, et al., 2004; Vecsey et al., 2007). These same mechanisms are involved in acquiring and stabilizing the long-term reinforcing effects of various rewards (e.g., drugs of abuse). In this dissertation, I examine various aspects of learning, memory, and addiction in relation to contextual cues, dorsal hippocampal activity, and one molecular modification. This chapter begins with an overview of learning and memory; the general processes that occur in the development, maintenance, and treatment of problematic behaviors (i.e., substance and non-substance addictions, Kardefelt-winther et al., 2017); the molecular underpinnings of reward-based learning, and the histone regulation that may be involved. I detail a specific histone-modifier that is known to interact with other epigenetic regulation, and exert significant control over long-term gene expression and plasticity. Lastly, I review current literature demonstrating how these mechanisms may be involved in various addiction processes similar to those investigated in this dissertation.

In Chapters 2, 3, and 4, I consider how manipulations of the context, the dorsal hippocampus, and one specific histone-modifying enzyme interact with behavior (acquisition and retrieval of memories, as well as extinction of reward-seeking behaviors) and review different theoretical perspectives that may account for these effects. Finally, I end this dissertation with a description of currently unresolved issues in the field of hippocampus-based theories of extinction and histone-mediated epigenetics in addiction and suggest future directions that may help to resolve some of these debates.

#### Learning processes involved in the development of addiction

One of the reasons that addiction is thought to involve learning and memory circuits is that drug seeking often occurs in the presence of specific cues. These cues can be contextual (e.g., drinking a beer in a favorite bar), social (e.g., using cocaine with a specific group of friends), and temporal (e.g., having a cigarette first thing in the morning), among other types of cues. Over time, drug-seeking occurs in various situations, broadening the cues that may be associated with drug intake. After even relatively few experiences, those cues will evoke powerful drug cravings when they are encountered. These cravings create a negative internal state that further motivates drug intake. From a learning and memory perspective, the challenge is in understanding how these cues become associated with drugs and what can be done to sever, or at the very least, suppress those powerful associations to support long-term abstinence.

Basic research on learning and memory processes in substance abuse has focused on mechanisms that underlie two very general learning processes: initial acquisition, in which the memory is initially formed and consolidated, and long-term maintenance, in which the memory is retrieved and modulated (i.e., extinction and reinstatement). Behavioral assays are often used in animals and humans to determine the mechanisms of these learning processes (i.e., acquisition, retrieval, extinction, reinstatement) and types (i.e., Pavlovian, operant). Animal models of reward-seeking behavior, such as conditioned place preference (CPP) and self-administration, are commonly used to investigate the reinforcing effects of drugs and infer the strength of drug-–cue or drug-response associations. In associative learning, CPP is most commonly used to study associative and multimodal drug-related learning and memory, often referred to as drugseeking. During CPP, a drug is administered to the subject and then paired with a particular place. The subject will often create a lasting memory, either aversive or rewarding, for the unconditioned stimulus (US) drug, and conditioned stimulus (CS) place. If the subject finds the drug rewarding they will often express this preference by spending a higher percentage of time near the previously paired place in subsequent testing.

In operant self-administration (SA), similar associative mechanisms occur in each learning process (i.e., acquisition, extinction, etc.), but it requires an additional response to be expressed and assessed. In drug self-administration, subjects self-administer substances (i.e., drug infusions, etc.). To determine the amount of instrumental learning that has accrued or memory that has remained, voluntary responses that are directly associated with an outcome are measured (i.e., active lever presses that lead to drug infusions).

Further research is needed to determine how spatial cues and contexts in these CPP and SA assays are used to help acquire, recall, or extinguish relevant associations and behaviors. Other assays, such as fear conditioning, objectlocation or recognition, mazes, and self-administration of natural rewards (i.e., pellets) are more commonly used to assess spatial or contextual learning effects. In contrast, it is less common for investigators using CPP and SA assays to compare the theoretical, behavioral, and molecular effects of the context on reward learning directly. The few CPP exceptions that exist show that spatial cues (Cunningham, Patel, & Milner, 2006; Cunningham & Zerizef, 2014) and the size of conditioning space (Vezina & Stewart, 1987a, 1987b) can alter preference for a previously drug paired area. In self-administration studies, testing the animal in a different context can increase lever pressing for a drug, depending on animal's previous history in that context (Crombag, Bossert, Koya, & Shaham, 2008a). This work and others (Crombag, Grimm, & Shaham, 2002; Crombag & Shaham, 2002; Todd, 2013; Todd, Vurbic, & Bouton, 2014) laid the groundwork for experiments in this dissertation, where CPP and self-administration contexts were directly modulated to test the control of context in reward seeking behavior and its underlying mechanism.

A new area of concentration in the substance use field is the study of epigenetic mechanisms (to be discussed later in this chapter). Briefly, epigenetics is the study of molecular pathways that alter the organization or accessibility of DNA, potentially affecting downstream transcription, plasticity, and behavior. In memory aspects of addiction, epigenetic research has largely focused on simple memory processes -- how cue-drug associations are initially encoded, consolidated, and retrieved. This research has revealed the critical importance of histone acetylation and gene expression in mediating several aspects of these memory processes. I begin this chapter by reviewing histonemediated epigenetics in addiction with a description of some of these memory processes in more detail.

# Initial establishment and epigenetic regulation of drug-associated memories

When patterns of drug use first begin, new associations are encoded between the drug and the user's environment, consolidated into a memory, and later retrieved when cues associated with drug seeking are encountered. The very first experience with a drug of abuse typically activates circuits that are involved in aversion or reward and in learning and memory. With this first exposure, the initial memory begins to form. This memory likely involves distal contexts and discrete cues associated with some aspect of the drug of abuse. Theoretical approaches to memory have found that upon this initial exposure to the drug, the memory is labile for a period of time before it is stabilized through a time-limited consolidation process (McGaugh, 2000). As these memories are being established, they can be modified by additional processes that are triggered by drugs of abuse, such as sensitization, tolerance, and withdrawal (Gould & Leach, 2014; Wise et al., 2011). Together, these processes ultimately result in habitual drug seeking that results from an interaction of circuits mediating contextual information (e.g., the hippocampus), response initiation and maintenance (e.g., the striatum), and reward value (e.g., nucleus accumbens and amygdala; Koob & Volkow, 2010).

These initial memory processes contribute to the development of the repeated binge/intoxication stage of drug addiction, driven by a collection of brain regions in an excitatory circuit. The first time drugs of abuse are used (e.g., psychostimulants, alcohol, opioids, nicotine,  $\Delta$  9 tetrahydro-cannabinol), brain

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regions in both reward (e.g., ventral tegmental area, striatum, nucleus accumbens core, thalamus) and learning (e.g., basolateral and central nucleus of the amygdala, medial prefrontal cortex, hippocampus) centers share excitatory information (Marchant, Millan, & McNally, 2012). Some of these structures serve a primary role in particular stages of addiction (e.g., binge/intoxication, withdrawal/negative affect, and preoccupation/ anticipation/ craving; Koob & Volkow, 2010) and memory processes (i.e., encoding, consolidation, and retrieval; Bernardi, Ryabinin, Berger, & Lattal, 2009; Lalumiere, Smith, & Kalivas, 2012), yet many of these regions can be recruited throughout each process. Importantly, the circuits mediating aspects of reward and aspects of memory overlap, with key processes in the amygdala (AMY), ventral tegmental area (VTA), and nucleus accumbens (NA) controlling consolidation of these drug memories.

# Retrieval of drug-associated memories and extinction of drug-seeking behavior

Once memories between environmental cues and drugs of abuse are established, several consequences occur with subsequent exposure to those cues and drugs. First, these memories may evoke cravings, causing an increase of drug-seeking behavior and drug consumption (Robinson & Berridge, 1993). Repeated cravings and drug administration result in tolerance and withdrawal, two processes that are key to maintaining addiction (Siegel, 1983). Second, the act of retrieval will trigger some of the initial processes – encoding and consolidation of the retrieved memory – and will potentially include new contextual components that were absent during initial acquisition. This additional consolidation may simply recapitulate the initial consolidation process (i.e., reconsolidation, Tronson & Taylor, 2013), and in addition, they almost certainly involve consolidation of new memories specific to this new experience (e.g., Badiani & Robinson, 2004). These consolidation and reconsolidation processes are thought to contribute to the long-term maintenance of addiction. Third, if the drug that is expected (based on the retrieval of a previous memory) is not consumed, extinction may begin to develop. If no drug is administered during repeated retrieval episodes, drug-seeking behavior may be extinguished due to the cue-no drug association developing alongside the original cue-drug association. The persistence and stability of this cue-no drug extinction association is often limited (reviewed in Dunsmoor, Niv, Daw, et al., 2015), as demonstrated by common forms of relapse.

The inhibitory learning that occurs during extinction requires similar encoding, consolidation, and retrieval processes as during the excitatory learning associated with initial acquisition. There are important similarities in the systems and molecular steps that are involved in initial memory formation and extinction (e.g., Lattal, Radulovic, & Lukowiak, 2006) but there also are critical differences. For example, initial memory formation and extinction may recruit specific and distinct subregions of the medial prefrontal cortex (PFC), AMY, and NA (e.g., Koob & Volkow, 2010; Peters, Kalivas, & Quirk, 2009; Stefanik, Kupchik, Brown, et al., 2013). It is thought that these excitatory and inhibitory circuits are usurped during the transition from drug use to eventual addiction (e.g., Hyman, 2005) and modulate inhibition of drug-seeking behavior during extinction or abstinence.

While extinction treatment (a model of clinical exposure therapy; Nic Dhonnchadha & Kantak, 2011) diminishes drug-seeking behavior, relapse often occurs over time (spontaneous recovery; e.g., Brooks, 2000) with the presentation of drug associated cues (reinstatement; e.g., Shaham, Adamson, Grocki, et al., 1997) or after leaving the extinction context (context-induced renewal, Crombag & Shaham, 2002). Renewal, or the return of an extinguished behavior once a subject is removed from the extinction context, is also commonly referred to as context-induced reinstatement in certain instrumental assays, especially in assays concerned with addictive instrumental behaviors (described further below, reviewed in Podlesnik, Kelley, Jimenez-Gomez, et al., 2017). The mechanisms of extinction and relapse are of particular interest in this chapter as they may apply to the treatment and potential prevention of many disorders where extinction learning may be impaired in post-traumatic stress disorder and addiction, for example (Tipps, Raybuck, & Lattal, 2014).

#### Contextual control of behavior and the dorsal hippocampus

Much of what we know about the contextual control of behavior is related to acquisition, extinction, and retrieval of Pavlovian fear conditioning (Barrientos, O'Reilly, & Rudy, 2002; Daumas, Halley, Francés, et al., 2005; Zelikowsky, Hersman, Chawla, et al., 2014; Vervliet, Craske, & Hermans, 2013) and in instrumental spatial learning assays (Balderas et al., 2008; Mizuno et al., 2002; Wartman & Holahan, 2013). Although a great deal is known about hippocampal function in these procedures (e.g., Ji & Maren, 2005; Ji & Maren, 2008; Todd, Jiang, DeAngeli, et al., 2017), fewer studies have linked the hippocampus and contextual control of behavior in reward-related assays, such as CPP and selfadministration (Groblewski, Franken, & Cunningham, 2011; Marinelli et al., 2007; Meyers, Zavala, Speer, & Neisewander, 2006; Raybuck, McCleery, Cunningham, Wood, & Lattal, 2013; Todd et al., 2014). Most of the work in drug and non-drug operant responding is related to the idea that the dorsal hippocampus (DH) is involved in context-induced reinstatement in animals and relapse in humans (e.g., McClernon et al., 2016, reviewed in Crombag, Bossert, Koya, & Shaham, 2008). In general, decreases in DH activity are associated with decreases in drug-related context-induced reinstatement or renewal (Fuchs et al., 2005). Nondrug operant procedures have also identified a similar role for the DH in contextbased behavior (Wilson, Brooks, & Bouton, 1995), although literature is still sparse. However, even fewer studies have extended their investigations to the potential interaction of epigenetic modifications in the dorsal hippocampus.

Although the role of the hippocampus in contextual modulation of drugseeking is not well established, there is good reason to think that it plays a key role. One reason is that, as described above, substance abuse seems to be controlled by the same contextual mechanisms as other Pavlovian and instrumental behaviors. A second reason is that, at a systems level, the hippocampus interacts with multiple brain regions that are involved in different aspects of acquisition, maintenance, extinction, and relapse of drug-seeking behaviors.

Experiments in this dissertation sought to determine how manipulations to the DH altered reward-based learning. The dorsal hippocampus (DH) and infralimbic cortex (IL) support extinction and reinstatement and may rely on specific modifications to transcription and the epigenome in these regions. More than a decade of research demonstrates that a mismatch in context is recognized by the DH (Kumaran & Maguire, 2007; Lang et al., 2009), which modulates the input from the DH to other brain regions (i.e., NA, AMY, and PFC) and contributes to the context-specificity of renewal (Knapska & Maren, 2009). Strong input from the entorhinal cortex (EC), bidirectional connections to the hippocampus from the postrhinal cortex (Agstera & Burwell, 2013; Burwell, 2004) and connections from the hippocampus to the NA core (Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004), prelimbic (Barker et al., 2017), ventral hippocampus (Sigurdsson & Duvarci, 2016), VTA (Luo, Tahsili-Fahadan, Wise, Lupica, & Aston-Jones, 2011; Penner & Mizumori, 2012), and the intermediate hippocampal areas to the IL (Fanselow & Dong, 2010) help connect sensory, spatial and limbic information. In addition, the IL connects to more inhibitory subregions of the NA and AMY (i.e., NA shell and intercalated cells of the AMY; (Peters, Vallone, Laurendi, Kalivas, 2008; Sotres-Bayon, Bush, & LeDoux, 2004), likely contributing to the inhibition of problematic behavior, the expression of extinction, and mediating context-dependent extinction in animals and humans (Kalisch et al., 2006).

Molecular mechanisms in learning, memory, and drug-seeking

We now know a great deal about the learning and memory processes that are involved in the establishment and maintenance of addiction. As an organism interacts with its environment, certain experiences trigger a cascade of events that lead to long-term memory formation. Depending on the nature of these experiences, different signals may be sent to various brain regions (e.g., tactile shock sends a pain signal to periaqueductal gray (PAG); spatial cues send a signal to the dorsal hippocampus). Within these different brain regions, any number of molecular signaling cascades is set into motion. Depending on the type of stimulus, signals trigger some action at the cellular level (e.g., binding of neurotransmitters to receptors, opening of ion channels) that is followed by movement of the signals into the cytoplasm, where different second-messenger signaling cascades are activated. This cascade may include activation of protein kinases that translocate into the nucleus and activate transcriptional machinery.

It is widely accepted that long-term learning critically depends on this downstream wave of transcription and protein synthesis, occurring for hours after stimulation (Hawk & Abel, 2011), as well as typical changes to dendritic spines (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). This process typically begins with the release of glutamate onto postsynaptic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, the release of calcium, second messenger G protein coupled receptor activation, elevated cyclic adenosine monophosphate (cAMP) production and activation of protein kinase A (PKA) or mitogen-activated protein kinase (MAPK) based on stimuli and cellular context. Once intracellular, PKA leads to phosphorylation of

cAMP responsive element binding (CREB) protein and recruitment of CREB binding protein/histone acetyl transferase (CBP/p300) and targeted transcription. These events may lead to an increase in transcription of immediate early genes, such as the activity-dependent family of nuclear orphan receptor and transcription factors (TFs) such as Nr4a or others (i.e., c-fos and arc). MAPK also leads to transcription, typically of Nr4a target genes, such as brain-derived neurotrophic factor (BDNF, Hawk & Abel, 2011). The transcription and translation of these genes into proteins is necessary for lasting memory consolidation (Rosenberg et al., 2014).

Many studies have found that inhibiting these intracellular mechanisms by pharmacological or genetic approaches leads to impairments in long-term memory consolidation (Abel & Lattal, 2001; Andre, Farahnaz, Schrick, Spiess, & Radulovic, 2004; Jarome et al., 2012; Jarome & Lubin, 2014; Kemenes, Kemenes, Michel, Papp, & Muller, 2006). These manipulations also lead to deficits in long-term potentiation in the hippocampus, a cellular analog of memory (Maity, Jarome, Blair, Lubin, & Nguyen, 2015; Nguyen et al., 1994). This necessary transcription may partially be regulated by epigenetic modifications, and is likely a critical component of memory storage.

#### General overview of histone mediated epigenetics

The field of neuroepigenetics has become a primary area of research for modulating transcription, protein synthesis, and the potential fate of learning, memory, and treatment of addictive behaviors. The term epigenetics refers to the regulation that occurs "epi" or "over" the genomic DNA. Although controversy about the term epigenetics remains, it commonly refers to reversible and nonheritable changes to the DNA or proteins (i.e., histones) that alter gene expression (Brumfiel, 2008; Deans & Maggert, 2015; Isles, 2015). Within the nucleus of a cell, DNA is wrapped around multiple nucleosomes. Nucleosomes are composed of and linked together by a collection of histone proteins. Histones are the small and positively charged building blocks that help package and organize DNA into a repeat bead-like structure. To allow for selective and modifiable outcomes through epigenetic processes, each histone is classified into one of two super-families (i.e., histone core or histone linker), five families (H2A, H2B, H3, H4, and H1/H5), and multiple subfamilies. Each of these have slightly different functions and cellular distribution patterns (Cheung, Allis, & Sassone-Corsi, 2000; Strahl & Allis, 2000). Two copies of each histone core (H2A, H2B, H3, and H4) are bound together by linker histones (H1/H5) to create one nucleosome (an octamer of core histones) for DNA to be carefully wrapped around, making the basic chromatin structure (i.e., nucleosome + DNA). Although not reviewed here, these histone variant modifications are also important for developmental, activity-dependent, and disease related regulation (reviewed in Maze, Noh, & Allis, 2012; Maze, Noh, Soshnev, et al., 2014). In this chapter, I will consider how histones may be modified during different stages of drug taking acute exposure, chronic drug taking, withdrawal, abstinence, and relapse. First, basic histone modifications will be reviewed, with evidence for their role in learning in memory, and then histone alterations that are thought to regulate addiction will be discussed in detail.

#### Histone-mediated epigenetics

There are several ways epigenetic and histone-specific changes may affect these learning and memory processes. Much of the work on histone modifications in addiction has focused on histone acetylation, but before reviewing those findings, it is important to consider other ways in which histones can be modulated by different molecular events. The study of histone modifications in addiction is in relative infancy compared to the study of these modifications (and epigenetic events in general) in other biological processes, such as cancer.

While epigenetic modifications to the genome are known to change molecular, cellular, and systemic function, they are being discovered as underlying mechanisms to many complex diseases, including developmental, neurodegenerative, and psychiatric (see Portela & Esteller, 2010; Tsankova, Renthal, Kumar, et al., 2007). Much of what is known about epigenetic mechanisms involved in cell biology comes from studies that have focused on the relation between these mechanisms and the causes of and cures for cancer (S. Sharma, Kelly, & Jones, 2009). These basic studies have led to the examination of epigenetic mechanisms in other processes, such as learning, memory, and addiction. The similarities between neural circuits, substrates, and many of the epigenetic factors that create long-term memories and those that cause long-term addiction suggest that common mechanisms are involved (e.g., (Malvaez, Sanchis-Segura, Vo, Lattal, & Wood, 2010b; Robison & Nestler, 2011; Zhou, Yuan, Mash, & Goldman, 2011). A major focus of current research is

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investigating how these mechanisms may contribute to developing persistent cellular and molecular changes that may translate into persistent behavioral changes, including lasting suppression of drug seeking.

There are at least five types of chemical modifications (i.e., methylation, phosphorylation, acetylation, poly-ADP-ribosylation, and SUMOylation) that take place on the amino acids of histone tails. These modifications allow access to certain genomic regions to be increased or decreased, which is associated with activation or repression of transcription of specific genes. The enzymes that complete these chemical modifications to DNA, transcription factors (TF), and histones, are typically recruited during development or after some type of stimulation to an organism (Lv, Xin, Zhou, & Qiu, 2013; Vecsey et al., 2007). With activity-dependent depolarization of neurons, activation of inter-and intracellular pathways leads to an interaction between these enzymatic coactivators and their substrate (e.g., DNA, TFs, or histones), largely regulating gene and protein expression, ultimately altering system function. The steps in this process are also modified by the physiological state of the organism and the type and extent of stimulation applied.

Research on histone-mediated epigenetic regulation of addiction has focused largely on the induction of factors downstream of histone modifications that are associated with increases or decreases of drug-seeking in rodents. Multiple studies have identified a major role for immediate early genes (IEGs; such as c-fos, c-jun, and fosB), TFs and coactivators [(such as cAMP, CREB, and kinases (such as protein kinase A, C, and Ras)] in the plasticity induced during learning or drug use (Darcy, Trouche, Jin, & Feig, 2014; Levenson, O'Riordan, et al., 2004; Shalin, Hernandez, Dougherty, Morrison, & Sweatt, 2006). The involvement of these key regulatory factors and the related gene targets relies on complex chemical modifications made to both DNA and histone proteins. In the last two decades, increasing recognition for the necessity of these modifications has led to advancements in the field. While both DNA and histone modifications interact and rely on each other (Cedar & Bergman, 2009) each substrate (DNA or histone protein) modification leads to a unique sequence of events.

Multiple types of chemical modifications can be made to histones, each of which is thought to create a novel surface to be recognized by effector proteins and specific downstream events. Although there are many types of modifications made to histones, such as phosphorylation, SUMOylation, ubiquitination, and poly-ADP-ribosylation, the majority of addiction-related research has investigated the effects of altering methylation and acetylation levels on histones 3 and 4 (Strahl & Allis, 2000). Broadly, research suggests that drug use stimulates many chemical modifications that are needed to repress or activate the transcription and translation of DNA into functional proteins.

Some of these marks can even have opposing effects on transcription. For example, methylation and phosphorylation (discussed in more detail below) are thought to participate in both closing and opening of chromatin and mediate the repression and activation of transcription (Cheung et al., 2000). Alterations to transcription after histone methylation or phosphorylation is based on many variables (e.g., organism's developmental stage, the onset and duration of stimulation, the type of tissue, cell, or histone residue that is being targeted for modification (Cheung et al., 2000; Greer & Shi, 2012; Smith & Shilatifard, 2010). In contrast, histone acetylation is primarily associated with active gene transcription with very few exceptions (e.g., Braunstein, Sobel, Allis, et al., 1996). The combination of these marks are in large part thought to be how environmental effects lead to individual variability, and how such great diversity can be created through epigenetic regulation. Yet, it is still unknown whether histone-mediated epigenetics, such as changes to histone deacetylase 3 (HDAC3) activity, can be used in the clinic to prevent and treat addiction. To give a more complete background of how context, hippocampal activity, and HDAC3 manipulations are likely leading to complex behavioral adaptions, a brief review of the various modifications, related research, and remaining questions pertaining to histone-mediated epigenetics in learning, memory, and addiction is below.

#### **Repressive histone modifications**

#### Methylation and phosphorylation

A repressed state is by and large the default structure of chromatin, preventing abnormal changes to the DNA's code (e.g., segregation, recombination and replication, (Grewal & Jia, 2007). The most common way that the tails of each histone remain tightly bound within the chromatin structure, repressing future gene transcription, is through methylation. Methylation is the act of adding a methyl group to a substrate or, in the case of histone-mediated epigenetics, to an amino acid in a histone tail, with three possible degrees of methylation (mono-, di-, and trimethylation). Methylation and additional phosphorylation and cross-linking between proteins (i.e., heterochromatin protein 1, HP1α), reinforce the stable and condensed chromatin structure, called heterochromatin. In this way and many others, methylation, phosphorylation, and their associated partners can result in transcriptional inhibition and decreased genome activity, yet these are not well characterized in the addiction field.

#### Active histone modifications

#### Methylation

In contrast to the more common repression of gene transcription by histone methylation, a number of studies have demonstrated that methylation can also activate gene transcription and contribute to behavioral changes (Chen, Kan, & Castranova, 2011; Santos-Rosa et al., 2002; Sims, Magazinnik, Houston, Wu, & Rice, 2008). As an example, the methyltransferase and demethylase (i.e., writer MLL1 and eraser kdm5c) responsible for di and trimethylation of H3K4 (histone 3 lysine 14) are thought to upregulate transcription of the oxytocin receptor and Fos protein in the NA, mediating methamphetamine-associated memory development and expression (Aguilar-Valles et al., 2014).

#### Acetylation

Histone acetylation occurs on the nitrogen-containing side chain of lysine amino acids and causes the otherwise positively charged histones to detach from negatively charged DNA in the chromatin structure (Jenuwein & Allis, 2001; Loidl, 1994). Acetylation levels are largely coordinated through histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes. HDACs are the enzymes that remove acetyl groups from the amino acid tails of histones. HDACs can be classified into four classes differing by the types of tissues they reside in, where they are active within the cell, the number and homology of catalytic sites, and what substrates and binding partners they interact with to determine functional outcomes (Dokmanovic, Clarke, & Marks, 2007). For example, class I HDACs reside within the nucleus of cells, are dispersed ubiquitously throughout the body, have one catalytic site, and have activity on DNA binding TFs and nuclear receptors, signaling mediators, and chromatinremodeling substrates. Each class and individual HDAC is thought to serve different functions. By decreasing class I HDAC function, alterations in cell survival and proliferation occur, whereas knockout analysis of class II HDACs may localize effects to specific tissue types. Within each class, individual HDACs contribute to a wide range of independent roles, from cardiac function and chondrocyte differentiation to changes in global histone acetylation and gene expression (Dokmanovic et al., 2007). The phosphorylation state of HDACs themselves can determine their permissibility to histones as well. For example, HDAC5 is known to be phosphorylated through activity-dependent mechanisms and after cocaine administration and then exported out of the nucleus, decreasing activity at specific histone sites (Dietrich, Takemori, Grosch-Dirrig, Bertorello, & Zwiller, 2012). Correspondingly, the repression of genes, such as NR4A1, a nerve growth factor involved in inflammation and cell survival, is increased by dephosphorylation of HDAC7. It is still unclear how HDAC function

is selectively recruited and how sequence specificity of histone tails helps determine and coordinate these regulatory factors. It is thought that these modifications may act alone or in concert with other modifications (methylation, phosphorylation, etc.) based on many variables (e.g., stimulus and cell type) to create a complex code that determines individual gene regulation, and behavioral outcomes.

#### Histone-mediated regulation in reward-related behavior

Because drugs of abuse often change cellular and systemic activities in animals and humans, simple measures of cellular and locomotor sensitization or tolerance are used to measure changes in physiology (e.g., receptor function) and overall behavior after acute or chronic drug administration. In addition, animal models like CPP and operant self-administration are commonly used to investigate the reinforcing effects of drugs and infer the strength of drug–cue or drug–response associations. The histone modifications discussed above contribute to many healthy functions, such as learning and memory, and to diseases, including drug addiction, and are initiated and terminated for many reasons (Bohacek & Mansuy, 2013; Moita, Rosis, Zhou, Ledoux, & Blair, 2003; Petronis, 2010). Within the drug abuse field, epigenetic researchers have predominantly discovered changes that occur to histone and DNA methylation and are beginning to delineate the associated acetylation status (Renthal & Nestler, 2009).

#### Histone methylation and addiction
One of the more prominent and recent discoveries on the mechanisms underlying addiction described how the epigenetic response to an initial or habitual dose of drug administration is often different. This difference is emphasized and depicted in the Figure 1, detailing an underlying hypothesis of histone-mediated regulation of addiction. After just one administration of cocaine (acute exposure) expression of the methyltransferase responsible for methylating H3K9 (histone 3 lysine 9) sites, G9a, is increased. Increased methylation at this site results in greater binding of G9a to the IEG FosB, an effect that seems to be counteracted after repeated cocaine use, where G9a levels and FosB binding decrease (Maze et al., 2010). As noted previously, methylation often leads to a heterochromatin, or an inaccessible structure, decreasing the likelihood of transcription. While  $\triangle$ FosB, a product of the FosB gene, accumulates with repeated cocaine exposure and is associated with increased cocaine reward (Renthal et al., 2008), removing this G9a hindrance at FosB sites enables  $\triangle$ FosB expression to be increased, perpetuating the accumulation of  $\triangle$ FosB and the addiction cycle (Maze et al., 2010).

Additional studies have demonstrated that chronic exposure to drugs of abuse, such as cocaine and opioids, reduces dimethylation of H3K9 (histone 3 lysine 9) by decreasing G9a and G9a-like protein (GLP) enzymes in the NA of mice (Aguilar-Valles et al., 2014; Renthal & Nestler, 2009; Sun et al., 2012). Similar effects occur in the mouse cortex and in cultures of human lymphocytes after repeated nicotine treatment (Chase & Sharma, 2012).

## Figure 1.



## Figure 1. Chapter 1 - Epigenetic changes in the cycle of addiction.

Some of the potential chromatin dynamics are shown for the cycle of addiction, which moves from acute exposure, to chronic drug taking, to withdrawal, to abstinence and recovery, and back to acute exposure in cases of relapse, which begins the cycle again. Three potential states of chromatin (i.e., OPEN, CLOSED, or INTERMEDIATE) and their associated nuclear changes are depicted within four small gray boxes (numbered 1-4). These chromatin states create a more accessible (Box 2), inaccessible (Box 4), or intermediate (Boxes 1 and 3) structure for DNA to be accessed and transcription to take place. The top half of this figure signifies chromatin in a more "adaptive and responsive" state. The bottom half signifies chromatin in a more "inflexible and unresponsive" state (splitting states 2 and 4 into both of these categories evenly). The left half of this figure signifies a more "heterochromatin" state, while the right half signifies a more "euchromatin" state (splitting states 1 and 3 into both of these categories). Dashed lines (e.g., + chronic, + relapse, and + treatment) represent the potential for associated changes to be accelerated by rate and/or intensity.

Box 1 (INTERMEDIATE chromatin before drug intake) represents a basal chromatin state with normal transcription (determined primarily by genetic and previous environmental interactions). In this state, the chromatin and associated nuclear changes are well-balanced and highly regulated. Box  $1 \rightarrow Box 2$  transition: With acute exposure to stress or drugs of abuse, brief and reversible changes (see bidirectional arrows) occur to select histone and DNA regions (increased histone acetyltransferases like CREB-binding protein [CBP], acetylation, DNA accessibility, and learning and memory-related gene transcription).

Box 2 (OPEN chromatin) represents the change that occurs with a single or acute insult to the system (e.g., acute stress or drug exposure). Chromatin expands, releasing repressive marks and tipping the balance of epigenetic regulation toward those associated with gene activation. Box  $2 \rightarrow Box 3$  transition: With repeated exposure to stress or drugs, a prolonged and less reversible change occurs to select histone and DNA regions.

Figure 1 (con't). Chapter 1 - Epigenetic changes in the cycle of addiction. Box 3 (INTERMEDIATE chromatin after drug intake) represents chromatin with dysregulated histone enzymes, marks, TFs, and transcription. The location where histone modifications occur, the type of modification, and the effect that histone modifications have on cellular and behavioral outcomes is altered to positively reinforce this chromatin state. Box 3 processes are similar to Box 1, yet the balance of regulation is shunted away from promoter regions that are associated with learning and adaption (c-Fos and BDNF) and shifted toward promoter regions that are associated with an altered chromatin state and positively reinforce altered gene regulation (e.g., Ras and AFosB). These changes are thought to induce increased cellular tolerance and maladaptive behavior. Box  $3 \rightarrow$  Box 4 transition: With acute drug abstinence, brief and reversible changes occur to select histone and DNA regions (increased histone deacetylases, methylation, DNA inaccessibility, and decreased gene transcription) in an attempt to rebalance the previous dysregulation. Yet after chronic or repeated insults to the system, recent drug abstinence induces withdrawal-associated effects (e.g., anxiety and depression), making the organism increasingly susceptible to relapse rather than recovery and long-term treatment.

Box 4 (CLOSED chromatin) represents the change that occurs with acute abstinence (without relapse) and the associated withdrawal from drugs of abuse. Here, chromatin begins the process of rebalancing enzyme levels, histone marks, and gene transcription by generally increasing the repression of prior imbalances related to addiction. The previous epigenetic and behavioral changes placed on the system (e.g., positive feedback of AFosB and behavioral depression) make this process slow, as the system is resistant to rebalancing and deprived of necessary proteins to counteract this state. As chromatin becomes more condensed, regulation is increased [although exceptions to this mechanism exist, such as decreases to repressive methylation with withdrawal; as noted in Chapter 6 (Combinatorial modifications and addiction)]. Transition from Box 4  $\rightarrow$  Box 1: With repeated and long-term abstinence from drug use, a prolonged and less reversible change occurs to select chromatin regions. rebalancing the location, type, and effect that histone modifications have on cellular and behavioral outcomes, recovering to a more normal and highly regulated level of transcription.

In parallel, G9 levels decrease as drug taking increases with chronic treatment (i.e., as administered and measured with an animal model of operant drug self-administration), but are increased after acute drug administration (Maze et al., 2010; Sun et al., 2012) potentially leading to initial drug-seeking and drug-sensitization behavior (as measured by animal models in CPP and locomotor sensitization assays). These effects suggest that increased levels of dimethylation of H3K9 impair drug reward initially but that these increases are mitigated with repeated drug use.

Trimethylation of H3K9 also plays an important role in the addiction process. While H3K9me1 resides in the euchromatin and is correlated to gene activation, H3K9me3 occurs in nongenomic or heterochromatin regions of the DNA and is correlated with gene repression (Barski et al., 2007; Greer & Shi, 2012). Repeated cocaine treatment increases the expression of this specific type of methylation, resulting in enhanced expression of transposons and typically silenced (heterochromatic) regions of the DNA by decreasing repressive methylation within the NA of mice (Maze et al., 2011). This work and others noted above suggest that repeated treatment with drugs of abuse causes the derepression of previously silenced DNA regions by inhibiting methylation at key nongenomic and genomic sites. This derepression is likely a key factor contributing to the altered gene expression and impaired physiological function after long-term use of drugs. Importantly, these alterations are thought to release the brakes placed on transcription and lead to a more permissive epigenetic environment (Covington et al., 2011; McQuown & Wood, 2011).

Covington et al. (2011) further investigated whether dimethylation of H3K9 could be related to the depressive-like phenotype expressed after chronic cocaine administration and social defeat stress (an assay inducing anxious, stressful, and depressive characteristics in animals. As expected, their results suggested that chronic cocaine administration leads to increased vulnerability to the detrimental effects of social stressors and that this is due to the removal of G9a, GLP, and subsequent demethylation of H3K9me2 and enhanced Ras–CREB signaling. This demethylation is likely a key mechanism of chromatin opening and the increase in expression of downstream proteins associated with addictive behavior (e.g., Ras G-proteins and △FosB TFs).

BDNF-Trk signaling increases with Ras signaling (Covington et al., 2011) and leads to greater drug reinforcement (Bahi, Boyer, & Dreyer, 2008), and is decreased with chronic cocaine use in humans (Corominas-Roso et al., 2013). BDNF's downregulation in reward-related regions of the brain (e.g., VTA and NA) after chronic cocaine use and its replenishment during abstinence (Corominas-Roso et al., 2013) are likely compensatory mechanisms of cocaine's effects. Interestingly, if the regulation of this factor contributes to the rewarding properties of cocaine and is increased during abstinence, this may account for slower rates of CPP extinction reported in rats (Bahi et al., 2008) and the positive correlations between BDNF, abstinence, anxiety, and depression during early abstinence (Corominas-Roso et al., 2013). These types of changes in gene regulation are often enabled by the combination of decreased methylation status and subsequent increased histone acetylation (Fuchikami, Yamamoto, Morinobu,

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Takei, & Yamawaki, 2010). Coordinated mechanisms between histone methylation and acetylation will likely be targeted for treatments of addiction and other disorders in the future (Kennedy et al., 2013; Sen, 2014).

## Histone acetylation and addiction

As more diseases are being attributed to novel histone-mediated mechanisms (e.g., palmitoylation, isomerization, deimination; Chavda, Arnott, & Planey, 2014: Dieker & Muller, 2010: Khanal et al., 2013: Kouzarides, 2007), alterations involving histone acetylation have been leading the charge in addiction research. Initial investigations with HDAC inhibitors focused on anticancer activity (Wagner, Hackanson, Lübbert, & Jung, 2010; Yoshida, Hoshikawa, Koseki, Mori, & Beppu, 1990; Zhang & Zhong, 2014), but more recent work has examined the potential of HDAC inhibitors in treating a variety of psychiatric disorders (e.g., addiction, PTSD, depression; reviewed in Renthal & Nestler, 2009). Major advances in understanding how occasional drug use can manifest into chronic problems have largely been made through the use of pharmacological agents that target HDACs. One example is Trichostatin A (TSA), a general HDAC inhibitor with antitumor activity (Drummond et al., 2005) that decreases the motivation for and intake of addictive drugs (Romieu et al., 2008). Such a finding has been observed with other HDAC inhibitors and variations of the time and route of drug administration (Arora et al., 2013; Kim et al., 2014).

In an operant measure of drug-seeking behavior, Romieu et al. (2008) demonstrated that rats that voluntarily self-administered high levels of cocaine would decrease responding when administered the nonspecific HDAC inhibitor, TSA. Repeated administration of TSA stably and selectively decreases cocaine administration but not sucrose intake while reducing cocaine induced locomotor sensitization. These effects were mediated through decreases in HDAC deacetylation activity in the PFC and NA, either with or without cocaine (IP or self-administration). These results and others (Malvaez et al., 2013; Malvaez et al., 2010b) suggest a potential use of HDAC inhibitors in relapsing addicts (Romieu et al., 2008). Although the circuits mediating these effects have yet to be fully described, research is focused on the nucleus accumbens, where drugs of abuse increase acetylation, locomotion, reinforcement, and reward (Kumar et al., 2005; Renthal et al., 2009; Schroeder et al., 2008; Sun et al., 2008; Wang, Krishnan, Ghezzi, Yin, & Atkinson, 2007).

Work from these groups and others have also demonstrated that chronic drug use (e.g., cocaine or alcohol abuse) will initiate decreases in HDAC activity in reward and learning-related brain regions (e.g., NA, PFC, hippocampus; Romieu et al., 2008; Zou & Crews, 2014), creating a more permissive genome for drug regulation to be imparted. Renthal et al. (2007) discovered a potential mediator for the transition from dysfunction or drug abuse to disease and drug addiction with HDAC5. HDAC5 is a class II HDAC that is phosphorylated minutes after activity-dependent stimulation (e.g., initial drug use or stress) and is exported from the nucleus, allowing gene transcription and behavioral adaption to take place. Without additional insult, HDAC5 will be returned to the nucleus within 24 hours, capping gene transcription. Chronic stimulation, such as chronic

cocaine-administration or chronic social-defeat stress, results in long-term HDAC5 inhibition, increases in cocaine-, or stress-associated gene transcription (i.e., Ras,  $\Delta$ FosB), and dysregulated sensitivity to subsequent challenges. These data demonstrate the importance of balancing acetylation to maintain flexible behavior.

As occasional drug use becomes chronic, many cellular and behavioral changes occur (Long-term Exposure in Figure 1). In light of the role that HDAC5 plays in chronic but not acute drug treatment or stress, the TF  $\Delta$ FosB is also thought to mark the transition from abuse to addiction (Renthal et al., 2008). With chronic amphetamine treatment,  $\Delta$ FosB is increased, leading to recruitment of HDAC1 and attenuation of the immediate early gene, c-fos (Renthal et al., 2008). In concert, dimethylation of histone 3 at lysine 9 is increased (recall that this type and location of methylation is repressive in nature). These data further demonstrate that the chronic use of drugs (i.e., amphetamine) not only swap methylation for acetylation at the c-fos promoter within the striatum (a region highly important to addictive behavior) but also lead to decreased expression of a methyltransferase (KMT1A) necessary to remedy this imbalance. As  $\Delta$ FosB increases with repeated drug use, G9a (another methyltransferase) is built to decrease the levels of this addiction marker by binding to the fosB promoter and suppressing its expression (Maze et al., 2010), though the coordinated tools put in place to rectify such epigenetic imbalances are in competition with drug effects that are inherently self-perpetuating.

While drug use impairs HDAC activity and positive feedback of  $\Delta$ FosB (by  $\Delta$ FosB) is initiated during chronic drug use (reviewed in Maze & Nestler, 2011) additional changes compound these imbalances. For example, an acute administration of alcohol or cocaine will briefly increase H4 acetylation and H3 phosphoacetylation (previously associated with active immediate early genes, cfos and c-jun, Clayton, Rose, Barratt, et al., 2000). Kumar et al. (2005) demonstrated mechanistic differences between acute and chronic covalent chemical changes in the brain. They determined that histone acetylation and phosphoacetylation influenced gene regulation in the striatum, an addiction mediating region of the brain, and behavior in mice and rats after acute and chronic cocaine administration (by investigator initiated intraperitoneal injections and subject initiated self-administration infusions). Cocaine induced acetylation (on H4) and phosphoacetylation (on H3) at specific gene promoters, with acute effects at the c-fos and fosB sites on H4 and chronic effects at the fosB, cyclindependent kinase 5 (cdk5), and BDNF sites on H3. These modifications create an overall pattern of hypoacetylation and desensitization on H4 at the c-fos promoter but an exaggerated state of acetylation on H3 at  $\Delta$ FosB promoters (only partially desensitizing  $\Delta$ FosB, Kumar et al., 2005; Renthal et al., 2008). Similar patterns of gene regulation have been demonstrated with c-Fos and BDNF in the hippocampus (rather than c-Fos and fosB in the striatum) after acute and chronic electroconvulsive seizure induction (a procedure that decreases long-term plasticity in the hippocampus of rodents and be an effective treatment for depression in humans; Tsankova, Kumar, & Nestler, 2004).

Similar to previous studies mentioned, alcohol can also modify acetylation patterns with temporal and spatial specificity (Sakharkar et al., 2014; Shepard et al., 2008). Acute doses of alcohol can alleviate minor levels of stress or anxietylike behavior (Starkman, Sakharkar, Ph, & Pandey, 2012). This effect may be due to alcohol's ability to decrease HDAC levels in the AMY, a brain region necessary for affective and emotional associations (Gruber & McDonald, 2012; Pandey, Ugale, Zhang, Tang, & Prakash, 2008; Sakharkar, Zhang, Tang, Shi, & Pandey, 2012). Pandey et al. (2008) demonstrated that decreases in HDAC activity (i.e., following acute ethanol) correspond to increases in H3 and H4 acetylation and increases in anxiolytic and plasticity-related protein levels [i.e., CBP and neuropeptide Y (NPY)]. In contrast, increases in HDAC activity and subsequent decreases, or rebalancing of H3 acetylation, CBP, and NPY likely mediate alcohol withdrawal and the associated anxiety. As previous data would predict, inhibition of HDAC activity (by TSA) normalizes the sharp decreases in H3ac, H4ac, NPY expression and the corresponding anxiogenic effects of withdrawal (Pandey et al., 2008).

Although there are nuances, and many other modifications that need investigating (phosphorylation, ubiquitination, SUMOylation, and poly(ADPribosylation)), the majority of research thus far supports the idea that drugs of abuse and stressors generally repress methylation which can be detrimental and lead to addiction and depressive-like behaviors, while acetylation tends to be protective and lead to adaptive behaviors. Interestingly, these two common mechanisms can lead to an increase in transcription, yet the timing and location of these modifications play a critical role in their behavioral outcomes.

### Histone deacetylase 3 (HDAC3)

Evidence suggests that one HDAC in particular, HDAC3, is a negative regulator of immediate early genes (IEGs), cocaine-related learning, and longterm memory (McQuown et al., 2011). Like other epigenetic regulators, HDAC3 is thought to require a learning event, to have later effects on long-term memory (McQuown et al., 2011). For example, systemic application of a novel and selective HDAC3 inhibitor, RGFP966, crosses the blood brain barrier within 15 minutes, with peak biochemical effects at 30 minutes. This drug leads to an increase histone acetylation (histones 3 and 4, lysine sites 14 and 8, respectively) and corresponding enhancements in learning in mice (Malvaez et al., 2013). HDAC3 inhibition (by RGFP966) also has effects in auditory tuning and cortical plasticity (Bieszczad et al., 2015). RGFP966 enhances late LTP and the production of plasticity-related proteins (e.g., phosphorylated-p65) in the CA1 region of the hippocampus in aged rats and ameliorates age-related deficits in associative processing (Sharma et al., 2015). Most recently, a site-selective inhibition of HDAC3 activity was initiated with a viral vector (using nearly identical methods to those in Chapter 5), enhancing recall of fear formation (Kwapis et al., 2017).

To my knowledge, there has been no report of systemic or site-selective manipulation of HDAC3 in drug or pellet self-administering rats. By targeting the deacetylase function of HDAC3 with RGFP966 or a selectively designed vector the specific role that HDAC3 deacetylation plays in reward-related behaviors can be determined. This method's specificity contrasts with designs which use global HDAC inhibition with pan-inhibitors, or the overall amount of HDAC3 and therefore binding function of HDAC3 with deletion models, which may result in indirect effects of HDAC3, such as binding to transcription dependent cofactors. **Summary** 

This document reviews the primary literature (Chapter 1) and dissertation research (Chapters 2-5) on the contextual control of behavior and two key factors that interact to influence context-dependent learning and memory in reward-based behavior. Chapter 2 concentrates on the role of contextual cues in the development and expression of acquisition and extinction of conditioned place preference. Findings from this work implicate configural cues in the extent of learning and recall (Hitchcock et al. 2014). This work leads to that discussed in Chapter 3, where the role of the DH in these behaviors was tested and found to vary depending on the configuration of the apparatus. This effect demonstrated that both context and learning process controls the contribution of the DH to the mediation of drug seeking (Hitchcock & Lattal, in preparation).

In a second group of experiments (Chapters 4-5), context-specific extinction was demonstrated after cocaine self-administration (SA), allowing renewal of operant reward behavior to be tested in our lab, and the selective and systemic RGFP966 (HDAC3i) was administered to determine long-term effects on extinction and reinstatement (Hitchcock, Raybuck, Wood, & Lattal, in preparation). In a final project (Chapter 5), dorsal hippocampal HDAC3 activity was altered indefinitely, leading to altered acquisition and extinction of pellet selfadministration. These results implicated systemic and DH-specific HDAC3 as a regulator of operant and contextual reward learning. In Chapter 6, I review and summarize results, the implications and limitations of findings, and the related considerations for future epigenetic research in reward-related behavior.



Portions of Chapter 1 are adapted from the publication:

Hitchcock, L. N., Cunningham, C. L., & Lattal, K. M. (2014). Cue configuration effects in acquisition and extinction of a cocaine-induced place preference. *Behavioral Neuroscience*, *128*(2), 217–27. https://doi.org/10.1037/a0036287

# Cue configuration effects in acquisition and extinction of a cocaine-induced place preference

## Introduction

Conditioned place preference (CPP) is a tool for investigating how neutral environmental cues (conditioned stimuli, CSs) become associated with drugs of abuse (unconditioned stimuli, USs). The process of cocaine-induced CPP involves an animal associating cocaine with specific cues (e.g., tactile, spatial) within a CPP apparatus. When animals are subsequently given a choice between a place that was previously paired with cocaine (CS+) and a place paired with saline (CS-), they often choose to spend more time with the CS that was paired with cocaine. The animal's performance (conditioned response) at the time of memory retrieval reflects the degree of CPP learning. Repeated exposure to the CS+ in the absence of cocaine (CS-no US) will result in extinction, during which the preference for the CS+ will be weakened. Extinction is thought to leave acquisition memories intact while new inhibitory learning occurs and suppresses expression of CPP.

CPP is widely used to assess the conditioned reinforcing properties of cues associated with drugs of abuse (Bevins, 2000; Napier, Herrold, & De Wit, 2013). Even a cursory reading of the CPP literature reveals that there are very different physical characteristics associated with the apparatus across laboratories. One of the more obvious differences is whether the apparatus is configured to have one or two compartments during conditioning. In a one-

compartment configuration with no divider, one of two tactile floor types which can be changed between trials serves as the CS+ or CS-, which results in exposure to either cue across the entire apparatus (e.g., Bernardi, Ryabinin, Berger, & Lattal, 2009; Cunningham, Ferree, & Howard, 2003; Raybuck, McCleery, Cunningham, Wood, & Lattal, 2013; Vezina & Stewart, 1987a, 1987b). In a two-compartment configuration, the chamber is divided into at least two compartments and the animal is confined to one chamber position during CS+ trials and to another chamber position during CS- trials (e.g.; (Fuchs, Weber, Rice, & Neisewander, 2002; Malvaez, Sanchis-Segura, Vo, Lattal, & Wood, 2010a; Shimosato & Watanabe, 2003). Thus, in both procedures, the tactile cues predict drug or saline reinforcement, but an additional spatial component is present in two-compartment procedures, with position of apparatus potentially predicting drug or saline delivery. This predictive spatial component can be eliminated in a third type of procedure, by alternating the spatial position of tactile cues over acquisition trials (Cunningham, Patel, et al., 2006).

These three procedures differ in several ways. First, the two-compartment procedures expose the animal to only half of the chamber during each training trial, resulting in exposure to tactile cues in a more confined space compared to a one-compartment procedure. Second, the similarity between the training and testing condition differs between procedures. A one-compartment procedure provides the same amount of apparatus space in training and testing (i.e., mice can explore the entire apparatus), but a different floor configuration between training and testing (i.e., only the CS+ or the CS- floor cue is present and

accessible during training whereas both floor cues are present and accessible during testing). Third, a two-compartment procedure with drug delivered in a consistent location introduces a relevant spatial component to the task that is not present in either a one-or a two-compartment alternating position procedure.

These configurations may differentially affect acquisition or extinction of CPP. During acquisition, more cues (e.g., tactile and spatial) may be associated with cocaine in the consistent two- versus one-compartment procedure, allowing better retrieval of the memory post-acquisition (Pearce & Bouton, 2001). In contrast, more cues could also lead to one cue overshadowing another cue, resulting in weaker expression post-acquisition depending on which cues are sampled at test (Rescorla & Wagner, 1972). If a dominant training cue is not present in the testing configuration, then retrieval and performance will decline. An alternating two-compartment or one-compartment procedure may also increase CPP. In these procedures, the spatial cues are not predictive of drug state; therefore, animals may better isolate the tactile cues as the key predictive CS and increase performance.

Following acquisition, extinction is specific to the context in which it occurs, with conditioned responding showing renewal when testing occurs in a different context (e.g., Bouton, 2004). Changes in CPP apparatus configuration from extinction to post-extinction testing may alter the expression of the extinguished preference. For instance, changes in cue configuration between extinction and testing may effectively change the context of testing, which will result in renewal of drug seeking, whereas similar configurations between extinction and testing would lead to greater generalization of the extinction context to the testing context and greater extinction expression. Therefore, the application of a one- or two-compartment procedure (with consistent or alternating cues) may change the similarity of cues between training (acquisition or extinction) and testing conditions and may ultimately influence CPP.

In the following experiments, the effects of apparatus configuration on acquisition and extinction of cocaine-induced CPP were examined. In Experiment 1, a two-compartment procedure promoted acquisition, but impaired extinction, regardless of acquisition history. In Experiment 2, alternating a two-compartment procedure promoted acquisition, relative to both one-compartment and consistent two-compartment procedures. In Experiment 3, a one-compartment procedure promoted extinction, compared to either of the two compartment procedures. These findings have practical implications for how to generate and extinguish CPP in the laboratory, as well as theoretical implications for the processes that underlie acquisition, expression, and extinction of CPP. They also point to potentially different neurobiological mechanisms of CPP as a function of cue configuration during acquisition and extinction.

#### Experiment 1: Effects of configuration on CPP acquisition and extinction

This experiment examines the role of position cues in the acquisition and extinction of cocaine-induced CPP. Expression of drug preference may change depending on stimulus conditions and apparatus configuration during testing (White, Chai, & Hamdani, 2005), though less is known about the effect of different conditions during acquisition and extinction. In addition, few direct comparisons have been made to determine the effects of configuration between the common one- and two-compartment CPP approaches (Cunningham, Patel, et al., 2006).

## Methods

Animals. Sixty male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed four per cage in a controlled environment (12 hr light dark cycle, lights on at 6 am). Mice (8-18 weeks of age) had ad libitum access to food and water and weighed 20–30 grams. Experimental events occurred between 7 am and 12 pm. All experiments were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Apparatus. CPP was generated using an unbiased apparatus, consisting of clear acrylic walls (30 cm x 15 cm x 15 cm). The apparatus included interchangeable halves (left and right) of two floor types (Grid, G and Hole, H; (Cunningham, Gremel, & Groblewski, 2006). Grid floors consisted of 2.3-mm stainless steel rods mounted 6.4-mm apart in an acrylic frame. Hole floors consisted of perforated stainless steel with 6.4-mm round holes on 9.5-mm staggered centers. The CPP test chambers were housed in melamine shells (McCarthy Manufacturing, Gresham, OR) with air vents around the side of the chamber, allowing low levels of light to enter each chamber. A camera was mounted inside the center of the shell. During two-compartment training, the CPP chamber was bisected by a clear acrylic divider. This divider created a left and a right side and two positions for the mouse to be placed. During one-compartment training, the acrylic divider was removed. The one-compartment chamber was 30 cm wide x 15 cm deep x 15 cm high; each side of the two-compartment chamber was 15 cm wide x 15 cm deep x 15 cm high. The opposite floor type was visible through the divider during two-compartment training.

**Drugs.** Cocaine HCI (COC) obtained from Sigma was dissolved in physiological saline (SAL, 0.9% NaCl) for intraperitoneal (IP) injection (10 ml/kg), and administered at a dose of 20 mg/kg on conditioned stimulus positive days (CS+). This dose was chosen based on previous cocaine-induced CPP results in mice (Bernardi et al., 2009; Raybuck et al., 2013). Saline (SAL) was injected (IP) into animals on conditioned stimulus negative days (CS-), matching any volume and handling specific effects between COC CS+ and SAL CS- acquisition trials.

**Cocaine-Induced CPP Protocol.** A schematic of the experimental timeline and CPP configurations is shown in Figure 2. CPP involved the following phases: Habituation, Pretest, Acquisition (A), Post-Acquisition test (Test 1), Extinction (E), and Post-Extinction test (Test 2). Mice were assigned to one of four treatment groups that were categorized by the type of apparatus configuration used (one-compartment or two-compartments with consistent position cues) during acquisition (A) and extinction (E). Locomotion and cocaine-induced CPP were compared in these four groups.

## Figure 2.

# **CPP Cue Configurations**



## Figure 2. Chapter 2 - Conditioned Place Preference (CPP) Cue

**Configurations.** Abbreviations for each configuration are based on the number of CPP compartments (one-,1 or two-compartments, 2) and the type of spatial cue (consistent, c or alternating, a) during CPP acquisition (A) and extinction (E). CPP preparations consisted of tactile cues (grid, G or hole, H floors) for all animals. Two-compartment chambers were identical to one-compartment chambers except that a clear divider bisected the chamber area, leaving the opposite conditioned stimulus (CS) flooring/position visible.

During acquisition (A), mice were injected IP with 20 mg/kg cocaine (+) or saline (-) and placed on their assigned CS+ or CS- paired floor in a one- or twocompartment apparatus. Cocaine was paired with the grid floor (G+/H-) for half of the animals and with the hole floor (G-/H+) for the other half (not shown).

During extinction (E), CPP was extinguished by placing mice on the previously paired CS+ floor without a cocaine injection (mice did not receive tactile exposure to the previous CS- floor during extinction). Preference for the CS+ paired floor was determined by a Choice Test before and after acquisition or extinction.

Treatment groups based on apparatus configuration (1 or 2c) and learning phase (A or E) were designated as follows: A1 E1 (one-compartment acquisition and one-compartment extinction, N = 14); A1 E2c (one-compartment acquisition and two-compartment extinction with consistent position cues, N = 14); A2c E1 (two-compartment acquisition with consistent position cues and one-compartment extinction, N = 16), and A2c E2c (two-compartment acquisition and extinction with consistent position cues, N = 16).

## Habituation (three trials prior to Pretest)

Mice were habituated to the experimental room and to handling prior to cocaine-induced CPP pretesting. On each day, mice were transported in their home cage with three other mice from the colony to the experimental room. They were weighed, allowed to rest for one hour before and after handling (similar to subsequent experimental days), and returned to the colony room.

Testing [Pretest, Post-Acquisition test (Test 1); Post-Extinction test (Test 2)]

Testing (Pretest, Test 1, and Test 2) consisted of a 5-min session in which the mouse had access to both the CS+ and CS- floors. Floors (Grid and Hole) were configured the same for each individual mouse during all testing sessions. These were also consistent with the orientation from acquisition and/or extinction days if applicable (i.e., any two-compartment manipulation: A1 E2c, A2c E1, and A2c E2c groups), but without the divider in place.

Conditioned stimulus assignments were counterbalanced within and between configuration groups by floor type (Grid or Hole) and position (left or right). Pretesting pre-exposed the animals to the apparatus and allowed a baseline (naïve) measure of preference in each animal for comparison to their Post-Acquisition Test 1 and Post-Extinction Test 2 preference. Testing was completed 24 hr after the last habituation, acquisition, and extinction trial. *Acquisition (A, four trials prior to Test 1)* 

Mice were moved to the experimental room, weighed, and allowed to habituate to the room for 1 hr prior to acquisition trials. Mice were then injected with COC or SAL, immediately placed back in their home cage for less than 30 s (while other mice within the same cage were injected) and then placed in their assigned acquisition chamber. Half of the mice were assigned to one of two floor subgroups (injected with COC and placed on a Grid floor = G+) and half were assigned to the other floor subgroup (injected with COC and placed on a Hole floor = H+). Thus, on alternate days over four acquisition trials (two COC, two SAL), mice in the G+ subgroup received COC immediately prior to two separate 15-min acquisition trials on the Grid floor and SAL immediately prior to two separate 15-min trials on the Hole floor. Alternatively, mice in the H+ subgroup received COC immediately prior to 15-min acquisition trials on the Hole floor and SAL immediately prior to 15-min trials on the Grid floor. In all three experiments, mice were counterbalanced as best as possible so that any residual effect of drug or CPP condition was balanced between groups and within each home cage. For example, there were two A1 treated mice and two A2c treated mice per home cage. On each conditioning day half of the mice treated with cocaine were in the A1 group and half were in the A2c group.

Mice were assigned to a one- or two-compartment acquisition (A) group and pre-test preference was counterbalanced between these groups prior to acquisition trials. The group of mice that acquired a CPP in a two-compartment configuration with consistent position cues will hereafter be identified as the A2c group. In this configuration, the left and right floor types were different during all phases of CPP (1 Grid side and 1 Hole side). A tall clear divider restricted mice to one floor type and one position of the apparatus per trial. Each position of the chamber was consistently paired with COC or SAL but not both; therefore, each position, as well as each floor, consistently predicted COC or SAL during twocompartment acquisition with consistent position cues. The group of mice that acquired a CPP in a one-compartment configuration will hereafter be identified as the A1 group. In this configuration, the left and right floor types were identical during acquisition trials and mice had access to both sides of the apparatus, with no divider separating each chamber position. The assignment of CS+ floor (Grid or Hole), CS+ position (left or right, if applicable), and the order of drug injection (Days 1-4: COC, SAL, COC, SAL or SAL, COC, SAL, COC) were counterbalanced within each acquisition group.

## Extinction (E, 2 trials prior to Test 2)

During extinction (E), all mice were exposed to the previously paired (CS+) floor (Grid or Hole) for 30-min with no injection. This training occurred over two consecutive days followed by a Post-Extinction test (Test 2). Each acquisition apparatus configuration group (A1 and A2c) was divided into two extinction configuration groups (E1 and E2c). For half of the mice, CPP was

extinguished in a two-compartment configuration (E2c), resulting in exposure to the previously COC-associated floor on the CS+ chamber position. For the other half, CPP was extinguished in a one-compartment configuration (E1), resulting in exposure to the previously COC-associated floor, unrestricted by position. This design resulted in four groups of mice, distinguished by the apparatus configuration applied during acquisition and extinction trials (A1 E1, A1 E2c, A2c E1 and A2c E2c). It should be noted that half of the mice were extinguished in exactly the same apparatus configuration as during CS+ acquisition trials (A1 E1 and A2c E2c), and half were extinguished on the same floor but not the same apparatus configuration as during CS+ acquisition trials (A1 E2c and A2c E1).

**Data Analysis.** The locomotor activity and position of each animal in the CPP box (left/right position) were recorded by a camera mounted on the CPP shell ceiling and analyzed by Ethovision software (Noldus, Leesburg, VA). Place preference was defined as the amount of time that each animal spent on the CS+ associated floor during each test session. This measure was represented first by comparing the average seconds per minute (sec/m) that each floor subgroup spent on the Grid floor (mice conditioned to Grid floor with COC, G+ subgroup, compared to mice conditioned to Hole floor with COC, G- subgroup). In all three experiments, a statistically significant difference in the time spent on the Grid floor by each floor subgroup (G+ versus G-, ps < 0.05, data not shown) verified a place preference in each treatment group following acquisition (Cunningham et al, 2003). There were no differences in results if G or H was used as the CS+

floor; therefore, the percent of total time that mice spent on their assigned CS+ paired floor during testing was used to present CPP data.

Behavioral data were analyzed with Microsoft Office Excel and SPSS software. Dependent variables were place preference (average time on CS+, %) and locomotion (total distance traveled in cm). Independent variables were configuration group (one- or two-compartment) during acquisition and/or extinction, test session (Pretest, Test 1, or Test 2), injection drug type (COC CS+ or SAL CS-), and extinction trial (1st or 2nd). Analysis of variance (ANOVA) and planned LSD follow-up tests analyzed datasets with a significance level set at 0.05. For repeated measures ANOVAs, a Greenhouse-Geisser correction adjusted the degrees of freedom and p value for violations to sphericity (Mauchly sphericity test, p < 0.05).

## **Results and Conclusions**

There was no effect of configuration on distance traveled during any test session (ps > 0.05, Figures 3A, 4A, and 5A) or Pretest preference (ps > 0.05, Figures 3B, 4B, and 5B). These results were consistent in all three experiments.

**Locomotor Activity.** During acquisition, mice traveled a greater distance during CS+ trials (in the presence of cocaine) compared to CS- trials (in the presence of saline) and in the one-compartment configuration compared to the two-compartment configuration.





Figure 3B.

Preference



**Testing Session** 

**Figure 3. Chapter 2 - Experiment 1.** Treatment group abbreviations are based on apparatus configuration (1 or 2c) during acquisition (A) and extinction (E): A1 E1 (N = 14), A1 E2c (N = 14), A2c E1 (N = 16), and A2c E2c (N = 16). Error bars indicate the standard error of the mean ( $\pm$ SEM). Please see Results and Conclusions section (Locomotor Activity and Test Preference) for a description of statistical findings.

- (A) Total distance traveled in cm (mean, SEM) during behavioral sessions (Pretest, Acquisition, Test 1, Extinction, and Test 2). Acquisition data have been pooled over the two cocaine CS+ and two saline CS- trial types. Extinction data are from trials 1 (1<sup>st</sup>) and 2 (2<sup>nd</sup>). Note. ^Saline CS- < Cocaine CS+; \*Two-compartment acquisition < One-compartment acquisition; #Twocompartment extinction < One-compartment extinction; p < 0.05, Fig 3A.</li>
- (B) Cocaine-induced CPP represented as the percent of time spent on the cocaine paired (CS+) floor at the Pretest (Pretest), Post-Acquisition test (Test 1) and Post-Extinction test (Test 2). Note. ^Pretest < Test 1; \*A1 < A2c; #E1 < E2c; ps < 0.05, Fig 3B.</li>

These observations were confirmed by a 2 (acquisition configuration) x 2 (subsequent extinction configuration) x 2 (drug type-repeated) ANOVA, which revealed main effects of drug type and acquisition configuration, and an interaction of drug type x acquisition configuration [Fs (1, 56) > 96.9, ps < 0.001]. There was no effect of subsequent extinction configuration on distance traveled during acquisition. Follow-up tests revealed that the one-compartment group traveled a greater distance than the two-compartment group following either cocaine or saline injections, and that cocaine injections induced greater activity than saline injections in both configuration groups (ps < 0.001, Figure 3A).

Effects on activity by extinction configuration were similar to acquisition configuration effects (Figure 3A). Mice confined to a one-compartment extinction configuration traveled a greater distance than those confined to a twocompartment configuration. A 2 (acquisition configuration) x 2 (extinction configuration) x 2 (extinction trial-repeated) ANOVA revealed a significant main effect of extinction trial [F (1, 56) = 6.8, p = 0.01]. Separate 2 (acquisition configuration) x 2 (extinction configuration) ANOVAs on Extinction Days 1 and 2 revealed reliable main effects of extinction configuration [Fs > 55.7, ps < 0.001] on both days and an interaction between acquisition and extinction configuration on Day 1 [F (1, 56) = 4.2, p = 0.05]. Follow-up tests revealed that distance decreased from extinction trial Day 1 to Day 2, the E1 groups traveled more than the E2c groups, and the E2c groups traveled more on extinction Day 1 after an A1 versus A2c conditioning procedure (ps ≤ 0.01, Figure 3A).

**Test Preference.** The effect of acquisition and extinction configuration on test preference is clear in the analysis of percent time spent on the CS+ floor (Figure 3B). The change in preference across test session was different between configuration groups and supported with a repeated measures ANOVA [2 (acquisition configuration) x 2 (extinction configuration) x 3 (test sessionrepeated)]. This analysis indicated a reliable main effect of test session and interactions between test and acquisition configuration, and between test and extinction configuration (Fs > 8.4, ps < 0.005). Follow-up ANOVAs suggested a reliable difference between Pretest and Test 1 preference [F (1, 56) = 96.6, p < 1000.001] but no interaction based on acquisition or extinction configuration. During Test 1 (Post-Acquisition), preference was higher after two-compartment acquisition (A2c) than after one-compartment acquisition (A1: Figure 3B: Test 1). This observation was supported by a two-way ANOVA (acquisition configuration x subsequent extinction configuration), which found a significant main effect of acquisition configuration [F (1, 56) = 5.6, p = 0.02] with no effect of subsequent extinction configuration (Test 1 in Figure 3B).

Between Test 1 (Post-Acquisition) and Test 2 (Post-Extinction), preference decreased in the E1 groups relative to the E2c groups. This was demonstrated by a 2 (acquisition configuration) x 2 (extinction configuration) x 2 (test session-repeated) ANOVA indicating a significant interaction of test x extinction configuration [F (1, 56) = 5.0, p = 0.03]. A two-way ANOVA for percent preference at Test 2 (Post-Extinction) revealed a significant main effect of acquisition [F (1, 56) = 9.2, p = 0.004] and a reliable main effect of extinction configuration [F (1, 56) = 14.4, p < 0.001] with no interaction. Therefore, the effect of extinction configuration was not altered by prior acquisition configuration treatment (Test 2 in Figure 3B).

Experiment 1 demonstrated that cocaine-induced CPP was higher after acquisition with a consistent two-compartment procedure compared to onecompartment procedure. In contrast, a one-compartment extinction procedure decreased preference compared to a consistent two-compartment extinction procedure. Therefore, the expression of cocaine-induced CPP was greater after acquisition in a two-compartment configuration with cocaine consistently paired to an apparatus position and floor type, whereas expression of this preference was decreased to a greater degree after extinction in a one-compartment apparatus (floor CS only), regardless of prior acquisition configuration.

# Experiment 2: Effects of configuration and consistent spatial cues on CPP acquisition

In Experiment 1, stronger preference was demonstrated following a twocompartment relative to a one-compartment acquisition procedure. These acquisition results may be accounted for by at least two possible mechanisms. First, retrieval of cocaine-location pairings may be stronger when additional cues combine into one association, such as the tactile and CS+ position cues. In the two-compartment procedure, this would result in associations between either the spatial or the tactile cues and cocaine guiding a stronger test preference. Second, confinement in a smaller space during acquisition may increase expression of CPP at test, independent of the spatial cues that are available. There is some evidence that preference can be modulated by changes in available locomotion space (Swerdlow & Koob, 1984) or CS+ floor size (Vezina & Stewart, 1987b) between acquisition and testing. In Experiment 2, spatial cues were made irrelevant during acquisition by confining mice to one compartment, but alternating the location of the compartment within the apparatus during acquisition.

## Methods

Unless noted otherwise, Experiment 2 was identical to Experiment 1.

Animals. Forty-eight mice (8 weeks old) were used for this experiment.
Cocaine-Induced CPP Protocol. CPP involved the following phases:
Habituation, Pretest, Acquisition (A), and Post-Acquisition test (Test 1).
Acquisition (A, four trials prior to Test 1)

Following the Pretest, mice were assigned to one of three acquisition groups based on apparatus configuration (A1 N = 16; A2c N = 16 and A2a N = 16). In each group, one tactile CS was always paired with cocaine (CS+) and the other was always paired with saline (CS-). One acquisition group was trained in a one-compartment apparatus (A1) and another in a two-compartment apparatus with a consistent CS+ position (A2c), as described in Experiment 1. The third group was conditioned in a two-compartment apparatus with the CS+ associated position alternated (A2a) every other day. This design eliminated the consistent spatial contingency during cocaine pairings. This alternating configuration kept the total chamber area equal to that of the A2c group (in which each floor and position type was consistently paired to a COC and SAL injection in a twocompartment chamber) while keeping only the floor type matched with each injection (similar to the A1 group). During each 15-min acquisition trial, A2c and A2a configurations contained a tall clear divider that confined animals to one of two chamber positions (left or right) and to one of two floor types (Grid or Hole). During Days 1 and 2 of acquisition, mice in the A2a group received CS+ and CS-trials in the same position of the chamber (e.g., right side). During Days 3 and 4, mice in the A2a group received CS+ and CS-trials in the A2a group received CS+ and CS-trials in the same position. This treatment should have maintained the specific tactile CS-cocaine associations, while severing the predictive relation between positional cues and cocaine. The order of stimulus exposure (e.g., SAL or COC drug, left or right position, grid or hole texture) and CS+ testing configuration (e.g., consistent with Trial 1, 2, 3, or 4) was counterbalanced between groups to minimize any order effects on preference.

**Data Analysis.** Locomotor activity and test preference was measured during each test (Pretest and Test 1) and compared by acquisition configuration group (A1, A2c, and A2a).

### **Results and Conclusions**

**Locomotor Activity.** Figure 4A shows that activity was enhanced by cocaine injections and during a one-compartment acquisition procedure, similar to Experiment 1. A 3 (acquisition configuration) x 2 (drug type-repeated) ANOVA revealed reliable main effects of drug type [F (1, 45) = 464.9, p < 0.001] and acquisition configuration [F (2, 45) = 43.8, p < 0.001], as well as a reliable interaction of acquisition configuration x drug type [F (2, 45) = 15.1, p < 0.001].





Figure 4B.

Preference





**Figure 4. Chapter 2 - Experiment 2.** Treatment group abbreviations are based on apparatus configuration (1, 2c or 2a) during acquisition (A): A1 (N = 16); A2c (N = 16), A2a (N = 16). Error bars indicate the standard error of the mean ( $\pm$ SEM). Please see Results and Conclusions section (Locomotor Activity and Test Preference) for a description of statistical findings.

- (A) Total distance traveled in cm (mean, SEM) during behavioral sessions (Pretest, Acquisition, Test 1). Acquisition data have been pooled over the two cocaine CS+ and two saline CS- trial types. Note. ^Saline CS- < Cocaine CS+; \*Two-compartment acquisition < One-compartment acquisition; p < 0.05, Fig 4A.
- (B) Cocaine-induced CPP represented as the percent of time spent on the cocaine paired (CS+) floor at Pretest (Pretest) and Post-Acquisition test (Test 1). Note. ^Pretest < Test 1; \*A1 < A2c < A2a; ps < 0.05, Fig 4B.</li>
Follow-up tests confirmed that the one-compartment procedure induced greater activity than either two-compartment procedure (ps < 0.001). In addition, mice traveled a greater distance after cocaine injections compared to after saline injections (ps < 0.001).

**Test Preference.** As demonstrated in Figure 4B, preference expression increased from Pretest to Test 1 in all groups, was greatest in the A2a group, and was least in the A1 group. This observation was supported by a 2 (test session-repeated) x 3 (acquisition configuration) ANOVA, revealing a significant main effect of test [F (1, 45) = 135.5, p < 0.001] and an interaction between test and acquisition configuration [F (2, 45) = 13.9, p < 0.001]. During Test 1, a main effect of acquisition configuration was supported by a one-way ANOVA [F (2, 45) = 10.4, p < 0.001)]. Follow up tests confirmed that A1 preference was significantly less than A2a (p < 0.001) and A2c groups (p = 0.02). In addition, A2c preference was significantly less than A2a preference (p = 0.04).

In conclusion, acquisition of a cocaine-induced CPP was enhanced in both groups that were confined during acquisition via a two-compartment configuration (A2a and A2c preference greater than A1). Preference increased further when the location of cocaine pairings alternated between the two compartments (A2a group), demonstrating that eliminating predictive spatial cues promoted preference. By alternating the mouse location during cocaine and saline delivery, spatial cues within the apparatus did not predict the location of cocaine delivery, which may have resulted in less overshadowing of the tactile cues by the spatial cues. In Experiment 3, it was determined whether these same apparatus configuration effects occurred in extinction of CPP.

# Experiment 3: Effects of configuration and consistent spatial cues on CPP extinction

In Experiment 1 a one-compartment procedure produced greater extinction compared to a consistent two-compartment procedure. In Experiment 2 a two-compartment procedure with irrelevant spatial cues produced greater CPP expression compared to a one- or consistent two-compartment procedure. In Experiment 3, these effects were examined during extinction after training all mice in a consistent two-compartment procedure. Mice were exposed to a large tactile area using a one-compartment procedure (Group E1) or to a small tactile area using a two-compartment procedure with consistent (Group E2c) or alternating (Group E2a) spatial locations.

Previous studies of extinction in many different procedures have demonstrated that the learning that occurs during extinction is specific to the context of extinction; changes in context between extinction and testing often reveal renewal of conditioned responding (Bouton & Bolles, 1979; Crombag et al., 2008a). The design of Experiment 3 allowed me to assess whether renewal of cocaine seeking would occur when certain features of the context were held constant or changed between extinction and testing. The size of the apparatus varied (i.e., whole apparatus in the one-compartment groups, half apparatus in the two-compartment groups), the size of the floors (whole floors in one compartment; half floors in two-compartment), and the informativeness of the positional cues within the apparatus (informative in the E2c group; uninformative in the E1 and E2a groups). All groups were tested in the whole apparatus with both floors present, as in Experiments 1 and 2.

If apparatus size is a salient feature of the context, then extinction in the one-compartment procedure should result in the lowest preference during testing in the same-sized apparatus. If CS position is a salient feature of the context, then extinction in the consistent two-compartment procedure should result in lower preference during testing than the alternating procedure with CS position the same.

## Methods

Unless noted otherwise, Experiment 3 was conducted similar to Experiments 1 and 2.

Animals. Forty-eight mice (8 weeks old) were used for this experiment.

**Cocaine-Induced CPP Protocol.** CPP involved the following phases: Habituation, Pretest, Acquisition (A), Post-Acquisition test (Test 1), Extinction (E), and Post-Extinction test (Test 2).

#### Acquisition (A, four trials prior to Test 1)

All animals acquired a CPP in a two-compartment apparatus with consistent CS+ position cues (A2c), as described in Experiments 1 and 2. Experiment 1 demonstrated no interaction between acquisition and extinction configuration groups on preference; therefore, only one acquisition procedure was applied prior to extinction group assignment in order to match groups for future comparisons.

# Extinction (E, two trials prior to Test 2)

Following an A2c acquisition procedure, mice were assigned to one of three extinction groups categorized by apparatus configuration (E1 N = 16; E2c N = 16 and E2a N = 16). Groups E1 and E2c were handled identically to extinction procedures in Experiment 1. On both extinction days, mice were only permitted to stand on their previously conditioned floor type (either in a one- or a twocompartment configuration). No injections were given. In the E2a group, animal placement each day alternated between CS+ and CS- positions in a counterbalanced order while the opposing floor and position remained visible. This procedure was designed to resemble E1 extinction with exposure to CS+ floor cues on both positions of the apparatus and to resemble E2c extinction with a smaller extinction area. Therefore, E2a mice were exposed to the CS+ floor in both positions of the apparatus (similar to the E1 group), but were confined to one position of the apparatus per trial (similar to the E2c group). The order of CS+ testing configuration (e.g., consistent with Trial 1 or 2) was counterbalanced in the E2a group to minimize any order effects on preference.

**Data Analysis.** Locomotor activity and test preference was measured during each test (Pretest, Test 1 and Test 2) and compared by extinction configuration group (E1, E2c and E2a).

# **Results and Conclusions**

**Locomotor Activity.** As previously demonstrated during acquisition trials, activity increased after cocaine injections compared to saline injections (Figure 5A). Supported by a 3 (extinction configuration) x 2 (drug type-repeated) ANOVA,

reliable differences in activity emerged due to drug type [F (1, 44) = 131.2, p < 0.001), with no interaction due to subsequent extinction configuration (p > 0.8, Figure 5A).

During extinction, traveling distance increased in a one-compartment procedure compared to a two-compartment procedure, similar to Experiment 1. A 3 (extinction configuration) x 2 (extinction trial-repeated) ANOVA revealed a significant main effect of extinction trial [F (1, 45) = 40.6, p < 0.001], and a reliable interaction between extinction trial and configuration type [F (2, 45) = 3.1, p = 0.05; Figure 5A]. Separate ANOVAs on extinction trials 1 and 2 revealed reliable main effects of extinction configuration [Fs > 9.0, ps ≤ 0.001]. Follow up tests revealed that on both Days 1 and 2, more distance was traveled in the E1 group compared to the E2c and E2a groups (ps ≤ 0.002, Figure 5A).

**Test Preference.** Following acquisition, preference for the CS+ paired floor was expressed in all groups at Test 1 (Figure 5B). This was tested by a 3 (test-repeated) x 3 (extinction configuration) ANOVA indicating a significant main effect of test [F (2, 70) = 117.3, p < 0.001)] with no interaction of subsequent extinction configuration [F (2, 70) = 2.4, p = 0.07]. A follow up ANOVA confirmed that percent preference significantly differed from Pretest to Test 1 [F (1, 45) = 220.7, p < 0.001)] with no interaction of extinction configuration. As demonstrated in Figure 5B, preference for the CS+ paired floor was similar in all three groups during Test 1, prior to extinction treatment.



Figure 5B.



**Figure 5. Chapter 2 - Experiment 3.** Treatment group abbreviations are based on apparatus configuration (1, 2c or 2a) during acquisition (A) and extinction (E): A2c E1 (N = 16), A2c E2c (N = 16), A2c E2a (N = 16). Error bars indicate the standard error of the mean ( $\pm$ SEM). Please see Results and Conclusions section (Locomotor Activity and Test Preference) for a description of statistical findings.

- (A) Total distance traveled in cm (mean, SEM) during behavioral sessions (Pretest, Acquisition, Test 1, Extinction, and Test 2). Acquisition data have been pooled over the two cocaine CS+ and two saline CS- trial types. Extinction data are from trials 1 (1<sup>st</sup>) and 2 (2<sup>nd</sup>). Note. ^Saline CS- < Cocaine CS+; \*Two-compartment acquisition < One-compartment acquisition; #Twocompartment extinction < One-compartment extinction; p < 0.05, Fig 5A.</li>
- (B) Cocaine-induced CPP represented as the percent of time spent on the cocaine paired (CS+) floor at Pretest (Pretest), Post-Acquisition test (Test 1) and Post-Extinction test (Test 2). Note. ^Pretest < Test 1; \*A2c E1 < A2c E2c and A2c E2a; ps < 0.05, Fig 5B.</p>

From Test 1 to Test 2 a reliable main effect of test [F (1, 45) = 17.8, p < 0.001] and a reliable interaction between extinction configuration and test session [F (2, 45) = 7.1, p = 0.002] was revealed. At Test 2 (Post-Extinction), a significant effect of extinction between groups was confirmed [F (2, 45) = 5.3, p = 0.009] by a one-way ANOVA (Figure 5B). Follow-up tests determined that there was no difference in preference between E2c and E2a groups (p = 0.897) and that preference decreased in the E1 group compared to both E2c and E2a groups (p = 0.009 and p = 0.006, respectively). These findings are consistent with greater extinction induced by a one-compartment procedure, compared to either of the two-compartment procedures.

These extinction results replicated and extended those from Experiment 1, with the E1 configuration inducing the greatest decrease in preference compared to either two-compartment procedure. In Experiment 3, a preference for the cocaine-paired floor was expressed in all subsequent extinction groups after A2c acquisition. Following acquisition, I tested whether consistent spatial cues (E2c group) or an increase to CS+ confinement without consistent spatial cues (E2a group) would influence extinction. Preference was extinguished differentially based on extinction apparatus configuration. One-compartment extinction facilitated the largest decreases in CPP and the two- compartment groups (E2c and E2a) demonstrated very little change in preference following extinction.

The large difference between the E2a and E1 groups suggest that experiencing the CS+ in different positions is not enough to promote extinction. One possible theoretical explanation for these findings is that the size of the apparatus becomes encoded as a salient feature of the extinction context. When this size was changed between extinction and testing, as was the case for the E2 groups, the extinguished preference showed renewal. Of course, there are other possible explanations, including total amount of exposure to the CS+ during extinction, which may account for differences in extinction. Whatever the mechanism, these results are clear in showing greater loss of preference following a one-compartment extinction procedure.

## Discussion

In a series of three CPP experiments, it was demonstrated that acquisition and extinction of cocaine-induced CPP are affected by the configuration of drugassociated cues. Expression of cocaine-seeking behavior was increased after acquisition in a two-compartment configuration compared to conditioning in a one-compartment configuration. When the location of the tactile CS+ and CScues were alternated in a two-compartment configuration, CPP was further increased. Finally, extinction treatment in a one-compartment configuration led to the greatest decrease in preference, compared to either two-compartment configuration. These findings suggest that cue configuration may have opposite effects during acquisition and extinction of cocaine-induced CPP.

### Two-compartment acquisition enhances preference

Previous studies have confirmed that changes to CS configuration, either by modality (visual or tactile cues) or size, can alter the expression of preference for ethanol- or morphine-associated cues (Cunningham, Patel, et al., 2006; Vezina & Stewart, 1987a, 1987b; White et al., 2005). In the current study,

preference for cocaine-associated cues was enhanced following a twocompartment acquisition procedure compared to a one-compartment procedure. Many theories expect that expression of associative learning will be affected by the similarity between the conditions of learning and the conditions of testing. To evaluate the contributions of CS configuration to the expression of preference after acquisition, three variables were manipulated: 1) the location of the CS in the chamber, 2) the size of floor, and 3) whether CS+ and CS- cues were present on all trials. In our two-compartment acquisition procedure with consistent spatial cues (Group A2c), the CS+ and CS- floors were visible and in the same location during acquisition and testing. The only difference between acquisition and testing was the presence of a clear divider during acquisition, which prevented the mice from making contact with the tactile cues on the opposite side of the apparatus. This divider resulted in a difference in size of the confined area during acquisition – in A2 groups, mice were confined to half of the apparatus and could see both CS+ and CS- cues, whereas in A1 groups, mice were allowed to explore the tactile cues over the entire apparatus but could only see one cue per trial.

One study that investigated the effect of confinement on CPP found that restrained rats learn amphetamine-induced conditioned locomotion (sensitization) but do not acquire CPP (Swerdlow & Koob, 1984). A difference between that study and ours is that Swerdlow and Koob (1984) confined animals to a much smaller area during conditioning than the area used in my twocompartment apparatus. Extensive confinement in the Swerdlow and Koob (1984) study may have significantly decreased reward-related associations paired with the amphetamine environment and increased exploratory behavior in the testing environment, ultimately decreasing CPP. My finding that confinement to half of the apparatus during acquisition resulted in greater CPP compared to the A1 group suggests that high preferences can be expressed with a moderate decrease in chamber size between acquisition and testing. In fact, the decrease in chamber area paired with unconditioned stimuli, and the related decrease in distance traveled by mice during training, may have promoted the acquisition of tactile cue associations. Although it is plausible that floor cues are better sensed and given more attention when activity on them is increased, my results suggest that activity alone does not predict the preference because the two-compartment configuration induced less locomotor activity, but higher levels of CPP.

Correspondingly, animals in a larger area (such as a one-compartment procedure) during acquisition may distribute a greater proportion of their cocaine association to the larger environment in general (due to more visible and tangible contextual cues). This larger area may dilute the overall floor-cocaine association and ultimately lead to a decrease in preference expression during testing due to competing associations elicited by extraneous cues (e.g., irrelevant position, distant visual, and auditory cues). Thus, a smaller physical stimulus size in this experiment may have increased conditioned responding to the tactile CS+ by minimizing the development of associations with extraneous cues. This idea is analogous to results from many conditioning experiments that have found a promotion of conditioning by smaller temporal durations (e.g., Barela, 1999; Cunningham & Prather, 1992) or smaller stimulus size (Kosaki, Austen, & McGregor, 2013; Tommasi & Polli, 2004). A decrease in the continuous dimension of a CS, such as a shorter audible CS or a smaller place CS may increase the ability to identify it from other irrelevant background cues. Therefore, a smaller stimulus duration or size may be easier to identify or isolate as a predictive cue. Thus, in two-compartment groups, a decrease in size of the apparatus and proximal cues may generally promote preference, which may then be further promoted when spatial cues are made irrelevant in the alternating two-compartment group, leaving only proximal drug-predictive tactile cues.

Further observations determined that it was not confinement to half of the apparatus alone during acquisition that promotes CPP expression at test. The group that received confined exposure to alternating spatial locations (Group A2a) showed enhanced expression during testing compared to the group that received confined CS+ and CS- exposure to consistent spatial locations (Group A2c). By alternating cocaine exposure in the A2a group, the spatial location was eliminated as a predictive cue. Removal of this predictive spatial component may have allowed associative learning to the tactile cue to be enhanced further in the A2a animals compared to the A2c animals and led to increased CPP expression. Therefore, an increase in familiarity with the CPP configuration (CS+ and CS- cues visible during all trials) and a decrease in overall movement on the CS+ floor may have enhanced the association between cocaine and tactile cues in both A2c and A2a groups. When spatial cues were made irrelevant in the A2a

group, this further increased conditioning to the tactile floor, resulting in increased CPP compared to the A2c group.

This result may seem contradictory to previous experiments that found impairments in CPP when cue configuration was alternated (Cunningham, Patel, et al., 2006). During conditioning and testing, Cunningham, Patel et al. (2006) alternated the location of tactile cues in the dark or visual cues in the light within a two-compartment apparatus. In contrast, our study alternated the location of both tactile and visual cues in the light. Therefore, both tactile and visual cues remained relevant predictors of cocaine in our study, but the spatial cue relevance was eliminated. As demonstrated by Cunningham, Patel, et al. (2006) visual cues in the absence of consistent spatial cues fail to produce CPP in rodents. In contrast, tactile cues alone or tactile cues in combination with visual or spatial cues effectively produce CPP. After spatial cues were alternated in the current study, the effective tactile and visual cue combination remained a predictive CS for mice. Contradictory findings between these alternating cue studies may be accounted for by the different cues that remained after spatial cues were made irrelevant.

### Locomotion differences cannot fully account for post-acquisition preference

During acquisition, a one-compartment configuration consistently induced less preference and greater cocaine-induced locomotion compared to a twocompartment configuration. A combination of cocaine and an increase in activity enhances catecholamine, glucose, and lactose plasma levels, both during and after activity (Han, Kelly, Fellingham, & Conlee, 1996). This activity could alter reinforcement and withdrawal between one and two-compartment groups that significantly differed in locomotor activity. Although it was beyond the scope of this paper to determine if activity differences altered drug pharmacokinetics and drug-cue associations, our data demonstrate that activity alone cannot account for differences in preference expression. For example, if a decrease in activity induced pharmacokinetic changes that extended to preference results, both consistent and alternating two-compartment acquisition groups, with similar activity levels, would have been expected to express similar preference levels. This did not occur. During extinction, consistent and alternating groups expressed a similar amount of activity once again, but had similar levels of preference. Thus, the relation between activity and preference expression is not consistent.

#### One-compartment extinction inhibits preference

In contrast to acquisition (gain of preference), a one-compartment procedure enhanced extinction (loss of preference). There are different ways to think about this result. One could think of these findings as reflecting impairments in learning during acquisition and enhancements in learning during extinction. It also is possible that a performance process that generally leads to low levels of expression mediates preference after one-compartment acquisition or extinction. How this process works is unclear, but these effects could be mediated by context, with differential sensitivity to changes in context between acquisition, extinction, and testing. If apparatus size is the salient feature of the context, keeping size constant between extinction and testing may allow the size of the apparatus itself to serve as a retrieval cue for the learning that occurred during extinction, resulting in low levels of preference (i.e., weak renewal). Another way to think about the extinction results is that the one-compartment extinction procedure allowed the animal to associate extinction of the tactile cues with multiple spatial contexts, compared to extinction confined to a specific spatial location. This learning may have reduced the ambiguity of cues associated with extinction, creating additional retrieval cues for extinction (Bouton, 2004; Bouton & Bolles, 1979; Bouton, 1988), similar to what might occur when extinction occurs in multiple contexts (e.g., Gunther, Denniston, & Miller, 1998; Holmes & Westbrook, 2013).

This interpretation is complicated, however, by the finding that confined exposure to alternating positions during extinction (E2a) did not promote extinction, relative to a group that received confined exposure to a consistent position (E2c). This result suggests that exposures to CS+ cues in multiple spatial locations do not alone promote extinction. Instead, exposure to a single large CS+ floor during extinction promoted the loss of preference. The change in CS floor size and CS position that the E1 group experienced between extinction and testing may have allowed the animals to better detect the extinction contingencies, resulting in a persistent extinction effect not observed with the two-compartment procedures.

These findings extend what is known about these commonly used CPP approaches and the underlying differences between acquisition and extinction. My experiments leave several important issues unresolved. For example, in my

two-compartment procedure, animals were confined to either the CS+ or CS-, but were still able to see the opposite floor through the divider. It remains to be determined how observing the CS+ during CS- conditioning days (and vice versa) alters the strength of CPP, although previous work with visual cues suggests that the identity of the opposite floor does not influence preference expression (Cunningham, Patel, et al., 2006). My results suggest that the same cue configuration may have different effects on the learning that occurs during acquisition and extinction of CPP. These effects lay the groundwork for future neurobiological studies to investigate potentially different mechanisms that underlie acquisition and extinction of CPP with designs that may or may not include a spatial component.



# Involvement of the dorsal hippocampus in cocaine-induced conditioned place preference

# Introduction

Cocaine users often associate environmental cues with their drug-related experiences. Re-exposure to these cues can reactivate drug-related memories, induce cravings, and initiate drug-seeking behavior and relapse. By identifying the neural substrates that underlie changes in drug-seeking behavior, therapies for drug abuse may better target these highly cue-specific learning events. The cocaine-seeking behavior evoked by environmental cues, and the necessity of substrates involved, can be measured in the laboratory using a conditioned place preference (CPP) procedure.

CPP is a widely used procedure, but in contrast to other Pavlovian preparations, such as fear conditioning, the apparatus and procedures used to generate CPP vary widely from laboratory to laboratory (Cunningham, Gremel, et al., 2006; Cunningham, Patel, et al., 2006; Cunningham & Zerizef, 2014). One major difference is whether the animal has access to the entire CPP apparatus (one-compartment) or is confined to one side or the other (two-compartment). These procedures differ in several important ways, including the size, visual cues, and relevance of spatial location within the apparatus. As discussed in Chapter 2, others demonstrate that CPP is altered with different compartment configurations (Bevins, 2000; Cunningham, Patel, et al., 2006; Cunningham & Zerizef, 2014), yet little is known about the neural circuits that underlie these differences (Meyers, Zavala, & Neisewander, 2003; Meyers et al., 2006)

In Chapter 2, I found that when one side of the apparatus was paired with cocaine in a two-compartment procedure, acquisition was facilitated, but that the same two-compartment procedure impaired extinction. In contrast, a one-compartment procedure impaired acquisition but facilitated extinction. A major way in which these two procedures differ is in whether a spatial location (side of the apparatus) is consistently paired with cocaine; in a two-compartment procedure with consistent spatial cues, the animal always receives drug in the same location, but in a one-compartment procedure, the animal is free to move throughout the entire apparatus. Interestingly, acquisition was the highest when alternating spatial cues were given, yet extinction learning was the same with alternating or consistent cues. This result led me to question the underlying biology of these effects and to determine whether the dorsal hippocampus (DH) may be involved.

The DH is necessary for acquisition, retrieval, and extinction of spatial and contextual memories (Anagnostaras, Maren, & Fanselow, 1999; Gould & Leach, 2014; Riedel et al., 1999). There is also evidence that the DH mediates contextual renewal of reward-seeking behaviors (Fuchs et al., 2005; Neisewander et al., 2000; Todd, 2013) and it may be involved in detecting a mismatch between training and testing contexts (Gill, Mizumori, & Smith, 2011; Jezek, Henriksen, Treves, Moser, & Moser, 2011; Leutgeb et al., 2005). There are fewer studies of how the DH directly modulates different context-drug

memories (Gould & Leach, 2014), especially in a CPP procedure. There is also large variation in the methodology and reporting styles of CPP studies. This variation makes it difficult to know if the same neural mechanisms are engaged across similar treatment manipulations and results.

It is unclear if changes in DH recruitment are influenced by CPP configuration, and if these effects influence retrieval and encoding during extinction similarly. While previous studies have described the importance of the DH in other procedures (e.g., object location memory, water maze, etc.) during different phases of learning and testing (e.g., acquisition, consolidation, extinction, reinstatement), none to our knowledge has clarified the role of the DH in extinction and retrieval of cocaine conditioned place preference extinction in mice, nor have they investigated preference effects based on apparatus configuration during this training.

In one study, inactivating the DH demonstrated direct involvement in cocaine CPP expression when tested soon (recent memory) or long (remote memory) after acquisition (Raybuck et al., 2013) and another found that muscimol altered CPP for a selective CB1 cannabinoid receptor agonist but did not induce conditioned place preference or aversion when administered alone (Nasehi & Kamali-Dolatabadi, 2016). An additional study determined that inactivating the DH before acquisition or the first test impaired preference expression (Meyers et al., 2006). Other studies have focused more on the mechanism of CPP and determined that glutamatergic (Tan, 2008) and dopaminergic activity in the DH (Kramar et al., 2014), as well as long-term

potentiation in area CA1 (Portugal et al., 2014) are involved in CPP expression. Few studies to date have investigated the role of the DH during CPP extinction (Sadeghi, Ezzatpanah, & Haghparast, 2016), and none have compared effects of differing contextual environments (i.e., one or two-compartment CPP configurations).

In the following experiments, the GABAa (gamma-aminobutyric acid, ionotropic) receptor agonist muscimol was used to inactivate the DH. Muscimol was administered before initial expression (Test 1, Experiment 1), before extinction expression (Test 2, Experiment 2), and before extinction learning (Extinction, Experiment 3) of cocaine-induced CPP under conditions in one- and two-compartment CPP approaches that promote acquisition or extinction.

Experiment 1: Effects of DH inactivation prior to a post-acquisition test

(Test 1)

#### Methods

Animals. All experiments used naïve C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME) that were 8-12 weeks old. Each experiment was conducted with two cohorts of mice. After similar results were confirmed between the cohorts of animals, data were pooled for further analysis. Mice (N = 26) were housed in standard colony cages, four per cage until separated after surgery, and maintained on a 12-h light/dark cycle (lights on at 6 am) and received ad libitum food and water access. All experiments were conducted during the light phase, were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee, and were performed in accordance with the

National Institutes of Health guidelines for the care and use of laboratory animals.

**CPP Apparatus.** CPP was assessed using an unbiased apparatus, consisting of clear acrylic walls (30 cm wide x 15 cm deep x 15 cm high). The apparatus included interchangeable halves (left and right) of two floor types (Grid and Hole), each 15-cm x 15-cm x 15-cm (Cunningham, Patel, et al., 2006). Grid floors consisted of 2.3-mm stainless steel rods mounted 6.4-mm apart in an acrylic frame. Hole floors consisted of perforated stainless steel with 6.4-mm round holes on 9.5-mm staggered centers. The CPP chambers were housed in melamine shells (McCarthy Manufacturing, Gresham, OR) with air vents around the side of the chamber, allowing low levels of light to enter each chamber. A camera was mounted to the ceiling in the shell. A clear and removable acrylic barrier divided the CPP chamber into a two-compartment apparatus configuration. The acrylic divider was removed to create a one-compartment apparatus configuration. Each side of the two-compartment chamber was 15 cm wide; the one-compartment chamber was 30 cm wide.

**Drugs.** Cocaine hydrochloride (COC, Sigma-Aldrich, St. Louis, MO) was dissolved in physiological saline (SAL, 0.9% NaCl), for intraperitoneal (IP) injections (10 ml/kg), administered at a dose of 20 mg/kg on conditioned stimulus positive days (CS+). This dose was chosen based on our previous cocaine-induced CPP results in mice (Bernardi et al., 2009; Raybuck et al., 2013). Saline (SAL) was injected (IP) into animals on conditioned stimulus negative days (CS-),

matching volume (10 ml/kg) and handling specific effects between COC CS+ and SAL CS- acquisition trials.

Muscimol (M, Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) and bilaterally microinjected (0.25 ul/min/side) into the DH. Phosphate buffered saline (PBS) was microinjected (MI) into control animals, matching any volume (0.25 ul/min/side) and handling specific effects between Muscimol (M) and Vehicle (V) microinjections. Muscimol was administered in the first cohort at 0.5ug/side and in the second cohort at 0.25ug/side (previously demonstrated to be successful in the DH in similar studies: (Nasehi & Kamali-Dolatabadi, 2016; Stackman, Cohen, Lora, & Rios, 2016; Yousefi, Farjad, Nasehi, Reza, & Zarrindast, 2013). With no difference between groups in drug effects (on locomotion or preference), Cohorts 1 and 2 were pooled for all experiments. These doses were chosen based on previous findings in our lab and related literature (Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Nasehi & Kamali-Dolatabadi, 2016; Raybuck & Lattal, 2011).

**Surgical Procedures.** Guide cannula (26g) were designed and fabricated (Plastics One, Inc. Roanoke, VA) to target the DH at coordinates A/P -1.7, M/L ±1.5, D/V -2.3 mm from bregma. Mice were anesthetized with isoflurane (2%–5%) and mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The eyes of mice were lubricated and the scalp was scrubbed with betadine. A small portion of the scalp was cut away, exposing the skull from bregma to lambda. Bilateral holes were drilled through the skull, bilateral cannula were inserted 1.5 mm into the brain, held in place with the stereotax, and Ketac

dental cement was added to secure cannula in place and cover the exposed skull. Stainless steel stylets were inserted into the cannula (-2.0 mm into brain) to maintain patency during the 5d post-surgical recovery period. To infuse vehicle or muscimol solutions, stylets were removed and a microinjector was inserted into the cannula, extending .8 mm beyond the length of the cannula, to a total depth of -2.3 mm from bregma. These targets took into account the loss in depth due to the slight curvature of the brain using bilateral cannula and the thickness of the skull (injector targeted -2.3 to hit between -1.5 and -2.0mm).

**Cocaine-Induced CPP Protocol.** The following behavioral sessions were conducted: Habituation, Pretest, Acquisition, Test 1, and Test 2.

#### Habituation

On the two habituation days, mice were transported to the experimental room, weighed, and allowed to rest in their home cage for one hour (habituation to room). Each animal was then handled for two minutes (habituation to handling) and returned to their home cage for one hour before being returned to the colony room.

## Pretesting (PT) / Posttesting (Test 1 and Test 2)

Mice were removed from their cages and placed in the middle of a onecompartment apparatus configuration. One side of the apparatus had a Grid floor and the other side had a Hole floor. The side location of each floor within the apparatus was counterbalanced within groups. Each test (Pretest, Test 1, and Test 2) was completed 24h after the last Habituation, Acquisition, or Testing session. To minimize any potential latent inhibition effects the Pretest was 5 min in duration. Tests 1 and 2 were 30 min in duration.

#### Acquisition (A)

Mice were removed from their home cages, given an IP injection of SAL or COC, placed in their assigned acquisition chamber for 15 min, and then returned to their cages. Half of the mice were assigned to one of two floor subgroups (injected with COC and placed on a Grid floor = G+) and half were assigned to the other floor subgroup (injected with COC and placed on a Hole floor = G-). Thus, mice in the G+ subgroup received COC immediately prior to trials on the Grid floor and SAL immediately prior to trials on the Hole floor. Alternatively, mice in the H+ subgroup received COC immediately prior to trials on the Hole floor and SAL immediately prior to trials on the Grid floor.

In Experiment 1, mice were assigned to one of two acquisition subgroups (one-compartment acquisition or two-compartment acquisition). Groups of mice that acquired CPP in one-compartment will hereafter be identified as the A1 group (acquisition in one-compartment). In this configuration, the left and right floor types were identical during acquisition trials (either both Grid or both Hole) and mice had access to both sides of the apparatus, with no divider separating the chamber. Each floor type was consistently paired with COC or SAL during one-compartment acquisition, with no side cue pairing. Groups of mice that acquired CPP in two-compartments with consistent side cues were identified as the A2 group (acquisition in two-compartments). In this configuration, the left and right floor types were different from each other during all phases of CPP (1 Grid

side and 1 Hole side). A clear divider restricted mice to one floor type and one side of the apparatus per trial. Each side of the chamber was consistently paired with COC or SAL but not both; therefore, each side, as well as each floor, consistently predicted COC or SAL during two-compartment acquisition with consistent side cues. Pretest preference was balanced between subsequent acquisition groups. All mice were trained over a 4d period (two CS+ trials, two CS- trials) with one trial per day.

DH Microinjections. A microinfusion pump controlled Hamilton Syringes (10ul, Reno, NV) that were connected to PE20 tubing. Microinjectors connected the syringes and tubing to the DH cannula for DH microinjections of PBS or muscimol. Mice were able to move freely during the microinjection of PBS or muscimol. Injections occurred 30 min prior to the behavioral session (Test 1) at a rate of 0.25 ul/min for 1 minute. Injectors were left secured in place for an additional 30 seconds after the microinjection.

**Data Analysis.** Dependent variables were locomotion (average distance traveled in cm) and preference (average percent of time spent on the CS+ floor). Locomotor activity and position of each animal in the CPP chamber (left/right side) was recorded by a camera and analyzed by Ethovision software (Noldus, Leesburg, VA). As expected, CPP configuration had an effect on the distance traveled during training trials (i.e., acquisition and/or extinction), with onecompartment groups travelling significantly more in a larger area than twocompartment groups confined to a smaller area. Distance effects during training, between saline and cocaine and between one- and two-compartment CPP were similar to Chapter 2, therefore only distance traveled during testing sessions will be highlighted to demonstrate differences between saline and muscimol groups.

Preference levels and the assignment of CS+ floor (Grid or Hole), CS+ side (left or right, if applicable), microinjection drug (V or M), and order of IP injection (COC or SAL first or second) were counterbalanced between treatment groups. Independent variables [behavioral session (Pretest, Test 1, and Test 2)], type of training (one- or two-compartment) configuration, and microinjection drug (V or M) were analyzed separately or by repeated measures when applicable.

The magnitude of place preference was determined by the amount of time that each animal spent on the CS+ associated floor during each testing session. This preference was represented first by comparing the average seconds per minute (sec/m) that each floor subgroup spent on the Grid floor (animals conditioned to associate COC with the Grid floor, G+ subgroup, compared to animals conditioned to associate COC with the Hole floor, G- subgroup). To verify CPP acquisition (Cunningham et al., 2003), the time spent on the Grid floor between conditioning subgroups (G+ and G-) was verified in each experiment (data not shown). No differences emerged by flooring subgroup assignment (G+ versus G-) at Pretest yet reliable differences across all groups and experiments. As a consequence, these values (sec/min) were converted into a second measure of preference, the percent of time that animals spent on their cocaine-paired floor (G+ and H+ subgroups pooled). Preference by each treatment group will be

presented in this way (time on CS+ floor, %) for an easier interpretation throughout the paper.

Data were analyzed with Microsoft Office Excel programs and SPSS software. ANOVAs and planned Bonferroni follow-up tests compare differences at a significance level of 0.05 between behavioral sessions, bins of data within sessions, and by drug and configuration treatment group. Based on previous findings, the data were analyzed in two ways. First, data were collected and compared over the first 5 min of each session (0-5 min) to give a common point of comparison between tests and a measure of initial memory retrieval from the previous session. Second, data were collected and compared over the entire 30 min sessions (0-30 min) as a measure of within test session extinction (over six-5 min bins). All data are reported as the mean  $\pm$  SEM. A Greenhouse-Geisser correction adjusted the degrees of freedom and p value for any violations to sphericity (Mauchly sphericity test, p < 0.05) when applicable.

DH Histology. Figure 6 shows a representative cannula placement above the cornu ammonis area 1 (CA1) and injector tracks within the DH. Placement was confirmed for in the DH by observing gliosis along the cannula and/or infusion tracts. Mouse brains were fixed in 10%-buffered-formalin (4% formaldehyde, 1% methanol stabilizer, Thermo Fisher Scientific Inc.) for at least 48h, sliced on a cryostat (60 um), and Nissl stained with cresyl-violet. Microinjections were given prior to the first test (Test 1) with a composite of placements in Figure 6. There were no differences in placement based on treatment.



**Figure 6. Chapter 3 - Representative dorsal hippocampal (DH) cannulation**. Mouse section (60um) after microinjection and staining with cresyl violet.

## **Results and Conclusions**

In Experiment 1 (Figures 7A-8B), mice received acquisition of CPP with either a one- or two-compartment (A1 or A2, respectively) procedure. The DH was inactivated prior to a CPP test (24hrs after the final acquisition trial) to evaluate whether the DH is necessary for expression of CPP (see Figures 7A-C, timeline, cannulations, and 5-min preference). Further, because that test session (Test 1) is an extinction session the necessity of the DH for the development of extinction during that test can be determined by testing mice again 24h later (Test 2). It was expected that if one and two-compartment acquisition configuration groups activated the DH differently during initial CPP retrieval or extinction, then this pattern of behavioral results would differ. It was hypothesized that if DH inactivation impaired CPP retrieval, extinction may also be simultaneously diminished during Test 1, so preference for the CS+ paired floor would be low at Test 1, but then high at Test 2.

**Locomotor activity.** Mice that were given muscimol were more active than were the mice given PBS vehicle during Test 1, primarily driven by results in the first 5 min of the session (Fig 8A). During Test 2, a similar effect emerged, but to a lesser extent. To determine whether locomotion differed during initial retrieval (in the first 5 min of testing) over the three testing sessions, a 3 (testrepeated) by 2 (drug) by 2 (configuration) ANOVA revealed a main effect of test (F (1.1, 24.9) = 8.2, p = 0.007), drug (F(1, 22) = 6.4, p = 0.019), and a reliable interaction of test x drug (F (1, 44) = 3.4, p = 0.042).





Figure 7. Chapter 3 - Experiment 1. Muscimol intra-DH before CPP Test 1 (Experiment 1). Treatment group abbreviations are based on acquisition apparatus configuration in one-compartment (A1) or two-compartments with consistent cues (A2) with Vehicle (V) or Muscimol (M) pre-session DH drug treatment of A1V (N = 7); A1M (N = 7), A2V (N = 8), A2M (N = 4).

- (A) Experiment 1 design with microinjection prior to Test 1 (indicated by red arrow), Fig 7A.
- (B) Composite microinjection placements in the dorsal hippocampus (DH), Fig 7B.
- (C) Cocaine-induced CPP represented as the percent of time (CPP % by 5 min bin) spent on the cocaine paired (CS+) floor at Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-5 min), and Test 2 the following day (T2, 0-5 min). Error bars indicate the standard error of the mean (±SEM). Note. \*Drug effect, #Drug x Configuration effect; p < 0.05. Please see Results and Conclusions section (Test Preference) for a description of statistical findings, Fig 7C.</li>





Figure 8B.



**Figure 8. Chapter 3 - Experiment 1.** Treatment group abbreviations are based on acquisition apparatus configuration in one-compartment (A1) or twocompartments with consistent cues (A2) with Vehicle (V) or Muscimol (M) presession DH drug treatment of A1V (N = 7); A1M (N = 7), A2V (N = 8), A2M (N = 4). Error bars indicate the standard error of the mean (±SEM). Note. \*Drug effect, #Drug x Configuration effect; p < 0.05. Please see Results and Conclusions section (Locomotor Activity and Test Preference) for a description of statistical findings.

- (A) Total distance traveled (cm, by 5-min bin) during behavioral sessions Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-30 min), and Test 2 (T2, 0-30 min), Fig 8A.
- (B) Cocaine-induced CPP represented as the percent of time (CPP % by 5 min bin) spent on the cocaine paired (CS+) floor at Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-30 min), and Test 2 the following day (T2, 0-30 min), Fig 8B.

No differences emerged in locomotion between treatment groups at Pretest, but a main effect of drug was confirmed at Test 1 and Test 2 (F's (1, 22) > 4.456, ps < 0.046). As seen in Figure 8A, muscimol-treated mice compared to vehicle-treated mice traveled significantly more during the first 5 min of Test 1 and Test 2, with no effect of prior acquisition configuration.

Locomotion decreased over the course of Test 1 to Test 2 (30 min data). A 2 (test-repeated) x 2 (drug) x 2 (configuration) ANOVA revealed a reliable main effect of test (F (1, 22) = 4.693, p = 0.041, and a reliable interaction of test x configuration (F (1, 22) = 6.571, p = 0.018) with no main effect of drug or configuration. Follow-up one-way ANOVAs revealed no significant effects of configuration (Fs (1, 24)  $\leq$  3.6, ps  $\geq$  0.07) or drug (Fs (1, 24)  $\leq$  2.1, ps  $\geq$  0.162) treatment at Test 1 or 2.

**Test preference.** All groups showed approximately 50 % preference for the CS+ floor during the Pretest (see PT in Figure 8B). To determine whether inactivation of the DH alters recall of acquisition or recall of subsequent extinction, preference across the first 5 min of each test (Figure 7C and 8B) was compared. Muscimol impaired expression of CPP (Test 1) and prevented extinction of CPP (Test 2). This was revealed by a significant main effect of test and interactions between test x drug, test x configuration, and test x drug x configuration (F's (2, 44) = 21.28, 9.701, 4.083, 3.597; p's ≤ 0.001; 0.001, 0.024, 0.036).

Looking at preference differences at each individual test, no effect of drug or configuration at Pretest was found, but a main effect of drug (F (1, 22) = 29.810, p < 0.001) and a drug x configuration interaction (F (1, 22) = 7.274, p = 0.013) at Test 1, and a main effect of configuration at Test 2 (F (1, 22) = 8.609, p = 0.008). As seen in Figure 7C, muscimol administration impaired initial expression of CPP based on the previous acquisition configuration at Test 1 and correspondingly, Test 2 expression remained similar or rebounded in muscimol groups

To determine if extinction differed throughout Test 1 and Test 2, each 5 min bin of data within and between tests was examined. Figure 8B demonstrates the average CPP % over the full 30 min session from Test 1 to Test 2. A 2 (testrepeated) x 6 (bin-repeated) x 2 (drug) x 2 (configuration) ANOVA confirmed a main effect of configuration (F (1, 22) = 4.844, p = 0.039) and an interaction of test x drug (F (1, 22) = 13.434, p = 0.001), with Figure 8B showing an increase in the muscimol group and a decrease in the vehicle groups at Test 2. A decrease in preference would be expected if extinction learning had taken place during Test 1. No reliable bin effects occurred, although bin alone reached a trend level of significance (p = 0.08). A post hoc test for bin (bin x drug x configuration) reached significance (p = 0.027) from bin 1 (0-5 min) to bin 2 (5-10 min). Therefore, no reliable differences occurred across extinction learning curves (by bin), but differences were found in immediate recall (0-5 min) of CPP and in overall extinction (0-30 min), between Test 1 and Test 2. These results differed based on drug and configuration groups (see Fig. 8B).

In this first experiment, preference was altered by a DH microinjection before post-acquisition testing and by the apparatus configuration applied during
acquisition. This indicated a direct effect of inactivating the DH on the ability to express CPP and to extinguish CPP (at Test 1). The following day (Test 2), preference was found to be different based on acquisition configuration and prior DH microinjection, indicating an indirect effect of configuration and the DH on the ability to retrieve previous CPP acquisition (from Acquisition trials 1-4) and to extinguish CPP (from Test 1 to Test 2).

# Experiment 2: Effects of DH inactivation prior to post-extinction test (Test 2)

To test whether activity within the DH contributes to expression of CPP after extinction, the DH was inactivated prior to Test 2 (one day after extinction learning). It was hypothesized that all groups would demonstrate high preference for the conditioned floor at Test 1 (prior to microinjection treatment), but that preference would be maintained after extinction at Test 2, demonstrating an impairment in extinction if the DH was critical to decreasing preference. In addition, it was hypothesized that if one- and two-compartment extinction groups recruited the DH differently during extinction retrieval or expression, then this pattern of behavior would differ early in the session (first 5 min) or later in the 30 min session.

#### Methods

**Animals.** Mice (N = 23) were handled, underwent surgery, and were maintained on ad lib chow as noted previously.

Drugs. Muscimol was administered at 0.5ug/side.

**Cocaine-Induced CPP Protocol.** The following behavioral sessions were conducted: Habituation, Pretest, Acquisition, Test 1, Extinction, and Test 2. *Pretesting (PT) / Posttesting (Test 1 and Test 2)* 

Each test (Pretest, Test 1, and Test 2) was completed 24h after the last Habituation, Acquisition, or Extinction session. Test 1 was 5 min in duration to minimize extinction during the choice testing (Pretest was 5 min and Test 2 was 30 min).

# Acquisition (A)

Mice from Experiment 2 were trained in a two-compartment apparatus with consistent cues (A2) over a 4d period (two CS+ trials, two CS- trials) with one trial per day.

# Extinction (E)

After two-compartment acquisition (A2), mice were assigned to one of two extinction subgroups, one-compartment extinction (E1) or two-compartment extinction (E2). The one-compartment extinction configuration (E1) is further identified as the E1 group (extinction in one-compartment). In this configuration, the left and right floor types were identical to each other during extinction trials and mice had access to both sides of the apparatus, with no divider separating each chamber side. This resulted in exposure to the previously COC-associated floor, unrestricted by side. In the two-compartment extinction configuration with consistent cues (E2), the left and right floor types were different during all phases of CPP (1 Grid side and 1 Hole side). This tall clear divider restricted mice to one floor type and one side of the apparatus per trial. This resulted in visuospatial

exposure to the previously COC-associated floor on the CS+ chamber side. Preference at Test 1 (post-acquisition) was matched between subsequent extinction groups. During extinction, mice were removed from their home cage and placed on their previously cocaine-paired (CS+) floor (Grid or Hole) for 30min for two consecutive days (one trial per day).

**DH Histology.** Microinjections were given prior to the second test (Test 2, see Timeline Figure 9A) with a composite of placements from Experiment 2. There were no differences in placement based on treatment. Cannula placements are shown in Figure 9B.

## **Results and Conclusions**

In Experiment 2 (Figures 9A-10B), mice received acquisition of CPP with two-compartment (A2) procedure. The DH was inactivated prior to a CPP test 24h after two extinction trials to evaluate whether the DH is necessary for retrieval of CPP extinction (Test 2). If one and two-compartment extinction configuration groups activated the DH differently during initial retrieval of CPP extinction, then preference was expected to differ. It was hypothesized that if DH inactivation impaired extinction retrieval (5 min, Figure 9C), extinction within Test 2 may be simultaneously diminished throughout Test 2 (30 min, Figure 10B), so expression for the CS+ paired floor would begin and remain high compared to the saline group in one of both of the configuration groups.

**Locomotor activity.** To test whether activity differed over the initial 5 min of testing, distance traveled was compared over testing sessions (Figure 10A).



**Figure 9. Chapter 3 - Experiment 2** Treatment group abbreviations are based on extinction configuration in one-compartment (E1) or two-compartments with consistent cues (E2) with Vehicle (V) or Muscimol (M) pre-session DH drug treatment of E1V (N = 6); E1M (N = 6), E2V (N = 6), E2M (N = 5).

- (A) Experiment 2 design with microinjection prior to Test 2 (indicated by red arrow), Fig 9A.
- (B) Composite microinjection placements in the dorsal hippocampus (DH), Fig 9B.
- (C) Cocaine-induced CPP represented as the percent of time (CPP % by 5 min bin) spent on the cocaine paired (CS+) floor at Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-5 min), and Post-Extinction Test 2 (T2, 0-5 min). Error bars indicate the standard error of the mean (±SEM), Fig 9C.





Behavior session (5 min bins)

Figure 10B.



**Figure 10. Chapter 3 - Experiment 2.** Treatment group abbreviations are based on extinction apparatus configuration in one-compartment (E1) or two-compartments with consistent cues (E2) with Vehicle (V) or Muscimol (M) presession DH drug treatment of E1V (N = 6); E1M (N = 6), E2V (N = 6), E2M (N = 5). Error bars indicate the standard error of the mean ( $\pm$ SEM). Note. Please see Results and Conclusions section (Locomotor Activity and Test Preference) for a description of statistical findings.

- (A) Total distance traveled (cm, by 5 min bin) during behavioral sessions Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-5 min), and Post-Extinction Test 2 (T2, 0-30 min). Note. \*Drug effect, p < 0.05. Fig 10A.</li>
- (B) Cocaine-induced CPP represented as the percent of time (CPP % by 5 min bin) spent on the cocaine paired (CS+) floor at Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-5 min), and Post-Extinction Test 2 (T2, 0-30 min). Note. #Drug x Configuration x Bin effect, p < 0.05. Fig 10B.</p>

Locomotion was also compared by drug and configuration treatment (ANOVA: 3 (test-repeated) x 2 (drug) x 2 (configuration), Figure 10A). A main effect of test (F (1.493, 28.375) = 4.765, p = 0.025) and an interaction between test and drug (F (2, 38) = 5.316, p = 0.009) were confirmed, with no main effects of configuration or drug. When distance was compared across the extended 30 min duration of Test 2 (ANOVA: 6 (bin-repeated) x 2 (drug) x 2 (configuration)), there was a main effect of bin (F (3.102, 58.937) = 25.366, p < 0.001) as extinction occurred, and a bin x drug interaction (F (5, 95) = 4.086, p < 0.002) as seen in the first 5 min bin of data, with no effect of extinction configuration.

**Test Preference.** Expression of CPP did not differ during PT or during the first 5 min of the tests following acquisition (Test 1) or extinction (Test 2, Figure 9C). This was confirmed by a 3 (test-repeated) x 2 (drug) x 2 (configuration)) ANOVA, with a reliable effect of test (F (2, 38) = 44.117, p <0.001). After extinction, a slight decrease in preference occurred in the E1 group as expected from Chapter 2, although this did not reach reliable levels (F (1, 19) = 3.676, p = 0.070).

Comparing extinction curves over the entire 30 min Test 2 session (6 x 5 min bins), while muscimol was present in the DH, confirmed an interaction between bin, drug, and configuration (F (5, 95) = 3.002, p = 0.015 see Figure 10B). To follow-up the interaction, drug effects were compared at each Test 2 bin within each extinction configuration group.

A main effect of drug and an interaction was found between drug and bin in the E1 group (F (1, 10)=5.642, p=0.039; F (5, 50)=2.845, p=0.025, respectively) but

not in the E2 group (ps>0.28). This confirmed that Test 2 extinction occurred differently between E1 and E2 vehicle groups, and between drug groups in the those previously conditioned with one-compartment extinction but not in the groups previously extinguished in a two-compartment configuration.

In this second experiment, animals were trained in a two-compartment apparatus and extinguished in a one- or two-compartment apparatus. It was found that apparatus configuration during extinction and a DH microinjection prior to post-extinction testing mediated preference. This indicated a direct effect of DH inactivation on the ability to retrieve previous CPP learning (Extinction trials 1-2) and to further extinguish CPP (at Test 2).

## Experiment 3: Effect of DH inactivation prior to extinction training

To test whether activity within the DH contributes to either one or twocompartment extinction and subsequent retrieval and extended extinction (during drug-free testing), the DH was inactivated prior to an extinction session. It was hypothesized that impairment in DH activity during extinction would lead to maintained preference early at Test 2 (over 0-5 min) and typical extinction rates during the full test (over 30 min). It was also hypothesized that if one- and twocompartment configuration activated the DH differently during extinction, then the pattern of CPP expression may differ between configuration groups at Test 2. **Methods** 

**Animals.** Mice (N=30) were handled, underwent surgery, and were maintained on ad lib chow as noted previously.

**Cocaine-Induced CPP Protocol.** The following behavioral sessions were conducted: Habituation, Pretest, Acquisition, Test 1, Extinction, and Test 2. *Extinction (E)* 

Preference at Test 1 was balanced between subsequent extinction groups. Mice were removed from their home cage and placed on their previously cocaine-paired (CS+) floor (Grid or Hole) in a one- or two-compartment apparatus configuration (E1 or E2, respectively) for one trial. Extinction was 30 minutes in duration.

**DH Histology.** Cannula placement is shown in Figure 11B.

# **Results and Conclusions**

Experiment 3 investigated the effects of DH inactivation during extinction on retrieval at Test 2. A timeline for Experiment 3, cannulation placements, and preference in the first 5 minutes of each test is shown in Figures 11A-C.

**Locomotor activity.** Activity that occurred during extinction and Test 2 is shown in Figure 12A. During the first 5 min of extinction, there were reliable main effects of drug (F (1, 26)=6.278, p=0.02) and configuration (F (1, 26)=13.05, p= .001), as well as a reliable interaction between the two (F (1, 26)=4.78, p=0.038). When analyzing the entire 30 min extinction session, there were reliable main effects of configuration (F (1, 26)=9.76, p=0.004) and bin (F (1.81, 47.07)=17.84, p<0.001), and an interaction of bin and drug (F (5, 130)=3.01, p=0.013, Figure 12A).



Behavior session (retrieval in first 5 mins, bin 1)

**Figure 11. Chapter 3 - Experiment 3** Treatment group abbreviations are based on extinction configuration in one-compartment (E1) or two-compartments with consistent cues (E2) with Vehicle (V) or Muscimol (M) pre-session DH drug treatment of E1V (N = 7); E1M (N = 8), E2V (N = 7), E2M (N = 8).

- (A) Experiment 2 design with microinjection prior to Extinction (indicated by red arrow), Fig 11A.
- (B) Composite microinjection placements in the dorsal hippocampus (DH), Fig 11B.
- (C) Cocaine-induced CPP represented as the percent of time (CPP % by 5 min bin) spent on the cocaine paired (CS+) floor at Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-5 min), and Post-Extinction Test 2 (T2, 0-5 min). Error bars indicate the standard error of the mean (±SEM). Fig 11C.





Behavior session (5 min bins)

Figure 12B.



**Figure 12. Chapter 3 - Experiment 3.** Treatment group abbreviations are based on extinction apparatus configuration in one-compartment (E1) or two-compartments with consistent cues (E2) with Vehicle (V) or Muscimol (M) presession DH drug treatment: E1V (N = 7); E1M (N = 8), E2V (N = 7), E2M (N = 8). Error bars indicate the standard error of the mean (±SEM). Please see Results and Conclusions section (Locomotor Activity and Test Preference) for a description of statistical findings.

- (A) Total distance traveled (cm, by 5-min bin) during behavioral sessions Pretest (PT, 0-5 min), Test 1 (T1, 0-5 min), Extinction trial (EXT, 0-30 min), and Test 2 (T2, 0-30 min). Note. #Drug x Configuration effect, p < 0.05, Fig 12A.</li>
- (B) Cocaine-induced CPP represented as the percent of time (CPP % by 5 min bin) spent on the cocaine paired (CS+) floor at Pretest (PT, 0-5 min), Post-Acquisition Test 1 (T1, 0-5 min), and Test 2 the following day (T2, 0-30 min). Note. ^Drug x Session (T1 to T2) effect in one-compartment group, p < 0.05, Fig 12B.

As activity was higher in the muscimol group compared to the vehicle group (Figure 12A), especially in the first half of the session, and activity in the one-compartment group remained higher than the two-compartment group throughout extinction. Overall, results confirm that activity changed as expected, decreasing over testing sessions and within extinction, with higher rates of activity in muscimol and one-compartment groups during extinction only. There were no differences in activity between treatment groups when the entire duration of Test 2 was analyzed, although there was a main effect of bin (F (3.685, 95.812) = 12.303, p < 0.001) as activity decreased in all groups through the session.

**Test preference.** Test 2 preferences for the first 5 min of each session are shown in Figure 11C. There was no effect of drug, but a main effect of session (F (2, 52) = 53.227, p < 0.001), and a session by configuration interaction (F (2, 52) = 3.912, p < 0.026) over the three sessions. A follow-up test confirmed that preference changed from Test 1 and Test 2 differently between extinction configuration groups ((F (1, 26) = 6.257, p < 0.019), 2 (sessionsrepeated) x 2 (configuration)). In the first 5 min of Test 2 there was reliable difference between configuration groups (p = 0.027), with the one-compartment groups expressing less preference than the two-compartment group (difference of 12.41% ± 5.31, pooled drug groups).

As seen in Figure 12B, a main effect of extinction configuration group (F (1, 26) = 4.657, p < 0.040) and of bin (F (3.2, 84.04) = 4.530, p = 0.004) emerged over the 6 bins during Test 2, with no overall drug effect at Test 2 (F (1, 26) =

1.810, p = 0.190). Follow-up tests for configuration effects occurred in the first 5 (as noted above) and 10 min of Test 2 (ps < 0.023, Figure 12B) with no drug effect in any bin (ps > 0.11).

As an additional comparison, configuration groups were analyzed separately for total preference expressed before (Test 1, 5 min) and after (Test 2, 30 min test) extinction. As expected when comparing a short to a long session, there was a main effect of session (F (1, 13) = 72.78, p < 0.001) as well as a session by drug effect (1, 13) = 4.824, p = 0.047) in the one-compartment group. In the two-compartment group there was a session effect (F (1, 13) = 16.728, p = 0.001) but no session by drug effect (F (1, 13) = 0.000, p = 0.998). These effects indicated a minor limitation to extinction from Test 1 to the end of Test 2 in the muscimol-treated one-compartment group compared to the vehicle-treated one-compartment group.

In Experiment 3, a two-compartment apparatus was used to train animals during acquisition (similar to Experiment 2) and a one- or two-compartment apparatus (one trial) was used to extinguish preference. As a result, neither retrieval (5 min) nor extinction (30 min) at Test 2 was mediated reliably by a DH microinjection (administered before one extinction trial the day prior), but was altered by the extinction configuration and between Test 1 and the end of Test 2 in the one-compartment extinction group. This indicated no significant effect of temporary DH inactivation on retrieval of CPP extinction at Test 2 but a consistent effect of configuration.

#### Discussion

These experiments demonstrate that inactivation of the DH impairs the post-acquisition expression, extinction, and post-extinction expression of a cocaine-induced conditioned place preference. In general, these effects occurred with different apparatus configurations (one- or two-compartment) but were largest in those conditions that led to stronger acquisition (two-compartment) or stronger extinction (one-compartment) in vehicle-treated groups. These findings extend previous work in Chapter 2, showing that acquisition and extinction of CPP is sensitive to the configuration of the CPP chamber and that the DH is necessary for retrieval of drug-associated memories.

Hippocampal inactivation immediately prior to a post-acquisition expression test caused a general impairment in expression of CPP, but this effect was more pronounced in the consistent spatial location condition (twocompartment procedure). Previous work has found that, in general, a twocompartment acquisition procedure leads to stronger CPP compared to a onecompartment procedure. One difference between these procedures as used here is the role of spatial cues. In the two-compartment procedure, spatial cues consistently predicted the location in which drug was delivered; in the onecompartment procedure, spatial cues were irrelevant. In Experiment 1, inactivating the DH prior to an expression test resulted in a pronounced deficit in mice trained in the two-compartment (spatial) procedure. This effect suggests that, relative to one-compartment training, two-compartment training may result in a larger dependence on spatial cues compared to tactile cues, which may in turn require the DH for retrieval. During a subsequent test, however, the group with the largest impairment in expression showed a very high preference. This effect suggests that during the first test, the two-compartment inactivation group was not able to express or access a previously stored cocaine-context memory. While vehicle groups experienced extinction during the first test, the two-compartment muscimol group did not, resulting in high preference expression during the test the next day. This finding is consistent with others showing that impairments in performance during a first test impair the learning of extinction contingencies, resulting in poor extinction retrieval during the second test (Corcoran & Maren, 2001; Corcoran, Desmond, Frey, & Maren, 2005; Holt & Maren, 1999).

In contrast to acquisition, which is promoted by two-compartment training, extinction is promoted by one-compartment training (as demonstrated in Chapter 2), potentially due to the association between extinction and multiple spatial locations within the apparatus. When the DH was inactivated during the postacquisition expression test (Test 1 in Experiment 1), extinction was impaired most in the two-compartment group. This result suggests that in addition to being involved in the expression of CPP, the DH is involved in the consolidation of the learning that occurred during choice extinction. Finally, when extinction was allowed to occur with normal hippocampal involvement (Experiment 2), muscimol injected prior to a post-extinction test disrupted retrieval of extinction in the onecompartment group.

Our results support previous findings that recall of previous learning is necessary for new extinction (Corcoran & Maren, 2001; Corcoran et al., 2005;

Meyers et al., 2006). If it was common for recall and expression to be impaired or withheld, and extinction to still occur, decreases in Experiment 1 at Test 1 CPP and at Test 2 may have been expected (due to normal extinction at Test 1 and normal retrieval at Test 2). This would suggest that the DH may be needed for retrieval of CPP and expression but not for encoding or consolidation of choice extinction. This did not occur though, implying that the DH is critical for each aspect of this process (the retrieval and expression of CPP and the ongoing encoding, consolidation, and expression of extinction). Results from Experiment 2 help clarify this, since retrieval in the first 5 min of testing was similar between all groups at Test 2 (after extinction) and differed during choice extinction, suggesting that the DH is important for encoding choice extinction, independent of recall.

The DH appears to be needed to compare previous learning in one context with the current experience in a new context. We cannot say for sure whether the DH is necessary for the recall of previous learning, or if it is only necessary to recall the previous context compared to the current context. In either case, it is necessary for translating previous learning onto a new contextual map to complete the choice test. Accepting this role for the DH might mean that the previous learning configuration (one vs two) or type (excitatory acquisition versus inhibitory extinction) plays a role in subsequent learning. Results may have been different in our study if animals had all been trained in a one-compartment apparatus (rather than two-compartments) in Experiments 2 and 3. Similarly, these results would be informed by directly comparing one- and two-compartment forced CS+ exposure extinction to CS+/CS- choice extinction.

#### Locomotion differences cannot account for preference

# Role of DH inactivation by muscimol on activity

It is important to note the consistency of activity effects due to muscimol. In all experiments, activity was reliably higher in muscimol- compared to vehicletreated mice when the injections occurred immediately prior to testing. Inactivation or lesions of the DH in other studies increase, decrease, do not change, or did not have a reported effect on locomotor activity (Campese & Delamater, 2014; Chee, Menard, & Dringenberg, 2015; Corcoran & Maren, 2001; Douglas, 1967; Fujiwara et al., 2012; Meyers et al., 2003, 2006; Rezayof, Razavi, Haeri-Rohani, Rassouli, & Zarrindast, 2007). The DH is thought to regulate activity and interact with other brain regions, such as the entorhinal cortex (EC), ventral hippocampus (VH), prefrontal cortex (PFC), nucleus accumbens (NA) and amygdala (AMY; Fanselow & Dong, 2010; Gould & Leach, 2014; Hermann, Stark, Blecker, et al., 2017; Khoo, Gibson, Prasad, et al., 2017; Quirk & Mueller, 2008; Strange, Witter, Lein, et al., 2014; Wells et al., 201; Zelikowsky, Hersman, Chawla, et al., 2014). For example, inactivation in the DH inhibits activity in the lateral amygdala (LA), and decreases freezing behavior, especially in a context that was not previously extinguished (Maren & Hobin, 2007). In a similar way, previous context training and muscimol-treatment regulated locomotion and CPP expression, but often in different directions based on the experiment.

The timing of injection and spread may have imparted specific results between our studies and others, and but this is unlikely with the time and drug range used in this Chapter (consistent with others). Most studies have tested behavior 20 to 60 minutes after intra-DH infusions of muscimol (Corcoran et al., 2005; Matus-Amat et al., 2004; Riaz, Schumacher, Sivagurunathan, Van Der Meer, & Ito, 2017). There is evidence that muscimol binding peaks immediately after injection (samples collected at 0 min post-infusion) with a spread up to ±1.5 mm in the rostrocaudal direction, that slowly dissipates for approximately 1-2 hours, both in the DH (Corcoran et al., 2005) and similarly in the sensorimotor cortex (Martin & Ghez, 1999). These factors are worth considering for the potential effect on place preference in relation to activity changes. *Preference after DH inactivation not determined by activity* 

There are different views on how activity can interfere with or mask preference, and was discussed in Chapter 2. The most common concern is that increases in activity are often co-expressed with decreases in preference (i.e., due to a drug, or novelty, etc.) and can compete during testing. This possibility makes it less clear whether decreases in preference are due to a conditioned motivational response or an indirect result of increased locomotion (Gremel & Cunningham, 2007). In this study, activity was inversely related to preference in the first 5 min of Test 1 in Experiment 1 and in the first 5 min of extinction training in Experiment 3. Therefore, preference during testing may not be a complete measure of learning, memory, or conditioned motivation, but a consequence of competition induced by muscimol to increase activity. This effect may result in a more equal distribution of time spent on the CS+ and CS- floors and lower preference expression overall. However, this relationship between locomotion and preference was not consistent between configuration groups, over experiments, or different durations of testing and may be related to the learning that occurs (i.e., more activity may demonstrate less recall and necessary exploratory behavior, or lead to differences in context learning and extinction during the test). Therefore, it is possible that drug-induced changes in activity partially led to changes in preference expression, but cannot fully account for effects.

In our experiments, a DH microinjection of muscimol consistently increased activity but these activity effects corresponded to different effects on preference depending on the experiment. For example, preference expression was decreased (Experiment 1), increased (Experiment 2), and unchanged (Experiment 3) after a muscimol microinjection consistently increased activity. Similarly, a larger apparatus configuration increased activity during conditioning, with no consistent direction of effects on preference (highest activity  $\neq$  highest preference). The opposite was also found as well, with no change in activity between drug groups later in sessions, preference differences often occurred independently (Experiments 1-3). Therefore, it is unlikely that changes in activity, due to a microinjection or configuration treatment, independently explain the differences demonstrated in preference.

An open question is whether different CPP configurations recruit different circuits and cellular or molecular mechanisms. Our results suggest that the

involvement of the DH may change as a function of apparatus configuration, which is supported by other studies. Because the two-compartment training procedure involves a consistent spatial location, it is quite possible that mechanisms that have been identified to represent space in the DH (such as coordinated cell activity between subregions) come more into play than those in the one-compartment procedure, in which spatial cues are explicitly made irrelevant. This account of results could mean that the one-compartment group may recruit other brain regions at training, and then need the DH and other regions (e.g., NA, AMY, VTA) more critically at testing to determine preference in a new test floor configuration (Bouton, Westbrook, Corcoran, & Maren, 2006; Crombag, Bossert, Koya, & Shaham, 2008b; Dunsmoor et al., 2015). Therefore, differences in configuration, may change how the DH is activated and to what degree, potentially tilting the molecular processes that are related.

Perhaps regions that are activated to a greater degree may have more stability and molecular cascades being initiated. Spatial and non-spatial water maze learning leads to different expression levels of immediate early genes (IEGs) such as activity-regulated cytoskeleton-associated protein (arc) and (zif)) in the DH, but may or may not be correlated to other regions based on the type of IEG and experience (Guzowski, Setlow, Wagner, & McGaugh, 2001). Other cases have demonstrated that activity-dependent changes can differ based on IEGs, brain region, and changes in behavioral design and context exposure. For example, rats exposed to a previous cocaine self-administration context demonstrate increases in DG arc expression, independent of lever presentation or lever activity, whereas CA1 and CA3 arc increases only occurred when levers were presented. In this context, there were no differences between training and testing (Hearing, Schochet, See, & McGinty, 2010).

This suggests that arc induction in the DH may occur during repeated presentations of the same context during exploration of the same space, as context-drug associations are modified, such as during extinction and as consolidation of new contextual and drug information occurs (Cammarota, Bevilaqua, Barros, et al., 2005; Hearing et al., 2010; Ramirez et al., 2009; Vazdarjanova, 2004). Similar results have been noted in related structures, such as the intercalated cells of the AMY (Busti et al., 2011) where retrieval and extinction increase phosphorylated alpha Ca2+/calmodulin-dependent protein kinase II (Huang, Chen, Liang, & Hsu, 2014; Meins et al., 2010).

In another example, cocaine mouse CPP increases phospho-cAMPresponse element binding protein (pCREB) in both the DH and the NA, but in the DH there are increases to phosphorylated glutamate receptor 1 (pGluR1), that likely leads to increased calcium influx and downstream phosphorylated extracellular regulated kinase (pERK, (Tropea, Kosofsky, & Rajadhyaksha, 2008) Concurrently, in the NA, a slightly different set of cascades occurs (with increases to Thr34 phospho-dopamine- and cyclic AMP-regulated phosphoprotein 32 and decreases to Thr75PDARPP-32), likely leading to increased inhibition of protein phosphatase 1. It is then plausible that with a difference in previous learning (during acquisition or extinction) and expression of preference, there are likely differences in brain region recruitment, signaling cascades, and associated subsequent behavior (i.e., retrieval, choice extinction, etc.). Although this did not apply in all cases, which may be due to previous extinction learning already taking place.

This difference in effects could mean that the contextual changes between training and testing may identify as a larger mismatch by the DH after onecompartment training, requiring the DH at testing, but as a small mismatch after two-compartment training, requiring the DH less for expression of preference at testing. This hypothesis is consistent with previous studies (i.e., Meyers, Zavala, & Neisewander, 2003; Meyers, Zavala, Speer, & Neisewander, 2006), findings in this Chapter (large effect of muscimol on two-compartment CPP), and unpublished data in our lab (greater c-Fos activation in the DH and BLA after one- than two-compartment training, and greater preference with DH inactivation during one- than two-compartment acquisition). These results led me to believe that while both groups may be accessing information in the DH and BLA (among other regions), the one-compartment group may be recruiting the DH to a larger degree at testing but relying on it less, and the two-compartment group may be recruiting it more at training but relying on it more at testing, all based on the contextual cues presented during training and testing.

During extinction, salient tactile cues may form stronger associations with the surrounding visuospatial and contextual cues than during acquisition. This change towards a context-specific type of learning may be facilitated by the onecompartment configuration more than the two-compartment configuration. As extinction was enhanced by a one-compartment configuration but impaired by DH inactivation (Experiments 2 and 3), our results suggest that allowing extinction to occur in more than one location within the one-compartment but not two-compartment configuration enhanced extinction and the reliance on the DH during extinction training and testing. It is possible that the DH is supporting acquisition and extinction learning and retrieval of learning in different ways (i.e. recalling a contextual memory or navigating in a novel context). This difference in the role of the DH may be determined by the configuration used during training.

Together, these results demonstrate hippocampal involvement in cocaineinduced CPP interact with the behavioral conditions that are present at the time of acquisition, extinction, and testing. These effects imply that the dorsal hippocampus, as well as contextual cues (even when they are ambiguous, such as in the one-compartment group) are involved in acquiring and extinguishing Pavlovian reward-seeking behavior. Importantly, DH involvement was gated by the contextual cues and phase of learning. This results suggest that potential research and clinical interventions should be particularly cognizant of the different types of contextual cues that are associated with initial drug use and those that are available during behavioral and pharmacological treatments to target the appropriate neurobiology.



# A histone deacetylase 3 inhibitor enhances extinction and attenuates reinstatement of cocaine self-administration in rats

## Introduction

Substance use disorder is a chronic, often relapsing disease that leads to a loss of behavioral inhibition and overuse of addictive drugs (e.g., cocaine, methamphetamine, alcohol). In an attempt to better understand and counteract this disorder, preclinical approaches have focused on behavioral treatments such as extinction to promote the formation of inhibitory learning and memory (Cammarota, Bevilagua, Rossato, et al., 2005; Myers & Davis, 2002; Quirk & Mueller, 2008). The severing of relations among cues, responses, and drugs in rodents during extinction models aspects of exposure therapy in humans (Kiefer & Dinter, 2013; Morrison & Ressler, 2014; Peters et al., 2009). As in humans, animals will relapse, or reinstate excitatory drug-seeking behavior once removed from an extinction context (or clinical therapeutic setting), presented with stimuli previously associated with drug reinforcement (e.g., visual cues), or with the passage of time (Bossert, Marchant, Calu, & Shaham, 2013; Bouton, 2002; Fuchs, Tran-Nguyen, Specio, Groff, & Neisewander, 1998; Hermans, Craske, Mineka, & Lovibond, 2006; Lonsdorf, Haaker, & Kalisch, 2014; Shaham, Shalev, Lu, De Wit, & Stewart, 2003; Xue et al., 2012). The challenge for treatments is therefore to develop tools that may promote extinction and weaken relapse.

Recent attention has focused on epigenetic mechanisms that modulate interactions between DNA, histones, and other signaling factors (e.g., co-factors,

TFs) as potential treatments. These processes alter the structure of chromatin with posttranslational modifications and covalent modifications to DNA. Promoter regions can then become more accessible to TFs that help regulate transcription and protein synthesis, essential to long-term memory (Bourtchouladze et al., 1998; Federman, Zalcman, de la Fuente, Fustiñana, & Romano, 2014; Jarome & Helmstetter, 2014; Lattal, Radulovic, & Lukowiak, 2006; Woldemichael, Bohacek, Gapp, & Mansuy, 2014). Many studies have now documented enhancements in memory caused by administration of drugs that inhibit histone deacetylases (HDACs; e.g., Dagnas et al. 2015; Foley, Cassidy, and Regan 2014). These compounds inhibit deacetylation, or the removal of acetyl groups from specific amino acid sites of histones. This inhibited deacetylation, increase histone acetylation, reversing the compressed genome state and results in greater accessibility to DNA and altered recruitment of proteins (Furumai et al., 2011; Sarkar et al., 2011; Stolzenberg, Stevens, & Rissman, 2014). These dynamic chromatin changes are positively associated with transcription (Bousiges et al., 2013; Kouzarides, 2007), protein synthesis, synaptic plasticity (Arrar et al., 2013; Blank et al., 2014; Guan et al., 2009; Rosen et al., 2004; Rosenberg et al., 2014; Vecsey et al., 2007), associative learning, and cognition (reviewed in Barrett & Wood, 2008; Penney & Tsai, 2014).

Most of what is known about pharmacological modulation of HDACs during extinction comes from the use of nonspecific HDAC inhibitors (e.g., trichostatin A, sodium butyrate, vorinostat, and valproic acid) in Pavlovian conditioning assays. These have demonstrated enhanced extinction after fear or

place preference conditioning (e.g., Bredy et al., 2007; Gaglio et al., 2014; Lattal, Barrett, & Wood, 2007; Malvaez, Sanchis-Segura, Vo, et al., 2010; Raybuck, McCleery, Cunningham, et al., 2013; Wang et al., 2015). Few studies have targeted specific HDACs in extinction (Bowers, Xia, Carreiro, & Ressler, 2015). HDAC3 is one of twelve HDACs, with distinct connections to other complexes and HDACs that are associated with learning. HDAC3 is therefore hypothesized to be a critical negative regulator of learning. As a result, long-term inhibition of HDAC3 increases histone acetylation (e.g., on histone 3 (H3) at lysine site 8 (K8)) and acquisition of cocaine conditioned place preference (Rogge, Singh, Dang, & Wood, 2013) in mice. In addition, systemic and short-term application of RGFP966, a novel and selective HDAC3 inhibitor enhances histone acetylation (at H3K14 and H4K8) and extinction learning in mice, resulting in decreased drug-primed reinstatement after extinction of cocaine-induced conditioned place preference (CPP, Malvaez et al., 2013). While increases in learning and corresponding decreases in drug-seeking have been discovered with Pavlovian and operant learning (Malvaez et al., 2010 and Castino et al., 2015; Romieu et al., 2008, respectively), no studies to date have determined the effects of targeting just one HDAC with extinction after operant self-administration.

It is generally accepted that long-term effects of HDAC3 inhibition (as well as pan-HDACi) on Pavlovian behavior are largely mediated through experiencedependent cellular pathways and early consolidation phases of learning (Gaglio et al., 2014; Malvaez, Barrett, Wood, et al., 2009). The goal of this chapter was to investigate whether HDAC3 inhibition promotes extinction of operant responding for cocaine. In Experiment 1, the persistent effects of a single systemic injection of the HDAC3 inhibitor, RGFP966, was evaluated during extinction and reinstatement. In Experiment 2, it was determined that RGFP966 had no effect when delivered prior to a maintenance session of cocaine self-administration. In Experiment 3, RGFP966 did not alter motivation for cocaine, measured with a progressive ratio schedule of reinforcement. In the second and third phase of this experiment, the effects of a single post-extinction session administration of RGFP966 were determined. It was hypothesized that RGFP966 would create a persistent decrease in drug-seeking if administered before or immediately after extinction learning.

# Experiment 1: RGFP966 treatment before FR5 extinction reduces drugseeking and reinstatement

HDAC3 inhibition enhances extinction and decrease reinstatement one week after treatment in a cocaine conditioned place preference assay in mice (Malvaez et al., 2013). This experiment investigates whether RGFP966 treatment would similarly enhance extinction and reduce reinstatement after chronic cocaine self-administration in rats. HDAC3 is highly expressed in the dorsal hippocampus (DH) and object location memory is enhanced with genetic deletion of HDAC3 in the DH (McQuown et al., 2011) or with RGFP966 (Malvaez et al., 2013) administered systemically prior to training, therefore it was tested whether RGFP966 administration prior to extinction training would enhance long-term extinction and context-induced reinstatement.

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# Methods

Animals. Male Long-Evans rats (N = 21, Charles River Laboratories, Wilmington, NC) weighing 275-300 grams and aged 2-3 months were pair housed and allowed to habituate to the vivarium for one week after arrival. Rats had ad libitum access to food and water before behavioral training and then 4-5 pellets/day of rat chow (equivalent to 20-25g) to limit weight gain and potential complications with catheters and attached backpacks. Rats were maintained on a 12 hour light-dark cycle (lights on at 6 am and off at 6 pm), with behavioral sessions ( $\leq$  3 hours in duration) conducted in their light-cycle, between 8 am and 4 pm for 1-3 months (5-7 days/week). These procedures were approved by the OHSU IACUC, and were in accordance with the ethical guidelines set by NIH, Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985).

Intravenous catheter surgery. To induce general anesthesia for surgery, a mixture of Ketamine (66 mg/kg) and Xylazine (AnaSed, Lloyd, Shenandoah, IA USA 1.33 mg/kg) was given to rats by intraperitoneal (IP) injection at 10% body weight (0.5ml/500g). Anesthesia was maintained by inhalation of 1-2% isoflurane gas for the remainder of the surgery.

Once rats were immobile and unconscious each rat was placed on a sterile pad for surgery and a small patch of hair was shaved for a small incision at the catheter insertion and exit sites. Catheters were pre-made out of Silastic laboratory tubing (Dow Corning, Midland, MI USA, cut to10 cm, 0.55 mm I.D. x 0.94 mm O.D.), filled with filtered (0.22um) saline, and inserted into the jugular vein. One end of the catheter was secured into the right jugular vein, and the

other end was threaded around the shoulder and out the exit incision between the shoulder blades. Two internal and three external Sofsilk sutures (Covidien, Minneapolis, MN USA, coviden3-0, wax coated brained silk) were added to the front and one external sutures was added to the back of the rat at each incision point to secure the catheter in place and decrease the possibility of infection. Each catheter was briefly checked for patency by drawing blood back into the catheter and gently flushing it with filtered saline before attaching the catheter to an external backpack (Instech, Plymouth meeting, PA USA, Cat.No. CIH95AB), worn indefinitely by the rat. A steel cannula with a screw connector (Plastics One, Roanke, VA USA, 22GA, 5mm above and below pedestal) was placed inside the backpack to attach the jugular catheter tubing to the backpack and to the tether attachment within the behavioral self-administration chamber for infusions.

Rats were given a subcutaneous (SC) injection of an anti-inflammatory (carprofen-Rimadyl, Pfizer, New York NY USA, 5mg/ml) to decrease swelling and associated pain, an intravenous infusions of an antibiotic (Timentin, GlaxoSmithKline, Research Triangle Park, NC USA, 238mg/ml) in filtered saline to decrease the potential for post-operative infection, and an anticoagulant (heparin, West-Ward, 100U/ml) in filtered saline to increase the patency of the catheterization. They were placed in a clean cage (singly housed), with two nestlets, and pellet food after surgery. After waking with no signs of distress, each rat was returned to the vivarium. During recovery (5-7 days after surgery) rats were monitored for any signs of pain or weight loss and given carprofen if warranted. To maintain and verify catheter patency over time, catheters were flushed with heparin, Timentin, and Brevitol intravenously. Heparin was administered before and after each self-administration session and during nontesting days (10-100U/ml, respectively). Timentin was given after each selfadministration session and during non-testing days.

**Drugs.** For intravenous cocaine (COC) self-administration, (-)-cocaine hydrochloride (Sigma-Aldrich CASRN 53-21-4) was dissolved in 0.9% physiologic saline (4mg/ml) and filtered (0.22um Millipore Disposable Filtration System) to make a solution of 0.2g COC/50ml saline. Syringes (BD sterile Luer-Lok Tip, Franklin Lakes, NJ, 10 ml) were attached to MedPC pumps and filled with this solution daily. Each infusion occurred after completion of the active lever schedule (e.g., one (FR1) or five (FR5) lever presses) pumped into the attached tether and rat catheter over 5 seconds and dispensed 88.7ul of the cocaine solution (dose of 0.89mg/kg with a rat weight of 400g). For COC reinstatement, \COC was dissolved in saline (10mg/ml) and injected (IP) at a dose of 10mg/kg (1ml/kg).

RGFP966 (R966, selective HDAC3 inhibitor; provided by Repligen Corporation) was dissolved in dimethylsulfoxide (DMSO, <10% final volume), and diluted with a vehicle (VEH) solution of hydroxypropyl-β-cyclodextrin and sodium acetate (provided by Repligen, pH 5.4). R966 was administered (2ml/kg vol, SC), at a dose of 10 mg/kg (Bieszczad et al., 2015; Bowers et al., 2015; Malvaez et al., 2013) 20-min prior to extinction, after two days of habituation to injections (with VEH).

**Apparatus.** Behavioral experiments were conducted in twelve MedPC modular test chambers (12" L x 9.5" W x 11.5" H; 30.5 cm x 24.1 cm x 29.2 cm), equipped with two response levers (one of each was retractable, consistent in all chambers), two cue lights (1" white lens above each lever), one pellet receptacle between the two levers, a small hooded house light (28V DC) above the pellet receptacle, and a small opening at the top of the chamber for drug infusion equipment (swivel, tether, syringe, and pump). Each chamber side panel (left and right), grid floor, and waste pan was made of stainless steel. The top, back, and front chamber walls were made of clear plastic. Each chamber was enclosed in a sound attenuating cubicle, equipped with a fan on one side of the chamber to circulate air and provide ambient background noise during all behavioral sessions (28V DC). On the other side of the chamber a movable-metal arm connected the tether tubing to the internal rat chamber and external pump system, allowing the rat to move freely within the chamber when connected to the syringe tether.

To create two separate contexts (hereafter referred to as Context 1 and Context 2), the floor, visual cues, and spatial location of 6 out of 12 of the chambers were altered, so that half were of one type and the other half were of another type. For the tactile cue, a grid floor with either large diameter bars spaced far apart (4.8 mm diameter, 10.8 mm apart, 19 steel rods total), or a floor with smaller diameter bars spaced closer together (4.8 mm diameter, 10.8 mm apart, 19 steel rods total) were used for each context (MedPC, Cat.No. ENV-005 and ENV-005A-T, respectively, Crombag, Grimm, & Shaham, 2002; Crombag &

Shaham, 2002). For the visual cue, one of two contexts had a clear Plexiglas back wall so that the exterior shell remained visible to the rat. In contrast, the back wall of the other context consisted of an 8.5" x 11" sheet of with black and white stripes and stars. Spatially, each context was located in a different location (vertically and horizontally) within the testing room (i.e., Context 1 was 5' vertical x 3' horizontal from Context 2). The context assignment (training in Context 1 vs. Context 2) was counterbalanced between drug treatment groups (i.e., VEH vs. R966 administration) so that half of the rats in each treatment group were assigned to one context, while the other half of rats, in the same treatment group, were assigned to the other context. This assignment largely eliminated the chance that any potential differences due to context might influence drug treatment results, but allowed for testing context-induced reinstatement (i.e., renewal).

Self-administration Protocol. A schematic of the experimental timeline and summary of treatment for each experiment is in Figure 13 (see Experiment 1). Experiment 1 consisted of three primary Phases: 1) Acquisition and maintenance (M), 2) Extinction (E) before reinstatement, and 3) Reinstatement (R) with intervening extinction. Rats were split into two balanced groups and received VEH or R966 treatment prior to the first extinction session. Inactive and active lever presses were compared over each session.
### Figure 13.



**Figure 13. Chapter 4 – Multi-experimental timeline.** Drug treatment (Tx) group abbreviations are based on systemic (SC) drug injections of Vehicle (VEH) or RGFP966 (R966, histone deacetylase 3 inhibitor) with red arrows indicating whether drug treatment was paired with maintenance of cocaine self-administration (Experiments 2 and 3 (Phase 1)) or the first session of extinction (Experiments 2 and 3 (Phase 2)).

Experiments 1 and 2 were conducted under a fixed ratio 5 (FR5) schedule. Rats from Experiments 1 and 2 were pooled for within session comparisons during Phase 1 of Experiment 3 and reallocated into three groups for Phase 2 of Experiment 3.

Following stable maintenance of self-administration, drug treatment was given: Before extinction Session 1 (Vehicle or RGFP966; Experiment 1), Before FR5 self-administration maintenance sessions with intervening vehicle treatment (Maintenance, Vehicle, RGFP966, Vehicle, RGFP966, Experiment 2), Before PR self-administration maintenance session with vehicle treatment prior and no treatment during the following maintenance session (Maintenance, Vehicle, RGFP966, Maintenance, Experiment 3 (Phase 1)), or After extinction Session 1 (Vehicle or RGFP966; Experiment 3 (Phase 1 maintenance of PR selfadministration (Experiment 3 (Phase 2)).

#### Acquisition and Maintenance (M)

Rats were moved from the vivarium to the experimental room, their catheters were flushed with heparin (10U), and they were weighed and then placed in a pre-assigned operant chamber. Immediately after, the MedPC program was started, the house light turned on, and rats were infused with a short duration and small volume priming injection of cocaine (2s infusion). All acquisition sessions were 120-min long. Once in the self-administration chamber, 1 active lever press (fixed ratio 1 (FR1) schedule) illuminated the cue light above each active lever, activated the syringe pump, and infused the cocaine solution into the catheter for 5s. After each active lever press and subsequent cocaine infusion, there was a 20s timeout period. Any active lever press that occurred after an infusion during this 20s timeout period had no scheduled consequence. In addition, there was no scheduled consequence for inactive lever presses. The cocaine dose, infusion rate, and timeout period remained constant in all self-administration sessions.

An FR5 schedule followed the previously described FR1 schedule. The criterion to increase from FR1 to a FR5 was that rats self-administer cocaine for  $\geq$ 1 week and with  $\geq$ 10 infusions/120-min session (Bongiovanni 2008, Fuchs 2007) for the last 2 days of FR1 training. After  $\geq$  2 weeks of cocaine SA acquisition and  $\geq$  3 days of consistent operant responding at the set schedule (i.e., an average of  $\geq$ 10 infusions/FR5 session) rats were assigned to an extinction treatment group. Once rats maintained stable and high levels of FR5 self-administration for at least 10 days, inactive and active lever presses were

averaged over the last 5 days of FR5 self-administration (FR5-M, R966 N = 10 or VEH N = 11) to create two balanced treatment groups prior to drug administration.

#### Initial and Early Extinction

All extinction sessions, in which lever presses had no programmed consequences, were 2h in duration. Rats received R966 or VEH 20-min prior to the first extinction session. Subsequent extinction sessions occurred until active lever responding had consistently decreased ( $\leq$  25 active lever presses/session for  $\geq$  2 days, with no significant difference between treatment groups). Extinction prior to reinstatement consisted of two sub-phases: 1) *Initial Extinction:* Initial extinction consisted of the first session (E1) that occurred during drug treatment and the one that followed 24 hours after treatment (E2), and 2) *Early Extinction:* Early extinction consisted of the remaining sessions (E3-E7) that occurred prior to reinstatement testing.

#### Reinstatement (R) and Late Extinction

After early extinction, rats began reinstatement testing with intervening extinction sessions (Late Extinction, described below). Reinstatement testing consisted of three sub-phases: 1) *Context-induced reinstatement (CTX-R):* Rats were placed into a novel context (Context B) compared to their original context (Context A). The two contexts were counterbalanced between Contexts 1 and 2. *2) Cue-induced reinstatement (CUE-R)*: Rats were placed original drug-taking context (Context A) and reintroduced to the conditioned reinforcer (5s cue-light presentation) with each active lever press. 3) *Cocaine-primed reinstatement* 

*(COC-R):* Rats were administered a priming injection of cocaine (10mg/kg, 1ml/kg) prior to the test prior to placement in their original drug-taking context (Context A).

The late extinction sessions that followed reinstatement consisted of two sub-phases: 1) Post-CTX-R Extinction, and 2) Post-CUE-R Extinction. Sessions were identical to previous extinction and served to return responding to equally low levels in treatment groups before potential differences emerged in subsequent reinstatement. This pattern of reinstatement and intervening late extinction was designed to test the effect of R966 treatment on reactivity of drugseeking behavior, such that reinstatement with the least amount of expected responses would be tested first (based on pilot data and previous studies on context-induced renewal (Crombag et al., 2002; reviewed in Crombag & Shaham, 2002; Fuchs et al., 2005) and the highest would be tested last (cocaineprimed). Similar methods have been used before (Berglind et al., 2007; Castino et al., 2015; Fuchs et al., 2005, 1998; Venniro, Caprioli, & Shaham, 2015).

**Data Collection and Statistical Analysis.** Behavioral data were collected with standard MED-PC power, control, and interface equipment, MED-PC IV control and data collection software, and MP2XL data transfer utility software. Once in Excel, the raw data were organized and the effects on lever pressing by drug treatment, session, and lever type were statistically assessed. Statistical software (SPSS v22) was used to make between subject comparisons with one and two-way analysis of variance (ANOVA) and within subject comparisons with repeated measure ANOVA, followed by post-hoc tests (Bonferroni corrected if

not denoted and when applicable, with a p value set at 0.05) and t-tests to determine the direction of effects. Any changes in the total N (due to equipment or procedural complications with individual rats over experimental sessions) were noted in results and not reintroduced back into the analysis.

#### **Results and Conclusions**

#### Acquisition and Maintenance (M)

Over the initial days of self-administration acquisition, rats began pressing the active lever more than the inactive lever and received reinforcing cocaine infusions on an FR1 and then FR5 schedule. Figure 14 shows the average lever pressing during the final maintenance sessions in treatment groups (N=10 R, N=11 V). A 2 (drug) x 2 (lever) ANOVA revealed a reliable main effect of lever (F (1, 19)=334.01, p<0.001), but no effect of subsequent drug treatment and no interaction (ps>0.5, Figure 14).

#### Initial and Early Extinction

**Initial Extinction**. On Extinction Session 1 (E1) rats received a VEH or R966 injection 20-min before the session began (Figure 15A). To determine how fast these initial drug effects occurred, data were compared at each thirty min interval of extinction Sessions 1 and 2. Figure 15A shows responding in 30-min blocks, as well as the total responding over the 2-hr session. When lever pressing was compared between drug groups in 30-min bins, there were reliable main effects of lever (F=34.7, p<0.001) and bin (F (1.6, 29.5)=22.6, p<0.001)), as well as a reliable interaction between bin and lever (F (1.6, 29.5)=16.0, p<0.001)).

Figure 14.



Self-administration (FR5-Maintenance, no drug TX)

Figure 14. Chapter 4 – Experiment 1. FR5 maintenance of cocaine selfadministration. Average ( $\pm$ SEM) inactive (not reinforced) and active (reinforced) lever presses during final fixed ratio (FR5) maintenance self-administration (120min duration). Drug treatment group abbreviations are based on subsequent systemic drug injections of Vehicle (VEH, N = 11) or RGFP966 (R966, N = 10) prior to the first extinction session. Error bars indicate the standard error of the mean ( $\pm$ SEM).





Extinction Session 1 (30 min bins and totals)

Figure 15B.



Extinction Session 2 (30 min bins and totals)

Figure 15. Chapter 4 – Experiment 1. Initial extinction after FR5 selfadministration (Pre-Extinction 1 Treatment). Average ( $\pm$ SEM) inactive (not reinforced, triangles) and active (reinforced, circles) lever presses during extinction (shown in 30-min bins and the sum of bins in 120-min total duration). Drug treatment (Tx) group abbreviations are based on systemic (SC) drug injections of Vehicle (VEH, N = 9) or RGFP966 (R966, histone deacetylase 3 inhibitor, N = 9) 20 min prior to the first extinction session (E1). Error bars indicate the standard error of the mean ( $\pm$ SEM).

- (A) Extinction Session 1 (E1) lever pressing after injection (red arrow) of Vehicle or RGFP966, Fig 15A.
- (B) Extinction Session 2 (E2) lever pressing (no drug Tx). Note. \*Drug effect; p < 0.05. Please see Results and Conclusions section (Initial Extinction) for a description of statistical findings, Fig 15B.</p>

This interaction occurred only within the first hour of extinction (between Bins 1 and 2, p = 0.003, and Bins 2 and 3 p = 0.03), when the largest decrease in active lever pressing occurred. Importantly, there was no main effect or an interaction with drug group (Fs < 1.75, ps > 0.2; ANOVA: 2 (drug treatments) x 2 (levers-repeated) x 4 (bins-repeated) during Extinction 1 (Figure 15A).

Figure 15B shows responding during Extinction 2 (E2) in 30-min blocks, as well as the total responding over the 2-hr session. A drug x lever x bin ANOVA revealed reliable main effects of lever and bin, as well as a reliable lever x bin interaction (Fs > 4.92, ps < 0.015). There also was a reliable main effect of drug (F (1, 19) = 9.245, p < 0.007), as well as reliable interactions between lever and drug (F (1, 19) = 11.049, p < 0.004), drug x bin (Fs (3, 57) = 3.76, p < 0.016) and drug x bin x lever (F (3, 57) = 3.15, p < 0.032). Follow-up tests by bin determined that a reliable difference existed between drug groups in Bin 1 (0-30 min, active lever, t (19) = 2.744, p = 0.013, Figure 15B).

**Early Extinction.** Figure 16 shows responding that occurred during Extinction Sessions 3-7 (N = 9 R, N = 9 V). A 2 (drug) x 2 (levers) x 5 (sessions) ANOVA revealed reliable main effects of lever (F (1, 16) = 24.783, p < 0.001) and drug treatment (F (1, 16) = 6.456, p = 0.022), as well as reliable interactions of session x treatment (F (4, 64) = 4.143, p = 0.005), and session x treatment x lever (F (4, 64) = 4.518, p = .003). Follow-up tests for each of these early extinction sessions determined that the main effect of lever was reliable on each extinction session (Fs > 4.850, ps < 0.05), suggesting that extinction was not complete by the seventh extinction session.





**Figure 16. Chapter 4 – Experiment 1. Early extinction after FR5 selfadministration.** Average (±SEM) inactive (not reinforced, triangles) and active (reinforced, circles) lever presses during early extinction sessions (E3-E7, 120min duration) and prior to reinstatement. Drug treatment group abbreviations are based on systemic drug injections of Vehicle (VEH, N = 9) or RGFP966 (R966, N = 9) prior to the first extinction session (E1). Error bars indicate the standard error of the mean (±SEM). Note. \*Drug effect in active lever. Please see Results and Conclusions section (Early Extinction) for a description of statistical findings.

Reliable main effects of drug occurred in the active lever during extinction Sessions 3 and 4 (Fs > 7.656, ps < 0.014) with a trend at Session 6 (F (1, 16) = 3.877, p = 0.067) and no effects in E5 (p = 0.099) or E7 (p = 0.158). There were no drug effects in the inactive lever (Fs < 0.911, ps > 0.354, Figure 16). *Reinstatement and Late extinction* 

As seen in Figure 17 (N = 9 V, N = 9 R) extinction sessions 8, 10, and 12 served as the lowest baseline measure achieved by rats to compare to subsequent context, cue, and cocaine-induced reinstatement tests, respectively (i.e., E8 to CTX-R, E10 to CUE-R, and E12 to COC-R).

**Context-Induced Reinstatement (CTX-R).** After early extinction (E3-11), rats received one additional extinction session prior to context-induced reinstatement. Responding during this final baseline extinction session (E8) and context-induced reinstatement (CTX) is shown in Figure 17. Between Extinction 8 and context-induced reinstatement, a repeated measures ANOVA demonstrated a reliable main effect of session (F (1, 16) = 16.862, p = 0.001), lever (F (1, 16) = 9.379, p = 0.007), and an interaction between session and drug group (F (1, 16) = 17.428 p = 0.015). Follow-up tests for each session determined that no reliable main effects or interactions occurred during Extinction 8 (main effect of drug p = 0.63, main effect of lever p = 0.16, and lever x drug interaction p = 0.6) yet when rats were tested in a novel context (CTX-R), a reliable main effect of drug (F (1,16) = 8.204, p = 0.011) and lever (F (1, 16) = 7.313, p = 0.016) occurred, with no lever x drug interaction (p = 0.2).





**Extinction-Reinstatment Sessions** 

Figure 17. Chapter 4 – Experiment 1. Extinction-Reinstatement sessions after FR5 cocaine self-administration and extinction (E). Average ( $\pm$ SEM) inactive (not reinforced, triangles) and active (reinforced, circles) lever presses during baseline extinction session and context (CTX, in Context B), cue (CUE, in Context A), and cocaine (COC, in Context A)-primed reinstatement (120-min duration). Drug treatment group abbreviations are based on systemic drug injections of Vehicle (VEH, N = 9) or RGFP966 (R966, N = 9) prior to the first extinction session (E1). Error bars indicate the standard error of the mean ( $\pm$ SEM). Note. \*Drug effect, # Session x Drug effect in active lever, ^Drug x lever effect; p < 0.05. Please see Results and Conclusions section (Reinstatement and Late Extinction) for a description of statistical findings. Therefore, both drug groups pressed the active lever more than the inactive lever, but the R966 group pressed the levers less than the VEH group. Additionally, when looking at results by lever type, a session x drug interaction occurred for the active lever responses from E8 to CTX-R (F (1, 16) = 4.993, p = 0.040), but not for the inactive lever responses (p = 0.303). This effect can be seen in Figure 17, as active lever presses increased in the VEH group, while there was no corresponding increase in the R966 rats.

Cue-Induced Reinstatement (CUE-R). Two additional extinction sessions occurred between context- and cue-induced reinstatement. Figure 17 shows responding during the last extinction sessions (E10) and the cue-induced reinstatement session (CUE). A drug (R966 or VEH) x session (E10 or cueinduced reinstatement) x lever (active or inactive) ANOVA revealed reliable main effects of session (F (1,16) = 58.260, p < 0.001), lever (F (1,16) = 86.408, p < 0.001) (0.001), and interactions between session and drug (F (1,16) = 5.672, p = (0.030), lever and drug (F (1,16) = 5.457, p = 0.033), and session and lever (F (1,16)=75.006, p < 0.001). Follow-up tests by session determined that no main effects or interactions occurred during Extinction 10 (drug factor p = 0.935, lever factor p = 0.961, and lever x drug interaction p = 0.524) but during cue-induced reinstatement a reliable main effect of lever (F (1,16) = 88.882, p = 0.000) and an interaction of lever x drug was observed (F (1,16) = 4.668, p = 0.046), with a trend of main a drug effect (p = 0.055). One way ANOVAs for each type of lever demonstrated a significant interaction between session and drug group on active (F(1, 16) = 5.007, p = 0.04) but not inactive responses (p = 0.115). As a result of

cue presentation, drug-seeking increased in both drug treatment groups, as measured by increased active lever pressing, but the group given R966 just prior to their first extinction session (11 days earlier) pressed the active lever reliably less than the VEH group (Figure 17).

**Cocaine-Induced Reinstatement (COC-R).** Extinction occurred again for two more days (E11 and E12) before rats re-established low extinction criterion and cocaine-induced reinstatement was tested. Reliable main effects of session (F (1, 16) =9.131, p < 0.008), lever (F (1,160= 10.545, p < 0.005), and a session x lever interaction (F (1, 16) =7.853, p < 0.013) was demonstrated, but no effects of drug group occurred as seen in Figure17. Therefore, drug-seeking increased in both drug treatment groups, as measured by increased active lever pressing during cocaine-primed reinstatement, yet there was no difference in responding between rats given R966 or VEH prior to their first extinction session (Figure 17).

Late Extinction. Late extinction (Figure 18) comparisons captured any influence of the previous drug treatment that remained following initial extinction (24h effect at E2), early extinction (E3-E7), and reactivation during reinstatement (e.g., CTX, CUE, and COC-R). There was no effect of drug treatment on late extinction (after reinstatement began), confirmed with a repeated measure ANOVA comparing extinction sessions 9 through 12 (Figure 18). There were main effects of session (F (3, 48) = 15.111, p < 0.001) and lever (F (3, 16) = 14.563, p < 0.002). There was also an interaction between the session and lever (F (3, 48) =7.299, p = < 0.001).





Figure 18. Chapter 4 – Experiment 1. Late extinction (E) sessions

**intervening reinstatement.** Average ( $\pm$ SEM) inactive (not reinforced, triangles) and active (reinforced, circles) lever presses during late extinction session (120-min duration) that followed context reinstatement (before extinction session E9) and cue reinstatement (before extinction session E11). Drug treatment group abbreviations are based on systemic drug injections of Vehicle (VEH, N = 9) or RGFP966 (R966, N = 9) prior to the first extinction session (E1). Error bars indicate the standard error of the mean ( $\pm$ SEM).

However, there was no difference between drug groups (ps > 0.481, ANOVA: 2 drug treatment x 2 levers-repeated x 4 sessions-repeated, Figure18). Follow-up tests by session determined that the active lever was pressed more than the inactive lever in sessions E11 (t (17) = 3.773, p < 0.002) and E12 (t(17) = 3.494, p < 0.003), and that active presses decreased reliably from Sessions 10 to 11 (F (1,417) = 48.554, p = 0.001) and 11 to 12 (F (1,17) = 21.608, p = 0.001), with no reliable differences over session in the inactive lever. Results demonstrate that rats decreased drug-seeking in the earlier sessions of extinction if given R966 before extinction compared to rats given a VEH injection but that this extinction effect did not persist during later extinction following reinstatement (E9-12, Figure 18).

In summary (Figures 14-18), one injection (SC) of R966 treatment before the first extinction session following FR5 self-administration, led to a reliable reduction in drug-seeking compared to VEH treatment during initial extinction (E2) following administration (E1), during subsequent early extinction (E3 and 4), and during context (CTX-R) and cued reinstatement (CUE-R) tests. However, there was no effect of this one-time treatment of R966 on later extinction that followed reinstatement (E9-12) or during cocaine-primed reinstatement (COC-R).

# Experiment 2: RGFP966 treatment before FR5 self-administration does not reduce drug-seeking

Experiment 1 found that a single injection led to a rapid loss of responding that persisted during early extinction and context- and cue-induced

reinstatement. In Experiment 2, it was determined whether RGFP966 led to

performance impairments that may disrupt lever pressing to respond for cocaine.

#### Methods

Unless noted otherwise, Experiment 2 methods were identical to Experiment 1.

**Animals.** Male Long-Evans rats (N = 7) were used for Experiment 2.

Drugs. RGFP966 (R966) was administered with an injection (2ml/kg vol,

SC) at a dose of 10 mg/kg 20-min before cocaine self-administration

maintenance sessions.

**Self-administration Protocol.** Experiment 2 consisted of Acquisition and Maintenance (M) sessions (see multi-experimental timeline, Figure 13, Experiment 2).

#### Acquisition and Maintenance (M)

All acquisition criteria were identical to Experiment 1. After reaching criterion, all rats received injections of R966 or VEH 20-min prior to FR5 self-administration maintenance sessions on alternating days. Inactive and active lever pressing was compared over sessions (Figure 19).

#### **Results and Conclusions**

#### Acquisition and maintenance (M)

Figure 19 shows responding during the final sessions of maintenance (FR5-M) and the sessions with RGFP966 (R966) or Vehicle (VEH) treatment in Experiment 2. A drug x lever x session ANOVA revealed only a reliable main effect of lever (F (1, 6) =74.640, p =<0.00); all other main effects and interactions were not reliable (Fs  $\leq$  0.554, ps > 0.697).





Figure 19. Chapter 4 – Experiment 2. Drug treatment before fixed ratio maintenance (FR5-M) of cocaine self-administration. Average ( $\pm$ SEM) active (A, reinforced) and inactive (IA, not reinforced) lever presses during final fixed ratio (FR5) maintenance self-administration (120-min duration). Drug treatment (Tx, N = 7) group abbreviations are based on systemic drug injections (SC, one injection per day, red arrows) of Vehicle (VEH) or RGFP966 (R966, histone deacetylase 3 inhibitor) prior to each cocaine self-administration session (Maintenance with no drug treatment  $\rightarrow$  Vehicle  $\rightarrow$  RGFP966  $\rightarrow$  Vehicle  $\rightarrow$ RGFP966). Error bars indicate the standard error of the mean ( $\pm$ SEM). Follow-up tests by lever for each session revealed that active lever presses were significantly higher than inactive levers (ts (6)  $\leq$  6.444, ps  $\leq$  0.001). Results here demonstrate that neither VEH nor R966 induced a deviation in operant responses during FR5 self-administration, suggesting that neither VEH nor R966 drug treatment creates a performance deficit or general motor impairment and that rats were still motivated to self-administer cocaine (Figure 19).

# Experiment 3: RGFP966 treatment does not reduce motivation for drugseeking but enhances late extinction

In Experiment 2, RGFP966 did not alter cocaine self-administration behavior under an FR5 schedule of reinforcement. In Experiment 3, there were to goals: (1) to further evaluate the effects of RGFP966 on motivation using the standard progressive ratio (PR) task and (2) to examine whether post-extinction treatment of RGFP966 would enhance extinction and weaken reinstatement.

#### Methods

Unless noted otherwise, Experiment 3 was conducted similar to Experiments 1 and 2.

**Animals.** Rats (N = 23) from Experiments 1 and 2 were regrouped and balanced for treatment and behavior history for Experiment 3 (Phases 2 and 3, see Figure 13). Separate ANOVAs were completed to test if active and inactive lever responses were different in Experiment 3 based on previous drug or behavioral history in Experiment 1 or 2, and no effects were found (ps > 0.05).

**Drugs.** Vehicle (VEH) or RGFP966 (R966, 2ml/kg vol, 10 mg/kg) was administered to rats (SC) as previously.

Self-administration Protocol. A schematic of the experimental timeline and summary of treatment for each experiment is in Figure 13 (see Experiment 3). Similar to Experiment 1, Experiment 3 consisted of three primary Phases: 1) Acquisition and maintenance, 2) Extinction before reinstatement and, 3) Reinstatement with intervening extinction. All rats received drug treatment 20-min prior to a PR self-administration session on alternating days (Figure20), were assigned to three balanced groups, and given VEH or R966 immediately after the first extinction session. Inactive and active lever presses were compared over each Phase.

#### Acquisition and Maintenance (M)

Initial FR1 and FR5 training occurred similar to Experiments 1 and 2, followed by two FR10 training sessions and then rats began the progressive ratio (PR) schedule in a 180-min session for 12 sessions prior to VEH and R966 treatment. During the PR schedule, an increasing number of lever presses was required to deliver each cocaine infusion (e.g., first infusion required 2 active lever presses, second infusion required 4 active lever presses, third infusion required 8 active lever presses, etc.) using a standard response ratio equation (Richardson & Roberts, 1996; with j = 0.32). This ratio was chosen to escalate the required active lever presses fast enough to reach a breaking point (when rats fail to receive a drug infusion within a 1 hour period, Richardson & Roberts, 1996) within a 3 hour session. The cocaine dose and the presentation of the cue light above the active lever were as in Experiments 1 and 2.

Once rats (N = 23) maintained stable and high levels of PR selfadministration (after  $\geq$  2 weeks of cocaine SA acquisition and  $\geq$  3 days of consistent operant responding at the set schedule (i.e., an average of  $\geq$ 5 infusions/PR session) the response number (inactive and active lever presses) was averaged over the last 5 days of PR self-administration to create a selfadministration maintenance level (PR-M) to compare to subsequent drug treatment. Following maintenance of PR self-administration levels (PR-M), VEH drug treatment was administered 20-min prior to a PR self-administration session (with reinforcement). To determine if R966 had an effect on the motivation for cocaine reinforcement, this pattern of drug treatment 20-min prior to selfadministration was repeated again but with the R966 drug administered for one day followed by another maintenance PR session (with no drug administered) on the following day (comparing across sessions: PR-M, VEH, R966, and the final PR maintenance session with no injection).

#### Initial and Early Extinction

Following the tests of R966 on PR responding, rats remained in their home cage for 27 days. After this, the effects of R966 administered immediately after the first extinction session (E1) were determined. This time in the home cage served two purposes: (1) to allow ample time to washout R966 from the system, and (2) to introduce a forced abstinence period that may increase responding for cocaine (Berglind et al., 2007; Gabriele, Pacchioni, & See, 2012; Kuntz-Melcavage, Brucklacher, Grigson, Freeman, & Vrana, 2009; Neisewander et al., 2000; Weiss et al., 2001). Rats were assigned to one of three subsequent treatment groups that were matched for their responding during the last PR session (PR self-administration maintenance session with no drug treatment (last session of PR self-administration). These subsequent groups received different drug and extinction treatments: (1) 120-min of extinction, followed by a vehicle injection (VEH 120 N = 8), (2) 30-mins of extinction, followed immediately by a vehicle injection (VEH 30 N = 8), (3) 30-min of extinction, followed immediately by a R966 injection (R966 30 N = 7, see Figures 20 and 21).

Our hypothesis was that the group that received 30-min of extinction and a vehicle injection would have the slowest rate of extinction, achieve the least amount of extinction, or have the greatest amount of reinstatement compared to the 120-min group with a vehicle injection and the 30-min group with an HDAC3 inhibitor. The two extinction durations were used to determine if the effects of the HDAC3 inhibitor could turn a weak behavioral extinction experience (30-min) into a strong behavioral extinction experience (120-min), as our lab has found with HDAC inhibitors (HDACi) and session duration effects in extinction of fear (Stafford, Raybuck, Ryabinin, & Lattal, 2012). All other extinction sessions were identical to previous sessions (120-min in duration).

#### **Results and Conclusions**

#### Acquisition and maintenance of PR self-administration

The effect of drug administration was investigated over 4 PR sessions, on active and inactive lever responses during the PR maintenance (PR-M, N = 23) (Figure 20). A main effect of lever (F (1, 22) = 25.776, p< 0.001) occurred, but there was no reliable effect of session and no interaction between lever and drug

treatment (Fs < 0.445, ps > 0.654, ANOVA: 2 levers-repeated x 4 sessionsrepeated). Paired follow-up tests at each session revealed that the active lever was pressed significantly more than inactive lever (ts > 5.326, ps < 0.001), with no difference between sessions.

The number of active lever presses required to receive the last infusion was determined with no effect of session (F (3, 66) = 0.408, p = 0.748, data not shown). It was also determined that there were no differences in inactive or active responses over the four days of testing (PR-M, Vehicle, R966, PR-M) in Phase 1 of Experiment 3, based on prior drug or experimental histories (ANOVA: 4(session-repeated) x 2 (lever-repeated) x 3 (History); Fs < 1.7, ps > 0.14). Results here demonstrate that neither current drug treatment (vehicle or R966), nor experimental history of drug altered motivation to respond on the inactive or active lever during a progressive ratio schedule of reinforcement in Experiment 3.

Figure 21 shows responding for cocaine self-administration in subsequent extinction and drug treatment groups (N = 8 VEH 120, N = 8 VEH 30, and N = 7 R966 30). There was a main effect of lever (F (1, 20) = 25.691, p < 0.001) with active lever presses greater than inactive (ps > 0.994) during this final PR session (no drug treatment) but no reliable effect of subsequent treatment (postextinction R966) or an interaction between subsequent treatment and lever.





Figure 20. Chapter 4 – Experiment 3. Drug treatment progressive ratio maintenance (PR-M) of cocaine self-administration. Average ( $\pm$ SEM) active (A, reinforced) and inactive (IA, not reinforced) lever presses during final PR maintenance cocaine self-administration (180-min duration) sessions. Drug treatment (Tx, N = 23) group abbreviations are based on drug injections (SC, one injection per day, red arrows) of Vehicle (VEH) or RGFP966 (R966, histone deacetylase 3 inhibitor) prior to each cocaine self-administration session (Maintenance with no drug treatment  $\rightarrow$  Vehicle  $\rightarrow$  RGFP966  $\rightarrow$  Maintenance with no drug treatment). Error bars indicate the standard error of the mean ( $\pm$ SEM).





Self-administration (PR-Maintenance, no drug TX)

**Figure 21. Chapter 4 – Experiment 3. Progressive ratio maintenance (PR-M) of cocaine self-administration.** Average (±SEM) active (reinforced) and inactive (not reinforced) lever presses during final PR maintenance cocaine selfadministration (180-min duration). Treatment abbreviations are based on subsequent drug injections of Vehicle before a long (120-min) extinction session (VEH, 120; N = 8), Vehicle before a short (30-min) extinction session (VEH, 30; N = 8) or RGFP966 before a short (30-min) extinction session (R966, 30; N = 7). Error bars indicate the standard error of the mean (±SEM).

#### Initial and Early Extinction

Initial Extinction. It was of interest to compare behavior during subsequent extinction and reinstatement (Figures 22-25) between groups of rats that had the vehicle administered 30-min after Extinction 1 to those that had R966 instead. It was also of interest to determine if shorter extinction (30-min) with an HDAC3i (R966) looked similar to longer extinction (120-min) without HDAC3i treatment.

It was not logical compare total lever presses between extinction groups during Sessions 1 and 2, as the 120-min vehicle group had much longer in the chamber than the two 30-min groups for E1 (N = 8 VEH 120, N = 8 VEH 30, or N = 7 R966 30, Figure 22A). Therefore, the initial 30-min of responses were compared during Extinction 1 to verify that groups were similar before drug treatment. On Extinction Session 1, there were no differences in the first 30-min between subsequent treatment groups F (2, 20) = 0.615, p = 0.550), due to our allocation of rats to each treatment group based on previous self-administration behavior, nor was there an interaction with lever presses (F (2, 20) = 1, p =0.386). There was however a main effect of lever (F (1, 20) = 77.522, p < 0.001), with all groups pressing the active more than the inactive lever during this initial extinction session (Figure 22A). Overall, groups responded similarly in the first 30-min of Extinction 1, prior to treatment. The 120-min group showed withinsession extinction during the four 30-min bins, with main effects of lever F (1, 7) =22.912, p =.002) and bin (F (1.898, 13.288) = 27.459, p < 0.001), and an interaction of lever x bin (F (1.280, 8.962) = 7.968, p = 0.016).

Figure 22A.



Figure 22B.



Figure 22. Chapter 4 – Experiment 3. Initial extinction after PR selfadministration (Post-Extinction 1 Treatment). Average ( $\pm$ SEM) inactive (IA, not reinforced, triangles) and active (A, reinforced, circles) lever presses during extinction (data shown in 30-min bins and the sum of bins in 120-min total duration). Treatment group abbreviations are based on extinction duration during Session 1 (120 or 30 minutes) and drug injection of Vehicle (VEH, 120 N = 8 or VEH, 30 N = 8) or RGFP966 (R966, 30 N = 7) immediately after the first extinction session (E1). Error bars indicate the standard error of the mean ( $\pm$ SEM).

- (A) Extinction 1 (E1) lever presses during 120- or 30-min session, followed immediately by and injection (red arrow) of Vehicle (VEH, 120 or VEH, 30) or RGFP966 (R966, 30), Fig 22A.
- (B) Extinction Session 2 (E2, 120-min) lever pressing (no drug Tx), Fig 22B.

During Extinction Session 2 (Figure 22B), all groups responded at similar levels, with a reliable main effect of lever (F (1, 20) =68.954, p < 0.001), bin (F (1.321, 26.416) =50.576, p < 0.001), and an interaction between bin and lever (F (1.378, 27.555) =44.161, p = 0.001). There was no effect of treatment, but a near trend for an interaction between lever and treatment (Fs < 2.617, ps > 0.098) during the entire 120-min of Extinction 2. As seen in Figure 22B, follow-up tests determined that the active lever pressing was significantly different between bins in the first and second bin (p < 0.001), with less of a difference between inactive and active presses in the other bins, as all rats responded less in the later part of the E2 session. As in E1, during the first 30-min of E2 there were main effects of lever (F (1, 20) =64.124, p < 0.001) but no effect of treatment or an interaction (Fs <1.213, ps > 0.235, Figure 22B).

**Early Extinction.** After initial extinction in the first 24 hrs (initial encoding and consolidation effects), the early extinction that followed was compared from Sessions 3 to 11 (Figure 23) between treatment groups (Figure 23, N = 7 VEH 120, N = 8 VEH 30, or N = 6 R966 30) and before reinstatement began. There was a main effect of lever (F (1, 18) = 76.603, p < 0.001) with active pressed more than inactive, and a main effect of session (F (3.714, 66.846) = 14.197, p < 0.001) with active presses decreased over sessions, as noted by the interaction (F (4.475, 80.546) = 10.084, p < 0.001). Therefore, there were not effects of drug treatment or extinction duration on responding in early extinction (Fs > 0.964, ps > 0.499). Additional sessions were conducted after this to determine whether drug treatment after E1 influenced reinstatement and later extinction.

Figures 23.



Figure 23. Chapter 4 – Experiment 3. Early extinction after PR selfadministration. Average ( $\pm$ SEM) inactive (IA, not reinforced, triangles) and active (A, reinforced, circles) lever presses during early extinction sessions (E3-E11, 120-min duration) and prior to reinstatement. Treatment group abbreviations are based on Extinction 1 (E1) duration (120- or 30-min) and the following drug injection of Vehicle (VEH, 120; N = 7 or VEH, 30; N = 8) or RGFP966 (R966, 30 N = 6). Error bars indicate the standard error of the mean ( $\pm$ SEM).

#### Reinstatement after PR self-administration

As seen in Figure 24 Extinction 12, 16, and 23 served as the lowest baseline measure achieved by rats to compare to subsequent context-, cue-, and cocaine-induced reinstatement tests, respectively (i.e., E12 to CTX-R, E16 to CUE-R, and E23 to COC-R).

**Context-induced Reinstatement (CTX-R).** Extinction Session 12 served as a low baseline for all groups, with no differences between groups (ANOVA: 3 treatments x 2 levers-repeated, main effect of lever p = 0.000, treatment p = 0.910, lever x treatment p = 0.852), and was therefore the last extinction session (in Context A) before context reinstatement testing (in Context B). During CTX testing all rats increased their active lever pressing during the 2 hour session with no effect of treatment. A 3 (treatment) x 2 (lever-repeated) x 2 (session-repeated) ANOVA confirmed main effects of session (F (1, 18) = 14.137, p = 0.001), lever ((F (1, 18) = 32.9, p < 0.001), but this interaction and all treatment effects (main and interactive) were not reliable (Fs < 1.172, ps >.293). While R966-treated rats demonstrated an ordinal decrease in responding compared to vehicle-treated, there was not a reliable treatment effect over the full session or in the first 30-min (ps > 0.193 for main and interactive treatment effects, Figure 24).

**Cue-induced Reinstatement (CUE-R).** After CTX-R, more extinction was completed (in Context A) to bring responses to a low level again, and by E16 active presses were again at a low baseline, with no differences between groups (ANOVA: 3 treatments x 2 levers-repeated, main effect of lever p < 0.001, treatment p = 0.441, lever x treatment p = 0.984).





**Extinction-Reinstatment Sessions** 

Figure 24. Chapter 4 – Experiment 1. Extinction-Reinstatement sessions after FR5 cocaine self-administration and extinction (E). Average ( $\pm$ SEM) inactive (IA, not reinforced, triangles) and active (A, reinforced, circles) lever presses during baseline extinction session and context (CTX, in Context B), cue (CUE, in Context A), and cocaine (COC, in Context A)-primed reinstatement (120-min duration). Treatment group abbreviations are based on Extinction 1 (E1) duration (120- or 30-min) and the following drug injection of Vehicle (VEH, 120 N = 7 or VEH, 30 N = 8) or RGFP966 (R966, 30 N = 6). Error bars indicate the standard error of the mean ( $\pm$ SEM). The next day rats were placed into the same context (Context A) and when they pressed the active lever, the cue light that was previously illuminated prior to each cocaine infusion during cocaine self-administration was lit with each active lever during CUE-R (Figure 24). All rats increased their active lever pressing during the 2 hour CUE-R session, with no effect of treatment. There were reliable main effects of session ((F (1, 18) = 29.478, p < 0.001), lever ((F (1, 18) = 34.297, p < 0.001), and an interaction between the two ((F (1, 18) = 29.900, p < 0.001), but all treatment effects (main and interactive) were not reliable (Fs <0 .791, ps > 0.469).

**Cocaine-induced Reinstatement (COC-R).** After CUE-R, more extinction was needed (in Context A) to bring responses to a low level again before COC-R (Figure 24). After 7 additional extinction sessions (E17-E23), the difference between groups was becoming larger rather than smaller, so testing began for the large expected increase in responding with a cocaine primed injection (COC-R). Although, with treatment groups significantly different on E23 (ANOVA: 3 treatments x 2 levers-repeated, main effect of lever p < 0.001, treatment p = 0.043, lever x treatment p = 0.099), it could be more difficult to compare the amount of change in responses between groups from one session to the other (E23 to COC). However, all groups increased their responses as expected and demonstrated COC-primed reinstatement (main effect of session: F (1, 18) = 15.427, p = 0.001, lever: F (1, 18) = 22.874, p < 0.001, and interaction of session x lever effect: F (1, 18) = 13.156, p = 0.002), with no effect of prior extinction or drug treatment (ps > 0.560). Therefore, one post-extinction (30-min session)

injection of R966 did not alter context-, cue-, or cocaine-induced reinstatement, as measured by inactive and active lever presses compared to a post-extinction (30-min or 120-min session) injection of vehicle (Figure 24). This is in contrast to the extinction that occurred between reinstatement sessions (E13-23, see below).

Late Extinction. Late extinction (E13-E23, Figures 25A and B) was compared to determine if treatment after E1 subsequently influenced the sensitivity to stimuli and the associated responses that followed reinstatement of drug-seeking. A repeated measures analysis was completed over late extinction (ANOVA: 11 (session-repeated) x 2 (lever-repeated) x 3 (treatments)). There was a main effect of lever (F (1, 18) = 74.541, p < 0.001) with active pressed more than inactive, and a main effect of session (F (4.513, 81.225) = 18.445, p < 0.001), such that active presses decreased over extinction sessions, as noted by the interaction (F (4.374, 78.737) = 10.914, p < 0.001). However, treatment at E1 had a reliable main effect on these later extinction sessions (F (2, 18) = 4.229, p = 0.031), one that interacted with the effect of lever (F (2, 18) = 3.874, p = 0.04), and as a three way interaction with lever and session (F (20,180) = 1.756, p = 0.029). This differed from effects in Experiment 1 (Figure 18), with the greatest extinction effects in initial and early extinction rather than late extinction.

Before doing additional follow-up tests, comparisons were made to determine if differences existed in inactive or active responses over the 11 days of late extinction in Experiment 3 based on prior drug or experimental histories.





Figure 25B.



## Figure 25. Chapter 4 – Experiment 3. Late extinction (E) sessions

**intervening reinstatement.** Average ( $\pm$ SEM) inactive (IA, not reinforced, triangles) and active (A, reinforced, circles) lever presses during late extinction session (120-min duration. Treatment group abbreviations are based on Extinction 1 (E1) duration (120- or 30-min) and the following drug injection of Vehicle (VEH, 120 N = 7 or VEH, 30 N = 8) or RGFP966 (R966, 30 N = 6). Error bars indicate the standard error of the mean ( $\pm$ SEM). Please see Results and Conclusions section (Reinstatement and Late Extinction) for a description of statistical findings.

- (A) Lever presses after context-induced reinstatement (CTX-R, in Context B), Fig 25A.
- (B) Lever presses after cue-induced reinstatement (CUE-R, in Context A). Note.
  \*Drug effect between 30-min extinction groups (R966, 30 and VEH, 30) in the active lever, p < 0.05, Fig 25B.</li>
There was no effect of history (main effect of treatment history p = .859, treatment history x lever p = .698, treatment history x session p = .102, treatment history x session x lever p = .308), only the effect of session and lever (described above). To follow-up the treatment effect noted in the repeated measures ANOVA during late extinction, a Bonferroni corrected post hoc test determined a reliable difference between VEH 30 and R 30 groups (p = 0.028) but not the VEH 120 (ps > 0.484) group over sessions E13-23 with both levers included. A follow-up test by lever (comparing all three groups) over sessions E13-23 determined that the active (p = 0.026) but not the inactive lever (p = 0.329) was different between groups. This effect was significant between R966 30 and VEH 30 again (p =(0.023) but not the VEH 120 (ps > 0.391) group. With lever and treatment group differences over many sessions, two separate repeated measures were completed for each set of late extinction sessions. One was completed after context reinstatement (E13-15, see Figure 25A) until criterion was reached at E16, and one after cue reinstatement (E17-23, see Figure 25B) until COC-primed reinstatement (criterion for the vehicle group was not reached after multiple days).

Between sessions E13 and E15 (Figure 25A), there were main effects of session (p = 0.000) and treatment (p = 0.044) on active lever presses, with a near effect between the R966 30 and the VEH 30 groups (p = 0.066) compared to the VEH 120 group (ps > 0.152). There was also a main effect of inactive presses over sessions (p = 0.013), but with no effect of treatment (p > 0.366). There was no interaction of session by treatment group between E13-15 for

active or inactive lever presses (ps >.476), suggesting that differences in active lever presses by treatment emerged by E13 and remained similar until E15. Oneway comparisons for each session determined that there was no treatment effect at E13 (p = 0.47), a trend at E14 (p = 0.082) on active responses (as the R966 group continued to extinguish to baseline levels), and no effects on E15 (no differences in responses, Figure 25A).

After a baseline extinction session (E16) and a CUE test (Figure 24) lever pressing was compared from E17-23 (Figure 25B). In the active lever there was main effects of session (p < 0.001) and treatment (p = 0.031), and a near interaction of treatment x session (p = 0.06) in active responses. In the inactive lever, there was an effect of session (p = 0.001), but no treatment effect (p =(0.450), or an interaction between session and treatment (p = 0.886). To determine which sessions and groups differed, further testing revealed this effect was not led by the VEH 120 group (ps > 0.215) but by differences between the R966 30 and VEH 30 (p = 0.029) group, with trends for treatment differences after CUE-R at E17 and E18 (ps < 0.064), and significant treatment effects in active responses at session E19-E23 (0.007  $\leq$  ps  $\geq$  0.046), compared with oneway ANOVAs for each session). In general, it took more extinction sessions in this experiment (than in Experiment 1) for the vehicle group to reach the same low and equal levels of responding as the RGFP966 group, before reinstatement (E3-11, Figure 23), and reliably after reinstatement (E17-23, Figure 25B).

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## Discussion

The development and persistence of memory and addiction is thought to be regulated by histone posttranslational modifications (reviewed in Maze, Noh, & Allis, 2012). The experiments in this chapter demonstrated that a specific histone deacetylase inhibitor (HDAC3i, RGFP966) could promote extinction of operant responding for cocaine. This was true when the HDAC3 inhibitor was delivered 20-min before (Experiment 1) or immediately after (Experiment 3) an extinction session, though these extinction enhancements were revealed in different ways in the two experiments.

A pre-session injection of RGFP966 was determined to promote extinction, measured in terms of rate of extinction across days and persistence of extinction in response to context- and cue-induced reinstatement challenges (Experiment 1, Figures 15-17). These effects were evident on the day following HDAC3 inhibitor treatment. The extinction-enhancing effects appear to not be due to general performance or motivational effects, because RGFP966 did not alter stable responding on an FR5 schedule or progressive ratio responding (Phase 1 in Experiments 2 and 3, Figures 19 and 20, respectively).

In Phases 2 and 3 of Experiment 3, in which rats received an injection of the HDAC3 inhibitor or vehicle immediately after the first extinction session (E1), there were no effects on extinction the next day (E2), nor were there reliable differences during the reinstatement tests. However, in this experiment an extinction enhancement emerged during additional extinction sessions that followed reinstatement sessions (Figure 25B), again consistent with a persistent enhancement in extinction. Together, these results suggest that extinction can be promoted by HDAC3 inhibitors and emphasize the importance of assessing potential extinction enhancements using a variety of behavioral measures (with effects on early extinction, reinstatement, and late extinction).

Our experiments are the first to show that HDAC3 inhibition enhances extinction after drug self-administration. While pan-inhibitors [such as sodium butyrate (NaB) and trichostatin A (TSA)] limit drug self-administration under different circumstances (Castino et al., 2015; Jeanblanc, Lemoine, Jeanblanc, Alaux-Cantin, & Naassila, 2015; Romieu et al., 2008, 2011; Simon-O'Brien et al., 2014) and RGFP966 weakens cocaine-primed reinstatement and extinction of cocaine CPP, it also promotes memory for location and auditory operant discrimination (Bieszczad et al., 2015; Malvaez et al., 2013; McQuown et al., 2011b). Our results demonstrate that a selective HDAC3 inhibitor can enhance extinction persistently and additional types of reinstatement can be weakened even after chronic and stable cocaine self-administration in rats.

# RGFP966 does not influence operant maintenance of cocaine selfadministration

In contrast to our findings in Experiments 2 and 3, pan-HDAC inhibitors alter operant maintenance levels of drug-taking (Romieu et al., 2008; Simon-O'Brien et al., 2014). Maintenance of self-administration (FR1 with 0.33 but not 0.75mg/kg/infusion dose) as well as the motivation (PR) to self-administer cocaine decreases with multiple pre-treatments of pan-inhibitors (TSA and phenylbutyrate, Romieu et al., 2008). Additional control studies by this group

demonstrated that these effects were not do to anhedonia (CPP preference for sucrose remained) or general locomotor preference (as seen in locomotor and cocaine-induced locomotor tasks). This groups findings coincide with ours in that self-administration of the larger dose of cocaine was not altered by HDAC treatment (0.89mg/kg/infusion in our study is more comparable to their 0.75 dose than their 0.33mg/kg dose) but contrasts with our PR results where we saw no effects. Another study demonstrated that rats with a history of heavy drinking (reaching ethanol levels near 1g/kg/30min) decreased ethanol self-administration on a PR schedule when given a pan HDACi (MS-275) at least two times and three hours prior to drug intake or reacquisition (Jeanblanc et al., 2015). MS-275 is a pan-HDACi but it is more selective for HDAC1 and 9 than other HDACs). This difference could mean that a pan-HDAC inhibitor, but not a selective HDAC3 inhibitor, decreases the motivation for drugs, or that a general HDACi increased the reinforcing property of the drug, such that animals received the same reinforcing effect from less drug.

It may also be that differences between drug exposure or total infusions in our study (~10 versus > 20/session) compared to Romieu et al. (2008) may lead to effects based on dependence, similar to the way in which dependent (but not non-dependent) rats limit ethanol intake with a pan-HDACi (Simon-O'Brien et al., 2014) or how rapid ethanol tolerance can be reversed with a pan HDACi (Sakharkar et al., 2012). These effects support the possibility that HDAC inhibition may lead to different behavioral and cellular outcomes due to the baseline cellular and behavioral environment during treatment (i.e., more or less tolerant, during drug taking or during extinction) and has the potential to enhance or weaken problematic behaviors such as addiction.

In addition, multiple consecutive injections of the pan-inhibitor and/or vehicle (10% DMSO, daily for ≥10 days) may underlie different results on PR self-administration as this study used fewer doses and greater spread between HDAC inhibitor doses over time than previous studies (e.g., Romieu et al., 2008). One of the benefits of targeting a specific HDAC is that pan-HDAC inhibitors target multiple HDACs and other non-histone proteins that may have other functions besides deacetylation (i.e., to increase net acetylation). Pan-inhibitors may lead to dynamic changes in the cellular environment, in the structure of chromatin, transcription targets, and to the life cycle of proteins that may be involved (Dokmanovic et al., 2007). For example, pan-inhibitors (e.g., NaB, RGFP963, TSA, etc.) occasionally lead to indirect peripheral and central nervous system effects that could more likely influence performance if given before behavioral testing (Andersen et al., 2013; Tran, Schulkin, Ligon, & Greenwood-Van Meerveld, 2014). Many pan-inhibitors (e.g., SAHA, Vorinostat) are thought to induce macroautophagy after changes to acetylation or transcription have occurred, and may sensitize cells to various forms of cell death (Qin, Li, & Liu, 2017; Wagner, Hackanson, Lübbert, & Jung, 2010; Zhang, Shang, Chen, & Li, 2017; Zhang & Zhong, 2014).

Here the focus was on HDAC3 because it is unique on many ways from other HDACs. This includes the ability of HDAC3 to move in and out of the nucleus to deacetylate and associate with other proteins (i.e., nuclear receptor corepressor, silencing mediator for retinoid and thyroid receptors, protooncogene c-Jun, nuclear cofactor kappa B, cAMP responsive-element binding protein, etc.), thought to lead to changes in acetylation (at particular lysine sites on H3 and H4), transcription, and enhancements in learning and memory (Chen & Greene, 2003; Karagianni & Wong, 2007; Krishna, Behnisch, & Sajikumar, 2016; Underhill, Qutob, Yee, & Torchia, 2000; McQuown & Wood, 2011). Together, this evidence suggests either non-specific effects or differences in HDAC effects based on previous drug history, dependence level or HDAC selectivity, and that HDAC3 could be applicable for treatment in other learning processes (i.e., extinction).

#### **RGFP966** enhances operant extinction and weakens reinstatement

Evidence exists of pan-HDAC inhibition enhancing Pavlovian extinction but there are fewer investigations on selective HDAC inhibition, or in translating this to operant behaviors. One study has demonstrated that extinction is enhanced with a pre-extinction administration of a pan-HDACi but not with RGFP966 (Bowers et al., 2015). An additional study found that pre-extinction administration of valproic acid (VPA, pan-HDACi and GABAergic enhancer) rescued retrieval and consolidation deficits of fear extinction in learning impaired mice, yet MS-275 (pan HDACi) did not enhance extinction acquisition (Whittle et al., 2013).

One of few studies to investigate the effects of HDACi on operant drug extinction found that a post-extinction injection (IP) of a pan-inhibitor (NaB) decreased nicotine-seeking during cued-extinction (one day after treatment) and that at least six days of post-extinction treatment increased the time it took to reach extinction criterion (Castino et al., 2015). This effect supports our results in Phase 3 of Experiment 3, with delayed rates of extinction criterion reached in the RGFP966-treatead rats. In addition, this group demonstrated that multiple NaB treatments after extinction sessions weakened nicotine-primed reinstatement, and cue + nicotine-primed reinstatement. With a few differences in drug and types of extinction and reinstatement tested, our work complements and extends these (Phases 2 and 3 in Experiment 1), in that both a pan-inhibitor and HDAC3 selective inhibitor persistently enhanced extinction and decreased reinstatement.

Similar to findings in this chapter, Malvaez et al. (2013) demonstrated that a single injection (SC) of RGFP966 after CPP extinction and followed 1 hour later by acquisition training for object location memory (i.e., prior RGFP966 injection) enhanced both consolidation of CPP extinction and initial memory (in spatial memory of object location). Our findings support and extend these, in that one pre-session injection of systemic RGFP966 had a reliable and long-term enhancement on early learning and encoding of inhibitory drug learning in Phases 2 and 3 of Experiment 1.

Supporting our Chapter 3 findings for a role of HDA3 in operant behavior, recent evidence demonstrated that post-session RGFP966 injections can enhance consolidation in a reward based auditory learning assay (Bieszczad et al., 2015). This task required increased discrimination of tones for natural reinforcers (water), and was enhanced (with corresponding changes in sensory A1 areas). With our results from Experiments 1 and 3, there is evidence that RGFP966 application in operant reward behavior can enhance learning for both natural and stimulant rewards, by increasing or decreasing responding (based on task requirement), during acquisition or extinction learning of complex behavior. This is further evidence that effects of HDAC3 (and potentially epigenetic regulation in general), directly relate to the learning event that occurs concurrently with drug treatment (McQuown & Wood, 2011) rather than altered behavior in general, or additional HDAC3 activities [e.g., TF recruitment (Liu & Bagchi, 2004; Nott et al., 2016)].

Findings in this Chapter are closely related to previous findings that RGFP966 promotes extinction and weakens reinstatement (Malvaez et al., 2013). That finding, as well as work with non-specific HDAC inhibitors suggests that a limited amount of behavioral extinction can lead to persistent changes when paired with an HDAC inhibitor. Our findings of weakened context and cued reinstatement after days of extinction following cocaine self-administration in rats (Experiment 1) support this idea, although cocaine-primed reinstatement was not weakened as Malvaez et al. (2013) demonstrated after cocaine-induced CPP in mice. The timing between treatment and primed reinstatement testing in cocaineinduced CPP compared to chronic self-administration may account for this difference in findings, yet only 2-4 days earlier context and cued reinstatement had been enhanced in our study. Therefore, it may be that prior cocaine exposure and additional response-cue associations in an operant selfadministration procedure may account for the more resistant reinstatement found in Experiment 1 compared to cocaine CPP.

Enhancements in extinction in Experiment 1 over multiple days and in a novel context and with a discrete cue implicate not only HDAC3, but the involvement of multiple neural circuits. With a systemic injection, conclusions are limited as to just one brain region or regions that may be mediating HDAC3 actions, but it is likely that regions recruited for learning are the same regions regulated by drug administration. Other studies have found spatial memory enhancements induced by RGFP966 treatment (Malvaez et al., 2013) and focal HDAC3 deletions in the DH have enhanced object location memory and cocaine CPP acquisition (McQuown et al., 2011; Rogge et al., 2013). It is therefore likely that context-induced reinstatement for cocaine seeking behavior may be particularity susceptible to prior HDAC3 modulation (as seen in Experiment 1).

Our data represent the first evidence that pre-extinction administration of an HDAC3 inhibitor promotes rapid decreases of drug-seeking, promotes longterm learning early in extinction, and reduces context- and cue-induced reinstatement in operant behavior. This research confirms that HDAC3 inhibition can not only enhance Pavlovian but also operant extinction, and create a less context dependent form of extinction or one that is less likely to renew behavior with a change in context or with exposure to drug cues. In addition, administration of the same HDAC3 inhibitor after extinction (rather than before extinction) and previous learning experiences, had a minimal effect on early extinction and reinstatement, but a pronounced effect on later extinction after reinstatement (extinction beyond zero). These findings suggest that effects of RGFP966 can extend beyond previous reports and are at least partially resistant to multiple protocol changes (i.e., time of drug treatment from pre to post Extinction 1, type of SA training from FR5 to PR, and amount of prior training using naïve versus non-naïve animals, and the addition of an abstinence period before extinction training). This research is an additional indicator of how reliable and applicable effects from RGFP966 may be for enhancing learning in previously unexplored behavioral treatments and clinical populations.



# Long-term inhibition of HDAC3 activity enhances acquisition and extinction of reward seeking

## Introduction

The dorsal hippocampus is the primary brain region thought to be involved in contextual learning (Gruber & McDonald, 2012; Kesner, 2013; Muller, Stead, & Pach, 1996), in helping to differentiate between contexts that signal different events (Kim, Lee, & Lee, 2012; Wiltgen & Tanaka, 2013; Wiltgen & Silva, 2007) and one of many regions that contributes to multiple learning and memory processes, such as acquisition and extinction, encoding, consolidation, and retrieval (Corcoran et al., 2005; Corcoran & Maren, 2004; Ji & Maren, 2008; Ji & Maren, 2007; Moita, Rosis, Zhou, et al., 2003). Contextual discrimination learning involves learning that different stimulus-outcome or response-outcome contingencies are in effect in different contexts. In Chapter 3, the dorsal hippocampus (DH) was determined to be involved in the expression of contextdrug associations after acquisition and extinction of cocaine-induced conditioned place preference (CPP). In this chapter, I examined whether histone deacetylase 3 (HDAC3) in the DH may be involved in the contextual modulation of acquisition and extinction of operant reward-seeking behaviors.

HDAC3 is highly expressed throughout the body, but has one of the highest global expression patterns in the brain (second highest to HDAC11) and in the cortex. It is also one of two with the high expression throughout the DH (CA1-3 and dentate gyrus) compared to other HDACs and brain regions (Broide

et al., 2007). HDAC3 is thought to be a negative regulator of learning and memory (McQuown et al., 2011), commonly recruited with learning events (due to increased cellular activity), binding to the promoter regions of immediate early genes (IEGs), and partnering with nuclear receptor corepressor (NCoR), a transcription factor associated with increased learning (Bhaskara et al., 2010; McQuown et al., 2011; Rawat, Goux, Piechaczyk, & D'Mello, 2015).

In Chapter 4, I found that a single systemic administration of the HDAC3 inhibitor RGFP966 enhanced extinction and weakened contextual and cue reinstatement of operant responding for cocaine. Previous studies targeting HDAC3 function have used focal homozygous deletions of HDAC3 in the nucleus accumbens (NA; Rogge et al. 2013) and the DH (McQuown et al., 2011) or the selective HDAC3 inhibitor (HDAC3i), RGFP966 (Bieszczad et al., 2015; Malvaez et al., 2013). These studies have demonstrated substantial enhancements in acquisition of cocaine CPP, and long lasting effects on object recognition, enhanced extinction, and decreased cocaine-primed reinstatement after cocaine CPP (Malvaez et al., 2013), as well as enhanced auditory discrimination (Bieszczad et al., 2015). The most recent manipulation of HDAC3 was induced with the same adeno-associated viral (AAV) methods used here, downregulating HDAC3 activity within the DH by inducing the expression of HDAC3 with a point mutation to the catalytic domain (Kwapis et al., 2017). This report complemented previous reports of enhanced learning and memory capability with HDAC3 activity decreased, enhancing fear formation in adult mice.

In this chapter, I take a viral approach to manipulate HDAC3 function in the DH. Because this manipulation occurs prior to acquisition, I use this approach to investigate how HDAC3 may be involved in contextual modulation of operant responding for a natural reward, in which lever pressing in one context led to a food pellet and lever pressing in a second context did not. Rats received infusions of one of three viruses: wildtype HDAC3 (to overexpress HDAC3), a point mutant of HDAC3 (to remove the deacetylase domain of HDAC3), or a control virus. My hypothesis was that overexpression of HDAC3 would impair contextual discrimination, whereas the point mutant of HDAC3 would enhance it. I further evaluated whether responding would generalize to a third context after this contextual discrimination.

#### Methods

Animals. Male Long-Evans rats (N = 32, weighing 275-300 grams, Charles River) arrived at the OHSU vivarium to habituate for two weeks prior to surgery. The vivarium was on a reverse light/dark cycle (lights on at 7a). Rats had access to water drippers in their home cages within the vivarium, and were given ~4 pellets per day (grain based natural reinforcer, 20mg pellet, Bio-Serv, Inc.) after each experimental procedure to maintain them at 95% of their freefeeding body weight throughout the experiment. There was no change in weight between virus groups from the start to the end of the study. Animals were housed in pairs until surgery, remained single housed during the initial recovery week after surgery, and then pair-housed again with their previous partner before behavioral testing began. All protocols conformed to the NIH guidelines for Care and Use of animals and the Institutional Animal Care and Use committee (IACUC) at Oregon Health & Science University and animals were housed and treated in accordance with the Department of Comparative Medicine at OHSU.

**Virus.** The design, preparation, and verification of two of the three viruses used have recently been published by (Kwapis et al., 2017). Briefly, mouse hippocampal cDNA for HDAC3 was amplified, cloned, and modified from the pAAV-IRES-hrGFP (Agilent) vector by removing the IRES element and hrGFP and adding the v5 epitope tag. This design allowed c-terminal fusion to HDAC3 (AAV-HDAC3-v5, referred to as WT). The expression of these genes was directed by the CMV promoter and  $\beta$ -globin intron. The vector contained the human growth hormone (hGH) poly adenylation signal (polyA tail).

A single amino acid substitution (Y289H) in the catalytic domain of HDAC3 (exon 11) directed production of histidine rather than tyrosine (AAV-HDAC3 (Y298H)-v5, referred to as PM). The HDAC3 coding sequence did not include a start codon in the empty vector, yet all other sequences were similar (AAV-EV, referred to as CON). The final titer for each was 3.46x10<sup>12</sup> (WT), 6.76x10<sup>12</sup> (PM), and 1.66x10<sup>12</sup> (CON) genome copies per ml.

The plasmid DNA, vector maps, sequence information, and the agarose gel blots verifying the correct sequence for all three of the plasmids were prepared by Dr. Marcelo Wood at UC Irvine. They were sent to the University of Pennsylvania Vector Core to be made using a serotype 2 AAV viral genome packaged within an AAV1 capsid (AAV2.1) for transduction of DH pyramidal neurons (Burger et al., 2004). Infusion of PM virus served to decrease the catalytic activity of HDAC3 (Kwapis et al., 2017) essentially leading to less HDAC3 deacetylation (more acetylation) in the injected site with normal function in all other ways (e.g., recruitment and release from gene promoter regions, binding to other partner proteins, etc.). This mechanism was tested in vitro among other possible mutations and conclusively found to have a significant and specific effects on HDAC3 activity (Lahm et al., 2007; Rai et al., 2010). Infusion of the WT virus served to increase total HDAC3 catalytic activity (more deacetylation), without altering function but increasing HDAC3 expression. Infusion of the CON virus served to have no effect on HDAC3 activity. Without a readable transcript (empty vector) it was expected that there would be no v5 expression and all levels of HDAC3 would remain at normal levels (Kwapis et al., 2017).

**Viral Surgery.** Rats were food and water restricted for 3 hours prior to surgery, anesthetized with a cocktail of xylazine and ketamine at 10% of their body weight (.1ml/100g). PE tubing was connected to two 10ml microinfusion syringes secured in a microinfusion pump. Once rats were unconscious, they were placed in the stereotaxis, with isoflurane flowing into their mouth and nose at 1% (range: 0.5-2%) to maintain a surgical plane for the duration of the surgery. Their eyes were lubricated, their head was shaved near the subsequent incision, and their skull was disinfected with betadine. A vertical incision was made in the scalp above bregma, two shallow holes were drilled into the skull above the CA1 subregion of the DH (target coordinates: ±2.5mm medial/lateral, -4mm ventral, and -3.8mm anterior/posterior from the skull and relative to bregma, Paxinos &

Watson, 1998) and a bilateral cannula was placed into the two holes and (Plastic One, 5.0mm wide, extending 3mm from the top of the skull). A dummy injector (Plastic One, extended 1mm beyond cannula) was slowly inserted and removed from the guide cannula three times to forge a path for the viral infusions, decreasing the potential for clogs when the viral injectors were inserted. An air bubble (1ul) was pulled into the injectors to separate and identify the distinction between distilled water (filling the majority of the PE tubing and each microsyringe) and the viral solutions that was pulled into the bilateral injector (2ul). The injector was tested prior to lowering and injecting into each animal.

The three AAV2.1 HDAC3 vectors were bilaterally infused (1ul/side) into the DH, with an empty, wildtype, or point mutant vector (University of Pennsylvania Vector Core). Each virus solution was stored at -80 degrees until the day of use, moved into the -20 freezer, and then thawed and vortexed immediately before use. The bilateral injector was then slowly lowered into the bilateral cannula at a rate of 0.2mm/15s; they remained in place for 2-min at final coordinates before infusing the virus solution (1ul of control, wildtype, or point mutation AAV) into the DH at a rate of 0.1ul/min. The injectors remained in place for an additional 2 minutes to allow for viral diffusion, the bilateral injectors were slowly lifted 0.1 mm and remained in place for an additional minute, and then the injectors and guide cannula were slowly removed at a rate of 0.1mm/15s.

Acrylic dental cement was mixed, placed over the drill holes, and allowed to dry, and then the scalp was sutured. The rat was given an infusion of carprofen (Rimadyl, Pfizer, 5mg/ml), placed into a clean cage with food and water, and checked for normal behavior after surgery. Each injector was emptied, rinsed, and soaked with ethanol and distilled water between surgeries.

**Apparatus.** Self-administration chambers were equipped with two levers, two cue lights, a main house light, and a pellet dispenser. Acquisition sessions began with illumination of the house light and a non-contingent delivery of a pellet. After this, the pellet dispenser and the cue light (above the active lever) were programmed to activate upon pressing the active lever (counterbalanced for side) one time (fixed-ratio (FR) 1). The cue light remained on for 5 sec and a 20 sec timeout began concurrently with pellet delivery. Inactive lever presses had no consequence. Extinction and Context testing sessions were identical but had no programmed consequence of lever presses (no cue light and no pellet delivery).

**Contexts.** Three separate rooms (Rooms 229A, B and C) and 24 selfadministration chambers (Med Associates, model ENV007) were used for behavior. Contexts A and B were located in different self-administration chambers, but within the same room (in Room 229A or in 229B). Context C was located in Room 229C. In short, this created three distinct contexts (detailed description below): 1) Room 229 A/B: wide floor bars, striped wall, higher location, 2) Room 229 A/B: thin floor bars, clear wall, lower location, and 3) Room 229 C: solid floor, gray wall, lowest location, and camera. Room assignment was counterbalanced between groups.

Contexts A and B differed by internal modular chamber cues (floor bar width, visual cue of back wall), and external cues (location within the room). The floor bars were larger or smaller in diameter and have been demonstrated to be distinguishable (Crombag et al., 2008a; Crombag & Shaham, 2002). The visual back wallpaper was either white and back striped or clear with the outer melamine shell visible. The location within the room was either closer to or farther from the ceiling and nearer to or farther from the room door.

Context C differed from A and B by the cues listed above (i.e., the floor was solid and white, the back wallpaper was a gray diamond with visible corners, the location was closer to the hallway and further from the vivarium, the location was closer to the floor), and the additional internal (direction and location of house light, pellet dispenser, video camera, direction of chamber) and external (location of room, melamine-coated shell box) cues. Inside the chamber the house light was moved to the opposite side of the chamber and flipped up instead of down, the pellet dispenser was a different model, a video camera hung from the chamber ceiling, and the chamber faced north instead of south. Outside of the chamber, the melamine shell was a larger size and a different color, and the room was in a different location in the vivarium.

**Pellet Self-administration Protocol.** A schematic of each context and the timeline is in Figure 26. Two cohorts of rats completed the study. Cohort 1 underwent surgery and began behavior testing before Cohort 2. To test all rats for final behavior over the same 2 day period in Context C, the second cohort of rats received 3 fewer days of A+ (acquisition in Context A) and B- (extinction in Context B) training (i.e., Cohort 1 completed three more days of training, each with one acquisition and one extinction session per day).



**Figure 26. Chapter 5 – Schematic of experimental timeline and contexts.** Please see Methods Section for a description of AAV, Behavior Contexts. Rats were injected with AAV-HDAC3 virus into the DH at least 2 weeks prior to completing four sessions of operant training in Context A, then began extinction training) in Context B, with reinforced acquisition maintenance training in Context A (denoted with bidirectional arrows) until testing with no reinforcement in Context A or C.

Two rooms (229A and 229B) and two contexts and were used (Context A: denoted in green, acquisition context, reinforced; Context B: denoted in red, extinction context, not reinforced). Each room was equipped with half Context A and half Context B features. Animals remained in the same room for acquisition and extinction sessions (Context A and Context B were located in both room). Context C was novel for all animals.

Groups 1 and 2 acquired in Context A (lever pressing was reinforced with on fixed ratio 1 schedule) and extinguished in Context B (no reinforcement), but only Group 1 was tested for renewal-like behavior in Context A and Context C (ABAC context exposure). Group 2 was identical to Group1 but did not test in Context A (ABC context exposure).

Each cohort was split into two groups, such that the first group received a test session in Contest A- (ABA-like renewal, described below) and then a session in Context C, while the second group was only tested in Context C (ABC-like renewal, see Figure 26 for schematic of behavior testing). Viral treatment (WT, CO, PM) was counterbalanced between cohorts, groups, incubation range (Mean  $\pm$  SEM: 19 d  $\pm$  1.1, Range: 2-5 weeks), and room (229A or B) assignment. *Habituation* 

Rats were habituated to handling (1-2 min each) for 2 d before behavior testing and were weighed prior to each behavioral session.

### Acquisition training (Context A)

Rats were placed into a novel context (Context A) to begin acquisition on an FR1 schedule. Day 1 consisted of one acquisition session, Day 2 consisted of two sessions separated by 4 hours (i.e., A2 at 10AM and A3 at 2PM), and Day 3 consisted of one session. The first four acquisition sessions were 60-min in duration. After four sessions of acquisition in Context A (minimum criterion of active lever presses: > 60) extinction began in Context B. The remaining acquisition maintenance sessions were 30-min in duration (A5-11 Cohort 1, A5-8 Cohort 2).

#### Extinction training (Context B)

During extinction, there was no consequence (no cue light or pellet reinforcer) of an active lever press. Day 1 consisted of one extinction session, Days 2-8 (E2-8 Cohort 1) or 2-5 (E2-5 Cohort 2) consisted of two sessions (one acquisition and one extinction session) separated by 4 hours with time of day counterbalanced. All extinction sessions were 30-min in duration. Once rats reached a minimum number of active lever presses (Context B: active lever presses < 30) testing in Context A and/or C began.

## Context Testing

There was no consequence of lever pressing (no cue light, no reinforcer) during testing in Context A (previously reinforced context) or C (novel context). Group 1 animals (CON, WT and PM) were tested for renewal-like responding in Context A (120-min duration), and in Context C (30-min duration) the following day. Group 2 animals (CON, WT, and PM) were trained identically but were not tested in Context A (only handled).

Immunohistochemistry. Immediately after the Context C test (30-min duration), rats were transported into another room (30-ft away), placed into an incubation chamber with isoflurane (0.25 ml/L) and deeply anesthetized. They were sacrificed by guillotine, their brain was carefully removed, flash frozen in 2-methylbutane on dry ice at -60C degrees for 1 min, double bagged, stored on dry ice for 2 hours, and transferred to a -80C freezer. For sectioning, brains were acclimated to -20 degrees, sectioned (20um) on a cryostat (Leica), thaw mounted on coated Superfrost slides, air dried, and placed in a slide box with desiccant beads and double bagged to protect slides and stored in a -20C freezer until staining commenced. To determine placements location prior CON, WT or PM HDAC3 intra-DH viral infusions, one rat was cannulated and the brain was sectioned at 60 um and stained with cresyl violate acetate (three vector designs, target infusion area, and cannulation shown in Figure 27A and B).

Figure 27A.



Figure 27B.



# Figure 27. Chapter 5 – Schematic of AAV-HDAC3 vector design and dorsal hippocampal (DH) target.

- (A) Adeno-associated vector (serotype 2.1) with cytomegalovirus (CMV) promoter instructing expression of three different versions of histone deacetylase 3 (HDAC3) were infused into the dorsal hippocampus (DH) of adult rats prior to operant training. The three vectors induced (1) Control levels of HDAC3 (CON: empty vector, no v5 tag), (2) overexpression of functional, wildtype levels of activity (WT: wildtype HDAC3 promoter and transgene, v5 tag) or (3) overexpression of inhibited HDAC3 activity with a point mutation (PM: amino acid 298 switched from tyrosine to histidine (Y298H) within HDAC3 transgene to selectively inhibiting deacetylation at catalytic domain, v5 tag).
- (B) Long-Evans rat (pilot animal) brain section (60um) after microinfusion with identical cannula used for AAV infusions and staining with cresyl violet.

For immunofluorescence, two sections per rat were collected anterior and posterior to the DH target and were mounted on each slide. Each treatment group was represented twice per slide and the placement order of each section was counterbalanced between treatment groups to control for any variability in immunofluorescent staining per slide. Multiple slides from different sets of rats were piloted for IHC (primary and secondary titrations for single and double label staining) from all viral groups, from one rat that was sacrificed 9 days after a PM infusion (with no training for a preliminary assessment of viral staining), and from three additional rats that did not have AAV infusions (to pilot IHC protocol, antibodies and DH infusions).

The protocol for fluorescent immunohistochemistry closely followed that published by Kwapis et al (2017). Slide cases were used to incubate slides in 4% PFA (10-min, made fresh, ice-cold), rinsed slides in PBS (2 x 5-min, on a shaker), incubated slides in PBS with 0.1%Triton X-100 (30-min), rinsed with PBS (5min), laid slides flat, pipetted 400ul of 8% normal goat serum in PBS and cover slipped to block for background staining (60-min, on a shaker and in a humidity chamber).

For single labeled primary staining, coverslips were removed and 400ul of the v5 (monoclonal mouse anti-v5 or rabbit anti-v5) or H4K8ac (polyclonal rabbit anti-H4K8ac) primary solution (made in 2% normal goat serum), was pipetted at a concentration of 1:500 to each slide and incubated overnight (at 4C on a rotating rack at 30 RPM in a humidity chamber). For double stained slides, a combined primary solution with v5 and H4K8ac was made at 1:500 for each antibody in 2% normal goat serum.

For single labeled secondary staining, 400ul of the mouse (anti-mouse or rabbit 488) or H4K8ac (anti-rabbit 594) secondary solution was made in PBS and pipetted onto each slide at a concentration of 1:500 (incubated in the dark for 60min on a shaker and in a humidity chamber). For double stained secondary stationing, an additional block step was added prior (2% normal goat serum pipetted onto slides and incubated on shaker and in humidity chamber for 60min). A combined secondary solution in 2% normal goat serum with anti-mouse and anti-rabbit antibodies (at concentrations of 1:500 each) was applied to each slide and incubated in the dark (on a shaker and in a humidity chamber) for 2 hours. Coverslips were removed, slides were rinsed in PBS with 1% tween-20 (2) x 5-min) then PBS alone (5-min), counterstained with DAPI (4 drops) and sealed with clear nail polish. One slide (N = 3 rats, 2 sections per rat), in the CA1 pyramidal layer of the DH (at -3.5mm posterior, 2.5mm lateral, and -2.5 mm ventral to bregma). Sections were photographed on an Olympus BX51 microscope and Olympus Q-Color 3 digital camera for nucleic acid stain 4',6diamidino-2-phenylindole (DAPI), epitope tag v5, and downstream acetylation expression (at H4K8) to be reviewed.

**Data Analysis.** To determine whether differences in behavior (active and inactive lever pressing) or immunoreactivity (for v5, H4K8ac or DAPI) existed between viral treatment groups (control, wildtype or point mutation), all data were organized in Excel and compared with SPSS, followed by multivariate and LSD

corrected post-hoc tests. When applicable, the degrees of freedom in repeated measures were Greenhouse-Geisser corrected for any violations of sphericity to elicit a more accurate and conservative significance value. Data from the first four sessions of acquisition (A1-4), extinction (E1-4), and context testing (Baseline extinction in Context B compared to Context A and C) are described (no effects in later sessions).

One wildtype rat had extremely high outlier values of active lever presses during the four initial acquisition days (two days of values were identified by SPSS as outliers between 1.5 and 3x the interquartile range and above 2 standard deviations from the group average, and on two other days SPSS labeled values as extreme values as more than 3x the interquartile range and nearly 3 standard deviations (SD) above the mean (i.e., WT group mean  $\pm$  3SD for active lever presses during Acquisition Sessions 1 and 2 = 19  $\pm$  121 and 93 $\pm$ 612 compared to WT outlier values = 121 and 600). Values were well within normal ranges for extinction and context testing. One control rat never reached the minimum acquisition criterion to qualify for extinction. These two subjects were removed from acquisition (WT rat) or extinction and context testing (CON rat) data analysis but results are noted within the text (N = 32/31: WT N = 11/10, CON N = 11/10, PM N = 10).

### **Results and Conclusions**

**Acquisition.** Acquisition Sessions 1-4 are shown in Figure 28. All groups acquired lever pressing, with the PM group showing the fastest acquisition.





Figure 28. Chapter 5 - Acquisition (Context A) of operant behavior for natural reward after AAV infusions into the dorsal hippocampus (DH). Average  $\pm$  SEM inactive (IA, not reinforced) and active (A, reinforced) lever presses during acquisition (60-min duration, fixed ratio 1) after treatment with AAV infusion (red arrow, at least two weeks prior) of Control (CON), wildtype (WT) or point mutant (PM) HDAC3. Note. \*\*Point-mutant HDAC3 (PM) > Control Empty HDAC3 (CON) and Wildtype HDAC3 (WT) in active lever, p < 0.05. Please see Results and Conclusions section (Acquisition) for a description of statistical findings. A session x lever x 3 virus ANOVA revealed reliable main effects of session (F (1.5, 40.7) = 101.107, p < 0.001) and lever (F (1, 2) = 126.541, p < 0.001), as well as session x lever (F (1.4, 39.8) = 102.162, p < 0.001) and lever x virus interactions (F (2, 28) = 3.692, p = 0.038) with more active lever presses completed by the point mutation group compared to the WT group and the control group (post hoc LSD simple contrast p = 0.033 and 0.040 respectively). There were trends for a reliable main effect of virus (F (2, 28) = 3.210, p = 0.056), as well as interactions between session x virus (p = 0.072), but no trend for a three-way interaction for session x lever x virus (p = 0.093).

All groups began with similar acquisition rates on the first session (A1) and multivariate analysis by session and lever determined a reliable viral effect on the active lever during Session 2 (F (2, 28) = 5.044, p = 0.013) and Session 3 (F (2, 28) = 4.522, p = 0.020). Looking at each virus group, significant differences emerged among viral groups on the active lever during A2 between the PM and CON group (p = 0.006) and the PM and WT group (p = 0.018) and during A3 (PM to CON p = 0.020, PM to WT p = 0.011, see Figure 28).

The number of reinforcers earned on these days was determined and similar effects were found between groups (4 sessions-repeated x virus, session p < 0.001, session x virus p = 0.043, virus p = 0.041), with post-hoc differences between PM and CON across sessions (p = 0.017, with a near effect between PM and WT, p = 0.055) and differences between PM and CON and PM and WT within sessions (A3: PM less than CON p = 0.044, A2: PM less than CON p =0.004 and WT p = 0.009, data not shown). Without removing the extreme outlier from the WT group, there were no reliable effects on lever presses or reinforcers x virus in the two main ANOVA comparison (ps > 0.079). However, individual comparisons for the active lever remained reliable between PM and the CON group during the A3 session (p = 0.049), and for reinforcers earned between PM and the CON group (p = 0.035), with an increase between A1 and A2 in the PM compared to CON group (p = 0.022), and more pellets earned during the following sessions (A2: p = 0.030, A3: p = 0.052, A4: p = 0.054) by the PM group compared to the CON group.

Therefore, the rats that had a mutated version of HDAC3 infused into their DH (PM rats) had a reliably enhanced rate of acquisition of pellet selfadministration compared to the rats with no promoter driven changes in HDAC3 (CON rats, see Figure 28). This effect was particularly evident between Sessions 1 and 2 (demonstrated by higher active lever presses and reinforcers). Effects were similar if the outlier was included but not as reliable. There were also significant effects demonstrated between the PM and WT groups with one outlier rat removed, but none with this rat included.

**Extinction.** Extinction Sessions 1-4 are shown in Figure 29. After four acquisition sessions, all rats were placed into a different context (Context B) for extinction training. A session x lever x virus group ANOVA revealed reliable main effects of session (F (2.1, 59.8) = 23.546, p < 0.001), lever (F (1, 28) = 83.229, p < 0.001), and a reliable interactions of session x lever (F (3, 63.82) = 16.583, p < 0.001), with no reliable main effect or interaction with viral treatment (ps > 0.20).





Figure 29. Chapter 5 - Extinction (Context B) of operant behavior for natural reward after AAV infusions (DH) and acquisition (Context A). Average ±SEM inactive (IA, not reinforced) and active (A, previously reinforced) lever presses during extinction (30-min duration, no reinforcement) after treatment with AAV infusion (at least two and half weeks prior) of Control (CON), wildtype (WT) or point mutant (PM) HDAC3. Please see Results and Conclusions section (Extinction) for a description of statistical findings. Note. \*Point-mutant HDAC3 (PM) < Control Empty HDAC3 (CON) in active lever, p < 0.05. Fig 29.

Follow-up statistics determined that the main effects of session occurred between E1 and E2 (F (1, 28) = 5.816, p = 0.023) and between E2 and E3 (F (1, 28) = 37.15, p < 0.001), the session x lever interaction occurred between E2 and E3 (F (1, 28) = 28.42, p < 0.001, and no effect over session and lever by virus (ps > 0.097).

Impairing HDAC3 function was hypothesized to enhance learning and memory, and therefore differences were expected to emerge on the active lever during acquisition and extinction. Multivariate comparisons were used to determine if reliable differences existed at individual sessions or with certain levers between the three groups. No differences existed by viral group during E1 in the active lever (ps > 0.242), but a reliable main effect of virus on active lever pressing occurred during E3 (F (2, 28) = 3.904, p = 0.032), with a reliable difference in active lever presses between the PM and CON group (p = 0.010, but not in the WT group p = 0.095, Figure 29). To determine when this occurred, 5-min bins of the data were analyzed (not shown), with the greatest differences noted in the first three of six bins in the session (PM pressed less than CON group, t-test ps < 0.05) and decreased over time. There was not an overall effect across sessions and levers between groups but a trend at the end for WT animals to press on the active lever more than the control animals on E4 (p = 0.056).

As a final note, without removing the rat that did not reach criterion, our most significant effect in this phase of the experiment would have remained reliable (PM: CON in active presses during E3, p = 0.035). There was no effect of

cohort and or incubation duration x virus on lever pressing during the last acquisition or extinction session completed by all groups (Fs < 1.853, ps > 0.184).

**Context A test**. Context test effects are demonstrated in Figure 30 (A and B). During the Context A test session (Group 1 only), a significant effect occurred between the end of extinction in Context B and testing in Context A (main effects of session (F (1, 13) = 17.9, p = 0.001) and lever (F (1, 13) = 22.58, p < 0.001) and a reliable session x lever interaction (F (1, 13) = 19.40, p = 0.001). However, there were no effects of virus (ps > 0.5). There were also no viral effects (ps > 0.364) when looking at individual levers and sessions. Effects were nearly identical when comparing the entire 120-min duration of the test rather than the first 120-min duration, or with the previously removed data point included.

**Context C test.** Lever pressing in the novel Context C is shown in Figure 30 B. There were no reliable differences by group on baseline extinction by viral treatment so they were pooled for further analysis in Context C (ps > 0.1). A repeated measures ANOVA demonstrated an effect of lever (F (1, 29) = 20.566, p < 0.000), but only trends for a session effect (F (1, 29) = 3.327, p = 0.078), and the interaction for session x lever x treatment (F (2, 29) = 2.882, p = 0.072). A multivariate ANOVA compared levers, and virus groups in each context and determined no differences between groups in Context B (ps > 0.3), but higher active lever presses in Context C in the PM group compared to the WT group (p = 0.044), with the control group responding between both altered HDAC3 groups (Figure 30B).





Context Test





Figure 30. Chapter 5 – Context testing (no reinforcement) of operant behavior for natural reward after AAV infusions (DH), acquisition (Context A) and extinction (Context B). Average ±SEM lever presses (Active, Inactive) during session (30-min) and after treatment with AAV infusion of Control (CON), Wildtype (WT) or Point-mutant (PM) HDAC3. Please see Results and Conclusions section (Context Testing) for a description of statistical findings.

- (A) Average ± SEM inactive (IA, not reinforced) and active (A, previously reinforced) lever presses after treatment with AAV infusion of Control (CON, N = 5), Wildtype (WT, N = 6) or Point-mutant (PM, N = 5) HDAC3 during the last extinction session (Context B, 30-min, no previous reinforcement in this context) and during a test in Context A (first 30-min shown of 120-min test, previously reinforced context) for renewal-like behavior (ABA), Fig 30A.
- (B) Average ± SEM lever presses after treatment with AAV infusion of Control (CON, N = 10), Wildtype (WT, N = 11) or Point-mutant (PM, N = 10) HDAC3 during the last extinction session (Context B, 30-min, no previous reinforcement in this context) and during a test in Context C (novel context) for renewal-like behavior (ABC), Note. \*Point-mutant HDAC3 (PM) > Wildtype HDAC3 (WT) in active lever, p < 0.05, Fig 30B.</li>
Including the CON animal that never fully acquired and therefore could not extinguish or demonstrate renewal-like behavior, did not change these results (active lever PM to WT p = 0.04).

Immunohistochemistry. Figure 31 demonstrates DAPI, v5, and acetylation staining in one animal from each treatment group. To verify viral expression at different time points, many small single label fluorescent immunohistochemistry studies were completed to determine if viral expression could be verified and whether a double label IHC study was feasible with the available antibodies. Over these pilot studies, almost half of the animals in each group have been tested for immunoreactivity (data not shown). As expected, all rats demonstrated normal DAPI staining, H4K8 acetylation staining (qualitatively at different levels), and v5 was seen in all of the PM and WT groups reviewed (within the DH primarily).

In most cases, immunoreactivity looked to be heavier in the CA1 and dentate gyrus areas than the CA2/CA3 areas. There was limited but some disparate staining of v5 in all control animals with an empty vector (differing by antibody piloted). In all cases, a moderate level of background staining was dispersed ubiquitously in all slides (differing slightly by primary antibody). In the early test of viral expression (9 day expression from PM animal with no behavior training), DAPI, v5, and acetylation staining was clear (data not shown). Figure 31 shows the results from a double stain experiment. Figure 31.



Figure 31. Representative immunohistochemistry (IHC) images from the pyramidal layer of CA1 in the dorsal hippocampus (DH) of AAV-HDAC3 animals. Control (CON, white), wildtype (WT, gray), and point mutant (PM, black) infused rat sections (20um) stained for 4', 6-diamidino-2-phenylindole (DAPI, blue), small epitope tag (V5, green), and histone 4 lysine 8 acetylation (H4K8ac, red).

Immunoreactivity to DAPI, v5, and H4K8 acetylation is shown (with one animal from each virus group) following the Context C test (see Figures 29B for behavior in novel Context C test, 30-min duration).

#### Discussion

In Chapter 4, I used a pharmacological approach to show that systemic administration of an HDAC3 inhibitor could promote extinction and weaken postextinction reinstatement. I found that a viral approach that mutates HDAC3 in the DH enhanced initial learning and had moderate effects on subsequent extinction and context testing (renewal-like behavior). However, overexpressing HDAC3 had no effects on behavior. These findings with the point-mutant reinforce my findings from Chapter 4 and they show that long-term changes in HDAC3 can potentially promote learning and memory processes.

In general, little is known about how HDAC inhibitors alter operant reward behavior and even less is known about the effects of targeting a specific HDAC in a single brain region. It is known that many epigenetic modifications work together to create large changes in transcription and learning, yet HDAC3 has received increased investigation in recent years as a critical negative regulator of long-term memory formation (McQuown et al., 2011). Many studies since have investigated this protein for its deacetylase function in a variety of behaviors, with most studies supporting similar enhancements to those seen here (i.e., in learning and memory), with few exceptions. In one case, fear extinction was not enhanced with a post-extinction systemic injection of RGF966 (HDAC3i, Bowers et al. 2015) but was enhanced with RGFP963 (inhibiting HDAC1, 2, and 3). In another case, HDAC3 deletion in the DH enhanced object location memory, but had no effect on novel object recognition (McQuown et al., 2011). There is evidence for and against the role of epigenetic regulation within the DH for this object recognition task (Barrett et al., 2011; Haettig et al., 2011; Stefanko, Barrett, Ly, Reolon, & Wood, 2009) as well as for associated changes in HDAC2 and HDAC3 (Fortress, Kim, Poole, Gould, & Frick, 2014). These studies demonstrate that the role of HDAC3 in learning and memory may be sensitive to the extent of HDAC3 treatment and the behavioral approach applied.

# Acquisition was enhanced for self-administration of a natural reward with impaired HDAC3 in the DH

The enhancement demonstrated here in operant learning and memory was in line with evidence in related behaviors (Bieszczad et al., 2015; Blank et al., 2014; Bousiges et al., 2010, 2013; Kwapis et al., 2017; McQuown et al., 2011; Rogge et al., 2013). Our acquisition results extend previous findings and highlight the role of HDAC3 in the DH and HDAC3 in acquisition of reward learning. Discrimination for object location was improved by deletion of HDAC3 in the DH (McQuown et al., 2011), and discrimination for auditory tones was enhanced with multiple post-training and systemic injections of RGFP966 (selective HDAC3 in the DH (rather than deleting the entire protein or with a systemic effect) would be a strong enough manipulation to create similar learning enhancements in operant reward learning. Rats that had been infused with the PM virus (HDAC3 inhibition) did in fact acquire the self-administration behavior faster that rats infused with the control (native HDAC3 expression) and wildtype (enhanced HDAC3 expression) vectors. Although acquisition of this behavior did not require rats to discriminate between contexts in this first phase of learning, it did begin in a novel context which is known to activate the DH (Kubik, Miyashita, & Guzowski, 2007). It also provided the opportunity for subjects to associate the novel context with a discrimination between levers for a natural reinforcer (active but not inactive resulted in a pellet reinforcer), and if successful this would require new learning that is often thought to involve episodic memory and DH (Gould & Leach, 2014).

Although the specific molecular mechanism underlying the enhancement in learning in the point mutant is unclear, other studies suggest some possibilities. Kwapis et al. (2017, see Figure 2a-b) found that the point mutation resulted in a 60% reduction in deacetylation activity, which is comparable to levels induced by RGFP966, and a similar absolute reduction in HDAC3 activity with both RGFP966 and AAV-HDAC3-PM. In addition, there was no additional inhibition of HDAC3 activity with application of RGFP966 to the HDAC3 PM, demonstrating a similar and selective mechanism of inhibition with both manipulations. Further, Kwapis et al. (2017, Figures 2c and 3b-c) verified expression of v5 intensity within the DH two weeks after the infusion of the point mutation virus (100% v5 compared to empty vector control ~15%) and a significant downstream increase in H4K8 acetylation intensity in the DH occurred in the PM rats (~140% H4K8ac compared to empty vector control set to 100%). This is similar to the qualitative results reported here.

At a behavioral level, Kwapis et al. (2017) found that the PM in the DH promoted contextual fear conditioning. Because the DH is necessary for identifying and recognizing a context and creating a spatial map of that context and associated experience within it (Daumas et al., 2005; Gruber & McDonald, 2012; Morris, Weeden, Churchwell, & Kesner, 2013; Rolls & Kesner, 2006; Wiltgen, Wood, & Levy, 2011), results from Kwapis et al. (2017) and this Chapter strongly suggest that impairing HDAC3 activity indefinitely with a point mutation (AAV-HDAC3-PM) leads to enhanced acquisition of contextually-associated behavior.

Enhancements in acquisition of reward-seeking behavior by inhibition of HDAC3 have also been demonstrated in an associative cocaine CPP assay (Rogge et al., 2013) and in an operant assay that required rats to discriminate between auditory signals to receive a contingent reward (Bieszczad et al., 2015). Both of these studies applied multiple post-training (consolidation) injections of RGFP966. Our results support these previous findings, and extend them, demonstrating manipulation of HDAC3 enhances reward behavior in an operant behavior assay that requires contextual discrimination for a natural reward. In addition, this occurred with one long-term and selective AAV infusion into the DH, compared to previous effects on behavior using a short-term systemic manipulation.

# Extinction for self-administration of a natural reward was marginally enhanced with impaired HDAC3 activity in the DH

Multiple injections of a pan-HDACi (sodium butyrate, NaB) enhance extinction and limit drug and cue-drug reinstatement after nicotine selfadministration (Castino et al., 2015). This change in behavior is similar to effects after multiple injections of RGFP966 (HDAC3i), which enhance extinction of cocaine-induced CPP and weaken cocaine-primed reinstatement (Malvaez et al., 2013). In addition, Castino et al. (2015) found no effect of NaB on extinction or reinstatement after self-administration of sucrose (as opposed to significant nicotine results above).

Research thus far has indicated that reward, behavioral approach, and selectivity of HDAC targets may alter extinction effects. Yet, in Chapter 4 just one injection of RGFP966, either before or after extinction training, was determined to enhance initial and early (for 1-4 days) or late extinction (delayed by weeks and after context and cued reinstatement). In this Chapter, enhancements were found in extinction of a contextual operant behavior for a natural reward. Our results confirm that the total number of time points of HDAC inhibition and the type of reward cannot fully account for differences noted in the literature. Our results then support and extend overall findings to date, demonstrating a consistent enhancement in acquisition, extinction, and reinstatement in associative or operant reward procedures with HDAC3 impairment (Bieszczad et al., 2015; Malvaez et al., 2013; Rogge et al., 2013).

This is also consistent with findings by Kwapis et al. (2017), who demonstrated enhanced decreases in freezing during fear testing (within test extinction) in animals with a point mutant version of HDAC3 compared to controls (AAV-HDAC3-PM and AAV-HDAC3-empty vector). In combination with our results, this further implicated involvement of HDAC3 activity in the DH in extinction of both aversive and rewarding behaviors. In our current findings, the PM rats learned extinction at a faster rate than CON rats, occurring only during E3 and not between the WT and Control groups, as expected. In this case, effects were not as strong as originally expected, therefore it may be that other regions or learning challenges may be needed to uncover stronger effects.

For example, Corbit, Ostlund, and Balleine (2002) provided compelling evidence that the DH may not mediate operant responses as contingencies change, but instead it may be the entorhinal cortex (EC) that is required for this learning. While the DH is a downstream target of EC activity and both regions may mediate the learning that was tested here, this could mean that activity in other regions could be altered or intercepted by the DH function. Other regions may also be equally or more receptive to an HDAC3 manipulation (i.e., ventral hippocampus (VH), amygdala (AMY), and NA). For example, enhancements in extinction have been found by impairing or deleting HDAC3 in the basolateral amygdala (BLA), lateral amygdala (LA, Kwapis et al., 2017), and NA (Malvaez et al. 2013; Rogge et al. 2013).

There is additional evidence that perhaps a region that has not been studied for HDAC3 function might be involved in the behaviors tested here as

well, and warrant further investigation. It was recently demonstrated that previous contextual learning (bidirectional contextual discrimination) could be retrieved without the DH but not without the VH. The DH may be more necessary for the encoding and consolidation of this learning rather than the retrieval of this learning (Riaz et al., 2017). Further work is needed to clarify how the different subregions of the hippocampus contribute to contextual modulation of learning and memory.

Other brain regions (EC and VH) and partners that HDAC3 deacetylates or binds with (NCoR, nuclear factor kappa (NFkB)) may have similar effects on the learning (acquisition, extinction, renewal) and memory processes (encoding, consolidation, retrieval) as those discussed here (reviewed in Jarome, Thomas, & Lubin, 2014). With mounting evidence that HDAC3 in the DH, NA, and BLA are involved in spatial object location, contextual operant reward and Pavlovian cocaine conditioned place preference and fear, as well as auditory tone discrimination, it is plausible that many behavioral challenges and potential treatments may be susceptible to these type of manipulations. Yet, I am unaware of another study demonstrating the control that inhibiting activity of HDAC3 (with one point mutation versus gene deletion) and within the DH alone has on operant responding during both acquisition and extinction. This research demonstrates that inhibiting HDAC3 activity can both increase and decrease behavior depending on the task challenge, even if in competition (i.e., enhancements in acquisition required excitatory learning more behavioral responses days prior to

an enhancement in extinction that required inhibitory learning and less behavioral responses).

#### Contextual control of behavior

Contextual renewal of behavior after extinction and the underlying involvement of the DH have been studied in fear conditioning (Corcoran et al., 2005; Corcoran & Maren, 2004; Zelikowsky et al., 2014), appetitive responding in conditioned suppression (Hirsch, Regmi, Birnbaum, & Greene, 2015; Lengersdorf, Stüttgen, Uengoer, & Güntürkün, 2014; Todd et al., 2017) and drugrelated relapse in animals (Fuchs et al., 2005; Marchant et al., 2012; Venniro et al., 2015) as well as in drug or fear-related tasks in humans (Bisby et al., 2015; Hermann et al., 2017). Yet, the role that epigenetic modifiers may play in the acquisition, extinction, and the renewal process is relatively unknown. Our results are fairly consistent with others (Campese & Delamater, 2014; Campese & Delamater, 2013; Todd, Jiang, DeAngeli, & Bucci, 2017), who demonstrated that the DH was not necessary for ABC and ABA renewal in operant selfadministration of natural rewards. Although an increase in responding was demonstrated in the A- context test, there was not a clear difference in viral groups. This lack of a reliable effect demonstrates that like the DH as a whole, HDAC3 activity within the DH does not reliably alter ABC or ABA renewal of selfadministration of a natural reward.

#### System consolidation and molecular signaling may underlie effects

As a final note on the timing of HDAC3 manipulations, it is possible that certain contextual memories in the DH may transfer over time to other brain regions, such as to the cortex (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009; Wiltgen & Silva, 2007). With our DH treatment in mind, it would be useful to determine if effects like ours would differ if trained or tested in a shorter or longer period of time (independent of other variables that may change over time such as viral expression or training extent). Systems consolidation is the gradual process of creating a more stable memory for extended periods of time with the recruitment and storage of long-term memories in different brain regions (e.g., anterior cingulate cortex, anterior temporal lobe) from regions originally recruited for initial learning (Merhav, Karni, & Gilboa, 2015; Walters & Zovkic, 2015).

Wartman & Holahan (2013) demonstrated that learning multiple hippocampal-dependent tasks and recalling them 2 days later during testing was associated with enhanced c-Fos activation in the ACC 30 days later. This mimicked behavior and activation patterns in groups with remote testing and long-term memory but not groups with less training, testing, or recall, suggesting a role for systems consolidation. Similar to enhancements that are noted days after additional training, or with HDAC3 inhibition, it may be that effects of HDAC3 modulation shift enhancements in cortical recruitment over longer periods of time and brain regions. Evidence suggests that transcription-promoting histone acetylation and other epigenetic regulations (i.e., DNA methylation, histone variant exchange, poly-ubiquitination, and potentially protein degradation) cooperate to enhance the expression and recycling of pre- and post-learning proteins (e.g., early and late waves of c-Fos) in the hippocampus for faster transfer of memories to the cortex (Katche et al., 2010; Walters & Zovkic, 2015; Wiltgen & Tanaka, 2013).

While it was beyond the scope of the study to investigate molecular effects of HDAC3 viral expression quantitatively, a number of genes may have been altered to influence ultimate behavior. As HDACs exist in many types of tissues they can regulate gene expression in neuronal and non-neuronal cell lines of different types and alter the fate of gene expression within them (Hoeksema et al., 2014; Xia et al., 2017; Zhang et al., 2016). This distribution leads to different cellular and likely behavioral outcomes, albeit potentially towards the same direction of effects (i.e., more or less acetylation at numerous targets). For instance RGFP966 (HDAC3i) has led to decreased tumor necrosis factor alpha (TNF-a) and interleukin-6 (IL-6) in primary microglia cultures, leading to oligodendrocyte precursors transitioning into astrocytes all while astrocyte specific genes are repressed. There does seem to be a more prevalent occurrence in the influence of certain HDACs in certain tissue types or regions and subtypes of cells. For example, HDAC2 and 3 are more common in the CNS and limbic regions, while HDAC8 is specific to muscle (Lazo-Gómez, Ramírez-Jarquín, Tovar-y-Romo, & Tapia, 2013; Yoo, Larouche, & Goldowitz, 2013). Yet, the field has only begun discovering how expression patterns by cell type contribute to HDAC3s overall molecular and behavioral outcomes.

Although our work suggests a role for HDAC3 in behavior, it is important to consider alternative mechanisms that could lead to behavioral effects. For example, there is evidence to suggest that AAV infection causes increased cell death and apoptosis (Timpe, Verrill, Black, Ding, & Trempe, 2007), which could then lead to differences between viral groups compared to the control group in Chapter 5. However, differences were found between the two AAV groups, suggesting that the AAV itself was not causing a specific pattern of results. Other studies have demonstrated that AAV infusions in the DH (independent of transgene target) do not significantly increase toxicity, or alter cell survival or viability of cells (Bankiewicz et al., 2000; Barrett et al., 2011; Haettig et al., 2011; McKee et al., 2010) but that sham and AAV intracranial infusions can similarly activate an immunologic responses (Scammell et al., 2003). Therefore, intra-DH surgery alone may have activated microglia in each of the three groups similarly and potentially changed the course of downstream effects. However, the evidence is mixed as to whether cell survival may be altered by AAV or by different transgene effects from AAV infusion (Burger et al., 2004; Sharma, Kumar, & Kundu, 2010; Timpe et al., 2007) and few studies have conducted a full evaluation (i.e., TUNEL assays to label apoptotic or necrotic cell death).

In contrast, there is clear literature suggesting that overexpression of HDAC3 may mediate neurotoxicity in cerebellar granular neurons in a mouse model of Huntington's disease. Evidence suggests that neurotoxicity may be largely mediated by phosphorylation of HDAC3 from glycogen synthase kinase 3 (GSK3B) and be prevented by Akt (also known as protein kinase B) activity (Bardai & D'Mello, 2011) as determined by DAPI staining (i.e., condensed or fragmented nuclei were considered apoptotic and with decreased cellular viability with changes in phosphorylation). A similar dependence for HDAC3 activity has been noted on PP4 phosphorylation at serine site 424 of HDAC3 (Zhang et al., 2005). While the specific site was not noted on HDAC3 for GSK3B phosphorylation (Bardai & D'Mello, 2011) it is likely that phosphorylation (i.e., via GSK3B or PP4) and accessibility to other transcription factors was equally possible in both viral groups and could have been equally enhanced compared to the control group (with only the tyrosine site 298 changed to a histidine between WT and PM groups). Without a defined assay for cell viability in this study it is not certain whether differences in cell survival influenced results, there is minimal previous evidence to support the idea that differences between infection, phosphorylation, or interaction with transcription factors may be the variable leading to potential differences in cell survival and ultimate behavioral differences between groups.

Accordingly, results from this chapter demonstrated no qualitative differences in DAPI staining between control, WT, or PM groups, nor were there quantitative and consistent differences in behavior between control and viral groups (that were similar between viral groups). This result suggests that any potential differences by AAV or HDAC3 on cell survival did not likely control overall morphology (similar between all three groups) or behavior (different between control and one viral group typically but not both). If acetylation levels alone (and not infection, phosphorylation, or other protein interactions) directly relate to downstream increases or decreases in necessary protein synthesis for learning and memory, perhaps larger changes in acetylation could have a greater impact on cell survival and further contribute to differential downstream behavior.

# Conclusion

It may be that enhancing native HDAC3 activity with the WT vector recruited larger compensatory mechanisms (i.e., other HDAC inhibitors or decreases in histone acetylation) and therefore led to the minimal behavioral effects of this manipulation compared to the point mutation group (versus control group). In this case, differences between the PM and the control group (during acquisition and extinction) or wildtype and control group (during context testing) were revealed by task and intensity of challenge, such that a more challenging task may uncover the more subtle effects of WT overexpression compared to the more significant effects demonstrated by impairing HDAC3 activity. As the field is moving into cell-specific deciphering of epigenetic modulation, future research is warranted to uncover not only brain region and function, but cell specific differences in signaling cascades between HDAC3 manipulations (PM versus WT), learning processes (acquisition versus extinction), and learning type (i.e., reward, spatial, and operant).

With this variation in HDAC expression and influence, based on tissue, cell, and task, downstream effects likely affect behavior in a very selective and dynamic manner. Results here demonstrate that histone deacetylase 3 in the dorsal hippocampus is involved in contextual and reward based operant learning in rats, yet it is likely that other epigenetic modifications as well as other brain regions contribute to these processes. Together, our results support the role of histone deacetylase 3 as a negative regulator of early learning, both during acquisition and extinction.



Portions of Chapter 6 are adapted from the publication:

Hitchcock, L. N., & Lattal, K. M. (2014). *Histone-Mediated Epigenetics in Addiction. Epigenetics and Neuroplasticity - Evidence and Debate* (1st ed., Vol. 128).

#### **General Discussion**

## **Dissertation summary**

A pervasive finding in animal models of substance abuse is that associations form quickly between contexts and drugs of abuse, such as cocaine. The main questions addressed in this dissertation focused on the importance of learning and recalling associations in a particular context, the involvement of the dorsal hippocampus (DH) in this type of learning, and how histone deacetylase 3 (HDAC3) might regulate this learning. These questions were addressed with one of two main behavioral assays, conditioned place preference and self-administration. Behavioral manipulations targeted the context of learning or the type of learning, excitatory acquisition or inhibitory extinction. Neurobiological manipulations targeted activity within the DH, and/or the activity of HDAC3 to determine if either played a significant role in learning and memory under these conditions.

Key findings reported in this dissertation are: (1) the context and DH are involved in the acquisition and extinction of associative reward-seeking behavior, (2) a systemic and short-term inhibitor of HDAC3 activity promotes extinction and limits context and cue reinstatement but not maintenance of operant drug-taking behavior, and (3) viral inhibition of HDAC3 in the DH enhances operant acquisition and extinction of a natural reward. These findings are discussed in relation to previous literature and fill at least three gaps in the field, demonstrating the strong influence that context, HDAC3, and the DH have in reward learning. In conclusion, my research is put into perspective with the larger field of histone-mediated epigenetic regulation of addiction, with unresolved questions and suggestions for future research discussed.

#### Chapter 2

In Chapter 2, the effects of acquisition and extinction of cocaine-induced CPP were compared as a function of apparatus configuration. During CPP acquisition, mice received cocaine paired with one tactile floor (conditioned stimulus, CS+) and saline paired with the other (CS-) in one of three configurations: one-compartment (exposure to the entire apparatus during CS+ or CS-), two-compartment consistent position (exposure to CS+ or CS- in adjacent, spatially distinct compartments), or two-compartment alternating position (exposure to CS+ or CS- in adjacent compartments that alternated spatial locations across days). A stronger preference for the CS+ floor occurred in two-versus one-compartment groups, with the strongest preference observed when cocaine was paired with alternating chamber positions. In contrast, greater loss of preference occurred after extinction in a one-compartment procedure, regardless of one- or two-compartment acquisition history. These findings suggest that a two-compartment configuration facilitated acquisition but attenuated extinction of a cocaine-induced CPP. The use of different CPP configurations may distinguish the underlying substrates and relevant cues for acquisition and extinction processes in cocaine abuse.

Chapter 3

In Chapter 3, the effects of hippocampal inactivation on acquisition, expression, and extinction of cocaine-induced conditioned place preference (CPP) were compared in mice. Groups differed in whether the positive conditioned stimulus (CS+) and negative conditioned stimulus (CS-) cues were presented in the same large space (one-compartment procedure) or in distinct small spaces (two-compartment procedure). Acquisition of CPP was promoted by the two-compartment procedure. Extinction, when mice were exposed to the CS+ cues in the absence of cocaine, was promoted by the one-compartment procedure. These findings suggest that a two-compartment configuration facilitated acquisition and attenuated extinction of a cocaine-induced CPP. Expression of CPP decreased when a microinjection of the GABAa agonist, muscimol, inactivated the DH (DH) prior to a post-acquisition test, and increased with a microinjection prior to a post-extinction test and limited extinction when given prior to extinction. These effects differed depending on the spatial configuration (one- or two-compartment training configuration) suggesting that the dorsal hippocampus may differentially modulate drug seeking following CPP acquisition and extinction.

#### Chapter 4

In Chapter 4, it was investigated whether a novel and selective HDAC inhibitor (RGFP966) that modulates epigenetic mechanisms could promote extinction and weaken contextual control of operant drug-seeking extinction after cocaine self-administration. Injections of RGFP966 before the first extinction session led to significantly less responding during subsequent extinction and reinstatement tests compared to vehicle-injected rats. These effects were not likely due to a performance deficit or a change in motivation to self-administer cocaine, as injections of RGFP966 had no effect on stable responding during a fixed or progressive ratio schedule in subsequent studies. In addition, rats injected with RGFP966 just after the first extinction day had no effect during early extinction and reinstatement tests, but responded significantly less after reinstatement, during later extinction sessions. Results suggest that a systemic injection of a selective HDAC3 inhibitor can enhance extinction and suppress reinstatement after cocaine self-administration. The finding that behavioral and pharmacological manipulations can be combined to decrease drug-seeking provides further potential for treatment by epigenetic modulation.

#### Chapter 5

In Chapter 5, the role of HDAC3 in the DH was determined in multiple phases of reward learning (acquisition, extinction, and renewal). To create this long-term but brain region and protein selective effect a virus was infused into the DH that changed the function of HDAC3 prior to acquisition of operant responding for pellets. This virus altered HDAC3 activity longer than possible with a classical pharmacological approach (as in Chapter 4) and led to enhancements in contextual control of acquisition and extinction of reward seeking.

## Implications

Contextual control during acquisition and extinction

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This dissertation supports the idea that contextual stimuli modulate behavior in multiple ways (Bouton, 1993): 1) contextual stimuli guide retrieval, 2) different memories were differentially dependent on context, and 3) contradictions between contexts and history of learning influenced performance. In all three experiments from Chapter 2, CPP configuration during acquisition or extinction influenced retrieval at Tests 1 and 2, but the specific configuration effects differed between acquisition and extinction. In contrast to their enhancing effects on acquisition, two-compartment cues (that alternated or remained consistent) led to poor development of extinction, suggesting that different types of memories (context-drug or context-no drug) may be under different contextual control.

By comparing effects between configuration groups, I can begin to posit how contextual information is processed in each configuration group in different phases of learning. For example, mice in a one-compartment configuration (with floor cues only) may experience cocaine's (or saline's) effects on both sides of the box within and between CS+ (or CS-) sessions. This configuration may result in a specific association between the tactile cues and cocaine or saline, but no specific association between the spatial cues within the box and cocaine or an ambiguous association, as context area and spatial cues are present on CS+ and CS- trials.

In contrast, mice in a consistent two-compartment group may learn to associate specific visuospatial contextual cues within the box with cocaine or saline because those cues become reliable predictors of cocaine or saline. Thus, contextual cues may become differentially associated with cocaine in the two conditions in a hippocampus-dependent manner (Carew & Rudy, 1991; Matus-Amat et al., 2004; Rudy & Sutherland, 1989; Sutherland & McDonald, 1990; Sutherland, McDonald, Hill, & Rudy, 1989), as demonstrated in Chapter 3. When tested in the choice configuration, previous context cues (ambiguous for onecompartment or unambiguous for two-compartment) are now relevant to expression of preference acquisition (because the tactile cues are presented in different locations). This choice configuration may create an ambiguous test context that weakens expression of preference in a one-compartment group.

Similar logic may apply during extinction training, such that extinction training (with ambiguous context cues for one-compartment or unambiguous context cues for two-compartment) is relevant to expression of extinction. Therefore, when tested in the choice configuration, the previous associations formed about the context (ambiguous for one-compartment, or unambiguous for two-compartment) were relevant and applied to extinction expression. This manipulation may create another ambiguous test context for the one-compartment group, that weakens expression of preference (enhances extinction expression) due to prior extinction training with ambiguous context cues. However, in a two-compartment group that was trained with unambiguous context, limiting extinction, and retrieving and strengthening expression of preference due to prior learning. In general, our results demonstrate that learning that a cue (i.e., context

space) is either ambiguous or relevant during training determines how that cue will be used to retrieve or express learning in subsequent testing.

These effects may be accounted for by a difference in ambiguity between training and testing (Bouton & Bolles, 1979; Bouton, 1988, 2002; Gruber & McDonald, 2012). It may also be that the two-compartment group (as opposed to the one-compartment group) associates the entire context during extinction training (with clear and unambiguous visuospatial cues, contextual area, and tactile floor cues) with the US (absence of cocaine during extinction). This two-compartment configuration may create a particular strong context-CS association during extinction (Carew & Rudy, 1991; Rescorla, & Wagner, 1972). Therefore, when the exact extinction context from training is not presented or replicated in the Test 2 choice configuration (absence of wall with new ambiguous space cues), retrieval of extinction learning may be inhibited and renew previous acquisition conditioning in the two-compartment group more than the one-compartment group.

Differences between procedures in Chapter 2 and 3 may also be due to differences in interference, based on previous context associations. Extinction is a context-dependent type of retroactive interference assay (i.e., negative influence of Phase 1 (Acquisition) on Phase 2 (Extinction; Bouton, 1993), in which the current testing conditions compete for expression with the conditioning learned first. The differences between one and two-compartment configurations may facilitate other types of interference as well (e.g., proactive latent inhibition or proactive learned irrelevance), leading to preference differences between configuration groups and between acquisition and extinction. There is some support for the idea of different amounts of interference due to changes in configuration in human fear conditioning and related disorders (anxiety, PTSD) and due to phase of learning (acquisition versus extinction) but a clear conclusion from the literature is still mixed as to how or why this may be (Vansteenwegen et al., 2005).

As demonstrated in Chapters 2 and 5, both acquisition and extinction learning are influenced by the context, yet only extinction is thought to be context dependent (Bouton, 2004). When transferring to a new context, behavior from acquisition will reappear and extinction retrieval will be limited. This reappearance of behavior is referred to as renewal (Bouton & Bolles, 1979), often tested in Pavlovian and operant assays, but referred to as context-induced reinstatement after drug self-administration. Renewal is a common problem in cognitive behavioral therapies, as extinction may limit a problematic and addictive behavior but over time renewal is triggered by a change in context. The control of context of extinction is demonstrated with additional phenomena as well (i.e., rapid reacquisition with training, spontaneous recovery with time, and reinstatement with US exposure), each leading to an increase in conditioned responding based on the testing context (Bouton, 2004) as seen in Chapters 2-5.

In general, contextual renewal occurs whenever there is a change in context between extinction and testing. ABA renewal is typically the most robust, where acquisition occurs in Context A, extinction occurs in Context B, and testing occurs in Context A. There are fewer investigations on AAB and ABC renewal, where acquisition and extinction occurs in the same context, but testing occurs in a different context, or where all three phases are conducted in separate contexts. The first demonstration ABA renewal in operant drug-seeking (heroin-cocaine mixture) was completed by Crombag & Shaham, (2002), however AAB renewal was not evident in this study. The first evidence in operant behavior for AAB renewal did not occur until 2011 (Bouton, Todd, Vurbic, & Winterbauer) with responding for a natural reward (pellets). Importantly, a change in context, whether it is a return to the original acquisition context in ABA renewal or removal from the extinction context in AAB or ABC renewal, leads to a reversal of extinction both in humans and animals (Balooch, Neumann, & Boschen, 2012; Podlesnik et al., 2017; Vervliet et al., 2013).

In Chapters 2 and 3, there are different ways of thinking about context and its relation to acquisition and extinction. In all cases, there is the context in which CPP is established (the chamber that houses the CPP apparatus and the visuospatial cues within that chamber) and there is the tactile floor itself, which, because of its reliable association with the presence or absence of drug, is a CS that has direct associations with drug or saline. In contrast to auditory conditioning, the CS in tactile conditioning is present throughout the experimental session and likely becomes part of the contextual representation. In Chapters 2 and 3, the visuospatial cues within the chamber (on the walls and ceiling) remained consistent between acquisition, extinction, and testing. The configuration of the floor cues between those phases changed, potentially resulting in a changed context that resulted in renewal. For example, a key difference at testing was that animals had access to both tactile floors for the first time since pretest. This test may have been more similar to the conditions of extinction for either the one- or the two-compartment group. In the twocompartment extinction procedure, animals that were restricted to half of the apparatus during extinction were allowed to explore the entire apparatus during testing. In the one-compartment extinction procedure, animals that explored a large floor in the entire apparatus during extinction received that floor on only half of the apparatus during testing. Either of these stimulus changes could have resulted in renewal, but the highest CPP expression occurred in the twocompartment group.

One implication of this is that one-compartment extinction allowed more of the visuospatial cues (including box size) to become associated with extinction, resulting in less renewal in that group. This effect may be similar to extinction (of the tactile association) occurring in more contexts (different locations within the box), which could weaken renewal. Evidence has demonstrated that extinction in multiple contexts weakens the renewal effect (Balooch et al., 2012; Chelonis, Calton, Hart, & Schachtman, 1999; Gruber & McDonald, 2012), so it is possible that extinction in a one-compartment procedure, especially when tested in a onecompartment apparatus, effectively serves to extinguish the tactile cues in different spatial locations compared to the two-compartment procedure. Applying the logic of contexts and renewal is limited in the CPP literature (Thanos, Bermeo, Wang, & Volkow, 2009), but our results suggest that it will be useful to continue to examine how tactile cues interact with the larger context and configurations to alter expression of CPP.

In Chapter 4, the context remained the same between acquisition and extinction, but animals were tested in a novel context after extinction (AAB renewal), and only demonstrated renewal when treated with vehicle rather than RGFP966. This effect implicated HDAC3 in the AAB renewal process, when inhibited at initial extinction in the drug-seeking and extinction context. AAB is typically less common and robust (Bernal-Gamboa, Carrasco-López, & Nieto, 2014; Todd et al., 2014), than other forms of renewal, especially in drug-seeking literature. This study is one of few to demonstrate this type of renewal in the drug-seeking field and none that I know of has investigated underlying epigenetic regulation.

In Chapter 5, animals acquired in one context but extinguished in another, and then were tested in the drug-seeking context and in a novel context (similar to ABA and/or ABC renewal). In general, there was greater responding in the drug-seeking context (ABA) than in the novel context (ABC), which is consistent with the literature (Bouton et al., 2011; Todd et al., 2014). However, no major differences were noted between HDAC3 treated groups in the drug-seeking context. This null result potentially demonstrates a difference between the type of HDAC3 treatment administered between Chapters 4 (systemic) and 5 (DHspecific), or between reinforcers and the amount of responding during testing (i.e., strong cocaine reinforcer and large renewal in Chapter 4 compared to natural reward and less absolute renewal in Chapter 5). There are other demonstrations of relapse in drug-seeking, such as with reacquisition and cueand drug-primed reinstatement, but these may be less context-specific than with renewal.

#### Neurobiology (brain regions)

On a behavior and theoretical level, CPP and SA are clearly controlled by the context (Chapters 2-5), but it was of interest to know how the context might be influencing the underlying neurobiology. In animals and humans problematic behaviors like addiction are acquired through the action of multiple brain regions and connections between regions, with few successful pharmacologic or behavioral treatments (as reviewed in Goodman & Packard, 2016; Packard, 2009; Schwabe, Dickinson, & Wolf, 2011).

Common variants of extinction are applied in behavioral treatment in humans, referred to as exposure-based therapy, to decrease problematic actions or emotions related to behavioral disorders (e.g., in addiction, overeating, PTSD, Pizzimenti & Lattal, 2015). Outcomes in these treatments are often mixed or successful in a portion of participants (Hunt, Barnett, & Branch, 1971; Jacquart et al., 2017; Jansen, Schyns, Bongers, & Van Den Akker, 2015) but are typically still subject to contextual or cued forms of relapse. In animals, Pavlovian and operate acquisition and extinction learning are used to model these context sensitive reward memories. This contextual memory was demonstrated in multiple ways in this dissertation, with preference for drug-paired places, and targeted actions in drug or food paired contexts that increased with contextual changes.

Based on the type of stimuli, or learning that is activated, specific brain regions may be recruited and impart more or less control over the learning process (Koob & Volkow, 2010). Primary regions that are thought to regulate context and reward specific behaviors include the basolateral amygdala (BLA), prefrontal cortex (PFC), nuclear accumbens (NA), and DH (as reviewed in Crombag, Bossert, Koya, & Shaham, 2008; Gould & Leach, 2014; Khoo, Gibson, Prasad, & McNally, 2017; Peters, Kalivas, & Quirk, 2009). Connections between regions or disruptions and activity in certain regions have been implicated (indirectly or directly) in these context-specific behaviors (Khoo et al., 2017). Most notably, involvement of the NA (Bossert, Gray, Lu, & Shaham, 2005), PFC (Bossert et al., 2011), PFC with NA (Bossert et al., 2012), BLA and NA (Millan, Marchant, & McNally, 2011), DH and BLA, or DH and PFC (Berglind et al., 2007; Fuchs et al., 2005; Fuchs, Eaddy, Su, & Bell, 2007; Hearing et al., 2010; Zavala, Biswas, Harlan, & Neisewander, 2007) are thought to influence contextdependent reinstatement or extinction.

Due to the context specificity of extinction, extinguished behavior, and its renewal are particularly sensitive to DH disruption, as demonstrated in Chapters 3 and 5. Likewise, extensive research suggests that the DH is a primary source of spatial map formation (O'Keefe, 1990), adult neurogenesis involved with cocaine-seeking behavior (Castilla-Ortega et al., 2016), spatial information processing and topological representations (i.e., loose representations of space based upon connectivity or enclosure between stimuli, (Goodrich-Hunsaker, Hunsaker, & Kesner, 2008)), long-term potentiation, plasticity, and long-term memory (Lopez et al., 2016; Sharma et al., 2015). The role of the DH has also been demonstrated in human extinction (Hermann et al., 2017; Kalisch et al., 2006). Hippocampal inactivation studies in animals demonstrate impaired acquisition, contextual encoding and extinction retrieval (Corcoran et al., 2005). The recruitment and activity of DH is common for learning and memory consolidation during acquisition and extinction (Corcoran, Desmond, Frey, & Maren, 2005) especially with training procedures that use location cues (Gaskin & White, 2013; Holland, 1997; Kubik et al., 2007).

These results support results from Chapters 3 and 5 in this dissertation, with one exception in Chapter 3 (Experiment 3). There was no reliable drug effect of pre-extinction DH inactivation (in either configuration), such that one compartment groups demonstrated extinction (with no reliable differences between saline and muscimol groups), and no extinction was seen in twocompartment groups (with or without DH inactivation). This effect contradicts results after contextual fear conditioning, where pre-extinction DH inactivation has disrupted contextual encoding of fear extinction and led to fear renewal (Corcoran et al., 2005). However, it is difficult to conclude whether the DH is involved in two-compartment extinction without expression of extinction in saline groups. Perhaps extended inactivation, extinction, or a test in the extinction context could draw out slow decreases in preference, and determine DHindependent or dependent extinction differences. Additional evidence has demonstrated that inhibiting the DH (with tetrodotoxin, sodium-channel blocker) during extinction training impaired extinction retrieval at later drug-free test

(Lengersdorf et al., 2014). However, authors noted that this effect was only seen when tested in the same context that extinction training occurred, suggesting that the choice test configuration in our experiment may not have been similar enough to the extinction context (in one- or especially two-compartment extinction) to demonstrate effects of prior DH inactivation. In that regard, Experiments 1 and 2 (Chapter 3) not only support previous literature, with a role for the DH in acquisition and extinction retrieval (Bouton, Westbrook, Corcoran, & Maren, 2006; Corcoran, et al., 2005) but extend what is known about involvement of the DH in CPP retrieval (i.e., involved in one compartment extinction and differentially during acquisition) based on configuration.

In Chapter 5, impairing a selective epigenetic protein in the DH led to enhancements in acquisition and extinction learning. In general, our findings from CPP and SA are consistent with the literature, in that the DH regulates acquisition and extinction. The role of the DH in renewal of CPP or SA was not directly tested here, yet results are consistent with expectations from renewal literature such that the DH is a primary source of context-induced reinstatement (Fuchs et al., 2005, 2007). Similarly, in Chapters 4 and 5, changes to HDAC3 activity (systemic or DH-specific) altered context-induced reinstatement, and may have then been mediated, at least partially by DH activity.

#### HDACs, learning, and memory

Studies with systemic and intra-DH delivery of pan HDAC inhibitors (inhibiting multiple HDACs) show that they can create a long-term memory that lasts beyond the point at which normal memory fails (Alarcón et al., 2004; Bredy et al., 2007; Roozendaal et al., 2010). Memory enhancements may be due connections with other relevant structures (e.g., BLA (Blank et al., 2014), PFC (Siddiqui et al., 2017), increases in synaptic plasticity, hippocampal LTP (Alarcón et al., 2004; Guan et al., 2009) or altered cooperation among other epigenetic regulators (i.e., histone deacetylases (HDACs) or histone acetyltransferases (HATs, Legube & Trouche, 2003). For example, HDAC3 is known to regulate HDAC4 function (Fischle et al., 2002; McQuown et al., 2011) and a loss of HDAC1/2 function can lead to an increase in HDAC3 (Jamaladdin et al., 2014).

This evidence indirectly supports enhancements noted in Experiments 3 and 5 that were sensitive to changes in context and hippocampal function and may have similar underlying mechanisms. Additional reports that acetylation within the DH is increased with cocaine associated memories in CPP (twocompartment CPP, Itzhak, Liddie, & Anderson, 2013) and in the PFC after SA (Sadakierska-Chudy et al., 2017) extend this possibility. Correspondingly, changes in histone acetylation by genetic (i.e., overexpression of CBP histone acetyltransferase) or pharmacologic manipulation (i.e., sodium butyrate), increase acetylation in the DH (Itzhak et al., 2013) and NA (Malvaez, Sanchis-Segura, Vo, Lattal, & Wood, 2010), with corresponding enhancements in CPP acquisition (Itzhak et al., 2013; Malvaez, Mhillaj, Matheos, Palmery, & Wood, 2011; Raybuck, McCleery, Cunningham, Wood, & Lattal, 2013). There have also been reports of changes to histone acetylation leading to enhancements (Malvaez, Mhillaj, Matheos, Palmery, & Wood, 2011, after choice or twocompartment extinction), or limits (Itzhak et al., 2013, after choice extinction) to

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cocaine CPP extinction. This contrast in extinction findings may be due to differences in the timing and dose of HDAC changes (after extinction or before acquisition), and apparatus configuration.

It is thought that epigenetic regulation of learning necessitates a recent learning event (e.g., Malvaez et al., 2010; McQuown & Wood, 2011; Sadakierska-Chudy et al., 2017). In addition, a recent study demonstrated that PFC and hippocampal increases in gene transcription (i.e., bromodomain containing 1 (brd1) occurred after cocaine SA, and early extinction (3), days) with associated H4K8 and H3K14 acetylation, but not once extinction reached a baseline (10 days, Sadakierska-Chudy et al., 2017). Brd1 is a component of the histone acetyltransferase complex that leads to increases H3K14, H3K9 and H4K8 acetylation (Fryland et al., 2012, 2016; Sadakierska-Chudy et al., 2017). Importantly, these sites are targets of HDAC activity (Malvaez et al., 2013; Seto & Yoshida, 2014) in the DH, NA, and PFC. These findings indirectly support results from Chapters 4 and 5, in the role for histone acetylation and HDAC3 in particular, regulating acquisition and extinction of SA. Together, it seems plausible that different contexts may recruit the DH (among other regions, such as the NA and PFC) and alter acetylation in these regions during acquisition and/or extinction via HDAC3, to regulate subsequent context-dependent rewardseeking behavior. If this were the case, it might be possible for extinction to be enhanced and context-induced reinstatement to be limited with systemic changes in histone acetylation (as seen in Chapter 4, experiment 1) or DH-targeted HDAC3 inhibition.

Effects with pan inhibitors may differ from more selective HDAC inhibitors (like RGFP966) with major differences in HDACs themselves (Seto & Yoshida, 2014). Differences between expression patterns (e.g., high expression of HDAC3) relative to other HDACs in the hippocampus, Broide et al., 2007), deacetylation patterns (i.e., lysine site), downstream effects (i.e., RGFP966 associated with H4K8 acetylation and increased IEG Nr4a2 in DH, Malvaez et al., 2013), with different synaptic plasticity (enhancements with HDAC3 inhibition), may all influence reported behavioral effects. For instance, overexpression of HDAC2 but not 1 impairs memory (Guan et al., 2009; McQuown & Wood, 2011). In light of these differences DH-dependent behaviors and HDAC3 were targeted in Chapters 4 and 5, and may have induced more selective effects on hippocampal related learning and memory (i.e., enhanced acquisition, extinction, contextinduced reinstatement) more than other potential regions, HDACs, or behaviors. Our results support the idea that not only pan-inhibitors, but also HDAC3 inhibition can create stronger versions of extinction memory than normally possible (Chapter 4 Experiment 1 and Chapter 5) and at extended time points (Chapter 4 Experiment 3).

Overexpression of HDAC3 induced an ordinal deficit in extinction and potentially in ABC renewal in Chapter 5, but to a minimal effect compared to enhanced learning demonstrated in Chapters 4 and 5 and in other studies (Kwapis et al., 2017; Malvaez et al., 2013; McQuown et al., 2011a; Rogge, Singh, Dang, & Wood, 2013). These modest effects may be the result of multiple factors. For instance, mechanisms that are more compensatory may exist to circumvent hyperactive HDAC3 deacetylation, at the molecular or behavioral level. It is possible that related complexes correspondingly change expression (Guo et al., 2012), or other epigenetic proteins (i.e., HATs, discussed in more below) are recruited and can increase or decrease their function to a larger extent in lieu of increased HDAC3 function to mitigate hyper HDAC3 function. However, our results are supported by recent literature, where an AAV shorthairpin vector, resistant to HDAC3 was overexpressed and had no effect on conditioned freezing behavior but expressed qualitatively more HDAC3 expression as designed in the CA1-CA3 regions of hippocampus (Uchida et al., 2017). These results are similar to ours and may mean that impairments may not have been compensated for at the epigenetic level (i.e., maximal net upregulation of HDAC3 activity achieved) but at the synaptic or system level, potentially recruiting less common processes for learning (protein degradation, receptor insertion, etc.) or brain regions to account for hyperactive deacetylation in the DH, such as the BLA, NA shell, VH, or RSC (Fanselow & Dong, 2010; Jarome & Helmstetter, 2014; Khoo et al., 2017; Miller, Vedder, Law, & Smith, 2014; Peters et al., 2009; Strange et al., 2014).

Acquisition, extinction, and renewal of reward behaviors may be regulated by this selective HDAC3 pathway with high expression in the DH, while noncontextual or non-reward stimuli might recruit different epigenetic pathways or regions. This idea is only partially supported by the literature though with enhancements in fear extinction from HDAC1/2 but not HDAC3 inhibition (Bowers et al., 2015) and an enhancement in fear formation at testing with an

HDAC3 PM in either the BLA (auditory fear) or the DH (contextual fear, Kwapis et al., 2017). In the Kwapis et al. (2017) study, they also noted within session extinction differences between the CON (empty vector) and PM group but comparisons are difficult in this case as groups started the session at different levels (reliable retrieval effect from conditioning) and it is unknown if effects persisted. These effects are similar to enhancements noted in Chapter 5 during acquisition of operant responding with a natural reward and moderate effects during extinction. Therefore, independent and dependent functions of each region (Gould & Leach, 2014; Koob & Volkow, 2010), multiple effects of each epigenetic target (Kennedy et al., 2013; McQuown & Wood, 2011; Nott et al., 2016; examples described in below), and variability in attention to certain stimuli or memories during a critical learning event (i.e., retrieval of acquisition or extinction memory) may determine the effects of HDAC3 regulation on behavior (discussed more below). In addition, there is evidence that a combination of factors (beyond type of reward, learning, or associated stimuli) may influence HDAC3 activity, such as disease, time of day, age, or activity (Intlekofer et al., 2013; Shi et al., 2016; Yan, Chen, Wang, Tong, & Tao, 2015).

Our results extend the literature to include HDAC3 selective enhancements in drug (after cocaine SA in Chapter 4) and natural (after pellet SA in Chapter 5) operant extinction, as well as long-term reductions on context and cue reinstatement more than a week after HDAC3+extinction treatment (Chapter 4) and DH specific HDAC3 in acquisition enhancements of natural
rewards. These results implicate HDAC3 in three additional behaviors and with system and DH specificity.

#### Limitations

# Interplay between pharmacological manipulations and behavior

In Chapters 4 and 5, two different approaches were used to block deacetylase function of HDAC3 when the protein was recruited and activated within a cell. These approaches were taken in part to clarify an underlying question in the field, that is, were previous effects from HDAC inhibition due to just one function (i.e., deacetylation) of one protein (i.e., HDAC3). Since epigenetic manipulations work in combination (discussed in detail below), previous studies using general HDAC inhibitors (Castino et al., 2015), or knockdown approaches [i.e., HDAC3 -/- (McQuown et al., 2011; Rogge et al., 2013)], could not answer this selectivity question completely. Another possibility would be that effects after HDAC3 modulation (with knockdown or pan-inhibitor methods) demonstrated the cooperation of HDAC3 with other proteins, such as HDAC4 and 5 that lead to additional downstream effects, independent on HDAC3 deacetylation activity (Alenghat et al., 2008; Fischle et al., 2002; Guenther, Barak, & Lazar, 2001). By testing this selectivity theory on two different timescales (short-term inhibition before or after one event or long-term inhibition before many learning events), our results add substantial support to the idea that HDAC3 deacetylase function may have short- and long-term effects with a short- or long-term manipulation.

Related to this idea of multiple learning challenges affecting later testing, extra consideration must be given to results from Experiment 3 in Chapter 4 (post-E1 RGFP966). In that experiment, a persistent effect of a single postextinction session delivery of RGFP966 was revealed during late extinction sessions, after tests for reinstatement. This finding implicates another way of examining persistent extinction effects outside of typical time-dependent reinstatement tests (i.e., spontaneous activity, incubation,

reinstatement/extinction model of reinstatement) that are often used in combination after abstinence. For example, multiple studies have implicated the involvement of the DH in these types of behaviors (Freeman et al., 2008; Hearing et al., 2010; Li et al., 2015; Mandyam, 2013; Wells et al., 2011). However, a complication in this experiment is that all rats had a history of experience with RGFP966, so it is not clear whether this persistent effect would have been detected if rats had received less drug, behavioral, or HDAC3 treatment, or abstinence. A more typical laboratory approach is to evaluate drug treatment in animals that have never received that drug or behavioral testing, although there are many exceptions (Araujo, Chan, Winka, Seymour, & Milgram, 2004; V. Campese & Delamater, 2013; Jeanblanc et al., 2015; Millan & McNally, 2011; Pelloux, Dilleen, Economidou, Theobald, & Everitt, 2012; Takahashi, Vengeliene, & Spanagel, 2017; Wise et al., 2011). This more extensive history is perhaps a better model of human conditions, where tolerant and resistant subjects and effects are revealed after multiple treatments (both pharmacologically and behaviorally).

In Chapters 2, 3 and 5, a related issue was addressed, on how experiencing results in one phase may affect results in the next phase. For example, if motivation or the amount of training were significantly different during acquisition, would this alter results in extinction? In the CPP experiments, configural cues altered acquisition and extinction learning, but one was not dependent on the other; suggesting that the history of learning did not affect the subsequent rate of extinction learning (Figure 3B).

## Temporal characteristics of drug and viral approaches

It would be useful to see if AAV and RGFP966 effects are interchangeable. This work would suggest that the type and extent of initial learning (based on reward and learning process) and not the duration of HDAC3 manipulation determines subsequent effects. It was determined that pharmacologic and viral approaches can enhance learning, in multiple contexts, and with multiple learning challenges, but the limits to these effects (in both extent and timing of enhancements) were not yet clearly delineated. For example, future studies would do well to determine if a short-term effect in one brain region has the same effect as a long-term effect in the same region. This research would help decipher if the initial learning event determines later enhancements or if ongoing HDAC3 changes in following learning events are necessary for enhancements in non-addictive learning and memory (i.e., first session of acquisition or extinction in Chapter 5, or the subsequent second and third sessions where effects were noted). Results from Chapter 4 and prior studies would suggest this is not the case, but without logistical limitations in

future studies, the difference between systemic and site-specific effects could be compared directly.

#### Molecular endpoints

Measuring the specific molecular mechanisms underlying behavioral changes was beyond the scope of this research, yet the manipulations used in this dissertation are supported by previous literature to reliably alter learning, DH and HDAC3 activity, and the documented downstream cascade of events from each (Muscimol: Arcaro et al., 2016; Farrant & Nusser, 2005; Obrietan, Gao, & Van Den Pol, 2002; Schumacher, Coirini, & McEwen, 1989; Sotiriou, Papatheodoropoulos, & Angelatou, 2005; RGFP966: Bieszczad et al., 2015; Bowers et al., 2015; Collins, Ellis, & Holaska, 2017; Krishna, Behnisch, & Sajikumar, 2016; Malvaez et al., 2013; Sharma et al., 2015; Wells et al., 2013; Xia et al., 2017; AAVHDAC3: Kwapis et al., 2017; Lahm et al., 2007; Rai et al., 2010; Seto & Yoshida, 2014; Zhang et al., 2005) and the related necessity of protein interactions independent of HDAC3 deacetylase activity (Guenther, Barak, & Lazar, 2001; Lee et al., 2015; Nott et al., 2016; Seto & Yoshida, 2014; Sun et al., 2013; Zhang et al., 2016; Zhang et al., 2005). While these manipulations are unique by type or timing, and therefore imply slightly different accounts of learning, many additional studies using general HDAC inhibitors (e.g., Dagnas, Micheau, Decorte, Beracochea, & Mons, 2015; Raybuck et al., 2013; Stafford, Raybuck, Ryabinin, & Lattal, 2012), or muscimol/baclofen agonists (e.g., Rossato, Kohler, Radiske, Bevilaqua, & Cammarota, 2015; Veyrac et al., 2015), have demonstrated similar effects in brain activity, downstream effects, and learning.

What has not been demonstrated is the connection between our cited DH and HDAC3 manipulations and the specific behaviors tested here. Despite this, additional pilot experiments (data not shown) were conducted to identify previously undocumented changes that may underlie behavioral effects and to guide our experimental approach throughout this work. This includes conducting IHC for c-Fos in the DH, AMY, and NA following A1 and A2c cocaine CPP in mice, and IHC for c-Fos in the DH following novel context extinction and RGFP966 administration into the DH after cocaine self-administration in rats. In both cases, larger than previous increases in variability of behavior (i.e., limited differences in CPP preference between configuration groups, and equality in selfadministration between subsequent treatment groups) made inferences from IHC results less meaningful. The primary goal of this research was to gain valuable and long-term behavioral data that often occurred long after molecular endpoints would be recovered (i.e., IEGs and downstream acetylation from drug). Therefore, the usefulness of obtaining molecular endpoints was limited in a few ways: (1) other studies have already confirmed the mechanism of action, downstream effects and evidence of behavioral effects from these exact manipulations (Kwapis et al., 2017; Malvaez et al., 2013), (2) determining the meaning of molecular differences with large differences in behavior becomes problematic, and (3) the requirement that activity or learning be engaged similarly at the time of measurement without the ability to test learning later. With the

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primary goal of determining late behavioral endpoints in extinction and reinstatement, the potential benefit of obtaining molecular endpoints was limited.

Similar designer drugs from different companies (e.g., Abcam, Selleck Chemicals, ApexBio, and Santa Cruz) and advanced measures (i.e., ChiP, proteomic analysis) are continuing to elucidate the molecular effects of related endpoints. As a final point, it would be useful for these future studies to investigate other brain regions that may be involved in operant based learning, reliant on both contextual and reward stimuli. While evidence from this dissertation indicates that the DH is involved in these behaviors, there are other regions and types of structural changes that may be contributing, such as in the ventral hippocampus (Gould & Leach, 2014) or AMY (Kwapis et al., 2017), and with tonotopic plasticity of sensory regions (Bieszczad et al., 2015). Based on this previous literature and others noted, it is possible that HDAC3 changes in the VH (Hobin, Ji, & Maren, 2006), NA (Rogge et al., 2013) or AMY (Kwapis et al., 2017) may modulate acquisition drug-seeking or renewal in a similar way as DH modulation.

## Remaining epigenetic questions and future science directions

Additional histone modifications may be the best targets for research

Research in this dissertation and within the addiction field has largely focused on histone acetylation, and more recently in impairing HDAC3 to induce selective histone acetylation. Additional posttranslational modifications (i.e., phosphorylation, ubiquitination, SUMOylation, and poly(ADPribosylation)) are well understood in other areas of research, providing evidence that they may be additionally warranted targets for treatment and prevention in the field of learning, memory, and addiction. Phosphorylation of serine 10 on histone 3 induces acetylation at a nearby lysine site (H3K9) and potentially others (e.g., H3K14) and subsequent transcription (Brami-Cherrier, Roze, Girault, Betuing, & Caboche, 2009; Cheung et al., 2000; Clayton et al., 2000). It is here that histone phosphoacetylation is thought to occur, subserving a "subset of rapid transcriptional responses" for gene induction (Clayton et al., 2000). These are thought to be mediated by mitogen-activated protein kinase and to lead to CREB and CBP/p300 activation. Although, little research has connected histone phosphorylation or phosphoacetylation to addiction, phosphorylative steps may be initiating or potentiating other posttranslational cascades that are recognized to occur during or after drug use (i.e., acetylation and methylation of histones).

Histone ubiquitination is the addition of a covalently bonded ubiquitin protein to the lysine residue on the N-terminal tails of histones. The addition of these bulky ubiquitin moieties to substrates, such as histones, initiates protein trafficking, degradation or activity, communication, or location changes. Ubiquitination often works in a phosphorylation-dependent manner to control gene transcription. It may also be a signal for active versus inactive chromatin and recruit methyl and acetyltransferases to assist in silencing or activating gene transcription at specific loci, respectively.

SUMOylation is similar to ubiquitination, as it results in the addition of bulky peptides to substrates such as histones. SUMO stands for small ubiquitinlike modifier, one that can recruit HDACs and HP1 and lead to potent

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transcriptional repression (Aguilar-Valles et al., 2014), with the phosphorylation state of substrates (e.g., various kinases, TFs, and histones) inhibiting or enhancing SUMOylation (Wilkinson & Henley, 2012). While the temporal and spatial details are limited to date, it is clear that complex interactions exist between SUMOylation and other histone marks (e.g., acetylation, phosphorylation, and ubiquitination) to repress transcription.

Lastly, histone poly(ADP-ribosylation) is the addition of one or more ADP (adenosine diphosphate)-ribose moieties to acceptor sites such as lysine, arginine, glutamic acid, and aspartic acid of histones. Nuclear proteins, such as histones, interact with poly(ADP-ribose) polymerase 1 (PARP1) to get mono or poly-ADP-ribosylated. The activation of PARP1 can mediate chromatin structure, gene transcription, and environmental stimuli responses of cells, during both development and adulthood (Tulin, Chinenov, & Spradling, 2003). Accordingly, it is well established that PARP1 is required for long-term memory formation by targeting (e.g., p53 and Fos) and binding to a variety of TFs (e.g., NFkB and AP-2), each involved in plasticity or learning and memory processes (R. Liu et al., 2012; Salles, Romano, & Freudenthal, 2014).

While this is just a brief summary of what is still an emerging field, there is additional evidence that these modifications work in a systematic but coordinated fashion, making the interpretation of each modifications exponentially more complex. For example, H3K9me3 initiates and maintains repression of transcription by antagonizing active modifications (e.g., H3K9ac, H3S10p, and H3K4me3,Chen, Kan, & Castranova, 2011). In addition, extensive coordination occurs between histones and DNA to modify the epigenome, facilitating, impairing, or neutralizing transcription. Without the maintenance of DNA methylation, the nucleosome assumes a euchromatic structure and allows abnormal transcription. One example of DNA- and histone-mediated interactions exist (Chen et al., 2003) with the stimulation and calcium influx of fetal rat cortical neurons, resulting in phosphorylation of methyl CpG (cytosine–phosphate– guanine)-binding protein 2 (MeCP2), releasing it from methylated CpG sites of DNA and ultimately decreasing methylation and increasing acetylation at histone 3 lysine 9. Early results from Jones et al. (1998) also demonstrated that MeCP2 binds methylated DNA and recruits HDACs to further stabilize transcriptional repression of chromatin.

Therefore, when MeCP2 is bound to DNA, it is responsible for global gene repression, and when released, HATs and demethylases help remove methylation and acetylate nearby sites (i.e., H3K9) to increase activity at specific gene promoters and increase protein expression (e.g., promoter region of brainderived neurotrophic factor (brain-derived neurotrophic factor (BDNF), Chen et al., 2003). While this is just one example of how DNA and histone-mediated epigenetics can regulate chromatin accessibility and relevant learning and memory proteins, many examples of this coordination exist (discussed further in Lv, Xin, Zhou, & Qiu, 2013), yet fewer studies have targeted specific epigenetic modifiers or coordinated relations between modifiers in relation to addictive behavior. In light of this, many fields of research are now trying to target or at least consider epigenetic regulators that work synergistically to better target complex behaviors like addiction.

### Evidence of combinatorial modifications and addiction

Histone modifications are merely a part of the physiological changes that occur with drug addiction. It is becoming accepted that many histone modifications play a role in the development, maintenance, or potential treatment of drug addiction. Therefore, understanding the upstream and downstream coordination of each modification is becoming increasingly necessary and elaborate. As one might expect, dopamine receptor agonists elicit rewarding effects, but the combination of dopamine agonists and HDAC inhibitors seems to compound these effects, leading to a synergistic increase in cocaine-induced locomotion or sensitization and CPP (Schroeder et al., 2008)

Research by Schroeder et al. (2008) demonstrated that a class I/II HDAC inhibitor (sodium butyrate) and SKF82958 (D1 receptor agonist) increased H3 phosphoacetylation (i.e., phosphorylation at serine 10 and acetylation at lysine 14) in striatal homogenates and increased deacetylation in the substantia nigra/VTA at the promoter regions of BDNF and tyrosine hydroxylase (the ratelimiting enzyme for synthesis of catecholamines like dopamine and norepinephrine). In another study, a dopamine D2 receptor antagonist was found to induce similar changes (Li et al., 2004). Importantly, the molecular effects in the Schroeder et al. (2008) study were sensitive to acute versus repeated administration of these drugs and CPP effects only emerged when the drugs were administered directly after cocaine-induced place preference (presumably during consolidation of CPP learning). This highlights two important points: (1) the transient molecular effects of epigenetic modifications that are known to change after repeated drug use and (2) the experience or activity-dependent nature of histone modifications.

Like other types of post-translational modifications, methylation (i.e., repressive di- and tri-states) and acetylation coordinate to compound the effects of histone modifications (Qiang, Denny, Lieu, Carreon, & Li, 2011). For example, cortical neuron cultures from mice have large increases in activating acetylation at H3K9 that coincide with large decreases in repressive methylation during the time of withdrawal from ethanol. Surprisingly, global and local downregulation of HDMTs (i.e., G9a) at the NMDA receptor gene (NR2B) rather than changes in global or local HATs or HDACs seemed to underlie this effect, underscoring the idea that these modifications work in concert to regulate or dysregulate the system at specific chromatin sites. Correspondingly, intracranial administration of an NMDA antagonist (MK-801) into the NA (but not the medial PFC) decreased H3 phosphoacetylation and acquisition of heroin CPP, while heroin CPP dose dependently increased H3 phosphoacetylation in the NA (but not the PFC; Sheng, Lv, Wang, Zhou, & Hui, 2011). TSA (general HDAC inhibitor) infusions prior to each CPP acquisition session facilitated heroin's effects, further increasing H3 phosphoacetylation in the NA and CPP. Experiments like these clarify the idea that certain brain and chromatin regions and types of histone modifications are targeted based on drug type and administration patterns. While many of the links between ubiquitination, ribosylation, histones, and addiction

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have yet to be uncovered, these marks (like others) present plausible mechanisms by which drugs of abuse, epigenetic processes, and long-term changes take place.

Because histone-mediated epigenetics in addiction is such a new field, many unresolved issues are currently being debated in the literature. Some of these are general debates that are common in all fields in which epigenetic mechanisms are studied. Other debates are more specific to the addiction and learning and memory fields, where very different theoretical approaches have been offered for defining epigenetic mechanisms, the nature of cellular memory and how it relates to psychological memory, and the nature of psychological processes in general, which are open to many different interpretations. The goal in this section is to present a broad overview of some of the debates that are directly related to my dissertation research. A resolution for most of these issues cannot be provided yet, but a survey of the literature and some suggestions about the direction of research in these areas is provided below.

#### Unresolved topics

Are histone marks causal or correlational to downstream processes and do histone modifications change the structure and function of the genome?

Histone modifications, such as HDAC3 regulation, are a contributing factor in a larger dynamic process that regulates accessibility of DNA and transcription (Henikoff & Shilatifard, 2011). By changing the charge between nucleosomes and nearby DNA, histone modifications help maintain an unraveled or raveled chromatin structure and docking area for regulatory modules. This account of histone marks highlights their many correlated and few causative roles in transcriptional regulation (Zhang, Gao, Anandhakumar, & Gross, 2014). As partial justification for a correlative role, histone-modifying enzymes often have effects on other substrates besides histones, leading to multiple effects on the genome, rather than direct effects on transcription only. In addition, it seems that histone modifications play a larger role in stabilizing nucleosome occupancy and position rather than recruiting regulatory factors to DNA. It is unlikely that modifications, like methylation or acetylation, recruit, organize, and direct downstream binding and function of other molecules extensively, since they have minimal binding affinity to most binding modules or regulatory factors.

On the other hand, modifications likely help maintain a particular chromatin state and stability, or instability by altering nucleosome dynamics. This presumably helps to ensure high or low accessibility of DNA by other factors to influence transcription. Nucleosomes are thought to impede the binding of elements to DNA. Therefore, by maintaining the occupancy and position of nucleosomes, histone modifications contribute to changes in chromatin landscape, being one of many events that alter transcription regulation. This is in contrast to the idea, or overgeneralization perhaps, that histone modifications themselves increase and decrease DNA accessibility and are directly activating and repressing transcription (reviewed in Smith & Shilatifard, 2010). This logic corresponds to the idea that histone modifications are not just creating a simple change of charge between DNA and histones to maintain the overall structure of nucleosomes, but they actually create binding sites for regulatory elements necessary for transcription, with specific location and marks combining to recruit specific regulatory proteins and outcomes (Kouzarides, 2007; Liu et al., 2012; Nakamura et al., 2007).

What is the relation between memory at the cellular, organismal, and transgenerational levels?

One of the reasons that epigenetic approaches to addiction are potentially so promising is that successful treatment of addiction requires long-term changes in behavior. Epigenetic changes are associated with long-term changes at the cellular and molecular levels, such as cell fate and cellular memory. However, what is the relation between cellular memory and long-term memory that is distributed among different neurobiological circuits? What is the relation between memory at the neurobiological level and memory at the transgenerational level? These are key questions that are not yet resolved

At the level of the cell, histone-mediated epigenetics may create tags that allow the transcriptional machinery to operate on certain DNA sequences. This activity creates a specific cellular memory that may result in increased rates and levels of transcription and translation the next time the circuit is activated. This cellular memory need not correspond directly to memory on the level of the whole organism because psychological memories are widely distributed and are controlled by many molecular processes. However, the cellular memory created by histone marks in various neurobiological circuits triggers the downstream events that are needed to solidify and maintain these organismal level memory circuits.

There are three primary hypothesis I would like to highlight, each proposing how learning and memory processes may be enhanced through molecular epigenetic mechanisms such as those discussed in this dissertation. The first of which emphasizes that specific epigenetic modifications, such as HDAC3 release and downstream increases in learning related genes, are elicited based on the type of stimuli that activated learning (McQuown & Wood, 2011). The second and related hypothesis proposes enhancements through "informational capture", where the amount of information and specificity of information that is encoded and consolidated can be enhanced with selective inhibition of HDAC3, and potentially others (Bieszczad et al., 2015). Another review highlights the idea that releasing the brake on acetylation early in learning may enhance cellular consolidation in the hippocampus and the transition to system consolidation in the cortex over time, but is likely intertwined with essential protein degradation processes that also mediate long-term effects (Walters & Zovkic, 2015). These theories may support our findings, in that release on HDAC3 early in activated brain regions (i.e., hippocampus) may have enhanced the discrimination of each context or learning contingency and facilitated greater cellular and system consolidation for better recall and encoding in later phases of testing.

Over even greater periods, it has been asked if a histone-mediated epigenetic memory can be transferred across generations. This is a critical question that requires an agreed upon definition of epigenetics. A definition for epigenetics is often disputed, largely on the basis of whether posttranslational changes can be lasting and inherited from cell to daughter cell or parent to offspring (Brumfiel, 2008). While the term genetics implies the necessity of inheritance, less traditional views are incorporating the idea that changes to the transcription and translation of the genetic code may be inherited along with the DNA code itself.

Although some studies have demonstrated that histone-mediated alterations in parents can lead to effects in subsequent offspring during development (Hammoud et al., 2009; Heard & Martienssen, 2014) few addictionrelated studies have tested if offspring can inherit increased risk or protection from parental modifications due to drug use (Bohacek & Mansuy, 2013). In addition, the definitions of trans- and intergenerational inheritance have been broad. As defined by Heard & Martienssen (2014), intergenerational effects are those inherited in utero through parental effects or stimulus exposure. This is in contrast to epigenetic effects that are inherited generations later, without exposure to the stimuli that epigenetically altered gene expression or function initially, generations earlier.

Drug use alters the information inherited by offspring and subsequent brain function (e.g., (Vassoler, Johnson-Collins, Carini, & Byrnes, 2014; Vassoler, White, Schmidt, Sadri-Vakili, & Pierce, 2013) and this may be mediated through histone modifications (Vassoler et al., 2013). In this last study, a cocaineresistant phenotype (i.e., slower rate of acquisition and decreased motivation to administer high doses of cocaine as measured by a progressive ratio schedule of reinforcement) was inherited by male, but not female offspring of parents with extensive and voluntary use of cocaine. The authors showed that this effect was reliant on H3-mediated increases in acetylation levels on the BDNF promoter, subsequent increases in BDNF mRNA in the PFC, and BDNF protein expression. A review by the same group emphasized the likelihood of epigenetic inheritance involved in many diseases, but reiterated that further research on the inheritance of post-translational modifications and drug abuse was needed (Vassoler & Sadri-Vakili, 2014).

Evidence for human trans- and intergeneration epigenetic inheritance is still lagging and a key challenge is to demonstrate that experience-dependent changes, such as those outlined in this chapter, can be passed through multiple generations. One study by Norrholm et al. (2013) demonstrated that PTSD patients with an alleged PTSD risk genotype (i.e., Met/Met single nucleotide polymorphism) had greater fear to an experimental safety signal and were unable to extinguish this fear after training compared to PTSD patients without the risk genotype. This Met/Met versus Val/Met or Val/Val single nucleotide polymorphism at the catechol-o-methyltransferase [COMT] gene likely contributes to increased forebrain dopamine levels (Bilder, Volavka, Lachman, & Grace, 2004; Matsumoto et al., 2003) in carriers. Accordingly, the Met/Met genotype was associated with greater DNA methylation at CpG sites that were also associated with patients experiencing enhanced fear to safety signals. Another study in rodents demonstrated that fear conditioning in an F0 generation mouse can lead to rapid and specific fear learning in F1 and F2 generation mice (Dias & Ressler, 2014). Studies like these (e.g., Norrholm et al., 2013; Vassoler

et al., 2013) will contribute to understanding how epigenetic and DNA based modifications can be inherited and influence health and behavior in subsequent generations.

What theoretical processes are altered by drugs that target histone modifications during treatment of addiction?

Much of the work reviewed here on histone-mediated epigenetics and addiction points to lasting effects on the levels of the cell and behavior. The most widely demonstrated effects on addiction-related memory processes come from studies showing that administration of HDAC inhibitors can promote memory and synaptic plasticity. When thinking of addiction treatment, however, an interesting approach may be to consider how pharmacological administration may alter the ways in which memories are associated with drugs.

As noted above, when memories are retrieved, multiple theoretical processes may be triggered. The memory itself may become labile, necessitating a post-retrieval reconsolidation process that centers on the original drugassociated memory. However, if the expected drug is not administered, the behavior may begin to show extinction – the absence of expected drug leads to a weakening of behavior through the development of an inhibitory memory. A consistent finding from the literature on histone-mediated epigenetics is that HDAC inhibitors paired with drug-related memory retrieval will decrease drugseeking behavior and weaken subsequent relapse. The issue at a theoretical level is why does this occur? The answer to this question has focused on both extinction and reconsolidation, with the distinction between them often boiling down to assumptions that are made about these theoretical processes (Lattal & Wood, 2013).

On one level, behavioral evidence in favor of an impaired reconsolidation account often comes in the form of persistently weakened behavior. Because extinction is often transient, with the response showing spontaneous recovery with time, renewal with context changes, or re-establishment with exposure to drug, any persistent effect is interpreted as an effect on reconsolidation, rather than extinction. Work with HDAC inhibitors, however, challenges this behavioral definition. Because HDAC inhibitors promote memory in a variety of behavioral approaches, it is not difficult to imagine how long-term suppression of drugseeking behavior could result from an enhanced extinction process. Increased histone acetylation and the permissive state of chromatin that it creates should strengthen the consequence of extinction, thereby transforming a potentially weak and transient behavioral experience into one that is long-lasting. Thus, a long-lasting effect on extinction is entirely consistent with an enhanced extinction effect.

On another level, one might examine the molecular processes that are thought to control reconsolidation and extinction. The thought here is that because extinction enhancements involve new memory formation and reconsolidation impairments involve memory elimination, molecular events associated with memory storage and erasure should be uniquely associated with extinction and reconsolidation, respectively. This is a reasonable way to deal with this issue, but the challenge is that it is not specifically known which molecular processes are associated with memory storage or erasure. Identical molecular evidence has been offered for enhanced extinction and impaired reconsolidation (Stafford & Lattal, 2011) suggesting that the field does not yet have a handle on how these processes underlie memory.

Perhaps the most promising level of analysis is to examine the circuits that are activated by histone-mediated enhancements in extinction. The line of thinking with this approach is that if circuits that regulate extinction are hyperactivated by a drug such as an HDAC inhibitor, then this could be taken as evidence that extinction has been enhanced. Indeed, research from our laboratory has used this approach to identify how extinction circuits are regulated by HDAC inhibitors (Stafford et al., 2012). Again, the challenge here is that the circuits that mediate initial consolidation, reconsolidation, and extinction are highly overlapping and interactive. As more is understood about these circuits (e.g., specific AMY projections that mediate extinction, neural substrates recruited by different contextual and developmental influences) this approach will become even more powerful in distinguishing between enhanced extinction and impaired reconsolidation processes.

Beyond extinction and reconsolidation, future work on histone-mediated changes in addiction-related behaviors will need to identify other psychological processes that are altered by these mechanisms. For example, behavioral research has identified several critical variables in the establishment and maintenance of drug-seeking behavior. One critical variable is the prediction error between the abused substance and the cues in the environment, with unexpected outcomes (or absences of outcomes in the case of extinction) having a large impact on the effects of the drug itself (Siegel, 1983) and the strength of the cue-drug association (Schultz, 2007). A second variable is the context in which drug seeking occurs – even after long periods of abstinence, exposure to a drug-associated context is enough to trigger relapse of drug seeking. Although there is a great deal known about how these and other behavioral variables influence drug-seeking behavior, very little is known about how epigenetic events contribute to prediction error and contextual modulation of drug-seeking. *Considerations for histone-mediated treatment of addiction* 

Although researchers are starting to understand how histone modifications lead to downstream changes in transcription, there are considerable gaps that still need to be understood before complete treatment can be accomplished. The exact mechanism of modification recruitment, coordination, and differences between diseases is largely unknown. These gaps in knowledge limit the potential for therapeutics to target aberrant modifications with spatial and temporal specificity. For example, most HDAC and HDMT inhibitors affect multiple isoforms and other non-histone proteins with similar activity. This lack of specificity would limit the control that clinicians have over side effects.

At a psychological level, a great deal of attention has been directed toward the idea that histone deacetylase inhibitors may work in a variety of disorders by improving cognitive function. These drugs hold tremendous promise in treatment of any disorder that involves some type of impaired cognitive function. Research from the rodent laboratory has demonstrated that these drugs are often ineffective on their own – they need to be paired with a behavioral experience, such as extinction. When combined with behavioral interventions, these drugs have great potential to promote treatment outcomes (Davis, Myers, Chhatwal, & Ressler, 2006; Kiefer & Dinter, 2013). However, this promise comes with potential peril, as a cognitive enhancing drug could strengthen the impact of an episode of relapse by promoting the transcriptional events associated with the relapse episode. There is therefore a need to administer these drugs under close clinical supervision, during which the clinician has some control over the experiences that the patient has while on the medication. These basic mechanistic and psychological questions will need to be addressed in the future.

## Concluding statement

Literature reviewed in this chapter makes the argument that environmental stimuli and histone modifications play an important role in the development and maintenance of learning and memory, as well as for potential rehabilitation of addiction. Novel experimental findings presented in this dissertation suggest that contextual cues modulate multiple reward-based learning processes through HDAC3 and dorsal hippocampal activity. Though some questions are still unresolved, there is little doubt that a thorough understanding of the effects and mechanisms of histone modification with specific considerations to context and reward learning will lead to important scientific discoveries with a high likelihood for clinical translation.

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