EXTINCTION OF ETHANOL-SEEKING BEHAVIOR

The role of extracellular signal-regulated kinase signaling and medial prefrontal cortex circuitry in extinction of ethanol-induced conditioned place preference in mice

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By

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CERTIFICATE OF APPROVAL

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ABBREVIATIONS

AMPA *2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid*
ANOVA *Analysis of Variance*
AP-5 *(2R)-amino-5-phosphonovaleric acid*
BCA *Bicyinchoninic Acid*
BLA *Basolateral Amygdala*
BLC *Basolateral Amygdaloid Complex*
BSA *Bovine Serum Albumin*
cAMP *cyclic adenosine monophosphate*
CET *Cue-Exposure Therapy*
CO_2 *Carbon Dioxide*
CPP *Conditioned Place Preference*
CRE *cAMP Responsive Element*
CREB *cAMP Response Element Binding Protein*
CS *Conditioned Stimulus*
DA *Dopamine*
DCS *D-Cycloserine*
DMSO *Dimethyl Sulfoxide*
ECF *Enhanced Chemofluorescence*
ERK *Extracellular Signal-Regulated Kinase*
EtOH *Ethanol*
GABA *γ-Aminobutyric Acid*
HCl *Hydrochloric Acid*
IACUC *International Animal Care and Use Committee*
IEG *Immediate-Early Gene*
IHC *Immunohistochemistry*
IL *Infralimbic Medial Prefrontal Cortex*
IR *Immunoreactivity*
LTD *Long-Term Depression*
LTP *Long-Term Potentiation*
MAPK *Mitogen-Activated Protein Kinase*
MDMA *Methylenedioxymethamphetamine*
MEK *Mitogen-Activated Protein Kinase Kinase*
MFB *Medial Forebrain Bundle*
mGluR *Metabotropic Glutamate Receptor*
MK-801 *(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (dizocilpine)*
mPFC *Medial Prefrontal Cortex*
NAc *Nucleus Accumbens*
NaCl *Sodium Chloride*
NIDA *National Institute on Drug Abuse*
NMDA *N-Methyl-D-Aspartate*
PAGE *Polyacrylamide Gel Electrophoresis*

ABBREVIATIONS (cont.)

pCREB *Phosphorylated cAMP Response Element Binding Protein*
PD98059 *2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one*
pERK *Phosphorylated Extracellular Signal-Regulated Kinase*
PFC *Prefrontal Cortex*
PL *Prelimbic Medial Prefrontal Cortex*
PTSD *Post-Traumatic Stress Disorder*
PTZ *Pentylenetetrazol*
PVDF *Polyvinylidene Fluoride*
SA *Self-Administration*
SDS *Sodium Dodecyl Sulfate*
SL327 *α-[Amino-(4-aminophenylthio)methylene)-2-(trifluoromethyl)phenylacetonitrile)*
SRE *Serum Response Element*
SUD *Substance Use Disorder*
TBS *Tris-Buffered Solution*
THC *Δ9-tetrahydrocannabinol*
U0126 *1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monothanolate*
US *Unconditioned Stimulus*
VTA *Ventral Tegmental Area*
WHO *World Health Organization*

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ABSTRACT

Although studies have begun to elucidate the neurobiological underpinnings of ethanol-induced conditioned place preference (EtOH-CPP), they have primarily been limited to the acquisition and expression phases, leaving the mechanisms underlying extinction of the behavior unknown. Therefore, these studies were intended to further our understanding of the specific neurochemistry and neurocircuitry involved in extinction of EtOH-CPP.

Chapter 2. The first set of experiments examined the effects of the NMDA-receptor partial agonist, D-cycloserine (DCS), on extinction and reconditioning of EtOH-CPP. The results showed that systemic DCS did not alter extinction of both strong or weak EtOH-CPPs. However, DCS (when administered during the extinction phase) prevented subsequent reconditioning of EtOH-CPP.

Chapter 3. The extinction-facilitating effects of DCS depend on ERK signaling, therefore, the second set of experiments was intended to assess the involvement of the ERK pathway in extinction of EtOH-CPP. The MEK inhibitor, SL327, when systemically administered prior to extinction trials did not impair extinction of EtOH-CPP despite causing significant reduction of pERK levels in the brain and suppressing locomotor activity. Furthermore, SL327 did not alter acquisition or expression of EtOH-CPP, or EtOH-induced locomotor sensitization.

Chapter 4. One downstream effector of ERK is the transcription factor CREB. Therefore the next set of experiments used immunohistochemical (IHC) analysis of pCREB and CREB to assess the effects of extinction on EtOH-paired cue-induced regional activation. The results suggested that exposure to an EtOH-paired cue produced an increase in pCREB in the prelimbic (PL) and infralimbic (IL) subregions of the medial prefrontal cortex (mPFC), but not in the nucleus accumbens or amygdala. Extinction of the EtOH-cue association eliminated the activation of PL and IL, suggesting a role for these regions in expression and/or extinction of EtOH-CPP.

Chapter 5. The next set of experiments then examined the effects of electrolytic lesions of the PL and IL subregions of the mPFC on extinction of EtOH-CPP. The results showed that lesions of the mPFC blocked extinction, thereby confirming a role for the mPFC in extinction of EtOH-CPP.

Chapter 6. Although the previous experiments showed that the mPFC is required for extinction of EtOH-CPP, the receptor and/or signaling systems within the mPFC that are involved remain unknown. Therefore, these experiments examined the effects of bupivacaine-induced mPFC inactivation on extinction of EtOH-CPP. The results showed that despite multiple parametric manipulations, neither the vehicle- nor bupivacaine-injected groups showed significant

extinction. Therefore, assessment of the effects of intra-mPFC bupivacaine was obstructed by the incomplete extinction of the vehicle control group suggesting that a component of the procedure prevented normal extinction.

These experiments showed that extinction of EtOH-CPP was not impaired by manipulations of ERK signaling via DCS or SL327. In contrast, IHC and electrolytic lesion experiments strongly suggested that the mPFC is necessary for extinction of EtOH-CPP. Subsequent attempts to utilize an extinction-specific intra-mPFC microinjection procedure were hindered by incomplete extinction in the vehicle-injected group. In conclusion, this dissertation reports the findings of the first systematic examination of the neurochemistry and neurocircuitry underlying extinction of EtOH-CPP in mice.

1

REHABILITATION AND RELAPSE: AN INTRODUCTION TO DRUG-RELATED MEMORIES AND ANIMAL MODELS OF DRUG-SEEKING BEHAVIOR

Introduction

It is difficult to argue against the ongoing need for alcohol and drug research as the statistics on abuse and addiction are staggering. The World Health Organization has reported that over 140 million people throughout the world suffer from alcohol-related disorders, 4% of global deaths are attributable to alcohol, and alcohol is the third leading risk factor for disease behind childhood malnutrition and unsafe sex (WHO, Global Status Report on Alcohol and Health, 2011). The National Institute on Drug Abuse reported that in 2007 in the United States, over 23 million people needed treatment for alcohol or drug problems while upwards of 60% of those individuals who actually received treatment relapsed (NIDA, Principles of Drug Addiction Treatment, 2009). Other groups have reported that between 60 and 90% of drug-dependent patients will relapse at some point following treatment (Brownell et al., 1986). The causes underlying the transition from recreational alcohol and drug use to abuse and subsequent addiction are still not completely understood and depend on a myriad of environmental, biological and genetic factors (*for review see* Koob & Le Moal, 2008). Once the transition from recreational drug use to abuse and addiction has occurred, however, individuals begin an uphill battle involving extensive rehabilitation therapy in hopes of avoiding relapse.

Relapse to drug-taking behaviors remains one of the most difficult obstacles to overcome in the treatment of patients with substance use

disorders (SUDs) (Erb & Placenza, 2011). Relapse among rehabilitated addicts is so widespread because of the overwhelming number and diversity of triggers that are able to reinitiate this detrimental behavior. These triggers can include exposure to a previously drug-associated cue, stressful or traumatic events, or experience with the drug itself (Bossert et al., 2005). A single exposure to a drug-paired environment or a stressful life event is able to reinstate drug craving even after prolonged abstinence because of the persistence of long-lasting changes to the nervous system made during the development of addiction (*for review see* Volkow et al., 2004). Because patients suffering from SUDs most often enter the clinic only after years of drug use, extensive neuroadaptations of reward and learning systems have already occurred. Treatment of SUDs such as alcoholism is even further complicated by the common comorbidity with other psychiatric and mood disorders (Mariani & Levin, 2004). Thus, clinical rehabilitation of patients with SUDs remains a daunting task that constantly challenges the patient's years of drug use and drug-environment learning in hopes of preventing relapse.

Rehabilitation protocols for many disorders, including SUDs, have successfully included cue exposure therapy, or CET, during which patients are exposed to drug or alcohol-paired stimuli in an unreinforced manner (Drummond et al., 1990). For example, CET targeted towards recovering alcoholics has consisted of exposing the patient to the sight, smell and feel of an alcoholic beverage and/or role-play that requires the patient to act out

drinking the beverage (Drummond & Glautier, 1994). Although some reports have shown positive effects of CET, a meta-analysis of nearly 20 clinical trials employing CET for the treatment of SUDs showed that, on the whole, CETs most often fail at increasing rates of abstinence (Conklin & Tiffany, 2002). It is not clear why CET is ineffective but some have hypothesized that CET could be improved by performing CET in multiple contexts outside of the laboratory (resulting in contextual generalization), increasing the number of exposure trials, and/or including additional coping strategies that consist of techniques to actively reduce a patient's physiological reactivity to drug-paired cues (Childress et al., 1993). Interestingly, in contrast to CET in humans, nonreinforced exposure to drug-paired stimuli (experimental extinction) in animals has been shown to be very effective in reducing cue-induced drug-seeking behavior (*for review see*, Millan et al., 2011). Although the reasons for this discrepancy are not completely understood, experimental extinction in animals will certainly continue to provide valuable insight into the different factors that influence the efficacy of CET in humans (Conklin & Tiffany, 2002; Kaplan et al., 2011).

It is for these reasons that it remains imperative to better understand the learning processes that occur specifically during the rehabilitation phase of drug abuse and addiction. Comprehensive preclinical investigations of the neurobiological underpinnings of the post-acquisition phases (i.e., extinction and reinstatement) have continued to provide health care professionals with a

better understanding of how to optimize behavioral and pharmacological therapies (*for review see* Shaham et al., 2003; Taylor et al., 2009). By further weakening the behavioral response elicited by the drug and by its associated cues, manipulations of extinction could serve to help prevent the possible resurgence of the initial drug-memory, thereby helping to reduce the likelihood of relapse (Kaplan et al., 2011; Kalivas & Volkow, 2005). Combining extinction-strengthening and relapse-preventing therapies, including those that strengthen CET, will hopefully provide a way to better treat the overwhelming population of patients struggling to recover from years of substance abuse and addiction.

Therefore, the overarching goal of the experiments outlined in this dissertation is to provide an in-depth examination of the brain regions, and systems within those regions, that are required for the learning processes involved in extinction of cue-induced alcohol-seeking behavior. These experiments will help to identify the similarities and differences between extinction of drug-seeking and other associative behaviors, such as conditioned fear. Furthermore, these experiments will also serve to elucidate the shared and distinct mechanisms that are responsible for extinction of cue-induced approach behavior to different drugs of abuse, including alcohol.

Animal models of drug-seeking behavior

1. Introduction

Animal models of drug- and alcohol-seeking behavior typically fall into two categories: those that involve instrumental behaviors such as self-administration (SA), and those that involve Pavlovian conditioned-behaviors such as conditioned place preference (CPP). These two procedures possess unique and shared representations of the different aspects of drug-seeking and drug-taking behaviors in the clinical patient population and they have been reviewed extensively in the literature (e.g. Sanchis-Segura & Spanagel, 2006).

2. The Self-Administration (SA) model

The SA model of drug-seeking and -taking behavior consists primarily of two types of procedures that require experimental subjects to either initiate consumption behavior (non-operant) or to perform an instrumental response in order to obtain drug access (operant).

In oral consumption SA models, animals are typically provided with an opportunity to directly consume (e.g., drink) a drug solution *ad libitum*. This model is most popular with alcohol studies as it is thought to possess face validity with respect to the human route of administration (Paterson, 2011; Lynch et al., 2010). Although there are many variations of alcohol SA models (*for reviews see* Crabbe et al., 2011; Koob et al., 2003), one of the most

commonly reported models is the 2-bottle preference procedure during which subjects are presented with free access to a bottle of diluted ethanol (e.g., 10% in water) in addition to their existing water bottle. A frequently encountered problem with this model is that animals will not readily consume the novel-tasting solution, and thus these models often require some type of procedural manipulation in order to overcome neophobia and initiate oral intake of the ethanol solution. For instance, sweetening the ethanol solution can help to overcome taste neophobia, resulting in initiation and maintenance of increased ethanol consumption (Samson et al., 1999). Additionally, a sucrose-fading procedure that slowly increases ethanol concentration in a sucrose solution can also result in increased oral consumption (Samson, 1986). Furthermore, procedures that limit the availability of the ethanol solution have also increased consumption and have included intermittent access (e.g., Wise, 1973), limited access following liquid deprivation (e.g., Finn et al., 2005), and limited access during the dark cycle (e.g., Rhodes et al., 2005). However, despite these consumption-increasing manipulations, with only a few of these oral consumption procedures do animals actually reach “intoxicating” levels of blood alcohol concentrations (Crabbe et al., 2011). Therefore, although the oral consumption model of alcohol intake possesses theoretical face validity, in practice, rarely do animals consume enough, and in a short-enough period of time, to reach physiologically relevant blood concentrations.

Instrumental responding models of SA involve animals gaining access to drug only after completing a schedule of specific actions that can include single or multiple lever presses or nose-pokes (*for review see* Sanchis-Segura & Spanagel, 2006). Upon completing the experimenter-implemented instrumental requirement, the animal receives access to drug (e.g., ethanol solution) or a pre-determined infusion of drug via an implanted cannula (e.g. intravenous cocaine). Despite including a pre-training phase, some instrumental procedures still often require extensive drug training that can limit sample size and increase the likelihood of problems arising with the patency of the cannula that was initially implanted for drug delivery (Panlilio, 2011). Another obstacle inherent to some SA procedures is its sensitivity to alterations of locomotor performance. Since these procedures utilize an instrumental response to assess the reinforcing properties of a drug, any manipulation that impairs the animal's ability to accurately make the response could confound interpretation of the behavior—that is, it can be difficult to differentiate between effects on performance versus effects on motivation and learning (Beninger, 1989).

Therefore, although components of the SA model possess face validity (especially with alcohol), these procedures can be labor intensive, as well as susceptible to performance-impairing drug effects and difficulty achieving high levels of intoxication.

3. The Conditioned Place Preference (CPP) model.

In this model of drug-seeking behavior, a neutral stimulus attains significant incentive, motivational value after being repeatedly paired with a drug of abuse. The simplest of CPP procedures most often consists of an initial conditioning phase followed by a drug-free preference test. During the conditioning phase an animal receives passive exposure (generally by injection) to an experimenter-controlled amount of drug followed by exposure to a single or set of distinct, neutral cues (either contextual or discrete). Another set of neutral cues is then paired with passive exposure to the drug's vehicle. Following this straightforward Pavlovian-conditioning procedure, the drug-paired neutral context gains incentive properties, which during the preference test elicits approach behavior to the cue. The reason for this approach behavior remains disputed, but one interpretation is that the behavior expressed during the preference test represents the conditioned reinforcing properties of the drug-paired context (*for reviews see* Bardo & Bevins, 2000; Cunningham et al., 2011). Thus, the animal, upon learning that the context predicts the rewarding effects of a drug of abuse spends a significantly greater amount of time in proximity to the drug-paired context when compared to the saline-paired context (i.e., drug-seeking behavior). The CPP procedure possesses many advantages over SA models of drug-seeking behavior including that it has increased sensitivity to low doses of drug, it is more rapidly conditioned, it is tested in a drug-free state, and it

provides the experimenter with greater control of cue-exposure and administered drug dose (Bardo & Bevins, 2000). Additionally, because CPP uses intraperitoneal or subcutaneous injections and can be rapidly conditioned, it is not as labor or technically intensive as some SA models.

Although there are many advantages to the CPP model of drug-seeking behavior, disadvantages certainly exist. For instance, in contrast to SA, CPP has been criticized for lacking direct face validity as a model of drug-seeking and drug-taking behavior in humans (Bardo & Bevins, 2000). Unlike in the human condition and SA model, CPP involves experimenter-controlled, noncontingent presentation of the drug during acquisition. Although this aspect of the procedure can be valuable (e.g., intoxicating doses can be reliably delivered), it does not reflect human drug consumption. Another criticism of the CPP model is that unlike SA models, CPP lacks a graded dose-effect curve for most drugs of abuse (Bardo & Bevins, 2000). In contrast to the biphasic dose-effect curves produced with SA models, standard cocaine- and EtOH-induced CPP (EtOH-CPP) procedures have reported step-like dose-effect curves where low doses condition equal preferences (or no preference at all) and a wide range of high doses condition equally-strong preferences (Bevins, 2005; Cunningham et al., 1992).

A cursory analysis of these findings would suggest that 1) low doses of cocaine and EtOH are not rewarding and/or 2) that the CPP-procedure is not sensitive enough to detect the rewarding effects of low doses. However, a

modification of the standard-CPP procedure has helped to disprove these assertions. The “reference-dose” procedure utilizes a comparison between cues that have each been paired with the subjective effects of two doses of a drug instead of the standard CPP procedure that simply involves a comparison between drug- and vehicle-paired cues. More specifically, the reference-dose procedure consists of pairing one dose of a drug with a distinct cue and pairing a different dose of the same drug with another cue. A subsequent CPP expression test can then be used to assess the animal’s ability to distinguish between the rewarding effects of the two doses of drug instead of between a drug and its vehicle. By comparing multiple doses of ethanol, Groblewski et al. (2008) showed that the reference-dose procedure was able to detect the rewarding effects of very low doses of ethanol (e.g., 0.5 g/kg) that, in the standard CPP procedure, were unable to condition a place preference. Other groups have reported similar findings utilizing cocaine (Bevins, 2005) and morphine (Barr et al., 1986). By combining the standard and reference-dose procedures, it is therefore possible to use CPP to detect the rewarding effects of a range of doses of a variety of drugs of abuse. As previously mentioned, the CPP procedure is also rapidly conditioned with experimenter-controlled drug doses that can cause intoxication and it is tested in a drug-free state, making the CPP procedure a unique and valuable animal model of drug-seeking behavior.

4. Conclusion

Although each of these behavioral procedures possess many advantages and disadvantages, together, the SA and CPP models provide researchers with a variety of tools with which to model human drug-seeking and drug-taking behaviors. As both of these models represent drug-seeking behavior, it is not surprising that expression of drug-induced SA and CPP behaviors rely on many of the same mechanisms (e.g., D3-type dopamine receptor antagonists attenuate cocaine-CPP and cocaine-SA, *see* Vorel et al., 2002). On the other hand, as previously discussed, these behaviors are also distinct and as such, they also possess different underlying mechanisms (e.g., D2-type dopamine receptor antagonists block cocaine-SA but not cocaine-CPP, *see* Caine & Koob, 1994; Cervo & Samanin, 1995). Thus it is important to consider each of these procedures as unique and separate animal models in their ability to represent the many aspects of drug- and alcohol-seeking behavior in humans.

Because of its reliability, sensitivity, dose control, and lack of labor and technical intensiveness, the EtOH-CPP model is well suited for manipulating and studying aspects of acquisition, expression, and extinction of EtOH-seeking behavior in mice. It is for these reasons that the current set of experiments utilizes the EtOH-CPP procedure to model the different phases of alcohol abuse and addiction in humans.

The phases of addiction and drug-related learning

1. Alcohol and Drug Use and Addiction in Humans

The development of drug dependence and addictive behaviors typically occurs only after extensive, repeated drug exposures and involves biological adaptations that occur at the systems and cellular levels (*for review see* Koob & Volkow, 2010). In its simplest form, the progression from initial drug use to addiction, followed by recovery and relapse, is often modeled as a cycle of behavior similar to that illustrated in Figure 1 (e.g., Koob & Le Moal, 2008, 2001; Paterson, 2011).

This cycle begins with initial drug exposure, most often experienced during recreational drug use. The majority of individuals are able to control and discontinue drug use at this phase and never continue along in this cycle. However, for the individuals who continue to use, this repeated recreational use could lead to a level considered to be abuse. Substance abuse has been characterized by continued drug use despite physical and social harms (adapted from the DSM-IV, 1994). As drug use continues and intensifies during the abuse phase, an individual can become addicted. Addiction represents a complex set of criteria including substance dependence, which is characterized by 1) biological tolerance, 2) withdrawal symptoms and 3) continued, persistent use of a drug despite the knowledge and recognition of its harmful effects (adapted from the DSM-IV, 1994). As addictive use of

Figure 1.

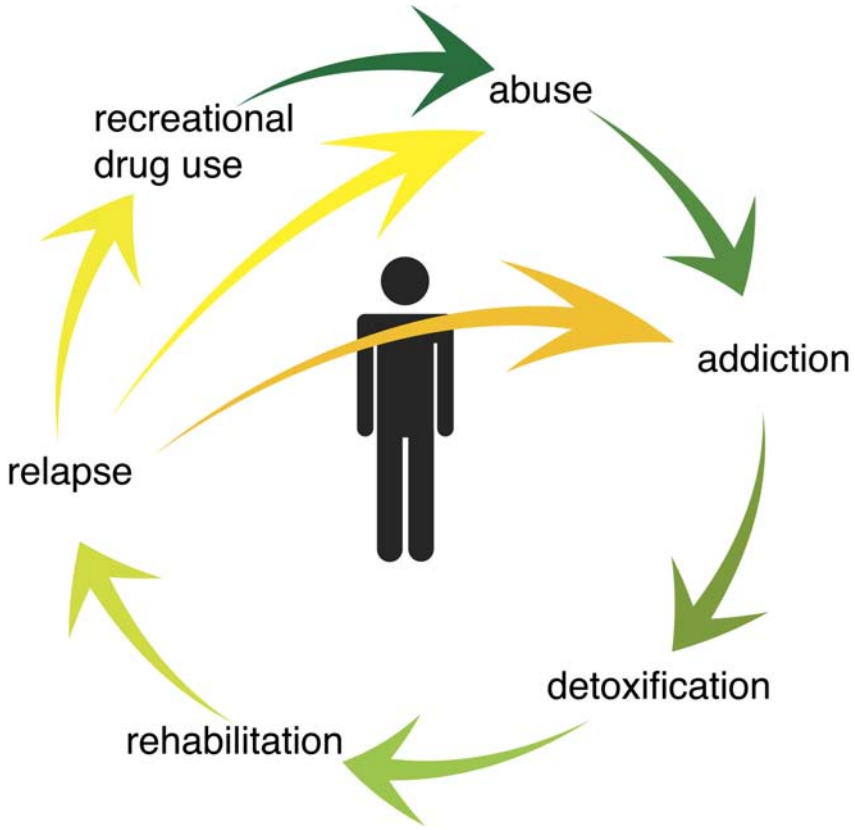

Figure 1. Diagram describing the progression of drug and alcohol addiction in humans. *Original artwork by PAG*

drugs persists, biological adaptations within the central nervous system continue to occur resulting in an altered biological (allostatic) state that is characterized by tolerance, withdrawal symptoms, and/or drug cravings (Koob & Le Moal, 2008, 2001). If an individual is able to discontinue drug use following detoxification, he/she will enter the rehabilitation phase during which the majority of individuals, through behavioral and sometimes pharmacological therapies, attempt to maintain complete abstinence from drug use (e.g., Alcoholics Anonymous). Unfortunately, it is most often the case that patients who are recovering from addiction relapse (Brownell et al., 1986). In some cases individuals who relapse may be able to return to abstinence, but in many instances these individuals re-enter the cycle of drug-use and can then advance back to the abuse or addiction phases.

2. Modeling Human Alcohol and Drug Abuse/Addiction in Animals

The SA and CPP procedures described previously in this chapter provide valuable, albeit simplified, animal models of the different phases of abuse and addiction in humans (Paterson, 2011; Shaham et al., 2003). Similar to the human condition, these animal procedures can also be represented as cyclic progressions of drug-seeking behavior (Figure 2).

The CPP procedure, for example, is capable of examining four general phases of drug-seeking behavior including acquisition, expression, extinction, and reinstatement (*for reviews see* Cunningham et al., 2011; Tzschentke,

Figure 2.

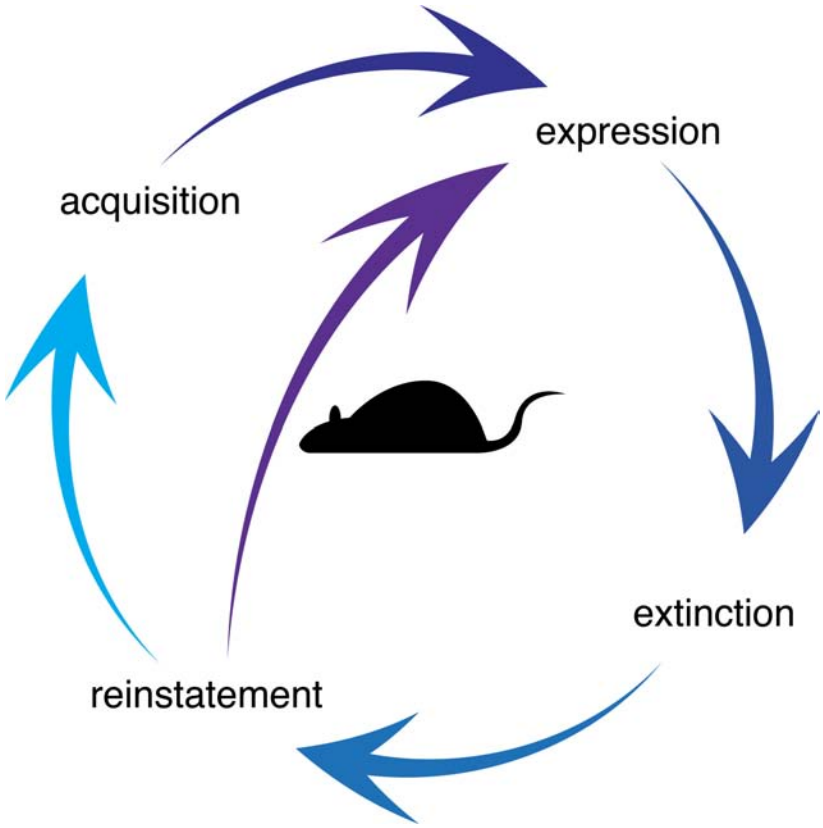

Figure 2. Diagram describing the progression of cue-induced drug- and ethanol-seeking behavior in animals as modeled by conditioned place preference (CPP). *Original artwork by PAG*

2007). The acquisition or conditioning phase represents the initial stages of drug exposure in humans. It is typically during this phase that the animal is first exposed to the drug as well as the drug-paired cues and environment. It is also during this phase that drug-related learning develops and any drug-induced biological changes (e.g., tolerance) may begin to occur. The second phase of CPP is the expression phase during which the animal exhibits the conditioned, cue-induced approach behavior (or drug-seeking behavior) that is learned as a result of the acquisition phase (Cunningham et al., 2011). The extinction phase of CPP follows acquisition and expression and corresponds to the rehabilitation stage in human patients. Similar to CET in humans, extinction of CPP is intended to weaken the cue-elicited behavioral response (resulting from the initial drug-cue association) through repeated exposures to the previously drug-paired cues in the absence of drug (e.g., Groblewski et al., 2009, 2011). Finally, following extinction, expression of CPP can be reestablished during the reinstatement phase. This phase is similar to the human relapse phase in that expression of CPP can be reinstated by a variety of triggers including stressors and drug exposure (*for review see* Aguilar et al., 2009).

3. Conclusion

Comparison of these two schematic representations of addiction and relapse behaviors helps to illustrate the value of animal models of drug-

seeking behavior in modeling the human condition. These models continue to provide important information regarding the neural mechanisms underlying the different phases of drug use and further support the notion that drug use and addiction, as well as rehabilitation and relapse, involve complex interactions between external and internal cues that rely on the convergence of learning, memory, stress and reward systems within the central and peripheral nervous systems (*for reviews see* Duka et al., 2010; Chao & Nestler, 2004; Nestler, 2001; Koob & Le Moal, 2000; Tzshentke, 2007).

Mechanisms of reward-related learning

1. Introduction

Addiction and drug-seeking behavior require recruitment and integration of a number of different systems in the brain and body. As the acquisition, expression, extinction, and reinstatement phases all involve active formation and/or recall of drug-related memories, it is of no surprise that establishing and maintaining drug-seeking behavior requires systems and pathways that are necessary for learning and memory as well as those involved in reward processing (*for review see* Tzschentke, 2007). How these two systems change and converge as an animal progresses through the different phases of drug-seeking behavior remains a key focus of the addiction field.

2. Mechanisms of learning and memory

Learning has been defined as "an enduring change in the mechanisms of behavior involving specific stimuli and/or responses that results from prior experience with similar stimuli and responses" (Domjan, 1998). Within a biological system these "enduring changes" can occur at many levels ranging from an organism's gross motor movements down to signaling at the synapse between two neurons.

Within the central nervous system, learning relies on the cellular changes that occur at the junction between neurons—also termed synaptic plasticity. Synaptic strength depends largely on how often signaling within the synapse occurs. This theory, initially proposed by Donald Hebb (1949) and fittingly termed "Hebbian Theory", suggests that synaptic strength and plasticity depends on how often a presynaptic cell stimulates a postsynaptic cell (*for reviews see* Cooper, 2005; Tsien, 2000). As the frequency of stimulation increases, so does the strength of that synapse. The result of these changes is an increased likelihood that a given postsynaptic cell will respond to stimulation and/or an altered response of the postsynaptic cell to a given stimulus intensity. One of the primary mechanisms underlying synaptic plasticity is long-term potentiation, or LTP—a phenomenon that is characterized by a prolonged period of enhanced synaptic signaling that occurs in a postsynaptic cell following rapid, repeated stimulation (*for review see* Cooke & Bliss, 2006). Conversely, long-term depression (LTD) can also

occur and is characterized by a prolonged period of inhibition, or weakening, of synaptic signaling following repeated, low-frequency stimulation (Dudek & Bear, 1992). Both LTP and LTD, and synaptic plasticity in general, are thought to underlie the phases of learning and memory (Martin & Morris, 2002) and both depend heavily on signaling via *N*-Methyl-D-aspartate (NMDA) receptors located in the synapse. Exogenously applied NMDA can trigger LTP while NMDA receptor antagonists such as (2R)-amino-5-phosphonovaleric acid (AP-5) block induction of LTP (Collingridge et al., 1983) and LTD (Dudek & Bear, 1992). Additionally, on a behavioral level, it is well established that NMDA-receptor activation is required for acquisition of a number of learned tasks including the Morris Water Maze (Morris et al., 1986).

On the cellular level, NMDA-receptor activation results in an influx of calcium that in turn depolarizes the intracellular space (*for review see* Dingledine et al., 1999). The calcium influx activates a number of signaling pathways via cAMP-dependent mechanisms that are thought to underlie the induction of LTP, also referred to as early-LTP (E-LTP) (Abel & Lattal, 2001; Huang, 1998). One group of pathways that is highly influenced by calcium influx is the mitogen-activated protein kinase (MAPK) signaling cascades, to which the extracellular signal-regulated kinases (ERK) pathway belongs. The ERK pathway has been implicated in a number of cellular functions that include cell growth, differentiation, development, and plasticity (including LTP)

(*for review see* Kyosseva, 2004) as well as in the formation of a variety of long-term associative memories (Adams & Sweatt, 2002). A simplified representation of the ERK pathway, including its upstream regulators and downstream effectors, is illustrated in Figure 3.

As the schematic shows, the ERK pathway consists of a cascade of three groups of kinases that each requires phosphorylation to successfully transmit a range of extracellular signals to their downstream effectors (Seger and Krebs, 1995). Specifically, activation of ERK requires the upstream phosphorylation of MAPK-kinase kinase (MAPKKK), which subsequently phosphorylates MAPK-kinase (MAPKK or MEK) leading to phosphorylation of ERK 1/2 and consequent regulation of cytoskeletal, signaling, and nuclear proteins (Adams and Sweatt, 2002). Inhibitors of MEK (e.g., α-[Amino-(4-aminophenylthio)methylene)-2-(trifluoromethyl)phenylacetonitrile; SL327) prevent the phosphorylation of ERK and terminate the signaling cascade by preventing the activation of these downstream targets that include, but are not limited to, transcription factors implicated in learning and memory such as cAMP-response element binding protein (CREB) (Adams et al., 2000). Phosphorylation of CREB in the nucleus results in expression of a number of CRE-containing genes including *c-fos*, *Zif268*, and *JunB* (Adams et al., 2000). Transcription of these and other genes as well as subsequent protein synthesis are thought to underlie the cellular and receptor changes required for maintenance, and expression of, LTP (referred to as late-LTP or L-LTP)

Figure 3.

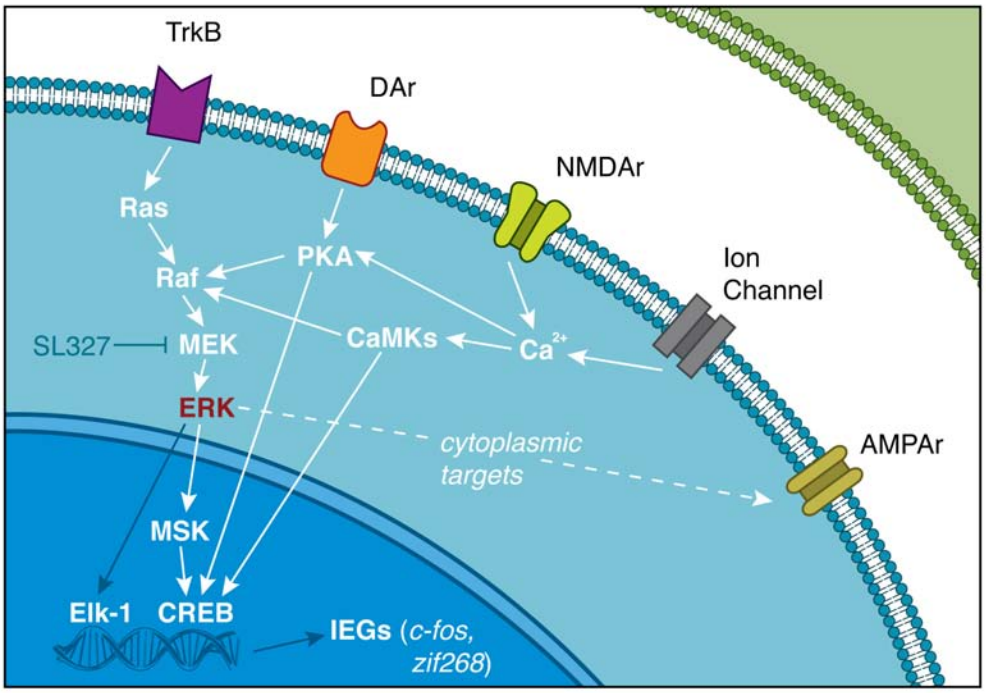

Figure 3. Schematic representation of the extracellular-signal regulated kinase (ERK) pathway including upstream regulators and downstream effectors. Direct intracellular upstream regulators of ERK include MEK (MAPKK) and Raf (MAPKKK) while downstream effectors include transcription factors (e.g., CREB). Notably, CREB can also be regulated directly by CaMKs and PKA. See text for complete description of pathway. *Original artwork by PAG*

whereas activity of the upstream kinases (e.g, ERK) are necessary for induction of E-LTP (*for review see* Sweatt, 1999). Interestingly, in addition to their roles in LTP and short- and long-term memory, both the ERK and CREB pathways play integral roles in the rewarding effects of drugs of abuse (*for reviews see,* Lu et al., 2006; Nestler, 2001).

3. Reward Pathways

Most current theories of reward are based around the basic tenet that the rewarding and/or reinforcing effects of a stimulus such as a drug of abuse rely on their ability to cause an increase in levels of dopamine (DA) in the brain, specifically in regions of the mesocorticolimbic pathway (Wise & Rompre, 1989; 1984). This pathway, colloquially referred to as the "reward pathway," originates in the ventral tegmental area (VTA). The VTA possesses the densest concentration of dopaminergic projecting neurons in the brain and connects with a number of brain regions (Oades & Halliday, 1987). Within the mesocorticolimbic reward pathway, the DA-containing VTA efferents project to the nucleus accumbens (NAc), amygdala, hippocampus, and prefrontal cortex (PFC) (Figure 4). Stimuli ranging from natural rewards to drugs of abuse all result in acute activation of dopaminergic pathways within the mesocorticolimbic pathway (*for review see* Spanagel & Weiss, 1999). The rewarding and reinforcing effects of these stimuli rely heavily on the connection between the VTA and NAc, also known as the medial

Figure 4.

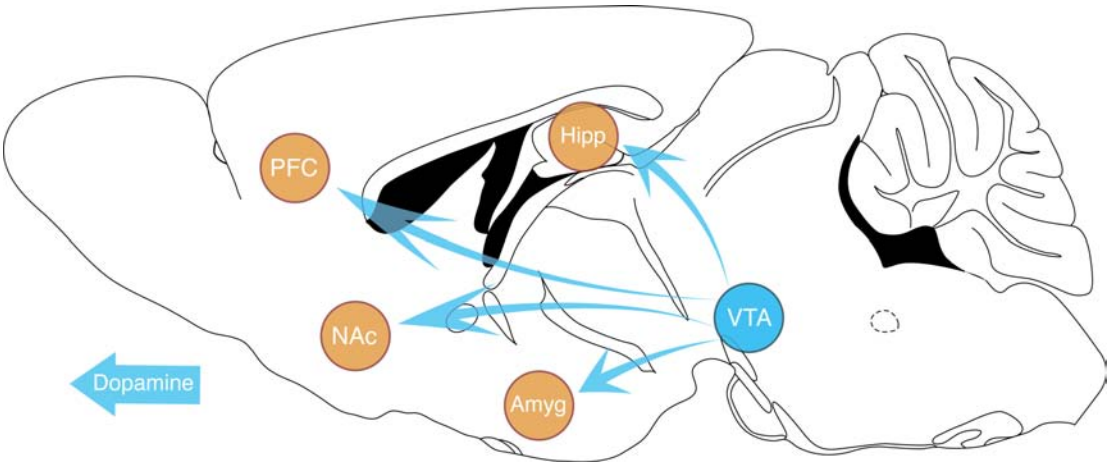

Figure 4. Diagram depicting the dopaminergic projections of the mesocorticolimbic pathway. Dopamine efferents that originate in the ventral tegmental area (VTA) project to the hippocampus (Hipp), prefrontal cortex (PFC), nucleus accumbens (NAc), and amygdala (Amyg). Adapted from Paxinos & Franklin, 2001.

forebrain bundle (MFB). Direct stimulation of the MFB has been shown to be intensely rewarding in both animals (Olds et al., 1960) and humans (Bishop et al., 1963). Drugs of abuse including alcohol, psychostimulants, and opioids are thought to activate the MFB as they all cause a significant increase in extracellular DA levels in the NAc (*for review see* Spanagel & Weiss, 1999). The MFB is not only involved in the acute, direct rewarding effects but also reflects reward-related learning—work most prolifically performed and reported by Wolfram Schultz, who has repeatedly demonstrated the dynamic properties of dopaminergic fiber activity (*for review see* Schultz, 2001).

In models of learning that are based on prediction error such as the Rescorla Wagner Model (1972), learning progresses as a function of the difference between what is expected and what is actually received. With respect to reward-related learning, learning occurs rapidly when the reward is initially presented unexpectedly. As the reward is repeatedly presented it becomes anticipated, thereby reducing the prediction error and slowing learning. Numerous experiments by Schultz et al. have shown that striatal dopaminergic fibers exhibit firing patterns that mimic this type of learning (*for reviews see* Schultz, 2001; 1998). Specifically, when rewards are presented unexpectedly, DA fibers exhibit increased firing rates—an effect that lessens following repeated presentations of the reward. When the reward is paired with presentation of a light or tone stimulus, the stimulus can attain reward value and independently elicit DA-fiber activation. Interestingly, when the

expected reward (or reward-paired stimulus) is omitted, DA fibers exhibit suppression in firing rate (*for review see* Shultz, 2001). These patterns of DA release have also been shown when using drugs of abuse such as cocaine as the reward (e.g., Phillips et al., 2003) and have also been recorded in other areas of the mesocorticolimbic pathway including the mPFC (*for review see* Rushworth & Behrens, 2008). The ability of drug-paired cues to attain control over DA firing may be one of the underlying reasons why cue-induced relapse is so prevalent in recovering drug addicts (Baler & Volkow, 2006). These data using drug and natural rewards show that the pattern of DA release in mesocorticolimbic reward pathway is directly influenced not only by the direct effects of the reward, but also by the reward-related learning that occurs based on a prediction error signal.

4. Integration of reward and learning systems with animal models of drug-seeking behavior

The progression from recreational drug use to abuse and subsequent addiction represents a complex integration of reward and learning systems over an extended period of time. Even during a person's first exposure, drugs of abuse are capable of activating many of the same intracellular signaling pathways that are required for non-drug related learning and memory formation (see Lu et al., 2006; Nestler, 2001). Thus, there exists significant overlap of the reward and learning systems within the brain—both of which

are integral to establishing the drug-seeking behavior that underlies the development of addictive behaviors.

The convergence of non-reward learning systems and reward pathways is most evident when considering the broad influence of drug-related learning. As previously mentioned, drug-seeking behavior during both the initial phases of abuse and relapse represents a combination of the control that drugs, as well as environmental and biological cues, exert over an individual's behavior. Over time, the environmental cues, which have gained incentive value following repeated pairings with the euphoric, biological effects of a drug of abuse, can elicit drug-craving and subsequent seeking behavior (Duka et al., 2010). Additionally, prolonged drug exposure can lead to increased withdrawal symptoms in the absence of the drug, triggering a cascade of dysphoric effects that mimic the compensatory biological responses characteristic of drug tolerance (Koob & Le Moal, 2008). The combination of this withdrawal-induced state and exposure to drug-paired environmental cues can drive drug-seeking behavior and/or trigger relapse (Koob & Le Moal, 2001). It is this type of drug-induced, cue-related learning that is represented in animal models of drug-seeking behavior such as CPP.

The NMDA receptor, which is necessary for induction of LTP, has been shown to be necessary for the acquisition of CPP to a number of drugs of abuse. Systemic administration of NMDA-receptor antagonists prevents the development of cocaine-CPP (Kotlinska & Biala, 2000), amphetamine-CPP

(Toth & Parker, 1999), and EtOH-CPP (Boyce-Rustay & Cunningham, 2004). Additionally, studies have also examined the involvement of intracellular pathways downstream of NMDA receptors in acquisition of CPP. For example, it has been reported that inhibition of ERK signaling with the MEK inhibitor SL327 impairs acquisition of CPP induced by cocaine (Valjent et al., 2000), Δ9-tetrahydrocannabinol (THC) (Valjent et al., 2001), and 3,4-methylenedioxymethamphetamine (MDMA) (Salzmann et al., 2003).

Interpretation of acquisition experiments is complicated, however, because NMDA receptors and their downstream signaling pathways are involved in both the learning component of CPP as well as the direct rewarding effects of a number of drugs of abuse. One of the primary pharmacological targets of EtOH, for example, is the NMDA-receptor (*for review see* Ron, 2004). Therefore, co-administration of an NMDA-receptor antagonist with EtOH during the conditioning phase of EtOH-CPP may interfere with the direct rewarding effects of EtOH, the general associative learning involved in CPP, or both. In some cases, inclusion of a control experiment that examines the effects of the antagonist on EtOH-independent learning, such as lithium chloride-induced conditioned place aversion, can aid in determining the specificity of the antagonist's CPP-preventing effects (e.g., Boyce-Rustay & Cunningham, 2004). For the most part, the literature strongly supports a role for general learning-related receptor systems (e.g. NMDA), intracellular signaling cascades (e.g., ERK), and nuclear signaling

pathways (e.g., CREB) in both the direct rewarding effects of drugs as well as drug-related learning (*for review sees* Tzschentke, 2007; Nestler, 2001).

Regarding acquisition and expression of EtOH-CPP specifically, a number of studies (including genetic and pharmacological manipulations) have shown both similarities and differences in the receptor systems that underlie EtOH-CPP and those induced by other drugs of abuse such as cocaine and morphine (*for review see* Tzschentke, 2007). For instance, the glutamate-receptor antagonist acamprosate blocked acquisition of CPP induced by both cocaine- and ethanol, but not morphine (McGeehan & Olive, 2003). In contrast, the opioid-receptor antagonist naloxone reduced expression of EtOH-CPP (an effect seen only in the later part of the test) while having no effect on expression of cocaine-CPP (Cunningham et al., 1998; Walters et al., 2005). It is important to note that while cocaine- and morphine-CPP studies (including those reviewed by Tzschentke, 2007) have utilized both rats and mice, EtOH-CPP is primarily performed in mice as EtOH has repeatedly been shown to condition a place aversion, instead of place preference, in rats (e.g., Cunningham et al., 1993). The discrepancy in EtOH-CPP between rats and mice has been suggested to result from species differences in the time course of the subjective, hedonic effects of EtOH and/or differences in the ability to associate these effects with the place conditioning cues (Cunningham et al., 1993). The existence of a species difference in CPP for EtOH but not cocaine suggests that these behaviors

may rely on both shared and distinct mechanisms (e.g., Groblewski et al., 2011; *but also* Valjent et al., 2000).

5. Conclusion

Examination of the mechanisms underlying learning and memory and those involved in reward and reinforcement shows a large amount of overlap at the extracellular and intracellular levels. Acquisition studies using CPP have shown that drug-related learning and memory requires many of the same pathways that are necessary for general, non-drug related memory formation. Although it is important to understand the acquisition and expression phases of CPP, it is also imperative to better understand the post-acquisition drug-related learning that occurs during extinction. In most cases patients seek professional treatment for addiction only after years of abuse, during which these patients acquired and established their alcohol- and drug-seeking behavior. Thus, the only opportunity that health care professionals have for manipulating a patient's drug-related learning is during the post-acquisition, or rehabilitation, phase. In some cases, rehabilitation consists of some form of CET, the clinical homolog of preclinical experimental extinction (Kaplan et al., 2011). Because extinction learning represents *de novo* memory formation that is dissimilar to acquisition in many ways, it is important to study the drug-related learning that occurs specifically during extinction of

CPP in hopes of identifying better behavioral and pharmacological treatments that aid in rehabilitation and relapse-prevention (Taylor et al., 2009).

Extinction

1. Introduction

Extinction has been defined as "a rapid and more or less smoothly progressive weakening of the reflex to a conditioned stimulus which is repeated a number of times without reinforcement" (Pavlov, 1927). With respect to the models of drug-seeking behavior described in the previous sections, instrumental (SA) or conditioned (CPP) behaviors are extinguished through repeated presentations of the operandum or conditioned stimulus in the absence of the drug, respectively (Millan et al., 2010; Myers & Carlezon, 2010). The loss of responding that occurs during extinction is not simply a result of "forgetting" the initial association, but instead requires active learning processes that work to inhibit the response elicited by said association (Bouton, 2004). It is during extinction, therefore, that the discrepancy between what a subject knows (i.e., the retained, initially-acquired learned behavior) versus how the subject performs (i.e., decreased responding during extinction) becomes increasingly evident (*for review see* Bouton & Moody, 2004).

The novel, inhibitory learning that occurs during extinction has been most convincingly demonstrated behaviorally using models of reinstatement.

Following extinction, performance of an extinguished behavior can be reinstated, sometimes to pre-extinction levels, upon exposure to any number of reinstatement procedures including contextual renewal, spontaneous recovery, rapid reconditioning, or drug-primed and stress-induced reinstatement (Bouton, 2004). Through differing mechanisms, each of these procedures is able to uncover the original learned association that persisted through extinction. These models of reinstatement further confirm that extinction does not erase the initial memory, but in fact represents a separate, and distinct learning process (Bouton, 2004).

2. Neurobiological mechanisms of extinction learning

Similar to the initial conditioning phase of an associative behavior, the extinction phase has been suggested to actually consist of three different stages of learning and memory: acquisition, consolidation, and retrieval (Abel & Lattal, 2001; Herry et al., 2010). Although these three phases of extinction do possess some distinct mechanisms, they also share many of the same underlying neurocircuits including relying on the BLA as well as receptor systems including requiring NMDA-receptor activation (*for review see* Quirk & Mueller, 2008). Moreover, the distinction between these different phases is not always clearly defined and with some types of extinction procedures (e.g., spaced extinction trials over multiple days) and/or manipulations (e.g., long-lasting pharmacological agents or genetic and lesion models), it is impossible

to differentiate between the phases of learning (Quirk & Mueller, 2008; Abel & Lattal, 2001). It is for these reasons that the extinction experiments described hereafter do not include specific discussion of extinction phases as it is beyond the scope of this dissertation.

Recently, another stage of post-acquisition learning termed "reconsolidation" has begun to receive attention. Reconsolidation refers to the "restabilization" of a memory that occurs when the memory is made labile following a brief, nonreinforced retrieval episode—a process that is thought to depend on the same molecular and cellular mechanisms as consolidation (*for review see* Nader & Orn Einarsson, 2010). In contrast to extinction of EtOH-CPP, our laboratory has been unable to identify procedural parameters that produce results consistent with an EtOH-CPP reconsolidation effect in mice despite other laboratories reporting CPP-reconsolidation with cocaine in rats (e.g., Miller & Marshall, 2005a).

Because extinction requires new learning, it is of no surprise that the molecular signaling pathways necessary for initial acquisition of a behavior are also involved in extinction. For instance, NMDA receptors within the amygdala complex have been shown to be necessary for both acquisition (Miserendino et al., 1990) and extinction (Falls et al., 1992) of conditioned fear. On the other hand, acquisition and extinction also have unique molecular characteristics evidenced by the findings that certain drugs can specifically alter one, but not both, of these learning processes. In contrast to

the antagonists, NMDA-receptor partial agonists such as D-cycloserine (DCS) facilitate extinction, but not initial acquisition, of conditioned fear (Davis et al., 2006). Interestingly, this extinction-facilitating effect of DCS has also been shown to be dependent upon the ERK signaling cascade (Yang and Lu, 2005; Matsuda et al., 2010).

Systemic drug-administration studies have shown that extinction of conditioned fear is impaired by antagonism of NMDA-, cannabinoid-, and opioid-receptor systems (*for review see* Quirk & Mueller, 2008). Interestingly, Cain et al. (2005) reported that the voltage-gated calcium channel blocker nifedipine impaired extinction, suggesting that similar to initial acquisition, extinction learning requires an influx of calcium into the intracellular space. In an attempt to identify possible therapeutic agents capable of strengthening rehabilitation in patients suffering from debilitating associative disorders such as post-traumatic stress disorder (PTSD), researchers have begun to focus on drugs that facilitate experimental extinction. Systemic administrations of drugs targeting the NMDA-, DA-, noradrenergic-, cannabinoid-, and glucocorticoid-receptor systems have all been shown to facilitate extinction of conditioned fear (*for review see* Quirk & Mueller, 2008).

On a circuit level, extinction of conditioned fear has been shown to depend on activity within a number of different brain regions including the amygdala, PFC, hippocampus and periacquaeductal gray (*for review see* Herry et al., 2010). Within the amygdala, NMDA-antagonists (Falls et al.,

1992), MEK-inhibitors (Herry et al., 2006), mGluR-antagonists (Kim et al., 2007) all block extinction of conditioned fear. Conversely, intra-amygdala injection of DCS facilitates extinction of fear (Mao et al., 2006). Within the periaquaeductal gray region, opioid-receptor antagonism with naloxone blocks extinction of conditioned fear (McNally & Westbrook, 2003).

Another region, the PFC, has recently begun to receive significant attention due to its dynamic control of extinction and expression of a number of behaviors including conditioned fear and instrumental drug-seeking behavior (*for review see* Peters et al., 2009). Of particular interest are the ventral subregions of the medial PFC (mPFC). Although the prelimbic (PL) and infralimbic (IL) subregions sit immediately adjacent to each other within the mPFC, their projections possess distinct subcortical targets. In rats, the PL projects primarily to the NAc, olfactory tubercle, amygdala, and dorsal raphe nucleus whereas the IL projects to the lateral septum, NAc, bed nucleus of stria terminalis, amygdala, hypothalamus, and brain stem (Vertes, 2004). Although both subregions project to areas of the amygdala and NAc, these projections do not overlap. The PL projects to the central and basolateral nuclei of the amygdaloid complex, whereas the IL projects to the medial, basomedial, and central nuclei (Vertes, 2004; Cassell, 1989). Similarly, the PL projects to the NAc core, while the IL projects to the NAc shell (Brog et al., 1993). The anatomical specificity of the projections from each of these subregions may be responsible for the differential roles the PL

and IL play in extinction. Specifically, while the PL stimulates expression of conditioned fear (Vidal-Gonzalez et al., 2006), the IL has been shown to inhibit expression of conditioned fear during extinction (Milad & Quirk, 2002). It has therefore been hypothesized that the reduction in expression of a conditioned behavior during extinction is initiated by a switch from PL-controlled excitation of amygdala output to IL-controlled inhibition of amygdala output required for extinction consolidation and subsequent retrieval (Vidal-Gonzalez et al., 2006; Quirk & Mueller, 2008). The vast number and diversity of recent studies investigating the role of mPFC in extinction strongly suggest that the PL and IL subregions of the mPFC play critical roles in the transition between expression and extinction of a conditioned behavior (*for review see* Peters et al., 2009).

It is clear from these studies that extinction represents a unique phase of learning that requires *de novo* memory formation. Studies like these further support the importance of treating acquisition and extinction as separate and independent phenomena; this is especially important when considering these two phases of learning in a drug-seeking framework.

3. Extinction of drug- and EtOH-seeking behavior

Similar to the literature on extinction of conditioned fear, there has been a recent surge in the number of published studies of drug-seeking behavior that include extinction manipulations although on a much smaller

scale (Tzschentke, 2007). Using CPP and SA procedures, these and other studies have begun to identify the receptor and signaling systems and neurocircuitry that underlie extinction of drug-seeking behavior (*for review see* Cleva & Gass, 2010). However, the majority of these studies have involved SA and there have been only a limited number of studies that have systematically examined the mechanisms underlying CPP extinction.

Pharmacological studies of extinction of drug-induced SA have produced results similar to those in the fear extinction literature. Areas including the mPFC, NAc, hypothalamus, hippocampus and amygdala have all been implicated in extinction of drug-induced SA (*for reviews see* Millan et al., 2010; Cleva & Gass, 2010). Similar to extinction of fear, extinction of drug-induced SA is altered by systemic pharmacological manipulations of the glutamatergic- (Cleva et al., 2011) and NMDA-receptor systems (Kelamangalath et al., 2009). Examination of this literature, however, also reveals a number of discrepancies between the mechanisms underlying extinction of aversive (i.e. fear) and appetitive (i.e. drug) behaviors. For instance, administration of cannabinoid receptor agonists facilitates extinction of conditioned fear (e.g., Pamplona et al., 2006) while actually inducing reinstatement of cocaine-SA (e.g., De Vries et al., 2001).

Although few in numbers, systemic and intracranial studies of CPP-extinction have identified some of the receptor and signaling systems to be involved in extinction (*for reviews see* Tzschentke, 2007; Myers & Carlezon,

2010; Cleva & Gass, 2010). CPP-extinction is impaired by systemic administration of NMDA-receptor antagonists (Gass & Olive, 2009), MEK inhibitors (Valjent et al., 2006), adrenergic-receptor antagonists (Davis et al., 2008) and cannabinoid-receptor agonists (Parker et al., 2004). Conversely, extinction of CPP is facilitated by systemic glucose injections (Schroeder & Packard, 2003), cholinergic muscarinic-receptor agonists (Schroeder & Packard, 2004), opioid-receptor antagonists (Cunningham et al., 1998), and NMDA-receptor partial agonists (Botreau et al., 2006). In addition to these systemic studies, the use of genetic models has suggested that the D1-type DA receptors and AMPA receptors are also involved in extinction of CPP (Zhang et al., 2006; Crombag et al., 2009).

Intracranial studies have only begun to expand upon the systemic findings in hopes of identifying the specific brain regions that are critical for extinction of CPP. Intra-amygdala injections of oxotremorine or glucose facilitate, while intra-mPFC injections of AP-5 block, extinction of amphetamine-CPP (Schroeder & Packard, 2003, 2004; Hsu & Packard, 2008). These findings suggest that the cholinergic and NMDA-receptor systems within the amygdala and mPFC are able to regulate CPP-extinction learning. However, it is important to note that these findings, all performed by a single laboratory, involved amphetamine-CPP in rats. Therefore, it remains unclear how these findings would generalize to other CPPs induced by other drugs of abuse (e.g., EtOH) in other species (e.g., mice).

Other studies have utilized temporary or permanent inactivation techniques to examine the involvement of different regions in extinction of CPP. Within the mPFC, Hsu & Packard (2008) showed that temporary inactivation with the sodium-channel blocker bupivacaine blocked extinction of amphetamine-CPP. In another study, Ovari & Leri (2008) showed that inactivation of the ventral mPFC with GABA-receptor agonists reinstated an extinguished heroin-CPP. These findings suggest that the mPFC plays a key role in expression of extinguished behavior. In contrast to these findings, however, Zavala et al. (2003) reported that permanent inactivation of the mPFC via electrolytic lesions had no effect on extinction of cocaine-CPP. Although the reason for these discrepant results is not completely clear, the large number of procedural differences may be an underlying cause (including the type of lesion, when the lesion was administered, and drug used to induce CPP).

The majority of the systemic, intracranial, and lesion studies have involved extinction of CPPs induced by stimulants such as cocaine and amphetamine. With regards to systematic examinations of extinction of EtOH-CPP, there are only two published study that exist outside of the work described in this dissertation. Cunningham et al. (1995, 1998) reported that the opioid-receptor antagonist, naloxone, facilitates extinction of EtOH-CPP when administered prior to repeated preference tests.

Due to the scarcity of existing published studies and the prevalence of discrepancies between other reports of extinction behavior, the mechanisms underlying extinction of EtOH-CPP remain largely unknown. Additionally, as previously described, the mechanisms responsible for extinction vary with the type of behavior (e.g., conditioned fear versus place preference) as well as unconditioned stimuli within a single behavior (e.g., cocaine- versus ethanol-induced CPP). It is therefore imperative to continue investigations of the similarities and differences involved in these diverse extinction processes in order to improve the rehabilitation of patients with SUDs, including alcoholism. Therefore, the following chapters include experiments that systematically examined the extinction phase of EtOH-CPP in mice.

Rationale

The overarching goal of this dissertation was to examine some of the possible receptor systems, intracellular signaling pathways, and neurocircuits that underlie extinction of EtOH-seeking behavior in mice. Analysis of the existing literature has exposed a number of discrepancies between the extinction processes involved in different conditioned behaviors as well as the differences within a single behavior. Furthermore, the mechanisms underlying extinction of EtOH-CPP have received little attention (e.g., Groblewski et al., 2009, 2011; Cunningham et al., 1995, 1998). Therefore the experiments described herein utilize a number of techniques and approaches

to better understand the mechanisms underlying extinction of EtOH-CPP in mice.

The first step in this approach was to begin at a systems level to investigate the involvement of the different receptor and signaling pathways in regulating extinction of EtOH-CPP. Because extinction involves *de novo* learning (Bouton, 2004), and learning depends on NMDA-receptor activation (e.g., Morris et al., 1986), the experiments of **Chapter 2** assessed the effects of the NMDA-receptor partial agonist DCS on extinction of EtOH-CPP. Because DCS has been reported to facilitate extinction of conditioned fear (e.g., Davis et al., 2006) and cocaine-CPP (e.g., Botreau et al., 2006), it was hypothesized that DCS would also facilitate extinction of EtOH-CPP.

The extinction-facilitating effects of DCS have been repeatedly shown to require activation of the intracellular ERK-signaling cascade (Yang & Lu, 2005; Matsuda et al., 2010). Furthermore, inhibitors of ERK-signaling have been shown to impair acquisition of CPP to a number of drugs of abuse (e.g., Valjent et al., 2000; Szapiro et al., 2003) and extinction of cocaine-CPP (Valjent et al., 2006). However, the data regarding expression of drug-seeking behavior is less clear, especially with respect to EtOH-induced behaviors. Specifically, it has been reported that expression of neither cocaine-CPP nor EtOH-SA is affected by inhibition of ERK-signaling (Lai et al., 2008; Carnicella et al., 2008). In contrast to the findings of Carnicella et al. (2008), Faccidomo et al. (2009) reported that administration of a MEK

inhibitor actually increased operant responding for ethanol. Thus, the involvement of ERK-signaling in drug- and alcohol-seeking behaviors including CPP and SA, remains unclear and certainly requires further investigation. Therefore, the experiments of **Chapter 3** examined the effects of inhibiting ERK-signaling with the MEK inhibitor SL327 on acquisition, expression, and extinction of EtOH-CPP. Considering the aforementioned studies, it was hypothesized that SL327 would impair both acquisition and extinction, but perhaps not expression, of EtOH-CPP.

In addition to utilizing systemic studies to identify the mechanisms underlying extinction, it is also important to identify the brain regional specificity of the effects of systemically administered drugs. As such, the remaining studies of this dissertation were focused on identifying and manipulating activity within specific brain regions. Previous studies have shown that exposure to drug- and EtOH-paired cues that are capable of eliciting approach behavior induced neuronal activation in the amygdala, mPFC, NAc, and VTA (e.g., Miller & Marshall, 2005b; Hill et al., 2007), however it remains unknown how extinction of the drug-cue contingency affects these regions. Therefore, **Chapter 4** examined the effect of extinction on neuronal activation induced by a brief exposure to an EtOH-paired cue. Cells that contained pCREB (a marker of neuronal activation) were counted in the amygdala, NAc core and shell, and the PL and IL subregions of the mPFC following cue exposure. As these regions have all been implicated in cue-

induced drug-seeking behavior and/or extinction, it was hypothesized that they would all show effects of extinction on cue-induced activation.

In agreement with the aforementioned immunohistochemistry results showing cue-induced activation of the mPFC, previously published reports have shown that the mPFC plays a key role in extinction of CPP (e.g., Hsu & Packard, 2008). However, other reports have shown that the mPFC lesions had no affect on CPP extinction (Zavala et al., 2003). In order to help clarify this discrepancy and try to confirm a causal role of the mPFC in extinction of EtOH-seeking behavior, **Chapter 5** describes experiments that involved electrolytic lesions of the mPFC prior to the extinction phase of EtOH-CPP. In light of reports supporting a role of the mPFC in extinction of a number of Pavlovian-conditioned behaviors (*for review see* Quirk & Mueller, 2008), it was hypothesized that mPFC lesions would impair extinction of EtOH-CPP. In order to further characterize the involvement of the mPFC in extinction of EtOH-CPP, it was necessary to identify the optimal parameters for extinction-specific intracranial injections, which would allow for subsequent pharmacological manipulations. Therefore, **Chapter 6** describes a series of experiments that attempted to assess the effects on extinction of EtOH-CPP of temporary inactivation of the mPFC by intracranial injections of bupivacaine. Because it has been reported that intra-mPFC bupivacaine impaired extinction of amphetamine-CPP (Hsu & Packard, 2008), it was

hypothesized that this same manipulation would also impair extinction of EtOH-CPP.

Together, the series of experiments described in this dissertation were designed to provide a better understanding of extinction of EtOH-seeking behavior. Successful rehabilitation and prevention of relapse continue to be the greatest obstacles that patients suffering from SUDs face. Through the use of animal models of drug- and alcohol-seeking behaviors including CPP, pre-clinical research such as the studies described hereafter, aim to shed light on novel and improved ways to help strengthen the rehabilitation process in hopes of reducing the likelihood, or even preventing, relapse.

2

EFFECTS OF D-CYCLOSERINE (DCS) ON EXTINCTION AND RECONDITIONING OF ETHANOL-SEEKING BEHAVIOR IN MICE

Contributions by:
Groblewski PA, Lattal KM, & Cunningham CL

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Groblewski PA, Lattal KM, Cunningham CL. Effects of D-cycloserine on extinction and reconditioning of ethanol-seeking behavior in mice. Alcoholism: Clinical and Experimental Research. 2009: 33(5): 772-82.

ABSTRACT

D-Cycloserine (DCS), a *N*-methyl-D-aspartate receptor partial agonist, has been shown to enhance the extinction of both cocaine and amphetamine-induced conditioned place preference (CPP). However, there have been no reports of the effects of DCS on the extinction of ethanol-conditioned behaviors in mice. Thus, the current experiments examined the effects of DCS on the extinction and subsequent reconditioning of ethanol-induced CPP in mice. Male DBA/2J mice received either 2 or 4 pairings of ethanol (2g/kg) with a CS+ floor cue (and an equal number of saline pairings with a CS- floor cue on alternate days) resulting in either a weak or strong ethanol CPP, respectively. Following conditioning of a strong ethanol CPP mice received saline or 30 mg/kg DCS prior to each of the twelve 30-min choice extinction trials administered at 48-hr intervals. Mice that had received conditioning of a weak ethanol CPP received saline, 30 or 60 mg/kg DCS immediately before each of six 30-min choice extinction trials. Following successful ethanol CPP extinction, mice received reconditioning trials similar to the initial conditioning trials. A final experiment examined the effects 12 DCS pre-exposures (15, 30, and 60 mg/kg) on initial conditioning of ethanol CPP. First, the results showed that neither of two doses of DCS (30 and 60 mg/kg) had aversive properties that could confound the effects on extinction of CPP (Exp. 1). Second, we showed that DCS (30 or 60 mg/kg) had no effect on the rate of extinction of either strong (Exp. 2) or weak (Exp. 3) ethanol-induced CPP. Interestingly, DCS administered during extinction interfered with reconditioning of ethanol-induced CPP—an effect specific to reconditioning, as DCS pre-exposure did not influence initial ethanol CPP conditioning (Exp. 4). These experiments show that although DCS showed no effect on extinction behavior, when given during extinction it interfered with subsequent reconditioning of ethanol CPP. The mechanisms of this effect were not, however, due to nonspecific interference with learning because repeated DCS pre-exposures did not impair initial conditioning of ethanol CPP.

INTRODUCTION

Successful attenuation of alcohol-seeking behavior, as well as prevention of relapse to these behaviors, continues to be a considerable challenge in the rehabilitation process in alcoholic patients. Pharmacotherapies, as an adjunct to cognitive behavioral therapy, may serve as a means by which the extinction of alcohol-seeking behaviors can be enhanced and strengthened, thereby reducing the likelihood of future relapse. Although a number of pharmacotherapies have recently been explored, one such drug, D-Cycloserine (DCS), has received a great amount of attention for its ability to augment exposure-based behavioral therapy in a number of cognitive disorders including social anxiety (Hofmann et al., 2006), obsessive-compulsive disorder (Kushner et al., 2007), and acrophobia (Ressler et al., 2004). However, to our knowledge, no published clinical studies have examined the effects of DCS on the extinction of alcohol-seeking behaviors. As such, the current set of experiments was aimed at examining the effects of DCS on extinction of, and relapse to, ethanol-seeking behavior using ethanol-induced conditioned place preference (CPP) in mice.

Because it has been firmly established that glutamatergic transmission via the *N*-methyl-D-aspartate (NMDA) receptor is important for the encoding and recall of memories (for review see Riedel et al., 2003), NMDA-receptor transmission may be an ideal target for pharmacological enhancement of the extinction learning process. Enhancing extinction of conditioned behavior, both fear and drug-related, may improve the rehabilitation process and increase the

long-term persistence of the extinguished behavior in humans, thereby reducing the potential of relapse.

One possible way to facilitate extinction via NMDA transmission may involve receptor partial agonists at the glycine site, as these compounds are capable of modulating channel activity (Johnson & Ascher, 1987) without resulting in the cellular toxicity seen with direct NMDA agonists (Deupree et al., 1996). One such partial agonist, DCS, has shown some promising results in its ability to enhance extinction in a variety of associative learning paradigms in rodents. For example, Walker et al. (2002) showed that DCS facilitated the extinction of freezing behavior in a conditioned fear procedure. This effect, however, was specific to the extinction phase, as DCS given during acquisition had no affect on the initial learning of the freezing behavior (Davis et al., 2006). Moreover, the facilitating effects of DCS on extinction of conditioned fear are blocked by co-administration of inhibitors of the extracellular signal-regulated kinase (ERK) pathway (Matsuda et al., 2010; Yang & Lu, 2005). Thus, it appears that DCS faciliates extinction by promoting NMDA-receptor activation of the ERK signaling cascade.

The majority of the reported experiments showing extinction-facilitating effects of DCS have involved conditioned fear and only a few studies have reported the effects of DCS on the extinction of learned behavior involving appetitive stimuli. Recently, however, DCS has been shown to accelerate extinction of a cocaine-induced place preference in rats when administered

immediately after repeated nonreinforced preference tests (Paolone et al., 2008; Botreau et al., 2006). Additionally, Kelley et al. (2007) showed that a DCS injection administered immediately before a 20-min preference test reduced the expression of cocaine-induced CPP, an effect that persisted to a second CPP test 7 days later. Further, Sakurai et al. (2007) found that a single set of bilateral, intrahippocampal DCS injections prior to the first of four non-reinforced amphetamine CPP preference tests facilitated extinction of the place preference compared to saline-injected controls—an effect that was significant only on the fourth test. Although these studies appear to indicate that DCS may facilitate the extinction of cocaine- and amphetamine CPP, this effect has not been examined using other more commonly abused drugs such as ethanol. Recently, however, it was reported that DCS facilitated extinction of an ethanol-associated operant behavior in rats (Vengeliene et al., 2008). Thus, it appears that the effects of DCS exist across a variety of drugs and drug-seeking behaviors.

The current set of experiments was conducted to determine the effect of DCS on the extinction of both strong and weak ethanol-induced CPP (achieved by varying the number of conditioning trials) in DBA/2J mice. Given that DCS has been shown to facilitate extinction of both cocaine- and amphetamine CPP and conditioned fear, we hypothesized that DCS would also facilitate extinction of ethanol-induced CPP. Additionally, these experiments examined the long-term effects of injections of DCS during extinction by examining the subsequent reconditioning of the initial place preference. Rapid reconditioning has been

used to show that extinguished behavior can be reinstated by a single presentation of the original Pavlovian association (Pavlov, 1927). This phenomenon, when examined in a CPP procedure, can be viewed as one of many models of relapse to drug-seeking behavior (Kehoe & Macrae, 1997). Because previous reports have shown that exposure to DCS during extinction hinders subsequent reinstatement (Ledgerwood et al. 2004), but not reconditioning (Ledgerwood et al. 2005) of conditioned fear, it was hypothesized that DCS given during extinction would have no effect on reconditioning of ethanol-induced CPP after extinction.

Also, to assess possible confounding effects of DCS on extinction of preference, we characterized the potential hedonic effects of two doses of DCS using our CPP procedure. This control study was included to confirm that any extinction-enhancing effects of DCS are due to its actions on extinction learning and not simply from counterconditioning the initial preference (as may be the case with the anxiogenic agent yohimbine, which has been shown to enhance extinction: File, 1986; Morris & Bouton, 2007). Finally, in order to examine the effects of chronic DCS exposure on ethanol-conditioned learning, animals were exposed to one of three doses of DCS prior to conditioning of ethanol CPP. The results of these experiments will serve to further elucidate the effects of DCS on extinction and reconditioning in hopes of characterizing potential clinical pharmacotherapies for aiding alcohol addiction rehabilitation and prevention of relapse.

MATERIALS AND METHODS

Subjects

Male DBA/2J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 6-7 weeks of age. DBA/2J mice were used because of the extensive literature showing the robustness with which this strain acquires ethanol-induced CPP (e.g. Cunningham et al., 2006). Upon arrival in the animal colony, mice were given a minimum of 2 weeks to acclimate before any experimental procedures. Mice were housed in groups of three to four with *ad libitum* food and water in polycarbonate cages that were ventilated in a Thoren rack. The colony temperature was maintained at 21±1° C and lights were on a 12-hr cycle with experimental procedures performed during the light cycle (between 0700 and 1900 hr). The Oregon Health & Science University IACUC approved all experimental procedures.

Drugs

Ethanol (20 % v/v in isotonic saline) was administered intraperitoneally at a dose of 2 g/kg (12.5 ml/kg). Vehicle injections consisted of isotonic saline administered in a volume of 12.5 ml/kg. D-Cycloserine (DCS) solutions were prepared daily in 1.5, 3, and 6 mg/ml concentrations by dissolving the powder (Sigma Aldrich, St. Louis, MO) in isotonic saline. DCS was administered in a volume of 10 ml/kg, yielding doses of 15, 30, and 60 mg/kg.

Place Preference Apparatus

The conditioning apparatus used in all experiments was identical to that described in detail by Cunningham et al. (2006). Briefly, acrylic and aluminum rectangular conditioning boxes (measuring 30 x 15 x 15 cm) were housed in sound and light attenuating chambers (Model E10-20, Colbourn Instruments, Allentown, PA). Each conditioning box was equipped with six, equally spaced, infrared emitter/detector pairs running the length of the box, 2.2 cm above the floor and 5 cm apart. These detectors provided activity counts (expressed as beam breaks per min) and side preference (expressed as time spent on the left and right sides) during all conditioning and test sessions. Activity and side preference data were collected using a computer. The conditioning boxes were equipped with removable grid and hole floors that served as the conditioned stimuli (CSs) for all experiments. The grid floors consisted of 2.3 mm stainless steel rods (spaced 6.4 mm apart) encased in an acrylic frame whereas the hole floors were made up of 16 gauge stainless steel sheets with 6.4 mm diameter holes on 9.5 mm staggered centers. Previous reports from our laboratory have shown that these cues are equally preferred when mice are given a pretest prior to any conditioning (i.e., the apparatus is unbiased: Cunningham et al., 2003).

Place Preference Procedure

Each experiment consisted of some, or all, of the following experimental phases: habituation, conditioning, preference tests, extinction and reconditioning.

For a detailed review of the standard conditioning procedure used in our laboratory see Cunningham et al. (2006).

Experiment 1: Unconditioned stimulus properties of DCS

The purpose of Experiment 1 was to determine whether DCS injections induce any confounding aversive or appetitive effects. Mice (n = 47) were randomly divided into two experimental groups (30 and 60 mg/kg DCS groups) that received habituation, conditioning and preference test phases. Beginning 24 hrs after a 5-min habituation session (animals injected with saline and placed in a conditioning chamber equipped with smooth paper flooring), mice received 8 days of place conditioning, with preference tests occurring 24 hrs after the fourth and eighth days. Each conditioning day consisted of one 20-min exposure to the conditioning chamber with either all grid or all hole flooring (i.e., a one compartment training procedure). This session duration was chosen in order to allow ample time for the drug to be absorbed and distributed as it has been shown that a dose of 400 mg/kg DCS has a half-life of 23 mins in mice (Conzelman & Jones, 1956). Mice were injected with either saline or DCS (30 or 60 mg/kg) immediately before placement on the CS- or CS+ floor, respectively. Exposure to the CS- and CS+ floors, as well as type of injection, alternated over the course of the conditioning sessions in a counterbalanced manner. Animals in the Grid+ (G+) group received drug paired with the grid floor and saline paired with the hole floor. Alternatively, animals in the Grid- (G-) group received saline paired with the grid floor and drug paired with the hole floor. Twenty-four hrs

after the fourth conditioning session, mice were injected with saline and placed in the center of the conditioning chamber prepared with both grid and hole flooring (preference test). Activity and side preference were monitored for 30 mins. Beginning 72 hrs after this test, mice received four additional conditioning sessions (two CS+ and two CS- trials) followed by another preference test.

Experiment 2: Effect of DCS on choice extinction of a strong preference

The purpose of Experiment 2 was to examine the effects of DCS pretreatments on the extinction of a strong ethanol-induced CPP. Mice (n = 48) were randomly divided into two experimental groups (Saline and 30 mg/kg DCS) that received habituation, conditioning, extinction and reconditioning. Animals received our standard ethanol-induced place conditioning procedure (Cunningham et al., 2006) that included a single 5-min habituation session, 8 days of ethanol place conditioning (5 min trials) with 2 g/kg ethanol and 12 preference tests (30-min each). These repeated preference tests, which served as the extinction trials, were given every 48 hrs. Mice in the Saline group received a saline injection immediately before each extinction trial whereas mice in the DCS group received a DCS injection before each extinction trial. Seventy-two hrs after the final preference test (Test 12), the two groups were further divided such that one half of each group received DCS before each reconditioning session and the remaining half received saline. Thus, four extinction-reconditioning groups (DCS-DCS, DCS-Sal, Sal-DCS, Sal-Sal) underwent the 2 days of reconditioning. Reconditioning consisted of one pairing

of the CS+ floor with ethanol and one pairing of the CS- floor with saline (counterbalanced order) followed 24 hrs later by a 30-min preference test. Analysis of the preference test after reconditioning revealed that DCS, when given immediately before reconditioning with ethanol, did not affect reconditioning (i.e., no significant interaction between Extinction Pretreatment and Reconditioning Pretreatment, $p > 0.3$). Specifically, the DCS-DCS group did not differ from the DCS-Sal group and the Sal-DCS group did not differ from the Sal-Sal group. Thus, data from these sets of groups were collapsed to the original two extinction treatment groups for analysis of Experiment 2 (Sal and DCS). Furthermore, because there was no effect of reconditioning treatment in Experiment 2, DCS injections were not administered before reconditioning trials in Experiment 3.

Experiment 3: Effect of DCS on choice extinction of a weak preference

The purpose of Experiment 3 was to extend the findings of Experiment 2 to the extinction of a weak ethanol-induced CPP using multiple doses of DCS. Mice (n=72) were randomly divided into three groups (Saline, 30 mg/kg DCS, and 60 mg/kg DCS) and, as in Experiment 2, underwent habituation, conditioning, extinction and reconditioning. However, animals received a shortened version of our standard ethanol-induced place conditioning procedure that involved only 4 days of conditioning (i.e., two CS+ and two CS- sessions) in contrast to the 8 days given in Experiment 2. After the fourth trial, animals received six extinction trials (30-min each) at 48-hr intervals. Each trial was preceded by either saline or

DCS using the same procedures described for Experiment 2. Extinction was followed by a reconditioning cycle (i.e., one CS+ and one CS- trial) and another 30-min choice test. Because no significant reconditioning was seen in the control group, two additional reconditioning cycles (i.e., a total of two CS+ and two CS- sessions over 4 days) were conducted, each followed by a 30-min preference test.

Experiment 4: Effect of chronic DCS pre-exposure on development of ethanol CPP

The purpose of Experiment 4 was to investigate the effects of repeated DCS injections, administered in the home cage before conditioning, on initial conditioning of ethanol CPP. Mice (n=96) were randomly divided into four groups (Saline, 15 mg/kg DCS, 30 mg/kg DCS, and 60 mg/kg DCS) and received chronic exposure to saline or drug before ethanol CPP conditioning. Specifically, animals received either saline or DCS injections every 48 hrs for 24 days, resulting in a total of 12 injections. This procedure was designed so that animals in Experiment 4 were matched for total amount and pattern of DCS exposure with animals from Experiment 2—the experiment that showed the strongest effect of DCS on reconditioning. Forty-eight hrs after the final pre-exposure injection, all animals underwent standard CPP conditioning for 4 days (2 CS+ and 2 CS- trials) followed by a 30-min preference test. Forty-eight hrs after this first preference test, animals underwent another 4 days of conditioning followed by a final, 30-min preference test.

Statistical Analysis

The primary dependent variable in these studies was the amount of time spent on the grid floor ("grid times") during the post-conditioning, extinction and post-reconditioning preference tests. In this unbiased, counterbalanced CPP procedure, differences between the G+ and G- conditioning subgroups in time spent on the grid floor are used to index strength of place conditioning (Cunningham et al., 2003, 2006). However, to simplify examination of the time-course of extinction across repeated preference tests (Experiments 2 and 3), we also converted these data to percent time spent on the drug-paired floor (collapsed across conditioning subgroups). All data were evaluated using analysis of variance (ANOVA). DCS dose (30 vs. 60 mg/kg in Experiment 1), Drug (Saline vs. DCS in Experiments 2, 3, and 4) and Conditioning Subgroup (G+ vs. G- in all experiments) were treated as between-group factors, whereas Trial Type (CS+ vs. CS-), Trial (conditioning phase) and Test (extinction phase) were treated as within-group factors. Alpha-level was set at 0.05 for all analyses. Additional information about follow-up strategies is reported in the results section for each experiment.

Because of two recent reports suggesting that the effects of DCS on extinction are more apparent in individual animals that showed the greatest amount of extinction learning, we also analyzed the results of Experiments 2 and 3 utilizing the "median-split" method described in these reports (Bouton et al., 2008; Weber et al., 2007). Specifically, a median-split was performed on

extinction data such that each drug-treatment group (Saline and DCS groups) was separated into “extinguishers” (those animals that showed above-median extinction, based on measures described below) and “non-extinguishers” (animals that showed below-median extinction). This median-split analysis was performed for three different measures of extinction including 1) Percent Time spent on the ethanol-paired floor on the final test of extinction (Extinction Tests 12 and 6 for Experiments 2 and 3, respectively), 2) Decrease in Percent Time spent on the ethanol-paired floor from the beginning (Test 1) to the end of extinction (Test 12 or 6 for Experiments 2 or 3, respectively), and finally, 3) Percent Time spent on the ethanol-paired floor during the second extinction trial (Test 2) for animals that showed above or below-median levels of within-session extinction during Test 1 (as indicated by the difference between the First 10-min and the Last 10-min blocks of the 30-min session). This last analysis was performed because it has been shown that the ability of DCS to facilitate extinction is most robust during the first few drug exposures. However, because these multiple statistical analyses did not reveal any new, significant effects of DCS on extinction and therefore added no additional insight, these data were not reported.

RESULTS

Unconditioned stimulus properties of DCS (Exp 1)

Experiment 1 was performed to assess the stimulus qualities of DCS by using two doses of DCS (30 or 60 mg/kg) as the unconditioned stimulus in a standard CPP procedure.

Conditioning Activity. Examination of activity levels during conditioning revealed decreases in activity for both groups over trials, but no effects of DCS dose or trial type. This observation was supported by a three-way repeated measures ANOVA (Dose Group x Trial Type x Trial) that revealed a significant main effect of Trial [$F(3,135) = 12.3$, $p < 0.0001$], but no effects of dose, trial type or interactions (p's > 0.4). Post-hoc analyses showed that activity for both dose groups was lower on the fourth trial (DCS mean = 34.0±1.3; Saline mean = 34.9±1.3) than on the first trial (DCS mean = 40.7±1.0; Saline mean = 40.2±0.9) on both CS+ [DCS: $t(46) = 5.2$, $p < 0.0001$] and CS- [saline: $t(46) = 5.5$, $p < 0.0001$] trials.

Preference Tests. There was no evidence of place conditioning on either the first or second test, with mice spending an average of 48.1±2.8% time on the DCS paired floor (averaged across both dose groups and tests). Two-way (Dose Group x Conditioning Subgroup) ANOVAs applied separately to the times spent on the grid floor in each test failed to yield any significant main effects or interactions, supporting the conclusion that DCS, at doses of either 30 or 60 mg/kg, did not condition either a place preference or aversion.

Test Activity. Prior exposure to DCS during conditioning trials did not affect activity during either drug-free preference test. Mean activity rates (counts/min) for the 30 and 60 mg/kg groups were 33.6±1.5 and 32.0±1.2, respectively (averaged across both conditioning subgroups and tests). One-way ANOVAs showed no group difference during either test.

Effect of DCS on extinction of a strong preference (Exp 2)

Experiment 2 was performed to assess the effects of DCS on the extinction of a strong, ethanol-induced CPP. DCS (30 mg/kg) or saline was administered before each preference test during the extinction phase. Three mice (from the Saline group) were removed from the study due to poor health.

Conditioning Activity. Consistent with previous studies in DBA/2J mice (e.g., Cunningham et al., 2003), activity was higher during CS+ (ethanol) trials than during CS- (saline) trials. Mean activity rates across all CS+ trials were 174.7±6.2 and 170.8±4.6 counts/min for the Saline and DCS groups, respectively. On CS- trials, activity rates for those groups were 45.9±2 and 46.6±1.7 counts/min, respectively. A two-way (Drug Group x Trial Type) repeated measures ANOVA confirmed the significant main effect of Trial Type [$F(1,43) = 930.6$, $p < 0.0001$] and the absence of a significant drug group effect or interaction. Thus, the groups did not differ in activity before the extinction phase.

First Preference Test. Performance on the first post-conditioning test is depicted in Figure 5 both as percent time spent on the ethanol-paired floor (Panel A) and as time (sec/min) spent on the grid floor (Panel B). As can be seen, both

drug groups expressed a robust conditioned preference, with the Saline and DCS groups spending 74.6±4.3% and 77.5±2.2% of the session on the ethanol-paired floor, respectively (averaged across conditioning subgroups; Test 1, Panel A). Conditioned preference was also apparent in the grid times, which showed that G+ subgroups spent more time on the grid floor than G- subgroups (T1, Panel B). Two-way (Drug Group x Conditioning Subgroup) ANOVA of grid time scores confirmed the development of a significant CPP [Conditioning Subgroup effect: $F(1,41) = 154.5$, $p < 0.0001$] that did not differ between drug groups [interaction: $F < 1$]. Unexpectedly, this ANOVA also yielded a significant main effect of Drug Group [$F(1,41) = 5.5$, $p < 0.05$], reflecting the fact that Saline-treated mice spent more time on the grid floor than DCS treated mice, regardless of conditioning subgroup. Overall, these data showed that DCS did not affect the initial magnitude of ethanol-induced CPP and that both groups showed a similar initial preference.

Extinction. Performance over the 12 extinction tests is shown as percent time on the ethanol-paired floor in Figure 5A. Preference declined steadily with repeated testing, but there was no effect of DCS pretreatment on extinction. A two-way (Drug Group x Test) ANOVA of percent time spent on the ethanol-paired floor during each test revealed a significant main effect of Test [$F(11,473) = 18.2$, $p < 0.0001$], indicating that repeated testing produced a decrease in place preference (i.e., extinction). However, there was no significant effect of drug group or interaction, suggesting that DCS did not affect CPP strength or the time

Figure 5.

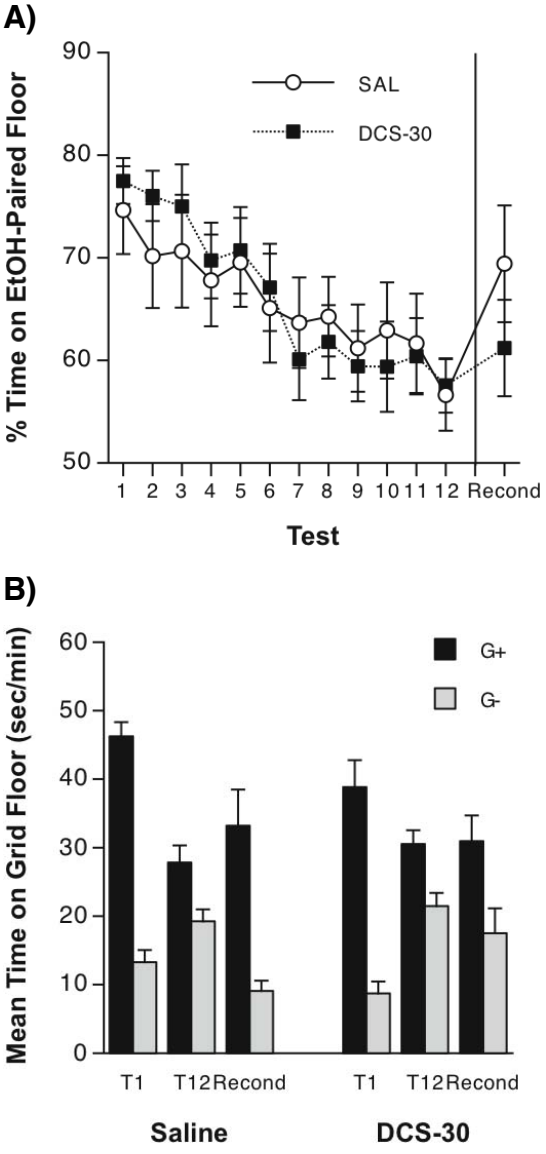


Figure 5. **(A)** Mean ± SEM percent time spent by Saline and DCS (30 mg/kg) groups on the ethanol-paired floor over the course of 12 extinction tests (Tests 1-12) and after reconditioning (Recond). Significant extinction of the strong preference occurred in both groups and was not affected by DCS. The DCS group showed impaired reconditioning (Test 12 vs Recond) of ethanol CPP when compared to the Saline group (a statistical trend toward a significant Group x Test interaction, p = . 051). **(B)** Mean ± SEM time spent on the grid floor for Grid+ (G+) and Grid- (G-) mice on the first extinction test (T1) and before (T12) and after reconditioning (Recond). DCS given before extinction trials impaired reconditioning of a strong ethanol-induced CPP.

course of extinction. A three-way (Drug Group x Conditioning Subgroup x Test) ANOVA comparing grid times on the first and 12th tests (i.e., T1 and T12 in Figure 5B) confirmed conclusions from the percent time analysis, yielding a significant Conditioning Subgroup x Test interaction [$F(1,41) = 78.2$, $p < 0.0001$] that reflected the decrease in CPP magnitude across tests. This analysis also produced significant main effects of Conditioning Subgroup [$F(1,41) = 109.2$, $p < 0.0001$] and Drug Group [$F(1,41) = 4.8$, $p < 0.05$], but no main effect of Test or other interactions. Thus, the analyses of both dependent variables indicated that the DCS and saline pretreatment groups performed similarly during extinction of ethanol-induced CPP.

Reconditioning. Results of the preference test conducted after the reconditioning cycle are shown on the right side of the panels in Figure 5 (Recond). As can be seen, DCS pretreatment on extinction trials impaired reconditioning when compared to the Saline group. A two-way (Drug Group x Test) repeated measures ANOVA of percent time spent on the ethanol-paired floor before and after reconditioning (i.e., Test 12 vs. Recond) revealed a significant Test effect [$F(1,43) = 13.1$, $p < 0.001$] and a trend toward a significant Drug Group x Test interaction [$F(1,43) = 4.0$, $p = 0.051$], consistent with the conclusion that the Saline group showed stronger reconditioning than the DCS group. A three-way (Drug Group x Conditioning Subgroup x Test) ANOVA applied to grid times (Figure 5B, T12 vs. Recond) offered additional support for this conclusion, yielding a significant three-way interaction [$F(1,41) = 4.3$, $p <$

0.05], as well as significant main effect of Conditioning Subgroup [$F(1,41) = 24.4$, $p < 0.0001$] and a Conditioning Subgroup x Test interaction [$F(1,41) = 13.6$, $p < 0.001$]. Two-way (Conditioning Subgroup x Test) follow-up ANOVAs conducted separately for each drug group showed a significant interaction in the Saline group [$F(1,19) = 15.9$, $p < 0.001$], but not in the DCS group [$F(1,22) = 1.4$, $p > 0.25$]. Thus, these analyses suggested that pretreatment with DCS during extinction interfered with subsequent reconditioning after extinction of a strong CPP.

Test Activity. Activity for both groups increased from the first to last of the 12 extinction tests, but there was no difference between groups (Table 1). These conclusions were supported by a two-way (Drug Group x Test) ANOVA of T_1 and T_{12} test activity that yielded a significant main effect of Test [$F(1,43) = 39.1$, $p < 0.0001$], but no significant main effect of drug group or interaction. Also, there was no effect of prior drug treatment during the reconditioning test. Thus, interpretation of the drug effect on reconditioning of a strong CPP was not complicated by group differences in test activity (Gremel & Cunningham, 2007).

Effect of DCS on extinction of a weak preference (Exp 3)

Experiment 3 was performed to assess the effects of saline or two DCS doses (30 and 60 mg/kg) on extinction of an ethanol-induced CPP weaker than that induced in Experiment 2. Saline or DCS was injected only before each extinction test. Two mice were removed from the study, one because of a

Table 1. Experimental Designs, Drug Doses, and Test Trial Activity (counts/min ± SEM)

Exp	Cond. CS+ drug	Cond. Subgroup	*n*	CS+ dose (g/kg)	DCS Dose (g/kg)	First Extinction Trial Activity (counts/min)	Last Extinction Trial Activity (counts/min)	Post-Reconditioning Test Activity (counts/min)
1	DCS	Grid+	12	.03	--	--	--	--
		Grid-	12					
	DCS	Grid+	12	.06	--	--	--	--
		Grid-	11					
2	EtOH	Grid+	11	2	0	**T_1= 29.7±1.9**	**T_{12}= 41.4±2.2**	T_{Recond}= 33.5±2.5
		Grid-	10					
	EtOH	Grid+	12	2	.03	**T_1= 32.7±1.3**	**T_{12}= 43.9±2.1**	T_{Recond}= 36.8±2.3
		Grid-	12					
3	EtOH	Grid+	12	2	0	T_1= 32.0±1.4	T_6= 30.2±1.7	T_{Recond}= 29.3±2.4
		Grid-	12					
	EtOH	Grid+	12	2	.03	T_1= 30.7±1.9	T_6= 28.7±2	T_{Recond}= 23.0±1.9
		Grid-	11					
	EtOH	Grid+	12	2	.06	T_1= 32.7±1.2	T_6= 32.6±1.6	T_{Recond}= 26.7±2.1
		Grid-	11					
4	EtOH	Grid+	12	2	0	--	--	--
		Grid-	12					
	EtOH	Grid+	12	2	.015	--	--	--
		Grid-	12					
	EtOH	Grid+	11	2	.03	--	--	--
		Grid-	12					
	EtOH	Grid+	12	2	.06	--	--	--
		Grid-	12					

NOTE: Values in **BOLD** indicate a significant ($p < 0.05$) main effect of Test from Drug Group x Test repeated measures ANOVA comparing activity from the first and last extinction tests.

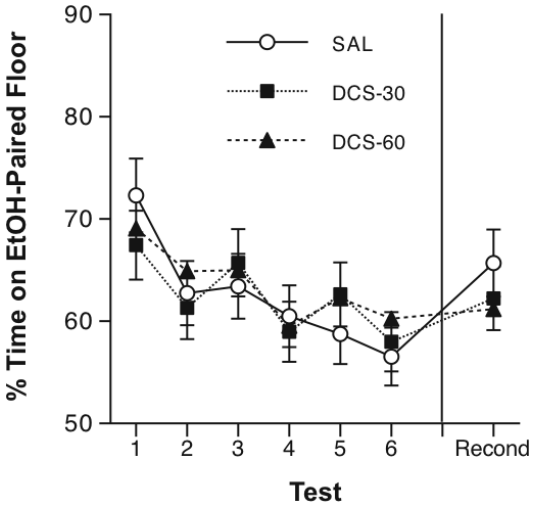
procedural error (DCS-30 group) and the other because of a health problem (DCS-60 group).

Conditioning Activity. As in Experiment 2, animals showed higher activity levels on CS+ (ethanol) trials than on CS- (saline) trials. Mean activity rates across both CS+ trials were 167.3±4.9, 171.6±4.0 and 173.6±5.5 counts/min for the Saline, DCS-30 and DCS-60 groups, respectively. On CS- trials, mean rates for those groups were 54.9± 3.4, 57.9±3.0 and 56.7±2.2, respectively. Ethanol's activating effect was confirmed by a Drug Group x Trial Type repeated-measures ANOVA that revealed a significant main effect of Trial Type [$F(1,67)=1891.3$, $p < .0001$], but no effect of drug group or interaction.

First Preference Test. Performance on the first post-conditioning test is shown in Figures 6A (percent time) and 6B (grid time). All three groups expressed a reliable preference, but (as expected) the preference produced by two CS+ and two CS- trials in Experiment 3 was generally weaker than that induced by four trials of each type in Experiment 2. Averaged across conditioning subgroups, the Saline, DCS-30 and DCS-60 groups spent 72.3±3.7%, 67.4±5.8% and 69.4±3.8% of the test session on the ethanol-paired floor, respectively (Test 1, Figure 6A). All three groups also showed significant place preference as indexed by higher grid time scores in the G+ subgroups than in the G- subgroups (T1, Figure 6B). Two-way (Drug Group x Conditioning Subgroup) ANOVA of grid times showed a significant Conditioning Subgroup effect [$F(2,64) = 63.1$, $p < 0.0001$], confirming development of CPP. However,

Figure 6.

A)



B)

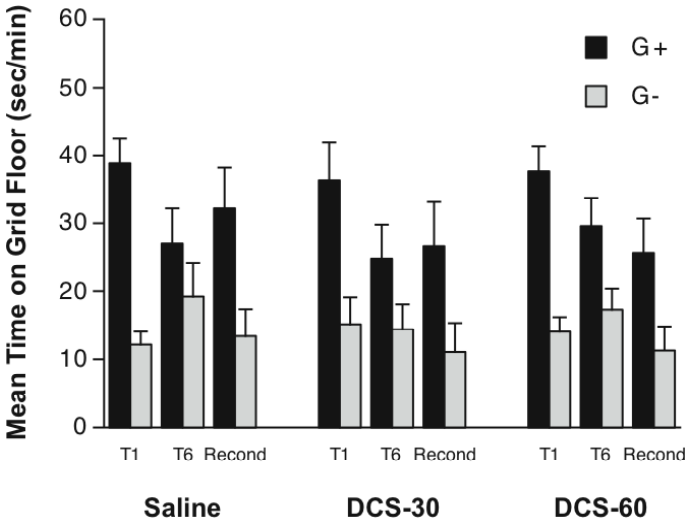


Figure 6. (A) Mean ± SEM percent time spent by both groups on the ethanol-paired floor over the course of six extinction tests (Tests 1-6) and after reconditioning (Recond). Significant extinction of the weak preference occurred in both groups and was not affected by DCS. Further analysis indicated that significant reconditioning occurred in only the Saline group. **(B)** Mean ± SEM time spent on the grid floor for Grid+ (G+) and Grid- (G-) mice on the first extinction test (T1) and before (T6) and after (Recond) the final reconditioning session. DCS given before extinction trials impaired reconditioning of a weak ethanol-induced CPP.

there was no significant effect of drug group or interaction, indicating that DCS did not affect the initial expression of ethanol-induced CPP.

Extinction. Figure 6A shows CPP expressed as percent time on the ethanol-paired floor over the six extinction tests (T1-T6). Although all groups showed a decrease in CPP across tests, neither DCS dose affected rate of extinction. A two-way (Drug Group x Test) repeated measures ANOVA of the percent time data supported this conclusion, yielding a significant main effect of Test [$F(5,335) = 6.8$, $p < 0.0001$], but no effect of drug group or interaction. This conclusion was also supported by a three-way (Drug Group x Conditioning Subgroup x Test) ANOVA of grid times on the first and sixth extinction tests (T1 and T6, Figure 6B), which produced a significant Conditioning Subgroup x Test interaction [$F(1,64) = 19.7$, $p < 0.0001$] and significant main effects of Conditioning Subgroup [$F(1,64) = 32.7$, $p < 0.0001$] and Test [$F(1,64) = 5.5$, $p < 0.05$], but no other effects. Thus, like Experiment 2, these data showed that DCS pretreatment had no effect on extinction of ethanol-induced place CPP.

Reconditioning. The right sides of the panels in Figure 6 show the outcome of the final post-reconditioning preference test. Visual inspection suggests that the Saline group showed a greater increase in CPP between the last extinction test (Test 6) and the reconditioning test (Recond). Although a two-way (Drug Group x Test) repeated measures ANOVA yielded a significant main effect of Test [$F(1,67) = 5.4$, $p < .03$], indicating that reconditioning was successful, the drug group x test interaction was not significant ($p > 0.3$).

Nevertheless, in light of the reconditioning test results in Experiment 2, we examined the reconditioning effect separately for each drug group using a paired t-test (i.e., Test 6 vs. Recond) with the Bonferonni-corrected alpha level set at 0.017. These analyses indicated that only the Saline group showed significant reconditioning [$t(23) = 2.7$, $p = 0.013$].

Our analysis of grid times (Figure 6B, T6 vs. Recond) yielded similar results. A three-way (Drug Group x Conditioning Subgroup x Test) repeated measures ANOVA revealed a significant Conditioning Subgroup x Test interaction [$F(1,64) = 5.9$, $p < 0.02$] and a significant main effect of Conditioning Subgroup [$F(1,64) = 12.7$, $p < 0.001$], but no main effects of drug group or test and no other interactions. Conditioning Subgroup x Test ANOVAs applied separately to data from each drug group indicated that only the Saline group showed significant reconditioning, as confirmed by a significant interaction [$F(1,22) = 7.0$, $p < .015$]. Thus, as in Experiment 2, DCS administration during extinction trials prevented subsequent reconditioning of CPP.

Test Activity. Analysis of test activity from the first (T_1) and last (T_6) extinction tests showed no significant differences in any of the groups (Table 1). This was supported by a two-way (Drug Group x Test) ANOVA that revealed no significant interaction or main effects. Moreover, there was no significant group difference in activity during the preference test after the last reconditioning session, eliminating test session activity differences as an explanation for the group difference in reconditioning of a weak CPP.

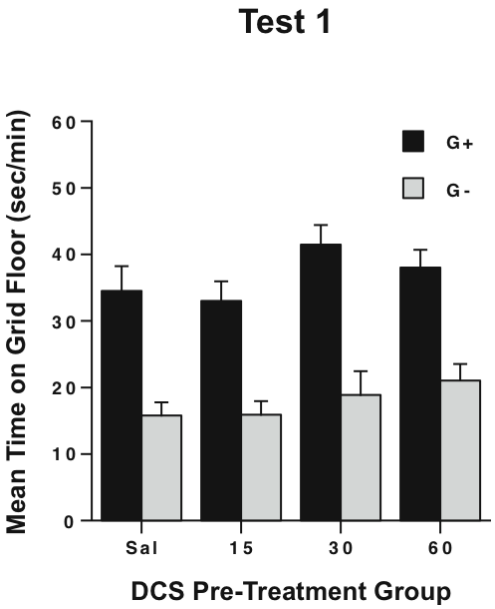
Effect of chronic DCS pre-exposure on development of ethanol CPP (Exp 4)

Experiment 4 was performed to assess the effects of chronic pre-exposure (12 injections every 48 hrs) of three DCS doses (15, 30 and 60 mg/kg) on subsequent development and expression of ethanol-induced CPP. One mouse was removed from the study because of a procedural error (DCS-30 group).

Conditioning Activity. As in Experiments 2 and 3, animals in all groups showed higher activity levels on CS+ (ethanol) trials than on CS- (saline) trials. Mean activity rates across CS+ trials were 141.1±3.8, 142.9±4.8, 137.5±5.2 and 141.7±4.0 counts/min for the Saline, DCS-15, DCS-30 and DCS-60 groups, respectively. On CS- trials, mean rates for those groups were 49.6±2.1, 45.1±1.5, 48.0±1.7 and 49.6±1.9, respectively. Ethanol's activating effect was confirmed by a Drug Group x Trial Type repeated-measures ANOVA that revealed a significant main effect of Trial Type [$F(1,91)=1918.8$, $p < .0001$], but no effect of drug group or interaction. Thus, DCS pre-exposure had no effect on either CS+ or CS- trial activity.

Preference Tests. Two CPP expression tests were conducted after the first two and then again after all four conditioning trials. Data from these two tests are shown in Figure 7 as time spent on the grid floor for animals in the G+ and G- conditioning subgroups. As can be seen, animals in the Saline group as well as all three DCS groups showed significant place preference on Test 1 (Figure 7A) that strengthened in Test 2 (Figure 7B). There was, however, no effect of DCS pre-exposure at any dose on the development of ethanol CPP.

Figure 7.
A)



B)

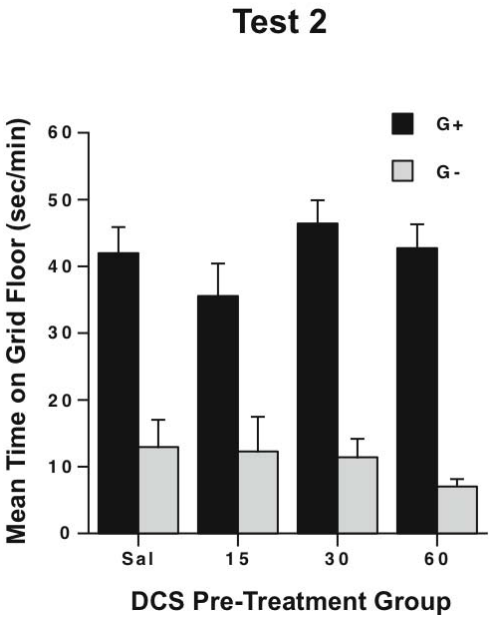


Figure 7. (A) Mean ± SEM time spent on the grid floor for Grid+ (G+) and Grid- (G-) mice on the first preference test (Test 1). Chronic DCS pre-exposure had no effect on development of ethanol CPP after 2 CS+ and 2 CS- trials. **(B)** Mean ± SEM time spent on the grid floor for Grid+ (G+) and Grid- (G-) mice on the second preference test (after a total of 4 CS+ and 4 CS- trials). All groups show signficantly stronger CPP on Test 2 when compared to Test 1 (a significant Conditioning Subgroup x Test interaction), but there was no effect of DCS pre-exposure at any of the tested doses.

The Drug Group x Conditioning Subgroup x Test ANOVA revealed a significant main effect of Conditioning Subgroup [$F(1,87)=141.3$, $p < .0001$] and a significant Conditioning Subgroup x Test interaction [$F(1,87)=26.4$, $p < .0001$], confirming that all groups showed similarly significant place preference that strengthened over the course of conditioning. There was no significant three-way interaction, indicating that DCS pre-exposure had no effect on develoment of ethanol CPP.

DISCUSSION

Although DCS did not enhance the rate of extinction, there was evidence that DCS enhanced the persistence of extinction, i.e., reconditioning of the initial place preference was impaired in DCS-treated mice. The lack of a facilitating effect of DCS on the rate of extinction was consistent across two doses (30 and 60 mg/kg) and two experiments that manipulated the strength of initial place preference by varying the number of conditioning trials. In both studies, however, administration of DCS prior to each extinction trial impaired subsequent reconditioning of the extinguished place preference. This effect on reconditioning was not due to unconditioned effects of DCS because neither dose conditioned a place aversion (Experiment 1) or had any effect on activity levels. Furthermore, the effects of DCS on reconditioning were not due to a nonspecific effect of chronic DCS exposure on learning because DCS pre-exposure before initial conditioning did not impair development of ethanol CPP (Experiment 4).

In Experiment 2, the strongly conditioned place preference resulted in a relatively slow rate of extinction, which should have allowed any extinction-facilitating effects of DCS to be observed. However, because extinction took such a long time in this experiment, it was hypothesized that DCS might be more effective in facilitating the extinction of a weaker, more susceptible, place preference. Because previous work from our laboratory had shown that testing after only two CS+ and two CS- conditioning trials produced a weaker CPP than that seen after four CS+ and four CS- trials (Cunningham et al., 2002), Experiment 3 examined DCS effects on extinction of a weaker ethanol-induced CPP. The results of that experiment showed that the weaker CPP extinguished more rapidly than CPP in Experiment 2 as indexed by the number of test trials required to reduce overall mean preference below 60% (6 tests in Experiment 3 vs. 12 tests in Experiment 2). However, despite beginning extinction with a weaker CPP, mice given DCS before each extinction test did not show an enhanced rate of extinction. Nevertheless, as in Experiment 2, DCS injections administered during extinction impaired subsequent reconditioning.

The results of Experiments 2 and 3 are not consistent with previous reports of the facilitating effects of DCS on extinction of conditioned fear and cocaine-induced CPP. In contrast to our experiments, those studies reported effects of DCS on rate of extinction (e.g., Botreau et al., 2006; Walker et al., 2002). Additionally, the effects of DCS on the reconditioning of an extinguished place preference in the current experiments are not in agreement with previous

reports showing that DCS given during extinction blocks reinstatement, but not reconditioning, of conditioned fear (Ledgerwood et al. 2004; Ledgerwood et al. 2005). Interestingly, in contrast to the results of Ledgerwood et al. (2004), Kelley et al. (2007) showed that extinction-specific DCS administration did not impair reinstatement of cocaine CPP. More recently, Paolone et al. (2008) showed that rats treated with DCS during extinction of cocaine CPP showed an inability to exhibit cocaine-induced reinstatement. However, because the control group (Saline-treated animals) showed no significant reinstatement of cocaine CPP (as indicated by a signficant increase in preference following the cocaine-priming injection), the interpretation of this effect is limited. Thus, considering the current reconditioning effects of DCS, as well as the data from Kelley et al. (2007), it appears that both reconditioning and reinstatement of drug-induced CPP may involve different mechanisms than those involved in the conditioned fear procedures used by Ledgerwood et al. (2004, 2005). However, a more systematic examination of the different methods of reinstatement and reconditioning within each of these behavioral procedures is required to clarify these discrepancies.

In contrast to most of the previously published reports of the extinction-facilitating effects of DCS, the current set of experiments involved examination of two doses of DCS (30 and 60 mg/kg) in the DBA/2J mouse strain. In previous reports, effective doses for the extinction-facilitating effects of DCS in rats have ranged from 5 to 30 mg/kg with the majority of experiments using 15 mg/kg,

whereas in mice, effective doses have ranged from 15 to 30 mg/kg (e.g., Kelley et al., 2007; Tomilenko & Dubrovina, 2007). Interestingly, doses of 15 and 30 mg/kg DCS have been shown to equally enhance extinction of a food-associated operant behavior in C57BL/6 mice (Shaw et al., 2008). However, because DCS can exhibit antagonist-like characteristics at high doses (for review see Lanthorn, 1994), as well as the fact that the cognitive enhancing effects of DCS are eliminated at both very low (i.e., 2.5 mg/kg) and high DCS doses (i.e., 50 mg/kg) in mice (Flood et al., 1992), we hypothesized that the extinction-facilitating effects of DCS in DBA/2J mice would be greatest at a dose of 30 mg/kg. Therefore, although Experiment 1 revealed no detectable stimulus properties or locomotor effects of either 30 or 60 mg/kg DCS, we decided to examine only the lower of these two doses in Experiment 2. Although this dose resulted in no effect on extinction of ethanol CPP (Experiment 2), it did significantly impair subsequent reconditioning and, as such, we are confident that DCS did reach biologically relevant levels. This conclusion is further supported by previous reports showing that an even lower dose of 20 mg/kg DCS has anticonvulsant effects against audiogenic seizures in DBA/2 mice (De Sarro et al., 2000). Therefore, we feel strongly that the doses used in Experiments 2 and 3 should have been sufficient to produce biologically relevant, potentially extinction-facilitating, levels of DCS in the brains of the DBA/2J mice used in these studies. It has been suggested, however, that the cognitive-enhancing effects of DCS may be strain dependent (Sunyer et al., 2008), and therefore, it is possible that DBA/2J mice, although

adept at expressing ethanol CPP, may not be as susceptible to the extinction-facilitating effects of DCS as other mouse strains and/or species.

Studies of DCS on extinction of ethanol-induced learning are particularly interesting because ethanol has direct interactions with the NMDA receptor. Ethanol inhibits glutamatergic transmission of the NMDA receptor by both glycine-reversible and glycine-independent mechanisms (Buller et al. 1995). Further, ethanol exposure can reduce the potency of glycine at its binding site, thereby inhibiting NMDA receptor transmission in rat cerebellar cells (Hoffman et al. 1994). In a study examining the effects of DCS on extinction during withdrawal from ethanol, Bertotto et al. (2006) showed that chronic ethanol exposure (14 days of a 6% v/v ethanol containing liquid diet) prior to acquisition impaired the subsequent extinction of conditioned fear in rats. Furthermore, the previous chronic exposure actually enhanced the extinction-facilitating effects of a sub-optimal dose of DCS. Similarily, in a recently published report, Vengeliene et al. (2008) showed that a low dose of DCS (5 mg/kg), administered 60 mins prior to the extinction trial, facilitated the extinction of an ethanol-paired operant behavior in rats that had previously received extensive ethanol exposure during saccharine-fading, training, and discrimination training. However, because in our study mice were administered only four or two injections of 2 g/kg ethanol over eight or four days (Experiments 2 and 3, respectively), it is unlikely that these sub-chronic ethanol exposures significantly altered NMDA-receptor function in such a way that impaired the effects of DCS during the initial extinction trials.

Because extinction required 12 trials in Experiment 2 and six trials in Experiment 3, we were able to examine the effects of DCS with different amounts of extinction. We found that DCS had no effect on extinction during the initial few trials, when preference was highest, nor did it have effects during later trials, when preference was lower. These findings suggest that the failure to observe effects of DCS on extinction were not due to ceiling or floor effects on preference. However, it is possible that the absence of an effect on the later extinction trials was due to previous exposure to DCS during initial extinction trials. Pre-exposure to DCS has been shown to reduce the learning-enhancing effects of DCS in the Porsolt Swim Test (Lopes et al. 1997), a linear maze apparatus (Quartermain et al 1994), and extinction of conditioned fear (Parnas et al. in 2005). All of these reports hypothesized that pre-exposure to DCS caused a desensitization of the NMDA receptor at the glycine-binding site, thereby reducing the effects of subsequent DCS exposures. Therefore, in the current experiments, the effects of DCS, expected to be the greatest during the first few extinction trials when the greatest amount of learning should occur, were either non-existent or undetectable with our behavioral assay. Any effects of DCS injections later in the extinction phase were most likely hindered by NMDA-receptor desensitization caused by the initial DCS exposures.

Although speculative, the impairment of reconditioning by DCS, evident in both Experiments 2 and 3, may have actually resulted from an extinction-facilitating effect of DCS. Specifically, despite showing no effect on extinction

behavior *per se*, DCS may have actually deepened extinction learning, uncovered by its ability to impair the subsequent reconditioning process. This hypothesis is supported, in part, by the finding that repeated exposure to DCS in Experiment 4 before conditioning had no affect on the new learning that occurs during initial ethanol CPP conditioning. Thus, it seems unlikely that the impaired reconditioning in the DCS groups was simply a result of NMDA-receptor desensitization caused by DCS exposure during extinction.

Given the complexity of the actions of DCS and the procedural sensitivity of the effects of DCS, it is not surprising that several reports have shown inconsistencies in the ability of DCS to enhance extinction. For example, extinction enhancing effects of DCS have not been observed in a variety of behavioral disorders including mild arachnophobia (Guastella et al., 2006) and obsessive-compulsive disorder (Storch et al., 2007). Further, in studies with rodents, DCS effects on extinction may sometimes be limited to low anxiety animals (Tomilenko & Dubrovina, 2007) or to animals that show large amounts of extinction within a session (e.g., Weber et al., 2007). In a recent report, Woods and Bouton (2006) demonstrated that DCS (30 mg/kg) enhanced the rate of fear extinction in rats, but did not weaken contextual renewal of conditioned fear. This effect, however, was not significant at a lower dose of 15 mg/kg. Moreover, when the authors attempted to replicate the significant findings using 30 and 60 mg/kg, they found no effect of either DCS dose.

In a more recent follow-up report, these authors showed that DCS did, in fact, have slight extinction-enhancing effects but only in animals that showed the greatest amount of overall extinction learning. Specifically, Bouton et al. reported that when analyzing only the rats that showed above-median extinction levels, DCS (30 mg/kg) showed signficant facilitation of extinction (Bouton et al., 2008). However, despite using multiple variants of this "median-split" technique to analyze our current set of experimental data, we were unable to detect any extinction-enhancing effects of DCS at any dose. In fact, we performed median-split analyses using three different measures of extinction including preference on the last test of extinction (Tests 12 and 6 for Experiments 2 and 3, respectively), decrease in preference over the course of extinction (calculated as a preference difference score from the first to the last test of extinction), and finally, preference on the second test of extinction (Test 2) for animals that showed above- and below-median within-session extinction on Test 1. This latter analysis was performed after considering previously published reports suggesting that the extinction-facilitating effects of DCS are greatest during the first few extinction trials. All in all, none of these median-split analyses revealed a signficant effect of DCS on extinction of ethanol CPP (data not shown).

In conclusion, these experiments demonstrated that DCS did not facilitate the rate of extinction of ethanol-induced CPP, regardless of the strength of the initial preference. Nevertheless, administration of DCS during extinction impaired subsequent reconditioning of ethanol-induced CPP, demonstrating that DCS did

enhance some aspect of the extinction experience. This was further supported by the findings of Experiment 4 that revealed no effect of chronic exposure to multiple doses of DCS on the initial development and expression of ethanol CPP. These findings emphasize the general importance of using multiple measures of learning to assess pharmacological effects on extinction. Additionally, these findings suggest that DCS may not be capable of facilitating the behavioral therapy used in the rehabilitation of alcoholic patients, though it may provide a means by which to reduce the potential for relapse to alcohol-seeking behavior.

3

INHIBITION OF EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) ACTIVITY WITH SL327 DOES NOT PREVENT ACQUISITION, EXPRESSION, AND EXTINCTION OF ETHANOL-SEEKING BEHAVIOR IN MICE

Contributions by:
Groblewski PA, Franken FH, & Cunningham CL

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Groblewski PA, Franken FH, Cunningham CL. Inhibition of extracellular signal-regulated kinase (ERK) activity with SL327 does not prevent acquisition, expression, and extinction of ethanol-seeking behavior in mice. Behavioural Brain Research. 2011: 317: 399-407.

ABSTRACT

Although extracellular signal-regulated kinase (ERK) activity is essential for the acquisition of a variety of associative learning tasks, its involvement in the acquisition and extinction of ethanol (EtOH)-induced conditioned place preference (CPP) remains unknown. Therefore, in these experiments we examined the effects of the ERK-kinase (MEK)-inhibitor SL327 on acquisition and expression of EtOH-CPP as well as the dose- and time-dependent effects of SL327 on CPP extinction. The parametric findings of Experiment 1 showed that three 30-min (but not 15- or 5-min) non-reinforced trials were required to completely extinguish EtOH-CPP in male, DBA/2J mice. In Experiments 2 & 3, SL327 (30 and 50 mg/kg), administered 30 or 90 mins prior to extinction trials, was unable to impair EtOH-CPP extinction. Experiment 4 showed that SL327 (50 mg/kg) had no effect on acquisition of EtOH-CPP or the development of EtOH-induced sensitization during conditioning. When administered prior to testing in Experiments 5 & 6, SL327 did not alter expression of EtOH-CPP but did reduce test activity. Importantly, SL327 significantly reduced pERK protein levels when assessed in the dorsal striatum and motor cortex (Experiment 7). Together, these data suggest that EtOH-related learning and EtOH reward in mice, as assessed with CPP, are not impaired by the systemically administered MEK-inhibitor SL327.

INTRODUCTION

Reinstatement procedures such as contextual renewal and spontaneous recovery have revealed that extinction of associative learning, like acquisition, requires the formation of new memories (Bouton, 2004). Furthermore, these procedures show that extinction is unique in that although it inhibits responding, the original association remains intact. As such, the processes underlying initial acquisition and subsequent extinction involve both shared and unique neurobiological substrates. For example, NMDA-receptor activation is necessary for both acquisition (Miserendino et al., 1990) and extinction (Falls et al., 1992), but D-cycloserine (DCS), a partial agonist of the NMDA-receptor, only facilitates extinction of conditioned fear (Davis et al., 2006). Interestingly, the extinction-facilitating effects of DCS are dependent on intracellular signaling through the mitogen-activated protein kinase (MAPK)-pathway (Yang and Lu, 2005). Signaling via one such MAPK, the extracellular signal-regulated kinase (ERK), requires phosphorylation of ERK1/2 via MAPK-kinase (MEK) in order to successfully transmit a range of extracellular signals, including those involved in learning and memory (Adams and Sweatt, 2002). Accordingly, MEK inhibitors block acquisition learning in a number of behavioral tasks, including the water maze (Selcher et al., 1999), cocaine conditioned place preference (Valjent et al., 2000) and cue- and context-conditioned fear (Atkins et al., 1998). Similarly, the extinction of conditioned fear is blocked by direct administration of MEK inhibitors into the amygdala, medial prefrontal cortex, or hippocampus (Herry et al., 2006;

Hugues et al., 2004; Szapiro et al., 2003, respectively). However, few studies have examined the involvement of ERK-signaling in extinction of appetitive associative behaviors, such as conditioned place preference.

Conditioned place preference (CPP) is an animal model of drug-seeking behavior that allows for direct manipulations of both acquisition and extinction learning as well as the rewarding properties of drugs of abuse. Understanding the differences between acquisition and extinction of CPP could help identify novel targets for pharmacotherapies that could facilitate the rehabilitation process and reduce the rate of relapse. Thus far, few studies have examined the involvement of ERK-signaling in drug-induced CPP. Inhibition of ERK-signaling with SL327, the only MEK inhibitor that can be systemically administered, impairs acquisition of cocaine-, Δ9-tetrahydrocannabinol (THC)-, and 3,4-methylenedioxymethamphetamine (MDMA)-induced CPP in mice (Valjent et al., 2000, 2001; Salzmann et al., 2003). The effect of MEK inhibition on extinction of CPP has received little attention. Although one study has reported that injection of SL327 before a single extinction session impaired partial extinction of cocaine-CPP (Valjent et al., 2006), it remains unclear how experimental parameters including route of administration, dose, and pre-treatment interval influence the effect of different MEK inhibitors on extinction learning and whether extinction of fear and CPP are differentially dependent on ERK activity. Furthermore, the involvement of the ERK pathway in the acquisition, expression, and extinction of alcohol-seeking behaviors in mice remains undetermined. Therefore, we

performed a series of experiments to examine the dose- and time-dependent effects of SL327 on the extinction of ethanol (EtOH)-induced CPP in mice. Additionally, we examined the effects of SL327 on acquisition and expression of EtOH-induced CPP. Finally, we used western immunoblot analysis of phosphorylated ERK (pERK) levels in multiple brain regions to confirm that SL327 had crossed the blood-brain-barrier and was actively inhibiting ERK signaling. These findings further characterize both the shared and unique biochemical substrates that underlie the acquisition and extinction of drug- and EtOH-induced associative learning and provide insight to the biochemical substrates of EtOH reward in mice.

MATERIALS AND METHODS

Subjects

Adult, male DBA/2J mice (n=432) were obtained from Jackson Laboratory (Sacramento, CA) at 6 weeks of age and allowed to acclimate to the animal colony for 2 weeks before experiments commenced. Mice were housed, four to a cage, in cob bedding in a Thoren rack with water and food available *ad libitum* throughout each experiment. All experiments were conducted during the light phase (7:00-19:00). The Oregon Health & Science University IACUC approved all experimental procedures.

Drugs

Ethanol (20% v/v in isotonic saline) was administered at a dose of 2 g/kg (12.5 ml/kg) in Experiments 1-6 and 2.5 g/kg (16 ml/kg) in Experiment 7. The MEK inhibitor SL327 (Ascent Scientific, Princeton, NJ) was first dissolved in 100% DMSO then diluted with dH_2O, prepared fresh daily. For Experiment 2, SL327 was prepared in 15% DMSO to concentrations of 1.5 and 2.5 mg/ml and administered at 20 ml/kg to achieve doses of 30 and 50 mg/kg, respectively. This drug preparation was identical to that used by Faccidomo et al. (2009). In Experiments 3-7, SL327 was prepared in 50% DMSO to concentrations of 6 and 10 mg/ml and administered at 5 ml/kg to achieve the same doses. This drug preparation was identical to that used by Mouledous et al. (2007) and Matsuda et al. (2010). Matched vehicles were administered at identical injection volumes and pre-treatment intervals for each experiment. All drug and vehicle injections were administered intraperitoneally (IP).

Place Preference Apparatus

All behavioral procedures were performed in custom made, acrylic and aluminum conditioning boxes (30 x 15 x 15 cm), each of which was enclosed in a sound-attenuating chamber (Model E10-20, Colbourn Instruments, Allentown, PA). A set of six infrared emitters and detectors, mounted 5 cm apart and 2.2 cm above the floor of the box, were used to obtain spatial location and locomotor activity data throughout conditioning, extinction and testing. The conditioned stimuli (CSs) consisted of two distinct tactile cues—grid and hole floors. Grid

floors (2.3 mm stainless steel rods, 6.4 mm apart) and hole floors (16-gauge stainless steel perforated with 6.4-mm round holes) were interchangeable allowing for either full- or split-cue configurations during conditioning/extinction and testing, respectively. These cues are unbiased in that naïve DBA/2J mice show equal preference for the two floors during drug-free preference tests (Cunningham et al., 2003).

Place Preference Procedure

All CPP experiments consisted of unbiased designs and procedures similar to those previously described in detail by this laboratory (Cunningham et al., 2006b).

Experiment 1: Effect of trial duration on extinction of EtOH-CPP

On Day 1, mice (n=96) were given a saline injection (12.5 ml/kg) and habituated to the conditioning apparatus, equipped with white paper flooring, for 5 mins. On Days 2-5, animals received daily CPP conditioning trials during which EtOH (2 g/kg) was paired with one of the tactile cues (e.g., Grid) while saline was paired with the other cue (e.g., Hole) on alternating days. The floor with which EtOH was paired (Conditioning Subgroup) and trial-type order (S-E-S-E or E-S-E-S) were fully counterbalanced. On Day 6, all animals received a drug-free, 15-min preference test during which both tactile cues were presented and place preference was assessed. Animals were matched for preference then divided into 4 groups that differed in extinction-trial duration: No Extinction, Ext-5 min, Ext-15 min, and Ext-30 min. During the 3 days of extinction (Days 7-9) the Ext-5

min, Ext-15 min, and Ext-30 min groups received three, non-reinforced exposures to each of the CS+ and CS- cues separately (each preceded by a saline injection) for their assigned durations. Trials occurred in the morning and afternoon of each day with order of cue exposure counterbalanced. The No Extinction group was weighed daily during this phase. On Day 10, all animals received a second, drug-free 15-min preference test.

Experiments 2 and 3: Effect of SL327 on extinction of EtOH-CPP

Animals (n=96 for each experiment) received EtOH-CPP conditioning and testing identical to that in Experiment 1 (see above). After Test 1, mice were matched for preference and assigned to one of four groups: No Extinction, Vehicle, SL-30, and SL-50. During the 4 days of extinction (Days 7-10), the Vehicle, SL-30, and SL-50 groups received four, 30-min, non-reinforced exposures to the CS+ and CS- cues separately. Animals received Vehicle (15% DMSO), 30 mg/kg SL327 (SL-30), or 50 mg/kg SL327 (SL-50) 30 mins before each CS+ trial; saline (5 ml/kg) was injected 30 mins before each CS- extinction trial. All mice received a saline injection (12.5 ml/kg) immediately before each CS+ and CS- trial. In order to eliminate any possible carry-over effects of SL327, cue exposure order was not counterbalanced during extinction (i.e., CS+ trials occurred in the afternoon). On Day 11 all animals received a second, drug-free 15-min preference test. Experiment 3 was identical to Experiment 2 except that SL327 was administered 90 mins prior to the non-reinforced CS+ extinction trials and it was administered in a 50% DMSO vehicle. The pre-trial interval was

increased in Experiment 3 in order to reduce the potential for any aversive effects of SL327 to enter into association with the CS+ cue during extinction (see Figure 2). The vehicle was changed in order to better reflect the administration parameters of other previously published studies (e.g. Mouledous et al., 2007).

Experiment 4: Effect of SL327 on acquisition of EtOH-CPP

Mice (n=48) were randomly assigned to one of two groups: Vehicle or SL-50. All animals received EtOH-CPP conditioning identical to that in the previous experiments with the exception that each group received either Vehicle (50% DMSO) or SL327 (50 mg/kg), 90 mins before each of the two EtOH (CS+) conditioning trials. On CS- trials, all animals received pre-injections of saline 90 mins before another injection of saline. On Days 6-11, both groups received daily 30-min, drug-free preference tests with only a saline pre-injection.

Experiments 5 & 6: Effect of SL327 on expression of EtOH-CPP

In Experiment 5, mice (n=48) received conditioning identical to that in previous experiments in that they received 2 CS+ and 2 CS- trials in an alternating manner over the course of 4 days. Following conditioning, mice received a 5 ml/kg injection of Vehicle (50% DMSO) or SL327 (50 mg/kg), 90 mins prior to a saline injection (12.5 ml/kg) followed immediately by a standard, 30-min place preference test. Experiment 6 used mice (n=48) from an unrelated experiment in which half of the mice had initially been trained using a one-compartment procedure (similar to that used in Experiments 1-5) or a two-compartment procedure (see Cunningham et al., 2006b). Consistent with

previous findings (Cunningham et al., 2006c), preliminary statistical analysis showed no difference in the CPP produced by these two training procedures. Thus, this factor was omitted in the analyses reported here. Mice received a total of 4 CS+ and 4 CS- conditioning trials, with 30-min preference tests (after saline pre-injection) on the day after the 4th and 8th days of conditioning. The final expression test was given one day later, preceded by either a Vehicle or SL327 (50 mg/kg) injection 90 mins before the saline pre-injection.

Western Blot Procedure

Experiment 7: Effect of SL327 on pERK levels in the dorsal striatum and motor cortex

One week following the conclusion of Experiment 3, animals from the No Extinction group (n=24) were randomly assigned to one of four groups: Vehicle-Saline, Vehicle-EtOH, SL-Saline, SL-EtOH. In Experiment 7, each group first received a pre-injection of Vehicle (50% DMSO) or SL327 (50 mg/kg) 90 mins before an injection of Saline or EtOH (2.5 g/kg). Five mins later, animals were euthanized by CO_2 asphyxiation, brains were extracted, and dorsal striatum and motor cortex were dissected from a 1 mm-thick brain slice corresponding to Bregma +0.86mm. Wet weights were taken and tissue was rapidly frozen in dry ice and stored at -80°C. Tissue samples were prepared by sonication in 20x w/v ice cold buffer containing 50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μl/ml phosphatase inhibitor cocktail and 10 μl/ml protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). Samples

were then incubated for 20 mins at 4°C, followed by centrifugation at 14,000*g* for 15 mins at 4°C. The supernatant was collected and stored at -80°C. Protein content was analyzed by BCA detection (Thermo Fisher Scientific, Rockford, IL, USA) and samples were incubated in Laemmli's Sample Loading buffer for 45 mins at 37°C. Following incubation, 20 μg of protein was loaded onto 10% SDS-PAGE gels and run for 2 hrs at 100 V in a running buffer containing 25mM Tris base, 20 mM glycine and 0.1% SDS. Proteins were then transferred to PVDF membrane at 30 V overnight at 4° C in a transfer buffer containing 50 mM Tris base, 40 mM glycine, and 20% methanol. Membranes were then blocked for 1 hr at room temperature in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) followed by a 1 hr incubation at room temperature with anti-pERK 44/42 or anti-ERK antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) in TBS containing 5% BSA. Membranes were washed three times for 10 mins in TBS and incubated for 45 mins at room temperature with anti-rabbit IgG-AP secondary (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS containing 5% BSA. Finally, membranes were washed three times for 10 mins in TBS and incubated for 5 mins with ECF substrate (GE Healthcare, Piscataway, NJ, USA). Membranes were scanned using an Ultralum imaging system and bands were quantified using Ultraquant 6.0 software (Ultralum, Claremont, CA, USA).

Statistical Analysis

Place preference data were presented and analyzed using one-way analysis of variance (ANOVA) of the percentage of each test spent on the EtOH-paired floor (% Time on EtOH-paired floor). When extinction of EtOH-CPP was assessed, Test was included as a factor in a repeated-measures ANOVA. Additionally, Table 1 includes the raw-score means and statistical comparisons between the counterbalanced Conditioning Subgroups (G+ and G-) within each group (Cunningham et al., 2003). Post-hoc comparisons of G+ and G- subgroups were Bonferroni-corrected (overall α=.05).

Conditioning and extinction locomotor activity was analyzed using repeated-measures ANOVAs with Trial and Group as factors. Ethanol-induced sensitization that occurred during conditioning of EtOH-CPP was analyzed using repeated-measures ANOVA with Trial, Trial-Type, and Group as factors. Post-hoc within- and between-subject comparisons were Bonferroni-corrected (overall α=.05).

Because the goal of Experiments 1-3 was to specifically manipulate extinction of an acquired EtOH-CPP, we decided, *a priori*, to remove animals that failed to express a place preference of greater than 50% on Test 1. These experiments revealed that approximately 25% of all subjects failed to express significant preference following the 2-trial conditioning procedure outlined above (total number of subjects removed from each experiment is reported in the figure legends). Removal of “non-learners” from analyses in order to examine the

effects of extinction-specific manipulations has previously been reported (e.g. Bouton et al., 2008; Weber et al., 2007).

For western blot analysis, pERK immunoreactivity was first normalized to ERK levels and presented and analyzed after being normalized to the Vehicle-Saline group for each gel. Separate two-way ANOVAs, with Pre-injection (Vehicle or SL327) and Injection (Saline or EtOH) as factors, were initially used for dorsal striatum and motor cortex analyses. However, following initial analysis, injection groups were collapsed, normalized to the Vehicle group, and the effect of SL327 was further analyzed by independent t-tests comparing the Pre-injection groups for each brain region.

RESULTS

Effect of trial duration on extinction of EtOH-CPP (Exp 1)

Experiment 1 was performed in order to determine the optimal extinction parameters in our procedure by varying extinction-trial durations. Figure 8 shows that on Test 1 following conditioning, all four groups showed significant preference for the EtOH-paired floor. However, after extinction (Test 2) only the group that had received the 30-min trial duration (Ext-30 min) showed significant extinction of EtOH-CPP. A two-way ANOVA revealed a significant Test x Group interaction [$F(3,67) = 4.8$, $p < .01$] and subsequent paired t-tests for each group revealed that only the Ext-30 min group showed significant extinction [$t(16) = 4.0$ $p < .005$]. As Table 2 shows, including the Conditioning Subgroups (G+ and G-)

Figure 8.

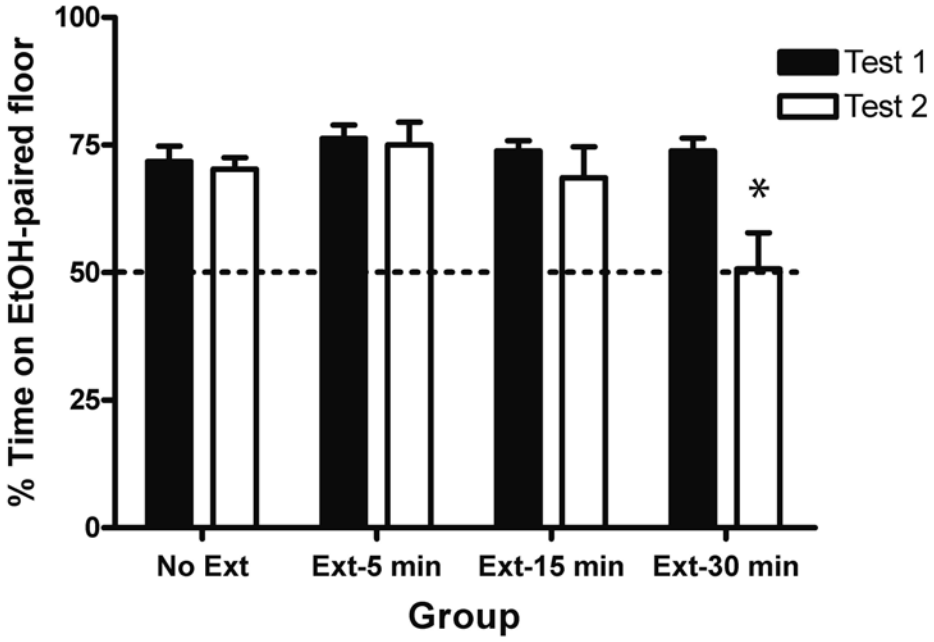


Figure 8. Extinction of EtOH-CPP was determined by trial duration.

All groups showed significant preference for the EtOH-paired floor following acquisition (Test 1). Following extinction, however, the group that received a 30-min (but not 15- or 5-min) extinction trial duration showed complete extinction of EtOH-CPP (Test 2). Error bars indicate standard error of the mean. A total of 23 subjects (24%) were removed because of failure to express >50% preference on Test 1. An additional two subjects were removed because of procedural errors.

* denotes a significant decrease in preference on Test 2 compared to Test 1 ($p < .05$).

Table 2. Preference Test Data including Conditioning Subgroup (expressed as Time on Grid Floor)

Exp.	Group	Conditioning Subgroup	*n*	Test 1: Time on Grid Floor (sec/min)	Post hoc tests	Test 2: Time on Grid Floor (sec/min)	Post hoc tests
1	No Ext	G+	9	44.1 ± 2.4	] *	42.7 ± 1.9	] *
		G-	9	17.9 ± 2.8		18.4 ± 2.0	
	Ext-5 min	G+	8	48.5 ± 1.9	] *	47.4 ± 2.9	] *
		G-	10	16.4 ± 2.3		16.8 ± 4.2	
	Ext-15 min	G+	9	44.1 ± 1.8	] *	40.1 ± 6.1	] *
		G-	9	15.5 ± 1.7		17.9 ± 4.4	
	Ext-30 min	G+	9	45.6 ± 1.9	] *	29.8 ± 5.6	
		G-	8	17.1 ± 2.4		28.9 ± 6.9	
2	No Ext	G+	10	50.3 ± 2.3	] *	45.8 ± 3.3	] *
		G-	10	16.8 ± 2.5		15.9 ± 3.8	
	Vehicle	G+	9	49.0 ± 1.3	] *	36.0 ± 5.7	
		G-	7	11.3 ± 2.1		27.5 ± 8.7	
	SL-30	G+	9	49.3 ± 2.0	] *	15.7 ± 5.1	
		G-	10	11.9 ± 1.9		20.8 ± 4.5	
	SL-50	G+	8	51.7 ± 1.9	] *	20.1 ± 4.2	] #
		G-	8	10.6 ± 1.4		44.6 ± 4.4	
3	No Ext	G+	10	52.0 ± 2.1	] *	51.4 ± 1.6	] *
		G-	8	13.3 ± 3.1		11.7 ± 2.2	
	Vehicle	G+	10	47.9 ± 2.3	] *	27.8 ± 7.5	
		G-	7	9.4 ± 1.7		12.9 ± 7.0	
	SL-30	G+	10	48.7 ± 2.0	] *	25.7 ± 7.1	
		G-	6	11.7 ± 2.2		37.8 ± 8.2	
	SL-50	G+	11	48.9 ± 2.1	] *	37.7 ± 6.2	
		G-	9	10.8 ± 2.3		29.7 ± 8.6	
4	Vehicle	G+	12	46.4 ± 3.0	] *	40.6 ± 2.7	] *
		G-	12	16.8 ± 3.8		18.8 ± 4.2	
	SL-50	G+	12	48.9 ± 3.0	] *	37.3 ± 3.7	] *
		G-	12	23.1 ± 4.6		22.0 ± 4.9	
5	Vehicle	G+	12	34.6 ± 3.5	] *	na	
		G-	12	20.3 ± 2.0		na	
	SL-50	G+	12	45.4 ± 4.5	] *	na	
		G-	11	21.2 ± 6.6		na	
6	Vehicle	G+	12	41.4 ± 2.1	] *	na	
		G-	12	16.4 ± 1.6		na	
	SL-50	G+	12	45.5 ± 4.1	] *	na	
		G-	12	19.1 ± 4.4		na	

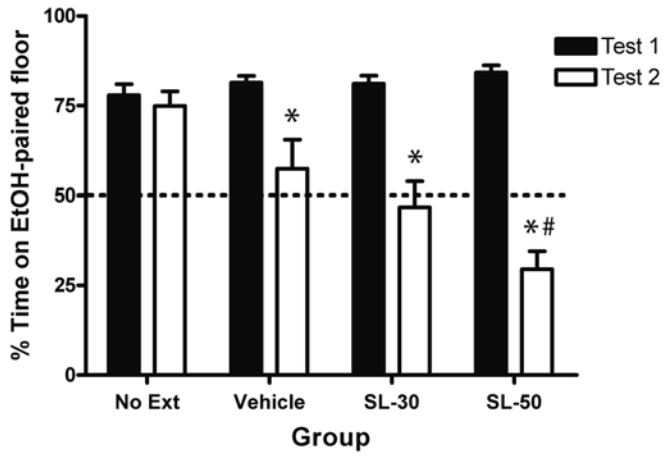
Significant place preference (*) and aversion (#) as determined by Bonferroni-corrected post-hoc comparisons of G+ and G- subgroups ($p < .05$).

in the analysis further confirmed these findings. Therefore, Experiment 1 revealed that a trial duration of 30 mins is necessary to completely extinguish EtOH-CPP. As such, all subsequent extinction experiments utilized a 30-min trial duration.

Effect of systemic administration of the MEK inhibitor, SL327, on extinction of EtOH-CPP (Exps 2 & 3)

In order to determine the involvement of ERK activity in extinction of EtOH-CPP, the MEK inhibitor SL327 was administered prior to the 30-min, non-reinforced CS+ cue exposures during each extinction trial. As Figure 9a shows, SL327 did not impair extinction of EtOH-CPP—that is, all groups that underwent extinction showed a significant decrease in preference on Test 2. These findings were supported by a significant Test x Group interaction [$F(3,67) = 12.4$, $p < .001$] and significant main effects of Test [$F(1,67) = 88.4$, $p < .001$] and Group [$F(3,67) = 5.5$, $p < .005$]. Paired t-tests comparing preference on Tests 1 and 2 revealed significant extinction in all but the No Extinction group (p's < .05). Further analysis revealed a simple main effect of Group only on Test 2 [$F(3,67) = 16.5$, $p < .001$]. Post-hoc comparisons of Test 2 preferences showed that the SL-50, but not SL-30, group differed significantly from the Vehicle group ($p < .05$). Therefore, the highest dose of SL327 appeared to be aversive and, when administered 30 mins before cue exposure, resulted in a decrease in preference below the indifference point (50% preference) on Test 2. This finding suggested that a dose of 50 mg/kg SL327 possessed aversive properties that may have

Figure 9.
A)



B)

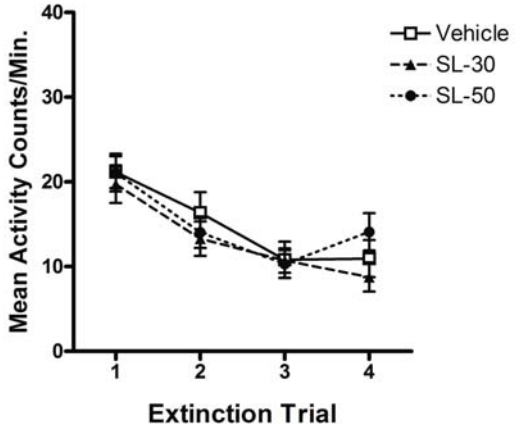


Figure 9. The systemic MEK inhibitor SL327 was unable to impair extinction of EtOH-CPP.

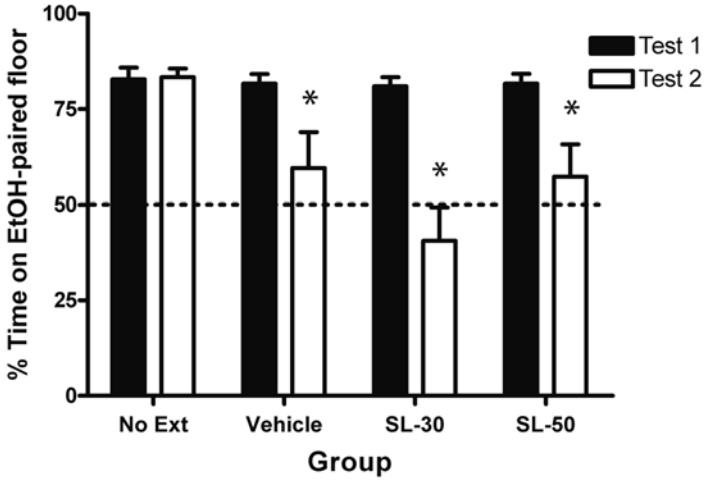
(A) All groups showed significant preference for the EtOH-paired floor following acquisition (Test 1). Administration of 30 mg/kg SL327 (SL-30) or 50 mg/kg SL327 (SL-50) 30-min before extinction trials did not impair normal extinction as seen in the Vehicle group (Test 2). The SL-50 group showed a significant place aversion following extinction. **(B)** All groups showed a decrease in activity over the course of extinction trials in Experiment 2. Pre-treatment with SL327 did not significantly reduce extinction activity as compared to the Vehicle group. Error bars indicate standard error of the mean. A total of 24 subjects (25%) were removed because of failure to express >50% preference on Test 1. An additional two subjects were removed because of procedural errors.

* denotes a significant decrease in preference on Test 2 compared to Test 1 ($p < .05$).
# denotes a significant difference in preference from Vehicle group on Test 2 ($p < .05$).

actually counter-conditioned the initial EtOH-CPP, resulting in a significant avoidance of, not just an indifference for, the previously EtOH-paired cue. As Figure 9b shows, CS+-trial locomotor activity decreased over the course of extinction (significant main effect of Trial [$F(3,144) = 34.5$, $p < .001$]), but did not differ between drug treatment groups (no significant Group x Trial interaction, $p > .05$).

Because the SL-50 group in Experiment 2 developed a place aversion during extinction, the parameters of SL327 administration in Experiment 3 were changed in hopes of eliminating this effect. By extending the pre-trial interval to 90 mins, we hoped to reduce the aversive properties of SL327 and/or weaken the ability of any aversive properties to enter into an association with the CS+ cue during extinction (i.e., prevent counter-conditioning a place aversion). The vehicle was also changed to 50% DMSO in order to better reflect the SL327 administration parameters used in previous experiments (e.g. Mouledous et al., 2007). As seen in Figure 10a, the results showed that although these manipulations eliminated the place aversion seen in Experiment 2, neither dose of SL327 interfered with normal extinction of EtOH-CPP. Analysis revealed a significant Test x Group interaction [$F(3,67) = 4.6$, $p < .01$] as well as main effects of both Test [$F(1,67) = 31.0$, $p < .001$] and Group [$F(3,67) = 4.4$, $p < .01$]. Paired t-tests comparing preference on Tests 1 and 2 revealed significant extinction in all but the No Extinction group (p's $< .05$). Further analysis revealed a simple main effect of Group only on Test 2 [$F(3,67) = 8.9$, $p < .001$]. Subsequent post-

Figure 10.
A)



B)

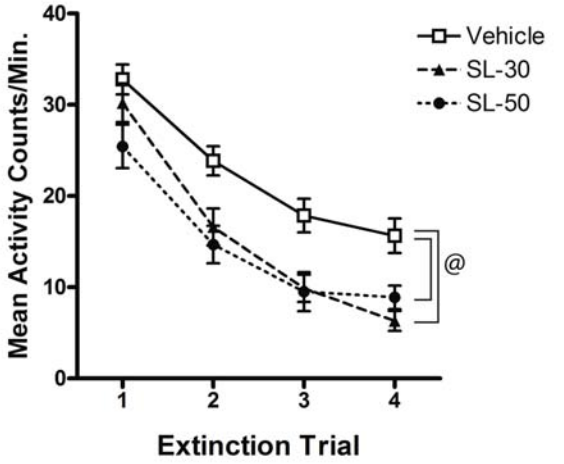


Figure 10. SL327, administered 90-min prior to trials, did not impair extinction of EtOH-CPP. **(A)** All groups showed significant preference for the EtOH-paired floor following acquisition (Test 1). Administration of SL327 (30 or 50 mg/kg) did not impair the normal extinction apparent in the Vehicle group (Test 2). **(B)** Extending the pre-treatment interval to 90 mins unmasked a general activity-suppressing effect of both doses of SL327 as compared to the Vehicle group. Error bars indicate standard error of the mean. A total of 22 subjects (23%) were removed because of failure to express >50% preference on Test 1. An additional three subjects were removed because of procedural errors.
* denotes a significant decrease in preference on Test 2 compared to Test 1 ($p < .05$).
@ denotes a significant difference in extinction activity as compared to the Vehicle group ($p < .05$).

hoc comparisons of Test 2 preferences revealed that neither the Ext-30 nor Ext-50 group differed from the Vehicle group (p's > .05). Therefore, when administered at 30- or 90-min pre-trial intervals, neither dose of SL327 impaired extinction of EtOH-CPP in mice.

In contrast to Experiment 2, however, both doses of SL327 reduced locomotor activity during extinction trials when compared to vehicle-treated animals (Figure 10b). A one-way ANOVA revealed significant main effects of Group [$F(2,50) = 8.3$, $p < .005$] and Trial [$F(3,150) = 127.8$, $p < .001$]. Subsequent post-hoc analysis showed that the Vehicle group exhibited higher locomotor activity than both the SL327 groups (p's < .01). These data suggest that although CS+-trial locomotor activity decreased over the course of extinction, both doses of SL327 caused a general reduction in activity.

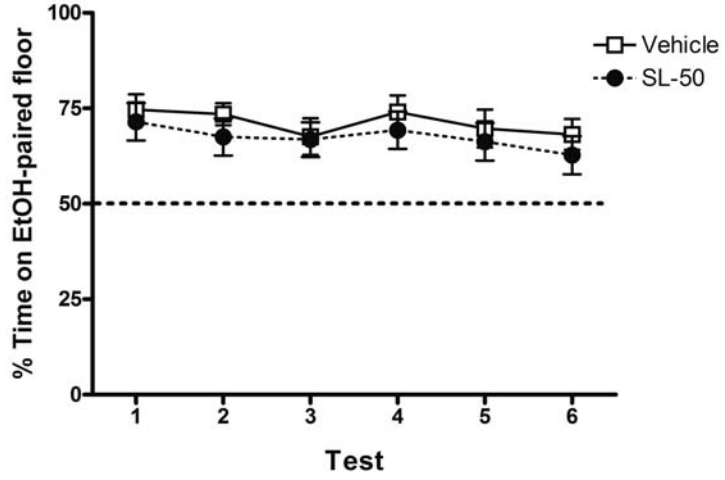
The combined results of Experiments 2 and 3 showed that inhibition of ERK-signaling with the systemic MEK inhibitor, SL327, did not impair extinction learning. This effect was evident at two doses that were administered in two vehicles, at two pre-trial intervals. However, despite failing to prevent extinction, these doses of SL327 did reduce locomotor activity when administered 90 mins before the non-reinforced extinction trials.

Effect of SL327 on acquisition of EtOH-CPP (Exp 4)

Although SL327 did not prevent extinction of EtOH-CPP, previously published experiments have reported that SL327 impairs acquisition of CPP (e.g., Valjent et al., 2000). Thus, Experiment 4 was performed to extend these

Figure 11.

A)



B)

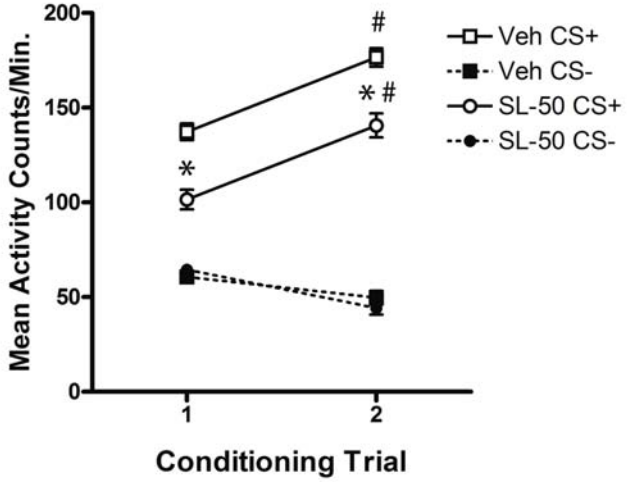


Figure 11. SL327 had no effect on acquisition of EtOH-CPP.

(A) SL327 (50 mg/kg) did not prevent the development of EtOH-CPP as both groups showed significant preference for the EtOH-paired floor following acquisition (Test 1). Significant EtOH-CPP of both the Vehicle and SL-50 groups persisted across five subsequent tests for both groups. **(B)** When administered during CPP acquisition, SL327 (50 mg/kg) caused a general reduction in EtOH-induced activity. However, SL327 did not impair the development of EtOH-induced sensitization across the two acquisition trials. Error bars indicate standard error of the mean.
* denotes a significant increase in EtOH-induced activity from CS+-trial 1 to 2 ($p < .05$).
# denotes a significant difference in EtOH-induced activity between the Vehicle and SL-50 group ($p < .05$).

previous findings by examining the effects of SL327 on acquisition of EtOH-CPP in mice. The results of Experiment 4 showed that SL327 (50 mg/kg) administered 90 mins prior to CS+ conditioning trials did not impair acquisition of EtOH-CPP (Figure 11, Test 1). In order to assess the persistence of CPP, both groups received five additional drug-free preference tests. Analysis of the preference across all six tests revealed no group differences in EtOH-CPP. This was confirmed by the absence of either a Test x Group interaction or main effect of Group (p's > .05). These data showed that SL327 was unable to impair either EtOH reward or the memory formation necessary for acquisition of EtOH-CPP in mice. Furthermore, this finding persisted across multiple, drug-free preference tests.

Analysis of the locomotor activity during conditioning revealed that, similar to Experiment 3, SL327 reduced locomotor activity. However, SL327 did not prevent the development of EtOH-induced sensitization that normally occurs during EtOH-CPP conditioning trials (Figure 11b). These findings were supported by significant interactions of Trial x Trial Type [$F(1,46) = 111.5$, $p < .001$] and Group x Trial Type [$F(1,46) = 25.0$, $p < .001$] as well as significant main effects of Group [$F(1,46) = 23.4$, $p < .001$], Trial [$F(1,46) = 34.2$, $p < .001$], and Trial Type [$F(1,46) = 578.7$, $p < .001$], but no significant Group x Trial x Trial Type interaction ($p > .05$). Paired t-tests comparing EtOH-induced activity levels on Trials 1 and 2 revealed significant increases for both the Vehicle [$t(23) = 6.5$, $p < .001$] and SL-50 [$t(23) = 7.6$, $p < .001$] groups. Furthermore, the Vehicle group

showed significantly greater levels of EtOH-induced activity than the SL-50 group on both Trial 1 [$t(46) = 5.2$, $p < .001$] and Trial 2 [$t(46) = 4.5$, $p < .001$]. These data showed that although SL327 reduced locomotor activity, it did not interfere with development of EtOH-induced sensitization.

Effect of SL327 on expression of EtOH-CPP following 2- and 4-trial conditioning procedures (Exps 5 & 6)

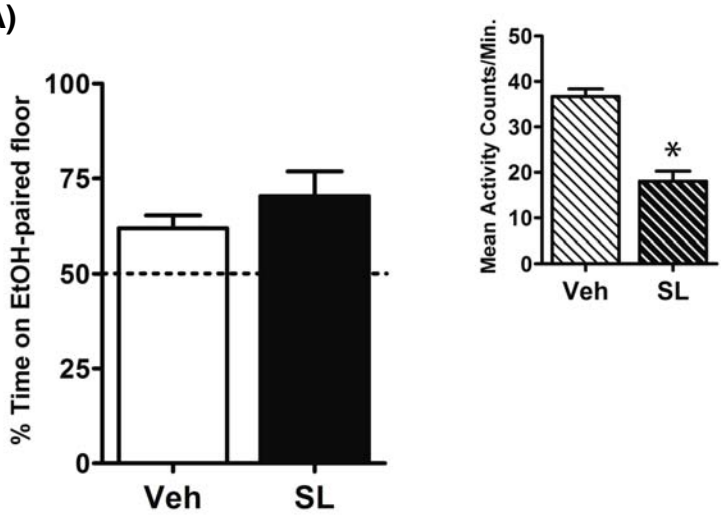
Experiments 5 and 6 were performed in order to assess the effect of SL327 on expression of EtOH-CPP following normal 2-trial, and extended 4-trial, conditioning. As Figure 12a shows the results of Experiment 5 revealed that both the Vehicle- and SL327-treated groups showed similar levels of preference for the EtOH-paired floor as confirmed by an independent t-test ($p > .05$). SL327 did, however, significantly reduce test activity [$t(45) = 6.8$, $p < .001$] (Figure 12a, inset). These effects were replicated when animals that had received extended CPP conditioning (4 trials) were tested in that SL327 had no effect on expression of EtOH-CPP ($p > .05$) while significantly reducing test activity [$t(46) = 5.5$, $p < .001$] (Figure 12b). These results suggest that inhibiting ERK activity with SL327 does not interfere with the conditioned motivational effects of EtOH or its retrieval from memory as assessed during a drug-free CPP test.

Effect of SL327 on pERK levels in the dorsal striatum and motor cortex (Exp 7)

Experiment 7 was performed in order to confirm that SL327 had crossed the blood-brain-barrier and was reducing ERK activity. Western immunoblot

Figure 12.

A)



B)

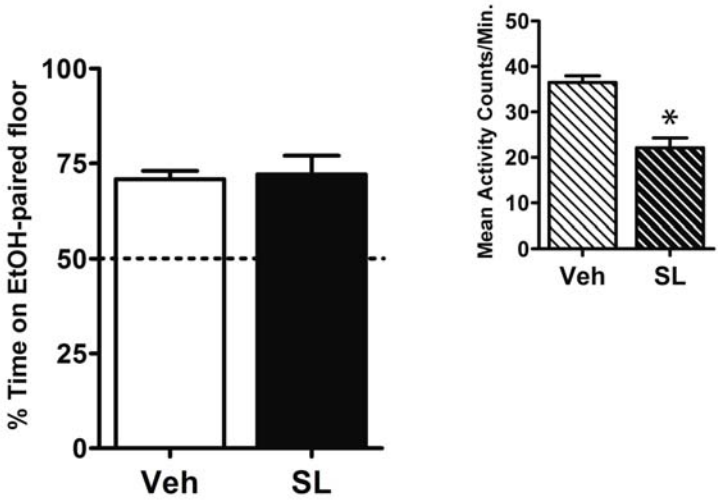


Figure 12. SL327 did not alter expression of EtOH-CPP following both 2- and 4-trial conditioning.

(A) SL327 (50 mg/kg), when administered prior to the CPP test did not alter expression of EtOH-CPP (after 2-trial conditioning) but did significantly reduce test activity (inset). **(B)** SL327 also failed to alter expression of EtOH-CPP following an extended conditioning (4-trials) procedure. As in 5A, SL327 significantly reduced test activity (inset). Error bars indicate standard error of the mean.

* denotes a significant decrease in activity as compared to the Vehicle-treated group ($p < .05$).

analysis of pERK protein levels in the dorsal striatum and motor cortex were examined as these regions have shown EtOH-induced activation (Asyyed et al., 2006) while the dorsal striatum has repeatedly been used to assess MEK-inhibition by SL327 in previous CPP experiments (Valjent et al., 2000 & 2001; Salzmann et al., 2003). As shown in Figure 13, although levels of pERK were not significantly increased 5 mins after an EtOH injection, pre-treatment with SL327 caused a significant reduction in both the dorsal striatum and motor cortex. Because the initial Pre-treatment x Injection ANOVA for each region revealed only a significant main effect of Pre-treatment (Dorsal Striatum: [$F(1,20) = 5.1$, $p < .05$], Motor Cortex: [$F(1,20) = 6.3$, $p < .05$]) and no significant interaction or main effect of EtOH injection (p's > .05), the EtOH and Saline injection groups were collapsed for further analysis of the SL327 effect. Subsequent independent t-tests comparing SL327 and Vehicle pre-treated animals revealed that SL327 significantly reduced pERK levels in both the dorsal striatum [$t(22) = 2.3$, $p < .05$] and motor cortex [$t(22) = 2.7$, $p < .05$] (Figure 13, insets). Specifically, SL327 administration resulted in a reduction of approximately 40% of the pERK levels in both brain regions. Therefore, despite having no effect on learning behavior in Experiments 2-4, or on expression of CPP in Experiments 5-6, SL327 was, in fact, crossing the blood-brain-barrier and significantly reducing pERK levels in multiple brain regions.

Figure 13.

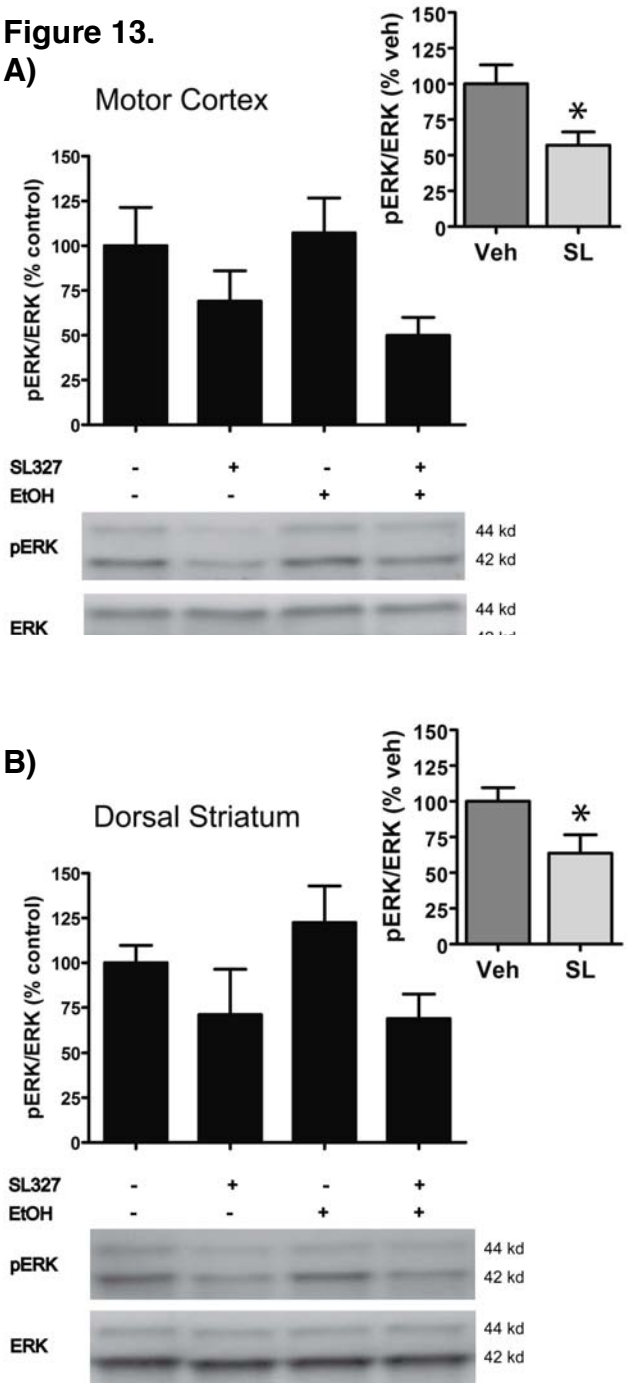


Figure 13. SL327 significantly reduced pERK levels in the motor cortex and dorsal striatum.

(A) Although EtOH pre-treatment did not significantly increase pERK levels in the motor cortex, SL327 (50 mg/kg) caused an approximate 40% decrease in pERK levels (inset). **(B)** As in the motor cortex, EtOH did not enhance pERK levels but SL327 caused an approximate 40% inhibition of pERK levels in the dorsal striatum (inset). Error bars indicate standard error of the mean.

* denotes a significant decrease in pERK/ERK levels as compared to the Vehicle-treated group ($p < .05$).

DISCUSSION

These experiments showed that systemic administration of the MEK-inhibitor SL327 had no effect on acquisition, expression, or extinction of EtOH-induced CPP in mice. Specifically, the results of Experiment 2 and 3 showed no effect of two doses of SL327 (30 and 50 mg/kg) on extinction of EtOH-CPP. This outcome was replicated when two different vehicles and pre-trial intervals were used. Additionally, Experiment 4 showed that SL327 (50 mg/kg) did not impair acquisition of EtOH-CPP or development of EtOH-induced sensitization when administered before each of two CS+ conditioning trials. Furthermore, when administered only before the CPP test, SL327 did not alter expression of EtOH-CPP after either 2- or 4-trial conditioning procedures. Importantly, these outcomes were not simply due to a lack of ERK-activity inhibition by SL327, as Experiment 7 showed that SL327 significantly reduced pERK levels in both the dorsal striatum and motor cortex by approximately 40%. Taken together, these data suggest that the initial memory formation involved in acquisition, as well as the inhibitory learning unique to extinction, of EtOH-seeking behavior in mice did not require intact ERK signaling. Additionally, these experiments showed that neither EtOH reward nor the conditioned rewarding effects of EtOH were altered by inhibition of ERK signaling with SL327.

The lack of an effect of SL327 on these different stages of EtOH-CPP is not easily attributed to insensitivity of our procedure and parameters to pharmacological and neurobiological manipulations. Indeed, our laboratory has

previously reported many studies showing significant effects of various treatments on the acquisition (Boyce-Rustay & Cunningham, 2004; Chester & Cunningham, 1999; Cunningham & Gremel, 2006a; Gremel & Cunningham, 2008), expression (Bechtholt & Cunningham, 2005; Gremel & Cunningham, 2007, 2008, 2009, 2010) and extinction (e.g., Cunningham et al., 1995, 1998) of EtOH-CPP using the same mouse strain, equipment and procedures described here. Thus, we are confident that the procedures used in the current set of experiments were appropriate for detecting potential effects of SL327 on EtOH-CPP.

ERK-signaling and extinction

Our findings are consistent with a previous study that showed that injection of SL327 (30 mg/kg IP) before 15 non-reinforced CS exposures had no impact on extinction of conditioned fear in mice (Matsuda et al., 2010). In contrast, Valjent et al. (2006) reported that injection of SL327 (50 mg/kg IP) before a single non-reinforced CS exposure impaired extinction of cocaine CPP in mice. However, because the control group in the Valjent et al. (2006) study showed only partial extinction, it is unclear whether the effects of SL327 would have persisted had the experiment included more trials that resulted in complete extinction in the control group. Nevertheless, the finding that extinction of EtOH-CPP does not depend on ERK signaling is consistent with previous studies (Groblewski et al., 2009) showing that extinction of EtOH-CPP was not altered by DCS, which has been reported to facilitate extinction of conditioned fear via the

ERK pathway (Yang and Lu, 2005). Taken together, such findings suggest that extinction learning may not depend on ERK signaling.

However, several previous studies have shown that extinction of conditioned fear is impaired by direct injection of the MEK inhibitors U0126 or PD98059 into the hippocampus, medial prefrontal cortex or basolateral amygdala (BLA) (Szapiro et al., 2003; Hugues et al., 2004; Herry et al., 2006). The discrepancy between these findings and the more recent finding that systemically administered SL327 had no effect on fear extinction (Matsuda et al., 2010) might be due to unknown differences in the impact of these various MEK inhibitors on ERK-signal transduction pathways or in the level of MEK inhibition produced by systemically administered SL327 in brain areas critical for fear extinction. Unfortunately, Matsuda et al. did not measure pERK levels in brain following SL327 injection, preventing direct comparison to the changes produced by SL327 in our study or previous studies. Nevertheless, those investigators found that SL327 reversed the extinction-enhancing effect of systemically administered D-serine, an effect that was attributed to a reduction of ERK phosphorylation in brain.

It is important to note that subjects received only a single exposure to the MEK inhibitor during extinction in all of the aforementioned studies. In contrast, Experiments 2 and 3 of the current manuscript included an SL327 injection before each of four consecutive extinction trials. This procedural difference raises the possibility that repeatedly administering SL327 could have diminished

its extinction-impairing effects (i.e., SL327 might have impaired extinction on the first or second trial, but did not prevent extinction on later trials). It would be difficult to evaluate this hypothesis in the EtOH-CPP procedure because a single extinction trial yields little to no extinction in control mice (unpublished findings), reducing the ability to detect impairment by SL327 exposure. Nevertheless, the finding that a single exposure to SL327 had no effect on fear extinction (Matsuda et al., 2010) suggests that SL327's inability to impair extinction is not unique to procedures that involve repeated drug exposure.

Future studies should address whether the inability of systemically administered SL327 to impact extinction is unique to this MEK inhibitor or can be explained by insufficient reduction in ERK phosphorylation in critical brain areas. Additionally, it would be important to know whether site-specific injections of SL327 into hippocampus, medial prefrontal cortex or BLA impair fear extinction and if these effects diminish following repeated exposures.

ERK-independent EtOH-related acquisition learning

A more general explanation for the lack of an SL327 effect on extinction of EtOH-CPP might be that ERK activation is not required for formation of any ethanol-related learned memories. This hypothesis receives support from the results of Experiment 4, which showed that administration of SL327 during conditioning did not impair acquisition of EtOH-CPP. This idea is further supported by data showing no effect of SL327 on development of EtOH-induced sensitization (see Fig. 4b), which has previously been reported to involve an

associative learning component in our procedure (Cunningham and Noble, 1992).

These findings contrast with previous studies showing that systemic pretreatment with similar doses of SL327 interfered with acquisition of cocaine-, THC- and MDMA-induced CPP (Valjent et al., 2000, 2001; Salzmann et al., 2003) and with development of cocaine- and d-amphetamine-induced locomotor sensitization (Valjent et al., 2006). Although the discrepancy between these findings and the present studies might be explained by differences in mouse genotype or various procedural variables, our overall pattern of findings suggests that the formation of ethanol-related memories, unlike those involving other drugs of abuse, occurs independently of the ERK signaling pathway. This conclusion is seemingly at odds with a recent study by Spina et al. (2010) who showed that intracerebroventricular (ICV) administration of the MEK inhibitor PD98059 impaired CPP induced by intragastric acetaldehyde, the primary metabolite of EtOH, in rats. However, although acetaldehyde and EtOH share some behavioral effects, studies suggest that the underlying pharmacological mechanisms are unique, and the presence of acetaldehyde in the central nervous system following EtOH consumption remains controversial (*for review see* Quertemont et al., 2005; but also Karahanian et al., 2011).

Additional evidence of the unique neurobiological mechanisms of EtOH-CPP comes from previously published data from our laboratory showing that acquisition of EtOH-CPP was not impaired by the NMDA-receptor antagonist MK-

801 (Boyce-Rustay & Cunningham, 2004) despite reports that MK-801 prevented cocaine-CPP (Kim et al., 1996). Furthermore, Kim et al. (1996) also showed that MK-801 blocked cocaine-induced locomotor sensitization, whereas Meyer and Phillips (2003) reported no such effect on EtOH-induced sensitization. Thus, it appears that EtOH-CPP and other EtOH-related behaviors, including sensitization, may depend upon signaling mechanisms and receptor systems that are distinct from those involved with other drugs of abuse including cocaine.

Involvement of ERK in EtOH reward and reinforcement

Not only was SL327 unable to interfere with acquisition and extinction learning, but it also did not alter the direct rewarding effects of EtOH. Specifically, if ERK signaling were necessary for EtOH reward, mice that received SL327 before EtOH-conditioning trials would have shown a weaker CPP on Test 1 of Experiment 4. Because both groups showed equally strong CPP, it can be concluded that inhibition of ERK signaling with SL327 altered neither the learning-component of EtOH-CPP acquisition nor the rewarding effects of EtOH. Additionally, our results showed that the conditioned rewarding effects of EtOH do not require ERK signaling as SL327 did not impair expression of EtOH-CPP in Experiments 5 and 6. These results are in agreement with a previous study showing that intra-BLA MEK inhibition did not alter the expression of cocaine-CPP despite causing a 40-50% reduction in BLA-pERK levels (Lai et al., 2008).

Recently, it was observed that SL327 biphasically altered expression of EtOH-self administration in mice—a result that the authors suggested supported

a role of ERK signaling in EtOH reinforcement (Faccidomo et al., 2009). Additionally, Radwanska et al. (2008) reported that presentation of relapse-inducing cues in an EtOH self-administration paradigm resulted in a significant increase in pERK levels in the BLA of rats. In contrast to these findings that link EtOH reinforcement with ERK signaling, Carnicella et al. (2008) showed that the MEK inhibitor U0126 failed to alter expression of EtOH self-administration in rats when administered directly into the ventral tegmental area. Although these reports provide mixed support of our current data showing that MEK inhibition does not interfere with the direct- or conditioned-rewarding effects of EtOH, it is important to note that self-administration and CPP involve distinct neural mechanisms and learning processes while incorporating different aspects of drug reinforcement and reward (Bardo and Bevins, 2000). Thus, it remains possible that these two behaviors are differentially dependent upon the ERK pathway.

Reduction in subcortical and cortical pERK levels by SL327

Although the results of the current behavioral experiments show that SL327 was unable to impair acquisition, expression, and extinction of EtOH-CPP in mice, the doses and pre-treatment intervals used were sufficient to significantly reduce pERK levels in the two brain regions examined. Specifically, Experiment 7 showed that SL327 (50 mg/kg) caused a substantial (40%) reduction of pERK levels in both the dorsal striatum and motor cortex. Contrary to expectation, EtOH (2.5 g/kg) did not result in a significant increase in pERK levels in either the dorsal striatum or motor cortex. This finding is in disagreement with the study by

Asyyed et al. (2006) that showed significant activation of the cAMP response element binding protein (CREB) pathway, a downstream effector of ERK, in both the dorsal striatum and motor cortex following an acute EtOH (3.2 g/kg) injection. Although it is not clear why this dose of EtOH did not alter pERK levels, it is clear that pre-treatment with SL327 significantly reduced pERK levels in these subcortical and cortical regions, regardless of whether or not animals received EtOH. Furthermore, these data are in complete agreement with those of Atkins et al. (1998) who showed that the same dose of SL327 (50 mg/kg) caused an approximately 40-50% reduction in hippocampal pERK levels at 60 mins post-injection—a reduction that was sufficient to impair acquisition of conditioned fear. Additionally, a previous examination of the time-course of MEK inhibition showed that SL327 caused a significant, and equivalent, reduction of pERK levels at all time points between 30 and 100 mins post injection (Selcher et al., 1999). Therefore, given the biochemical results of Experiment 7 in conjunction with the aforementioned published studies, we are confident that SL327 crossed the blood brain barrier and significantly reduced ERK activity during the extinction trials of Experiments 2 and 3 as well as the conditioning trials of Experiment 4 and preference tests of Experiments 5 and 6. This interpretation is further supported by the behavioral findings that SL327 pre-treatment resulted in a general depression of activity during extinction and preference tests, as well as a reduction in EtOH-induced locomotor activity during conditioning trials. Although we cannot rule out that the suppression of activity by SL327 was due to a

peripheral effect of the drug, it is more likely that this effect corresponds to a reduction in pERK levels in the brain, in particular the dorsal striatum as this area has been shown to be involved in control of movement (Kreitzer & Malenka, 2008).

In conclusion, SL327 had no affect on acquisition, expression, or extinction of EtOH-induced CPP in mice despite causing significant reduction of pERK levels in multiple brain regions. Additionally, although SL327 caused a generalized depression of locomotor activity, it did not prevent the development of EtOH-sensitization. In light of the current data, as well as previously published data from our laboratory and others, it appears that extinction-specific learning may be insensitive to inhibition of ERK-signaling via systemically administered SL327. Furthermore, our data also suggest that unlike other drugs of abuse, the EtOH-related associative learning components of EtOH-CPP, as well as the direct and conditioned rewarding properties of EtOH, may not require activation of the ERK pathway.

4

REGIONAL ANALYSIS OF CUE-INDUCED NEURONAL ACTIVATION FOLLOWING EXTINCTION OF ETHANOL-SEEKING BEHAVIOR IN MICE

Contributions by:
Groblewski PA, Ryabinin AE, & Cunningham CL

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Groblewski PA, Ryabinin AE, Cunningham CL. Regional analysis of cue-induced pCREB expression following extinction of an ethanol-seeking behavior in mice. Abstracts of the 39th Annual Meeting of the Society for Neuroscience. 2009.

ABSTRACT

Previous studies have shown that brief exposure to an ethanol-paired cue that is capable of eliciting a conditioned place preference (CPP) results in significant changes in regional expression of FOS protein in mouse brain. However, the effects of extinction training on these cue-induced changes remain unknown. Thus, the current experiment used immunohistochemistry (IHC) to analyze the effects of extinction on cue-induced changes in phosphorylated-cAMP response element binding (pCREB) protein in several brain regions. Forty-eight male, DBA/2J mice received either CPP training (2 pairings of ethanol (2 g/kg) with a grid floor, CS+, and 2 pairings of saline with a hole floor, CS-) or an equal number of unpaired ethanol and cue exposures (Paired and Unpaired Groups, respectively). Following conditioning, mice received a 15-min CPP expression test followed either by 4 days of non-reinforced exposure to the conditioning cues (Extinction Groups) or no cue exposure (No-Extinction Groups). A second CPP test administered upon completion of the extinction phase revealed that the Paired-No Extinction group showed a significant preference for the ethanol-paired cue while the Paired-Extinction animals did not (neither of the Unpaired groups showed significant CPP). Three days later, animals received a brief (5 mins) exposure to the CS+ and were subsequently sacrificed for IHC analysis of pCREB immunoreactivity (IR) in several brain regions that have been implicated in expression and extinction of drug-seeking behavior. The results show that the prelimbic (PL) and infralimbic (IL) subregions of the medial prefrontal cortex (mPFC) exhibit an increased number of pCREB-containing cells following exposure to the EtOH-paired cue. Interestingly, extinction of the EtOH-cue association eliminated this cue-induced increase in pCREB levels. Analyses of the basolateral amygdala and nucleus accumbens core and shell, showed no group differences in cue-induced pCREB expression. Number of CREB-positive cells did not change in any of the analyzed regions. These data suggest that the PL and IL subregions of the mPFC may play an important role in regulating cue-induced approach behavior before and after extinction of EtOH-CPP.

INTRODUCTION

In order to understand how drug-paired cues are able to initiate and control drug-seeking behavior, it is important to understand the brain regions that are first activated upon exposure to these cues. Many studies have examined the regional activation induced by exposure to cues that, following either Pavlovian or instrumental conditioning, are capable of eliciting approach or consumption behavior, respectively. Primary areas of interest have included, but have not been limited to, the interconnected regions of the mesocortical and mesolimbic reward circuits including the ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala, hippocampus and prefrontal cortex (PFC). These studies have shown that exposure to cues capable of initiating responding for ethanol (EtOH) in self-administration procedures produced increases in Fos expression in the prelimbic (PL) and infralimbic (IL) subregions of the medial PFC (mPFC), nucleus accumbens shell, hippocampus, and hypothalamic paraventricular nucleus (Dayas et al., 2006; Wedzony et al., 2003). Following extinction, exposure to cues capable of reinstating EtOH-seeking behavior cause significant activation of the PL, IL, Anterior Cingulate, NAc Core and Shell, basolateral amygdala, and lateral hypothalamus (Hamlin et al., 2007; Topple et al., 1998; Radwanska et al., 2008; Zavala et al., 2007). Finally, exposure to EtOH-paired cues capable of eliciting approach behavior in a CPP framework results in significant activation of the dorsomedial hypothalamus, bed nucleus of the stria terminalis and VTA (Hill et al., 2007). Although the aforementioned

studies, including that by Hill et al., have elucidated the regions activated by EtOH-paried cues in both self-administration and CPP expression, it remains to be shown how extinction directly effects regional activation induced by a Pavlovian-conditioned EtOH-paired cue.

It is widely accepted that immediate-early gene (IEG), such as *c-fos*, expression is correlated with neuronal activation (Herrera & Robertson, 1996). The transcription of *c-fos* is influenced by a number of regulatory elements, including the cAMP/Ca^{2+}-responsive element (Ca/CRE) and the serum response element (SRE). The primary substrates of Ca/CRE and SRE are CREB and Elk-1, respectively. Blocking phosphorylation of these two post-translationally activated transcription factors (CREB and Elk-1) with the MAPK-inhibitor PD98059 abolished glutamate-induction of Fos expression (Vanhoutte et al., 1999). Additionally, it has been shown that neuronal activation results not only in *c-fos* expression, but also a rapid phosphorylation of the CREB protein that is followed by a subsequent, delayed increase in Fos protein levels (Moore et al., 1996). Thus, assessment of pCREB can provide a more rapid method of detecting neuronal activation than the delayed expression of Fos protein. In fact, significant increases in pCREB immunoreactivity have been detected 15 mins after an ethanol injection in mice (Bachtell et al., 2002) and detected 8 mins after a pentylenetetrazol (PTZ)-induced seizure in rats (Moore et al., 1996). Therefore, regional immunohistochemical (IHC) analylsis of pCREB provides an

optimal marker of neuronal activation following a brief exposure to an EtOH-paired cue.

The current set of experiments utilized EtOH-CPP conditioning and extinction procedures combined with regional IHC analysis of the activated transcription factor pCREB to examine the effects of extinction on activation of multiple brain regions following a brief exposure to an EtOH-paired cue. This study was intended as a preliminary analysis of the brain regions that are immediately activated when conditioned animals are briefly exposed to an EtOH-paired cue capable of eliciting drug-free approach behavior. More importantly, this study was intended to better understand how extinction of the EtOH-cue association effects the cue-induced regional activation.

METHODS AND MATERIALS

Subjects

Adult, male DBA/2J mice (n=48) were obtained from Jackson Laboratory (Sacramento, CA) at 6 weeks of age and allowed to acclimate to the animal colony for 2 weeks before experiments commenced. Mice were housed, four to a cage, in cob bedding in a Thoren rack with water and food available *ad libitum* throughout each experiment. All experiments were conducted during the light phase (7:00-19:00). The Oregon Health & Science University IACUC approved all experimental procedures.

Drugs

Ethanol (20% v/v in isotonic saline) was administered intraperitoneally (IP) at a dose of 2 g/kg (12.5 ml/kg).

Place Preference Apparatus

All behavioral procedures were performed in custom made, acrylic and aluminum conditioning boxes (30 x 15 x 15 cm), each of which was enclosed in a sound-attenuating chamber (Model E10-20, Colbourn Instruments, Allentown, PA). Six infrared emitters and detectors, mounted 5 cm apart and 2.2 cm above the floor of the box, were used to obtain spatial location and locomotor activity data throughout conditioning, extinction and testing. The conditioned stimuli (CSs) consisted of two distinct tactile cues—grid and hole floors. Grid floors (2.3 mm stainless steel rods, 6.4 mm apart) and hole floors (16-gauge stainless steel perforated with 6.4-mm round holes) were interchangeable allowing for either full- or split-cue configurations during conditioning/extinction and testing, respectively. These cues are unbiased in that naïve DBA/2J mice show equal preference for the two floors during drug-free preference tests (Cunningham et al., 2003).

Place Preference Procedure

The CPP portion of the study involved procedures similar to those previously described in detail by this laboratory (Cunningham et al., 2006b). The experiment involved a 2 x 2 design with the following four groups: Paired-No Extinction, Paired-Extinction, Unpaired-No Extinction, Unpaired- Extinction.

Thus, each of the two Paired groups had a control group matched for total drug and cue exposure.

Habituation. On Day 1, mice were given a saline injection (12.5 ml/kg) and habituated to the conditioning apparatus, equipped with white paper flooring, for 5 mins.

Conditioning. On Days 2-5, Paired animals received daily CPP conditioning trials during which EtOH (2 g/kg) was paired with the Grid floor while saline was paired with the Hole floor on alternating days. As in the report by Hill et al. (2007), all animals received EtOH paired with the Grid floor, thus this factor was not counterbalanced. However, the order in which the animals received the pairings (S-E-S-E or E-S-E-S) was fully counterbalanced. Two to three hrs after the end of the conditioning trial, animals received a saline injection (12.5 ml/kg) in their home cage. During the CPP conditioning trials, Unpaired animals received saline injections before both of the cues (S-S-S-S) followed by an EtOH injection in the home cage 2-3 hrs later. Thus, the Unpaired groups received equal exposures to EtOH over the course of the experiment (Cunningham, 1993).

Test 1. On Day 6, all animals received a drug-free, 15-min preference test during which both tactile cues were presented and place preference was assessed.

Extinction. Three days after Test 1, animals in the Paired-Extinction and Unpaired-Extinction groups received 4 days of extinction (Days 7-10). Extinction consisted of four, non-reinforced exposures to each of the CS+ and CS- cues

separately (each preceded by a saline injection) for 30 mins. Trials occurred in the morning and afternoon of each day with order of cue exposure counterbalanced. The No Extinction groups were weighed daily during this phase.

Test 2. Twenty-four hrs after the last extinction trial (Day 11) all animals received a second, drug-free 15-min preference test.

Cue Exposure. Two days following Test 2, all animals received a brief, 5-min exposure to the CS+ cue and were sacrificed with CO_2 12-15 mins later and brains were processed for IHC.

Immunohistochemistry Procedure

The general IHC procedure has previously been described in detail (Bachtell et al., 2002). Briefly, brains were postfixed with 2% paraformaldehyde (PFA) in phophate-buffered solution (PBS), immersed in 20% & 30% sucrose, 0.1%NaN3, 1mM NaF in PBS, then frozen and sectioned (30μm) with a cryostat (Leica CM1900). The phosphatase inhibitor, NaF, was added to all buffers (1mM) and incubation solutions (0.1mM) in order preserve the protein phosphorylation state. Blocking was performed with 4% normal goat serum. CREB (1:500) and pCREB (1:250) (both from Cell Signaling Technology, Beverly, MA) immunoreactivity was detected with Vectastain ABC kit (Vector Laboratories, Burlingame, CA), and enzymatic development was performed with Metal Enhanced DAB kit (Pierce Chemical, Rockford, IL). For each area of interest,

separate adjacent coronal slices were used for pCREB and CREB staining and subsequent cell quantification.

Slice images were obtained using an Olympus BX51 microscope and Macintosh computer equipped with Q-Capture software. Slices were viewed, and cells counted, using Image J software. CREB- and pCREB-containing cells were counted in a rectangular fixed-size region (173 μm x 104 μm) randomly applied to an area well within each brain region's boundaries determined from Paxinos & Franklin, 2001 (Figure 14). Three counts were obtained in each area for every subject, averaged and pCREB counts were normalized to CREB for each subject. As Figure 14 shows, slices from the following approximate anterior/posterior (AP) coordinates (Paxinos & Franklin, 2001) were analyzed for each brain region: PL and IL subregions of the mPFC (AP: +1.70mm), BLA (AP: -1.46mm), Core and Shell subregions of the NAc (AP: +1.10mm). These coordinates were determined based off of previous IHC analysis of these regions (e.g., Hill et al., 2007).

Statistical Analysis

Place preference data were presented and analyzed using analysis of variance (ANOVA) of the percentage of each test spent on the EtOH-paired (CS+) floor (% Time on EtOH-paired floor). An initial 2-way ANOVA (Group x Test) was followed by Bonferroni-adjusted within-subjects comparisons (paired t-tests) for each group. The Time on Grid Floor data was also analyzed and reported in Table 1, but because all Paired animals had EtOH paired with the

Figure 14.

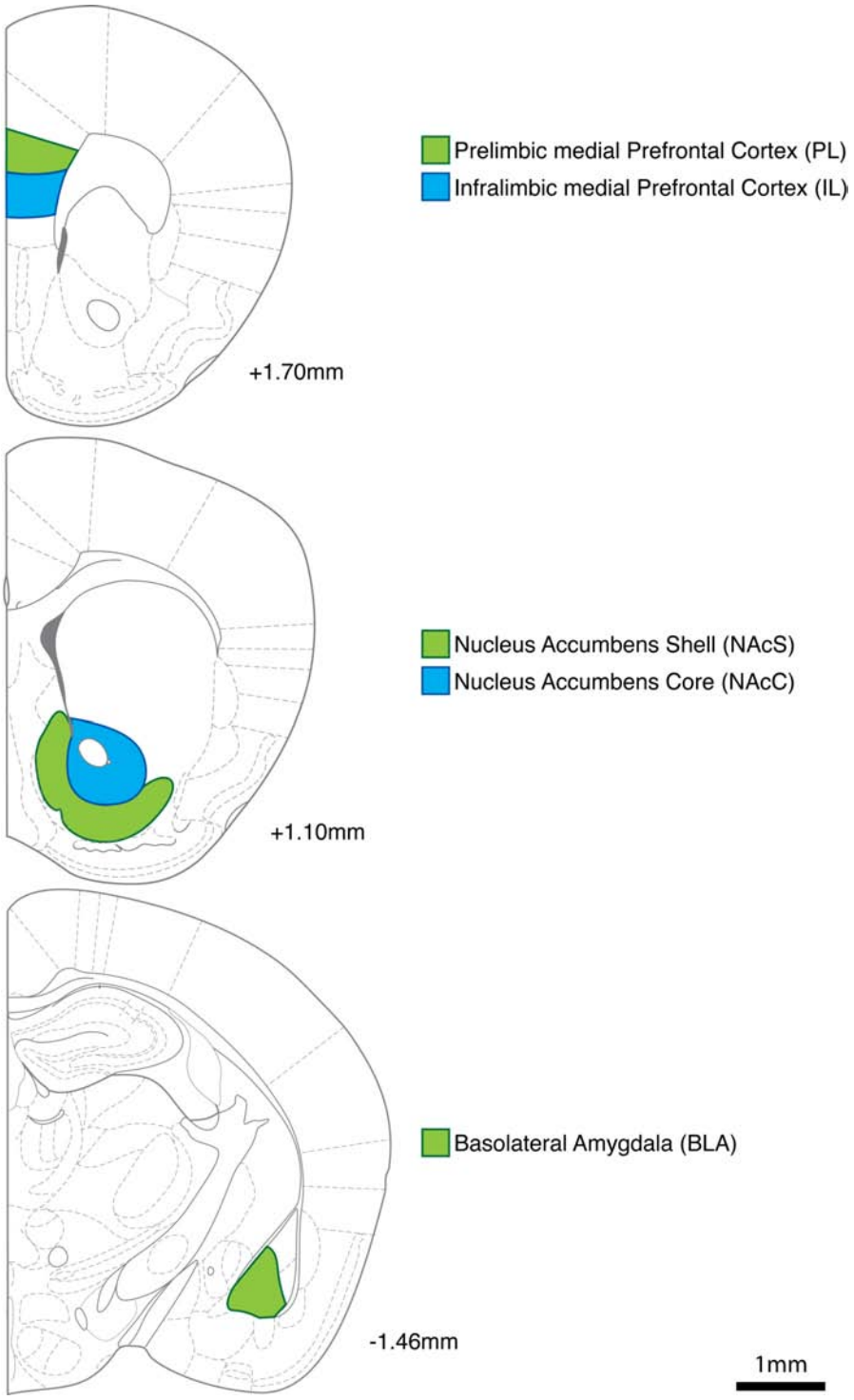


Figure 14. Regions of interest included in IHC analysis of pCREB- and CREB-containing cells (adapted from Paxinos & Franklin, 2001). Cells were counted in a rectangular area (173 μm x 104 μm) placed within the presented boundaries for each region/subregion.

Grid floor, the statistical analysis yielded the same results. IHC data were presented as pCREB/CREB Immunoreactivity (IR) and between-group differences were determined by 1-way ANOVAs followed by Bonferroni-adjusted between-groups comparisons (independent t-tests). Overall α was set at .05 for all statistical tests.

Because it was necessary that the EtOH-paired cue in these experiments was capable of eliciting approach behavior, it was decided, *a priori*, to remove animals that failed to express a place preference of greater than 50% on Test 1. These experiments revealed that less than 10% of all Paired subjects failed to express significant preference following the 2-trial conditioning procedure—a finding that is consistent with previous reports that have also removed non-learners from extinction analyses (e.g., Groblewski et al., 2011).

RESULTS

Conditioned Place Preference

A total of 2 animals was removed from analysis for exhibiting less than 50% preference on Test 1.

On Test 1, animals in both Paired groups showed significant preference for the EtOH-paired floor (Figure 15). As expected, neither of the Unpaired groups showed significant preference for either side of the chamber. Following the extinction phase (Test 2), the Paired-Extinction group exhibited a significant reduction in CPP (i.e., extinction) whereas the Paired-No Extinction group

Figure 15.

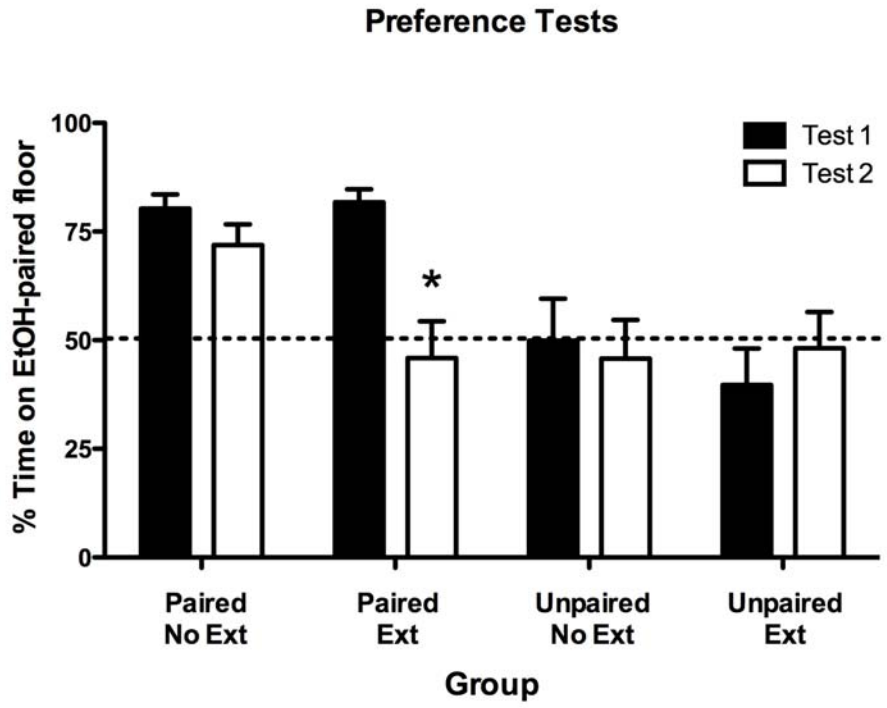


Figure 15. Preference Test data (expressed as Percent Time on EtOH-Paired Floor) before (Test 1) and after (Test 2) extinction. Significant reduction in place preference (*) as determined by Bonferroni-corrected post-hoc comparisons of Tests 1 and 2 for each group ($p < .05$). Data are expressed as mean (± SEM).

continued to express significant CPP equal in strength to that expressed on Test 1 (Figure 15). These findings were supported by a significant Group x Test interaction [$F(3,42) = 6.5$, $p < .01$] as well as significant main effects of Group [$F(3,42) = 4.9$, $p < .01$] and Test [$F(1,42) = 7.1$, $p < .05$]. Separate paired t-tests revealed that only the Paired-Extinction group showed a significant change in preference following the extinction phase [$t(11) = 3.8$ $p < .005$]. Both Unpaired groups continued to show an indifference for the two cues regardless of whether they underwent extinction or not. These results demonstrated the ability of these EtOH-paired cues to elicit Pavlovian approach behavior, which is eliminated following extinction (Paired-Extinction) yet is conserved following the passage of an equivalent amount of time (Paired-No Extinction).

These results were supported by the alternative analysis of Time on Grid Floor, which is presented in Table 3.

Immunohistochemistry

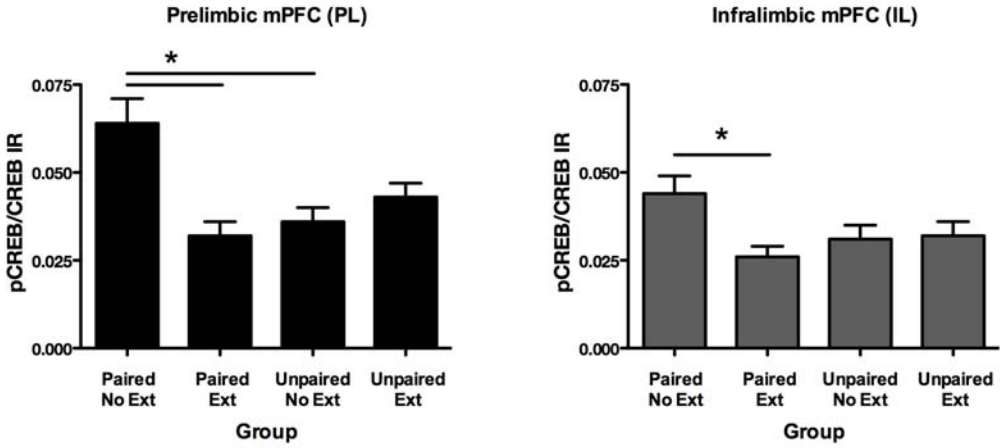
Medial Prefrontal Cortex: Analyses of the PL and IL subregions of the mPFC revealed a significant increase in the number of pCREB-containing cells in the Paired-No Extinction group (Figure 16a). This cue-induced increase was eliminated following extinction, as seen in the Paired-Extinction group. In the PL, an initial one-way ANOVA revealed a significant main effect of group [$F(3,38) = 7.8$, $p < .001$] and subsequent post-hoc comparisons showed that the Paired-No Extinction group had significantly more pCREB-containing cells in the PL than both the Paired-Extinction ($p < .001$) and Unpaired-No Extinction groups ($p <$

Table 3. Preference Test Data including Conditioning Subgroup (expressed as Time on Grid Floor)

Exp.	Group	Conditioning Subgroup	*n*	Test 1: Time on Grid Floor (sec/min)	Test 2: Time on Grid Floor (sec/min)	Post hoc tests
1	Paired-No Extinction	G+	10	49.0 ± 2.1	43.8 ± 3.1	
	Paired-Extinction	G+	12	49.7 ± 1.9	27.5 ± 5.3	*
	Unpaired-No Extinction	G+	12	29.9 ± 6.2	27.4 ± 5.7	
	Unpaired-Extinction	G+	12	23.6 ± 5.4	28.8 ± 5.5	

Significant reduction in place preference on (*) as determined by Bonferroni-corrected within-subject comparisons of Test 1 & 2 ($p < .05$).

Figure 16.
A)



(B)

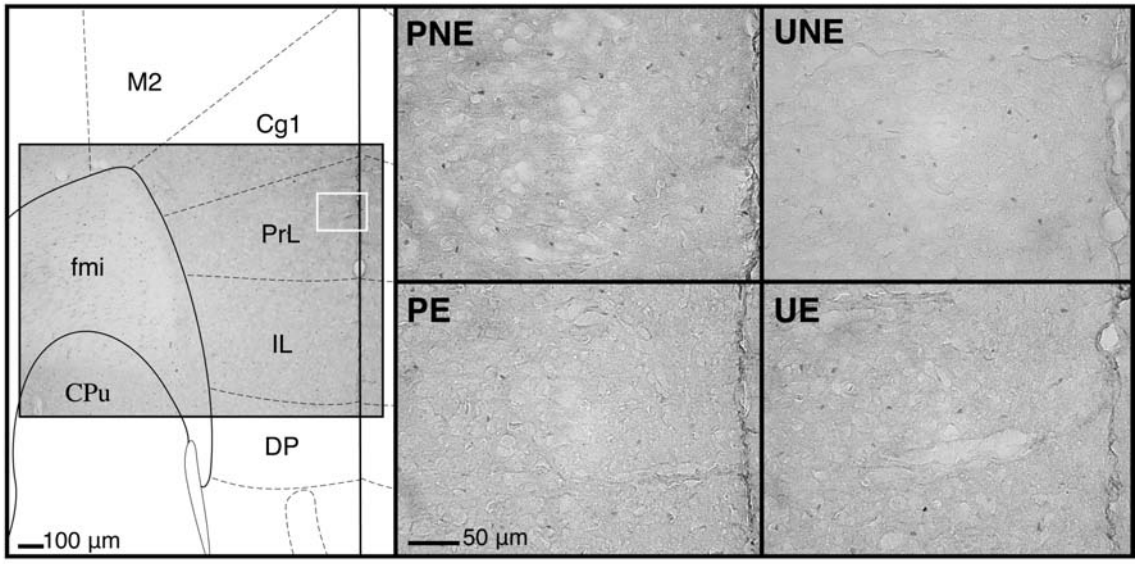


Figure 16. **(A)** Immunohistochemistry data from the prelimbic (PL) and infralimbic (IL) subregions of the mPFC. Data expressed as a ratio of pCREB-positive cells to CREB-positive cells (mean ± SEM). Significant differences (∗) as determined by Bonferroni-corrected post-hoc comparisons ($p < .05$). **(B)** Representative photomicrographs of regions of analysis within the PL of coronal sections approximately 1.7mm anterior (rostral) of Bregma (Paxinos & Franklin, 2001).

.005). In the IL, there was also a main effect of group [$F(3,38) = 2.9$, $p < .05$] and post-hoc comparisons revealed that the Paired-No Extinction group was significantly different than the Paired-Extinction group ($p < .05$). There were no group differences in the number of CREB-containing cells (p's > 0.05). Therefore, these results suggest that the cue-induced increases in pCREB expression in the mPFC were eliminated if the EtOH-cue association is extinguished.

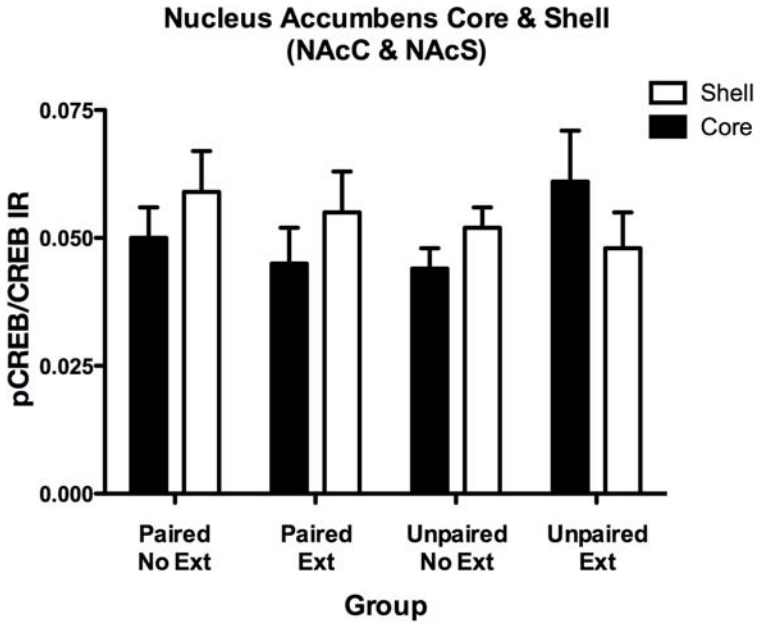
Nucleus Accumbens: There were no significant group differences in the number of pCREB- and CREB-positive cells in either the core or shell subregions of the NAc (p's > 0.05) (Figure 17).

Basolateral Amygdala: There were no significant group differences in the number of pCREB- and CREB-positive cells in the BLA (p's > 0.05) (Figure 17).

DISCUSSION

The results of the current experiments revealed that the PL and IL subregions of the mPFC are activated following a brief exposure to an EtOH-paired cue that is capable of eliciting Pavlovian approach behavior in a CPP procedure. Interestingly, the cue-induced activation in both of these regions is eliminated when the cue is no longer capable of eliciting approach behavior following extinction. Unlike the mPFC, neither the BLA nor NAc (core or shell) were significantly activated following cue exposure. These data suggest that the

Figure 17.
(A)



(B)

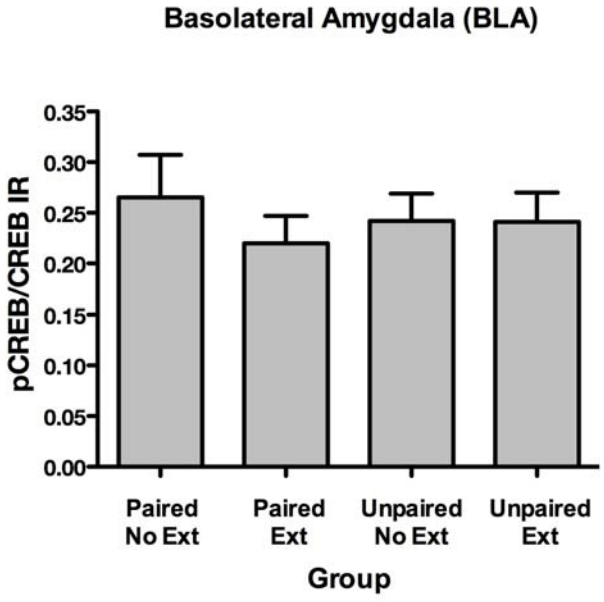


Figure 17. Immunohistochemistry data from **(A)** the nucleus accumbens (core and shell) and **(B)** basolateral amygdala. Data expressed as a ratio of pCREB-positive cells to CREB-positive cells (mean ± SEM).

PL and IL subregions of the mPFC may play a critical role in the expression and/or extinction of EtOH-induced cue-evoked approach behavior.

The use of pCREB-IR as an indicator of neuronal activation has been well documented in previous studies (e.g., Bachtell et al., 2002). It has been shown that phosphorylation of CREB results in transcription of a number of immediate early genes (IEGs), including *c-fos and Zif268* (Adams et al., 2000). In fact, blocking phosphorylation of CREB also prevents Fos expression (Vanhoutte et al., 1999). However, it is important to note that a significant advantage of utilizing pCREB instead of Fos as a marker of neuronal activation is its shorter detection window as phosphorylation of CREB can be detected within 10-15 mins whereas detection of Fos requires waiting upwards of 90 mins (Bachtell et al., 2002). Thus, pCREB-IR is a sensitive method of assessing neuronal activation that possesses better temporal resolution than Fos-IR.

The most intriguing finding of the current study is the extinction-sensitive conditioned increase in activation of two subregions (PL and IL) of the ventral mPFC. Activation of these regions following exposure to previously-conditioned cues is not surprising given the wealth of evidence implicating the PL and IL in expression and extinction of drug-seeking behavior (Peters et al., 2009). The current findings are in complete agreement with the findings of Zavala et al. (2007) that showed significant activation of the PL and IL by a cocaine-paired cue capable of eliciting drug-seeking behavior. Importantly, extinction eliminated the cue-induced activation of these mPFC regions. However, because the animals in

the study by Zavala et al. were tested for cue-induced lever pressing, it is unclear if the increases in mPFC activation were cue-induced or behavior-induced, or both. Regarding expression of CPP, it has been shown that a brief (15 mins) CPP expression test results in a significant increase in Fos levels in the PL (Miller and Marshall, 2004; 2005b). Furthermore, pharmacological lesions of both the PL and IL were shown to cause impairment in acquisition of CPP to a number of drugs (Tzschentke and Schmidt, 1999) and inactivation of the mPFC with the sodium-channel blocker bupivacaine blocked extinction of amphetamine-CPP (Hsu and Packard, 2008).

The findings that neither the BLA nor the NAc were significantly activated by exposure to a drug-paired cue are somewhat surprising given the extensive data that have implicated these two areas in drug-related behaviors. A study by Radwanska et al. (2008) showed that exposure to an EtOH-paired cue capable of reinstating self-administration resulted in a significant activation of the BLA (indicated by an increase in BLA-pERK levels). Additionally, lesions of both the BLA and NAc were shown to prevent acquisition of EtOH-CPP, whereas only BLA lesions eliminated CPP expression (Gremel and Cunningham, 2008). However, a previous experiment by Hill et al. (2007) involved a procedure nearly identical to that of the current study and revealed a similar finding, that neither the BLA nor the NAc showed significant conditioned neuronal activation (as indicated by Fos). This report did not, however, address the effects of extinction or include an analysis of the PL/IL subregions of the mPFC. In another similar

study, Bernardi et al. (2009) examined BLA activation following exposures to both the CS+ and CS- cues alone or the CS+/CS- cues together (as in the CPP expression test). The results of the IHC analyses showed that Fos expression was significantly increased in the BLA following the choice test (CS+/CS-) condition, but not after exposure to either of the cues alone. Bernardi et al. concluded that the BLA may play a more important role in the actual expression of approach behavior exhibited during a CPP test, but may not be significantly activated following simple presentation of the CPP-eliciting cue. The current results are in agreement with this assertion in that a brief exposure to the EtOH-paired cue did not induce changes in pCREB-IR within the BLA.

In conclusion, these IHC results suggest that areas of the mPFC, but not BLA or NAc, are activated by exposure to an EtOH-paired cue as indicated by an increase in the number of cells containing the activated form of the transcription factor, pCREB. Moreover, the conditioned increase of pCREB-IR in the PL and IL was eliminated when the EtOH-cue association was inhibited following extinction (as indicated by a reduction in place preference). Thus the mPFC represents a dynamic region in which cue-induced activation is sensitive to manipulations, such as extinction, that alter the drug-cue contingency. These results strongly suggest that the mPFC plays an integral role in the expression and/or extinction EtOH-CPP; however, additional studies are required to systematically test this assertion.

5

ELECTROLYTIC LESIONS OF THE MEDIAL PREFRONTAL CORTEX (mPFC) PREVENT EXTINCTION OF ETHANOL-SEEKING BEHAVIOR IN MICE

Contributions by:
Groblewski PA & Cunningham CL

The work described in this chapter has not yet been published.

ABSTRACT

Studies have shown that the prelimbic (PL) and infralimbic (IL) subregions of the medial prefrontal cortex (mPFC) play critical, and distinct, roles in the expression and extinction of drug-seeking behavior, respectively. The exact involvement of the mPFC in expression and extinction of ethanol (EtOH)-induced CPP, however, remains unknown. A previous study from our laboratory showed that a brief exposure to an EtOH-paired cue capable of eliciting approach behavior (as assessed with CPP) resulted in a significant increase in phospho-cAMP response element binding protein (pCREB) levels in the PL and IL of mice. Interestingly, extinction of the EtOH-cue association eliminated this cue-induced increase in pCREB, suggesting that the mPFC may play a critical role in the expression and/or extinction of EtOH-CPP. Therefore, the current study was intended to expand upon these findings by determining a causal role of the mPFC in extinction of EtOH-CPP. Electrolytic lesions of the mPFC (including both the PL and IL) were performed following conditioning but before extinction of EtOH-CPP. The results of this experiment suggest that focal, electrolytic lesions of the mPFC significantly impaired extinction of EtOH-CPP. These data support the notion that the mPFC plays an integral role in extinction of EtOH-seeking behavior.

INTRODUCTION

Rehabilitation from drug-associated, abusive and addictive behaviors represents a uniquely difficult task in the clinic. Because patients who are receiving rehabilitation therapy have most often spent many years with an addiction, breaking the control that these addictive behaviors exert over a patient's everyday life is a daunting task—one that more often than not ends in relapse. Understanding the mechanisms that underlie the post-acquisition (i.e., rehabilitation and relapse) phases of drug addiction is therefore paramount for successful treatment and persistent prevention of relapse. Experimental extinction of a previously learned drug-association is thought to model the cue-exposure therapy (CET) aspects of rehabilitation and provides an opportunity to observe and manipulate the unique learning processes that are involved in reducing the expression of the drug-seeking behavior once it has been learned (Kaplan et al., 2011).

Conditioned place preference (CPP) is one such model of drug-seeking behavior that consists of animals learning to associate the rewarding effect of a drug with a set of distinctive cues following their repeated, concurrent presentation. In the subsequent extinction phase of CPP, animals are then exposed to the same set of cues in the absence of drug, and over time, learn that the previously drug-paired cues no longer predict the drug's rewarding effect. The extinction phase of CPP is thought to require a unique type of inhibitory learning that instead of erasing the original drug-cue memory, works by

overlapping new memories that reduce cue-induced approach behavior. The extinction phase of CPP is therefore modeled after the rehabilitation phase of clinical treatment protocols that can involve CET. Therefore, understanding the brain regions responsible for regulating extinction-specific learning has significant implications for optimizing the current treatment options available for patients in the clinic.

Recent evidence suggests that the medial prefrontal cortex (mPFC) is involved in both the expression and extinction of conditioned fear and drug-seeking behavior (*for review see* Peters et al., 2009). Specifically, the prelimbic (PL) subregion has been suggested to be primarily involved in expression of conditioned fear and drug-seeking behaviors while the infralimbic (IL) subregion is more important for extinction. Within a CPP framework, a brief cocaine-CPP expression test causes significant activation of the PL (Miller & Marshall, 2004; 2005b) and inactivation of the mPFC (both PL & IL) impairs extinction of amphetamine-CPP (Hsu & Packard, 2008). Additionally, inactivation of the mPFC reinstated heroin-CPP, an effect the authors attribute to a disinhibition of place preference (Ovari & Leri, 2008). Contrary to these findings however, Zavala et al. (2003) reported that PL lesions had no effect on acquisition, expression, or extinction of cocaine-induced CPP.

Although the current literature generally supports an important role of the mPFC in drug-seeking behavior, it remains unknown whether the mPFC is necessary for extinction of ethanol-seeking behavior within a CPP framework. A

recent experiment from our laboratory has shown that a brief exposure to an EtOH-paired cue that is capable of eliciting approach behavior in a CPP expression test caused significant neuronal activation in the PL and IL subregions of the mPFC (Chapter 4). Interestingly, this cue-induced activation was eliminated in a group of animals that had received extinction of the EtOH-cue association. Thus, these data strongly suggest that the mPFC is involved in cue-induced behavior such as CPP and may play an important role in extinction of EtOH-CPP. Therefore, the current experiment was performed in order to examine the effects of electrolytic lesions of the mPFC on extinction of EtOH-induced CPP in mice. Animals received an electrolytic lesion of the mPFC, a sham insertion of an electrode into the mPFC, or no surgery at all. It was hypothesized that animals that received lesions of the mPFC would show impaired extinction when compared to the animals that received sham-lesions and no surgery.

METHODS AND MATERIALS

Subjects

Adult, male DBA/2J mice (n=63) were obtained from Jackson Laboratory (Sacramento, CA) at 7 or 6 weeks old and allowed to acclimate to the animal colony for 2 or 3 weeks before experiments commenced. Mice were housed, four to a cage, in cob bedding in a Thoren rack with water and food available *ad libitum* throughout each experiment. All experiments were conducted during the

light phase (7:00-19:00). The Oregon Health & Science University IACUC approved all experimental procedures.

Drugs

Ethanol (20% v/v in isotonic saline) was administered intraperitoneally (IP) at a dose of 2 g/kg (12.5 ml/kg).

Place Preference Apparatus

All behavioral procedures were performed in custom made, acrylic and aluminum conditioning boxes (30 x 15 x 15 cm), each of which was enclosed in a sound-attenuating chamber (Model E10-20, Colbourn Instruments, Allentown, PA). A set of six infrared emitters and detectors, mounted 5 cm apart and 2.2 cm above the floor of the box, was used to obtain spatial location and locomotor activity data throughout conditioning, extinction and testing. The conditioned stimuli (CSs) consisted of two distinct tactile cues—grid and hole floors. Grid floors (2.3 mm stainless steel rods, 6.4 mm apart) and hole floors (16-gauge stainless steel perforated with 6.4-mm round holes) were interchangeable allowing for either full- or split-cue configurations during conditioning/extinction and testing, respectively. These cues are unbiased in that naïve DBA/2J mice show equal preference for the two floors during drug-free preference tests (Cunningham et al., 2003).

Behavioral Procedures

The CPP portion of the study consisted of unbiased designs and procedures similar to those previously described in detail by this laboratory (Cunningham et al., 2006b).

Habituation. On Day 1, mice were given a saline injection (12.5 ml/kg) and habituated to the conditioning apparatus, equipped with white paper flooring, for 5 mins.

Conditioning. One day later animals received daily CPP conditioning trials during which EtOH (2 g/kg) was paired with one of the tactile cues (e.g., Grid) while saline was paired with the other cue (e.g., Hole) on alternating days using the standard, one-compartment procedure. The floor with which EtOH was paired (Conditioning Subgroup) and trial-type order (S-E-S-E or E-S-E-S) were fully counterbalanced.

Test 1. One day after the last conditioning trial, all animals received a drug-free, 30-min preference test during which both tactile cues were presented and place preference was assessed. Floor orientations were counterbalanced within each group.

Surgery. Animals received a single, midline electrolytic lesion of the mPFC (see *Surgical Procedures* for details) or sham surgery and received 6-7 days to recover. Animals in the No Sx group remained in the home cage undisturbed.

Extinction. After fully recovering from surgery, animals received 4 days of extinction. Extinction consisted of AM and PM sessions during which animals received four, 30-min non-reinforced exposures to each of the CS+ and CS- cues separately. Specifically, for the AM session, animals were weighed, injected with saline, and immediately placed on the CS- cue for 30 mins. Approximately 3 hrs later during the PM session, animals were again weighed and injected with saline and immediately placed on the CS+ floor for 30 mins. Cue exposure order was not counterbalanced during extinction (i.e., CS+ trials occurred in the afternoon for all animals) in order to mimic the other extinction experiments in this dissertation where drug carry-over effects are a potential issue (Chapters 3 & 6).

Test 2. One day after the last extinction trial all animals received a second, drug-free 30-min preference test identical to Test 1.

Surgical Procedures

Electrolytic Lesion Surgery. A single, midline electrolytic lesion of the mPFC was performed based on the lesion parameters described previously (*see* Gremel and Cunningham, 2008). Specifically, after receiving meloxicam (0.2 mg/kg, SC), animals were induced and maintained under isoflurane anesthesia. Animals were placed in a stereotax (Model 1900, KOPF Instruments, Tujunga, CA) with the skull horizontal. A midline burr hole was drilled 1.75 mm rostral to bregma and an electrode (Rhodes Medical Instruments, Woodland Hills, CA) was lowered into the mPFC (AP: 1.8 mm, ML: 0.0 mm, DV: -2.5 mm) determined from Paxinos & Franklin (2001). A 0.5 mA current was then passed through the tip of

the electrode for 5 secs. Sham animals received an identical surgery but no current was passed through the lowered electrode. Mice were given 5-6 days to recover before the behavioral procedure resumed. Animals in the No Sx group remained in the home cage, undisturbed, during the surgery and recovery period.

Histology. Upon completion of each experiment, animals received an overdose of pentobarbital (150 mg/kg), brains were removed and postfixed with paraformaldehyde (2% w/v in phosphate buffered solution, PBS). Brains were cryoprotected with 20% then 30% sucrose in PBS and 0.1% NaN_3. Frozen slices (40μm) were obtained on a cryostat (Leica CM1900) and thionin-stained for verification of cannula location and lesion location/size.

Statistical Analysis

Place preference data were presented and analyzed using analysis of variance (ANOVA) of the percentage of each test spent on the EtOH-paired (CS+) floor (% Time on EtOH-paired floor). An initial 2-way ANOVA (Group x Test) was followed by Bonferroni-adjusted within-subjects comparisons (paired t-tests) for each group. Additional analyses of the time spent on the Grid floor were performed including the Conditioning Subgroup (G+ and G-) as a third factor and these data are reported in Table 4. Overall α was set at .05 for all statistical tests.

Because the goal of these experiments was to specifically manipulate extinction of an acquired EtOH-CPP, we decided, *a priori*, to remove animals that failed to express a place preference of greater than 50% on Test 1. These

experiments revealed that less than 25% of all subjects failed to express significant preference following the 2-trial conditioning procedure—a finding that is consistent with previous reports that have also removed non-learners from extinction analyses (Groblewski et al., 2011).

RESULTS

A total of 14 animals (approximately 22%) were removed from analysis for exhibiting less than 50% preference on Test 1.

Histology

Histological analysis of the damage caused by the electrolytic lesion shows that tissue damage was primarily limited to the PL, IL, subregions of the mPFC (Figures 18 & 19). Although the extent of cell proliferation was primarily limited to the PL and IL, there was some damage that extended dorsally into the anterior cingulate subregion of the mPFC.

Conditioned Place Preference

The results of the CPP expression tests revealed that electrolytic lesions of the mPFC prevented the normal extinction of EtOH-CPP exhibited by the Sham and No Sx groups (Figure 20). Initial analysis of the place preference results (Percent Time on EtOH-Paired Floor) from Tests 1 and 2 revealed a significant main effect of Test [$F(1,46) = 13.3$, $p < .005$] but no Group x Test interaction ($p > .05$). Although not statistically justified, Bonferroni-corrected paired t-tests comparing preferences on Tests 1 and 2 for each group revealed a

Figure 18.

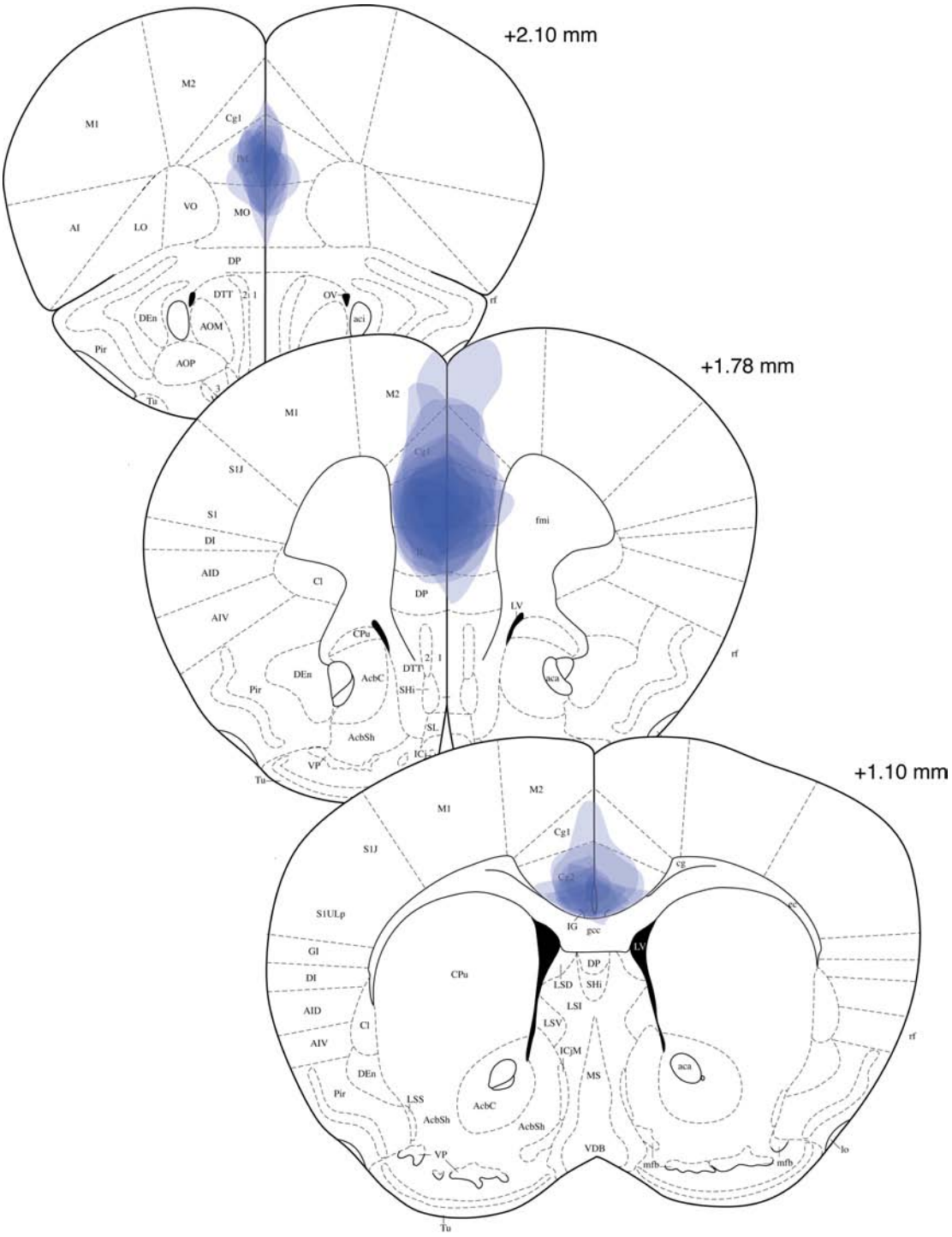


Figure 18. Lesion damage across three stereotaxic levels (adapted from Paxinos & Franklin, 2001) for each subject, represented at 15% opacity in order to show overlap.

Figure 19.

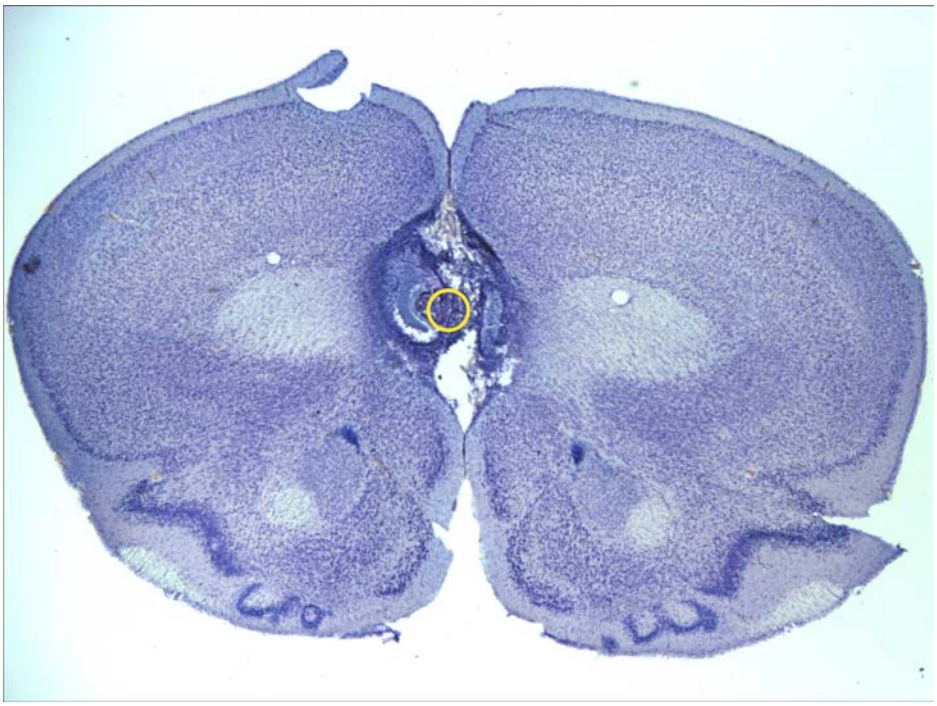


Figure 19. Representative photomicrograph of electrolytic lesion of the mPFC. Yellow circle depicts the intended stereotaxic mPFC target (AP: 1.8mm, ML: 0.0mm, DV: -2.5mm) from Paxinos & Franklin, (2001).

Figure 20.

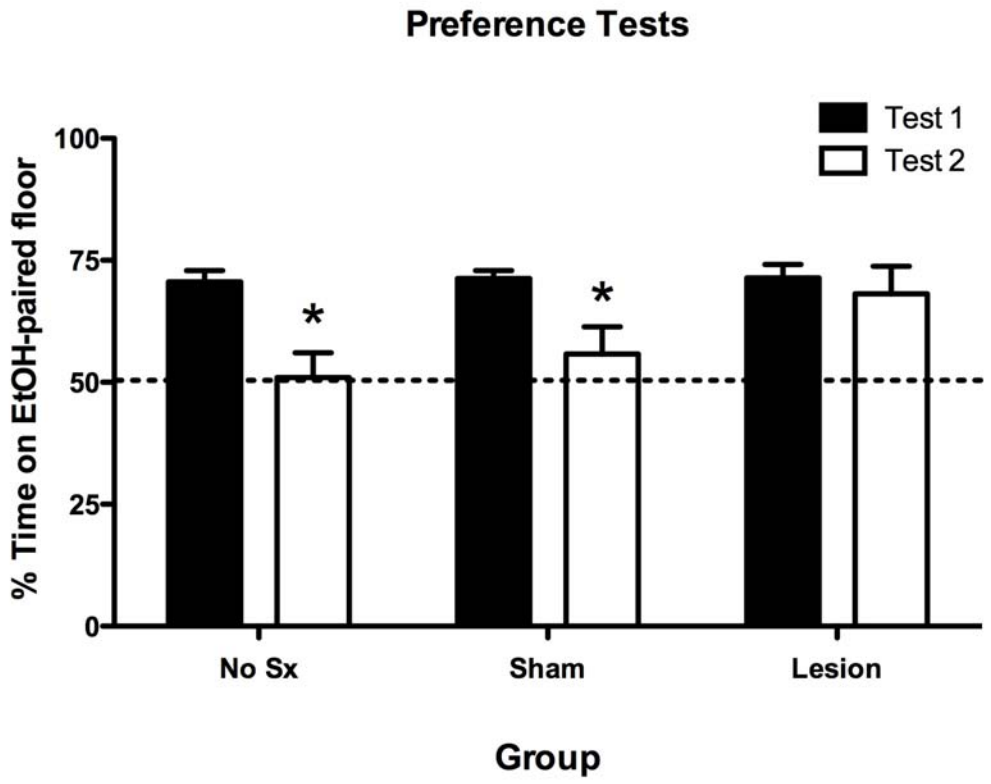


Figure 20. Preference Test data (expressed as Percent Time on EtOH-Paired Floor) before (Test 1) and after (Test 2) extinction. Significant reduction in place preference (*) as determined by Bonferroni-corrected post-hoc comparisons of Tests 1 and 2 for each group ($p < .05$). Data are expressed as mean (± SEM).

significant reduction in preference (i.e., extinction) in the No Sx ($t(13) = 4.8$ $p < .001$) and Sham ($t(22) = 2.8$ $p < .05$) groups. Most interestingly, the Lesion group showed no reduction in place preference on Test 2 ($p > .05$), suggesting that electrolytic lesions of the mPFC prevented extinction of EtOH-CPP. These findings were further supported by the additional analyses of Time on Grid Floor data for each of the Conditioning Subgroups (see Table 4).

DISCUSSION

The results of this experiment showed that electrolytic lesions of the mPFC that encompass both the PL and IL subregions significantly impaired extinction of EtOH-CPP in mice. These findings further support previous published results implicating a crucial role of the mPFC in extinction of drug-seeking behavior (e.g., Hsu & Packard, 2008). Furthermore, these data are in agreement with the immunohistochemistry data previously reported by our laboratory that showed significant effects of extinction on cue-induced neuronal activation (using pCREB) in areas of the mPFC (Chapter 4). If a reduction in mPFC CREB activation is required for extinction of EtOH-CPP, then it is possible that the electrolytic lesions in the current experiment prevented extinction by eliminating the dynamic nature of mPFC activity.

The current experiment is in complete agreement with the findings of Hsu & Packard (2008) showing that reversible lesions of the mPFC, achieved by temporary inactivation with bupivacaine, blocked extinction of amphetamine-

Table 4. Preference Test Data including Conditioning Subgroup (expressed as Time on Grid Floor). Data are expressed as mean (± SEM).

Group	Conditioning Subgroup	*n*	Test 1: Time on Grid Floor (sec/min)	Post hoc tests	Test 2: Time on Grid Floor (sec/min)	Post hoc tests
No Sx	G+	7	41.1 ± 2.1	] *	27.0 ± 5.1	
	G-	7	16.4 ± 1.9		25.9 ± 3.2	
Sham	G+	12	42.8 ± 1.3	] *	29.7 ± 4.5	
	G-	11	17.2 ± 1.5		22.4 ± 4.8	
Lesion	G+	7	44.6 ± 2.2	] *	38.7 ± 5.4	] *
	G-	5	19.7 ± 2.2		16.1 ± 3.3	

Significant place preference (*) as determined by Bonferroni-corrected post-hoc comparisons of G+ and G- subgroups ($p < .05$).

CPP. The current findings are also in agreement with the suggestion that the mPFC plays a key role in actively inhibiting expression of CPP during extinction (Ovari & Leri, 2008). In contrast to these findings, Zavala et al. (2003) showed that pre-conditioning quinolinic acid lesions of the PL region of the mPFC did not prevent acquisition or subsequent extinction of cocaine-CPP in rats. In fact, the extinction data reported by Zavala et al. (2003) suggests that these PL lesions may have even facilitated extinction of cocaine-CPP. Although the reasons for this discrepancy in results are not completely clear, it is important to note that the lesion administered by Zavala et al. was primarily limited to the PL subregion of the mPFC and was administered prior to conditioning. On the other hand, the areas affected by the manipulations reported here, as well as by Hsu & Packard, encompass both the PL and IL subregions of the mPFC. As previously mentioned, it is thought that the IL is more important for extinction of a conditioned response whereas the PL is more involved in expression of that behavior (Peters et al., 2009). Thus, it is possible that these two subregions are differentially involved in extinction of CPP. Because the current study involved mice instead of rats, it is difficult, if not impossible, to accurately and reproducibly lesion only one of the subregions of the mPFC. Therefore, it is not possible to discern the differential effects of PL and IL lesions on extinction of CPP with this procedure. However, given the extensive data from the conditioned fear literature, it is expected that PL lesions would affect expression, while IL lesions would affect extinction, of EtOH-CPP.

In conclusion, the current experiment showing that electrolytic lesions of the mPFC impair extinction of EtOH-CPP is in agreement with previously reported experiments by our laboratory (Chapter 4) and other laboratories (Hsu & Packard, 2008; Ovari & Leri, 2008). Together with the immunohistochemistry data presented in the previous chapter, these data show that the mPFC plays an integral role in extinction of EtOH-CPP in mice. Future studies will further examine the role of mPFC in extinction of EtOH-CPP by manipulating specific receptor systems and signaling pathways via intracranial microinjections of pharmacological agents.

6

EFFECTS OF PHARMACOLOGICAL INACTIVATION OF THE MEDIAL PREFRONTAL CORTEX (mPFC) ON EXTINCTION OF ETHANOL-SEEKING BEHAVIOR IN MICE

Contributions by:
Groblewski PA & Cunningham CL

The work described in this chapter has not yet been published.

ABSTRACT

Recent immunohistochemistry and lesion data from our laboratory suggest that the prelimbic (PL) and infralimbic (IL) subregions of the medial prefrontal cortex (mPFC) are necessary for extinction of ethanol-induced conditioned place preference (EtOH-CPP). However, the exact mechanism by which the mPFC is exerting control over extinction of EtOH-CPP remains unclear. In order to explore the different receptor systems and signaling pathways within the mPFC that are involved in extinction of EtOH-CPP, parametric assessment of a microinjection procedure are required. The current set of experiments attempted to determine the optimal parameters for extinction-specific intra-mPFC microninjections by first assessing the effect of temporary inactivation of the mPFC (via microinjections of the long-lasting sodium-channel blocker, bupivacaine) on extinction of EtOH-CPP. A single, midline guide cannula was implanted aimed at the PL and IL and injections of bupivacaine or vehicle were administered before extinction sessions. The results of this study showed that despite multiple parametric manipulations, neither the vehicle- or bupivacaine-injected groups showed significant extinction whereas animals that did not receive surgery (or microinjections) extinguished normally. As such, assessment of the effects of intra-mPFC bupivacaine on extinction was obstructed by the inability of the control microinjection group to extinguish. One explanation for these results is that the microinjection procedure, regardless of what compound was injected, caused excessive tissue damage to the mPFC. By effectively lesioning the mPFC, this microinjection procedure mimicked the effects of the electrolytic lesion previously reported by this laboratory. These results further support the findings that the mPFC is necessary for extinction of EtOH-CPP.

INTRODUCTION

Recent evidence from our laboratory has shown that the medial prefrontal cortex (mPFC) plays an integral role in extinction of ethanol (EtOH)-induced conditioned place preference (CPP). Specifically, using the phosphorylated transcription factor, phospho-cAMP response element binding protein (pCREB), as an indicator of neuronal activation, we have shown that the prelimbic (PL) and infralimbic (IL) subregions of the mPFC show significant activation following a brief exposure to an EtOH-paired cue. However, this increase in the number of pCREB-positive cells in the mPFC was not evident in animals that had had the EtOH-cue association extinguished (Chapter 4). Additionally, our laboratory has shown that electrolytic lesions of the mPFC following conditioning significantly impaired extinction of EtOH-CPP (Chapter 5). Therefore, we have shown both a correlative and causal role for the mPFC in extinction of EtOH-CPP.

The lesion study presented in Chapter 5 is in agreement with previously published reports that have shown that pharmacological inactivation of the mPFC with the long-lasting sodium channel blocker bupivacaine prevented extinction of amphetamine-CPP (Hsu & Packard, 2008). Additional evidence for the involvement of the mPFC in extinction of CPP came from a report by Ovari & Leri (2008). In this study, Ovari & Leri showed that intra-mPFC injection of the GABA-receptor agonists baclofen and muscimol actually reinstated an extinguished heroin-CPP—presumably caused by a removal of the inhibition of CPP-expression by the mPFC. Although these findings strongly support a

general role of the mPFC in extinction of EtOH-CPP, the receptor/signaling systems within the mPFC that are involved in extinction of CPP remain unknown. Studies involving conditioned fear have reported extinction-altering effects following mPFC manipulations of metabotropic glutamate and AMPA receptors (Fontanez-Nuin et al., 2011; Zushida et al., 2007), alpha-1 adrenergic receptors (Do-Monte et al., 2010), D1-type dopamine receptors (Hikind & Maroun, 2008), and extracellular signal-regulated kinase (ERK) signaling (Hugues et al., 2006). Although these findings have begun to illuminate the different receptor systems and signaling pathways involved in extinction of conditioned fear, whether these findings would generalize to extinction of CPP is unknown.

In order to begin to explore the different mechanisms underlying mPFC control of extinction of EtOH-CPP, it is first necessary to identify the optimal parameters for intra-mPFC micromanipulations. Therefore the current set of experiments attempted to examine the effects of temporary mPFC-inactivation on extinction of EtOH-CPP via microinjections of bupivacaine. Similar to lidocaine, bupivacaine is a sodium channel blocker that, when injected, temporarily inactivates neuronal signaling by preventing membrane depolarization and subsequent action potentials (Catterall & Mackie, 2006). Subjects received surgical implantation of a single, midline guide cannula aimed at the mPFC and subsequently received intra-mPFC infusions of bupivacaine or vehicle prior to the nonreinforced CS+ cue exposures during extinction of EtOH-CPP. It is hypothesized that intra-mPFC injection of bupivacaine prior to each of the

extinction trials will mimic the effects of an electrolytic lesion by eliminating the dynamic characteristics of mPFC activity that are required for extinction learning, thereby preventing extinction of EtOH-CPP. These experiments were intended to identify the necessary parameters required for future explorations of the receptor systems and signaling pathways in the mPFC that are required for extinction of EtOH-CPP.

METHODS AND MATERIALS

Subjects

Adult, male DBA/2J mice (n=108) were obtained from Jackson Laboratory (Sacramento, CA) at 6-7 weeks old and allowed to acclimate to the animal colony for 2 or 3 weeks before experiments commenced. Mice were housed, four to a cage, in cob bedding in a Thoren rack with water and food available *ad libitum* throughout each experiment. All experiments were conducted during the light phase (7:00-19:00). The Oregon Health & Science University IACUC approved all experimental procedures.

Drugs

Ethanol (20% v/v in isotonic saline) was administered intraperitoneally (IP) at a dose of 2 g/kg (12.5 ml/kg).

Bupivacaine hydrochloride (Sigma Aldrich, St. Louis, MO) was diluted in a vehicle of isotonic saline at 2% w/v (i.e., 20 mg/ml). These parameters were based on previous studies utilizing intracranial lidocaine injections in mice

(Daumas et al., 2005) as well as parametric studies that have quantified the diffusion characteristics of intracranially administered anesthetics (Tehovnik & Sommer, 1997).

Place Preference Apparatus

All behavioral procedures were performed in custom made, acrylic and aluminum conditioning boxes (30 x 15 x 15 cm), each of which was enclosed in a sound-attenuating chamber (Model E10-20, Colbourn Instruments, Allentown, PA). Six infrared emitters and detectors, mounted 5 cm apart and 2.2 cm above the floor of the box, were used to obtain spatial location and locomotor activity data throughout conditioning, extinction and testing. The conditioned stimuli (CSs) consisted of two distinct tactile cues—grid and hole floors. Grid floors (2.3 mm stainless steel rods, 6.4 mm apart) and hole floors (16-gauge stainless steel perforated with 6.4-mm round holes) were interchangeable allowing for either full- or split-cue configurations during conditioning/extinction and testing, respectively. These cues are unbiased in that naïve DBA/2J mice show equal preference for the two floors during drug-free preference tests (Cunningham et al., 2003).

Behavioral Procedures

The CPP portion of the study consisted of unbiased designs and procedures similar to those previously described in detail by this laboratory (Cunningham et al., 2006b). Each experiment outlined below consisted of a combination of the following phases: Habituation, Conditioning, Extinction, Preference tests, and Surgery. In Experiment 1, Surgery was performed after

Conditioning but before Extinction whereas in Experiment 2, Surgery was performed before Habituation. These procedures are described in more detail below.

Experiment 1

Habituation. On Day 1, mice were given a saline injection (12.5 ml/kg) and habituated to the conditioning apparatus, equipped with white paper flooring, for 5 mins.

Conditioning. Twenty-four hrs later animals received daily CPP conditioning trials during which EtOH (2 g/kg) was paired with one of the tactile cues while saline was paired with the other cue on alternating days. Animals in the "G+" group had EtOH paired with the Grid floor and saline paired with the Hole floor while the "G-" group received EtOH with the Hole floor and saline with the Grid floor. Conditioning subgroup (G+ and G-) and trial-type order (S-E-S-E or E-S-E-S) were fully counterbalanced.

Test 1. One day after the last conditioning trial, all animals received a drug-free, 30-min preference test during which both tactile cues were presented and place preference was assessed.

Surgery. Three days following Test 1, all animals underwent surgical implantation of a single, microinjection guide-cannula targeting the mPFC (see *Surgical Procedures* for details).

Extinction. After fully recovering from surgery, animals received 3 days of extinction, each of which consisted of a morning (AM) and afternoon (PM)

session. During AM sessions, all animals received a sham microinjection followed by a saline injection (12.5 ml/kg IP) and immediate placement onto the CS- floor for 30 mins. In the PM sessions, animals received a microinjection of Vehicle or Bupivacaine followed by a saline injection and immediate placement onto the CS+ floor for 30 mins (see *Microinjection Procedure* for details). Intracranial injections of bupivacaine have been shown to effectively prevent >90% of neuronal activation for up 90 mins (Alam & Mallick, 1990).

Test 2. Twenty-four hrs after the last extinction trial all animals received a second, drug-free 30-min preference test.

Experiment 2

Surgery. Animals in the Bupivacaine and Vehicle groups underwent surgical implantation of a single, microinjection guide cannula targeting the mPFC (see *Cannula Implantation Surgery* for details). Animals in the No Sx group were separated into 2 mice per cage and remained in the home cage undisturbed until Habituation.

Habituation. After fully recovering from surgery, mice were given a habituation session identical to that in Experiment 1.

Conditioning. Twenty-four hrs later animals received CPP conditioning identical to that in Experiment 1.

Test 1. One day after the last conditioning trial, all animals received a drug-free, 30-min preference test identical to that in Experiment 1.

Extinction. Three days after Test 1, animals received 4 days of extinction. Extinction was performed identically to that in Experiment 1 with the exception that an additional day was included. The purpose of adding an additional day of extinction was to increase the amount of nonreinforced cue exposure in hopes of increasing the amount of extinction. Animals in the No Sx group were matched for handling (time being scruffed) but no injection was administered.

Test 2. Twenty-four hrs after the last extinction trial all animals received a second, drug-free 30-min preference test.

Surgical Procedures

Cannula Implantation Surgery. A single, midline, indwelling guide cannula was implanted in each animal following a similar procedure to that previously described by Gremel and Cunningham (2009). Animals received an injection of meloxicam (0.2 mg/kg, SC) prior to being placed and maintained under deep anesthesia with isoflurane. Animals were placed in a stereotax (Model 1900, KOPF Instruments, Tujunga, CA) with the skull horizontal and an anchor screw was affixed. A single, midline burr hole was drilled 1.8 mm rostral to bregma. Indwelling, stainless steel guide cannulae (10 mm, 28g) were implanted, using a stereotax, aimed 1 mm dorsal to the target area in the mPFC (AP: +1.8 mm, ML: 0.0 mm, DV: -2.5 mm) using the mouse brain atlas (Paxinos and Franklin, 2001). Guide cannulae were secured to the skull with carboxylate dental cement (Durelon, 3M, St. Paul, MN) and a 32 g stainless steel stylet was inserted.

Animals were given 5-7 days to recover before any behavioral procedure commenced or resumed.

Histology. Upon completion of each experiment, animals received an overdose of pentobarbital (150 mg/kg), brains were removed and postfixed with paraformaldehyde (2% w/v in phosphate buffered solution, PBS). Brains were cryoprotected with 20% then 30% sucrose in PBS and 0.1% NaN_3. Frozen slices (40 μm) were obtained on a cryostat (Leica CM1900) and thionin-stained for verification of cannula location and lesion location/size.

Microinjection Procedure

Microinjections into the mPFC occurred during the extinction phase only. Mice were gently restrained, the stylet was removed and replaced with the injector (11 mm, 32 g stainless steel tubing attached inside of a 25 g tube) attached to a Hamilton syringe (10 μL) with polyethylene tubing (PE20). A syringe pump (Model A-74900-10, Cole Parmer, Vernon Hills, IL) delivered 0.2 μL of Vehicle or Bupivacaine over 2 mins (0.1 μL/min) and injectors remained in place for 30 secs after the injection was complete in order to prevent diffusion up the cannula. Based on previous parametric studies of effective diffusion, this injection volume of bupivacaine is expected to cause neuronal inactivation in the PL and IL subregions of the mPFC (Tehovnik & Sommer, 1997). Injectors were then removed and a clean stylet was inserted. Sham microinjections were performed identically except that no solution was pumped through the lowered injector.

Statistical Analysis

Place preference data were presented and analyzed using analysis of variance (ANOVA) of the percentage of each test spent on the EtOH-paired (CS+) floor (% Time on EtOH-paired floor). An initial 2-way ANOVA (Group x Test) was followed by Bonferroni-adjusted within-subjects comparisons (paired t-tests) for each group. Additional analyses were performed on the amount of time spent on the Grid floor that included the Conditioning Subgroup (G+ and G-) as a third factor (Table 5). Overall α was set at .05 for all statistical tests.

Because the goal of these experiments was to specifically manipulate extinction of an acquired EtOH-CPP, we decided, *a priori*, to remove animals that failed to express a place preference of greater than 50% on Test 1. These experiments revealed that less than 25% of all subjects failed to express significant preference following the 2-trial conditioning procedure—a finding that is consistent with previous reports that have also removed non-learners from extinction analyses (Groblewski et al., 2011).

RESULTS

Experiment 1

A total of 18 animals were removed from analysis (9 showed less than 50% preference on Test 1 and an additional 9 were removed for not meeting histological criteria).

Histolology

Histological analysis revealed that for the most part, injector tracks terminated at a point within the target region between the PL and IL subregions of the mPFC (Figure 21 & 22). There was no difference in the location of injector tips for the Bupivacaine and Vehicle groups (Figure 21). A total of 9 subjects were removed because injector tracks were located too lateral, ventral, and/or rostral from the target region.

Conditioned Place Preference

The place preference results from Tests 1 and 2 revealed that neither the Vehicle nor Bupivacaine group showed a significant reduction in preference following extinction (Figure 23). Specifically, the 2-way ANOVA of the Percent Time Spent on the EtOH-paired floor revealed a main effect of Test [$F(1,33) = 4.3$, $p < .05$] but no main effect of Group or a Group x Test interaction ($p's > .05$). Although not justified statistically, paired t-tests of preferences on Test 1 and 2 revealed no significant reduction for either group ($p's > .05$). Thus, neither the control (Vehicle) nor the experimental group (Bupivacaine) showed significant extinction of EtOH-CPP. Inclusion of Conditioning Subgroup in the analysis of Time On Grid Floor (Table 5) yielded a significant Conditioning Subgroup x Test [$F(1,26) = 6.0$, $p < .05$] interaction and significant main effects of Conditioning Subgroup [$F(1,26) = 50.4$, $p < .001$] and Test [$F(1,26) = 4.5$, $p < .05$] but no Group x Conditioning Subgroup x Test interaction ($p > .05$). Although not statistically justified, in order to pursue the Conditioning Subgroup x Test

Figure 21.

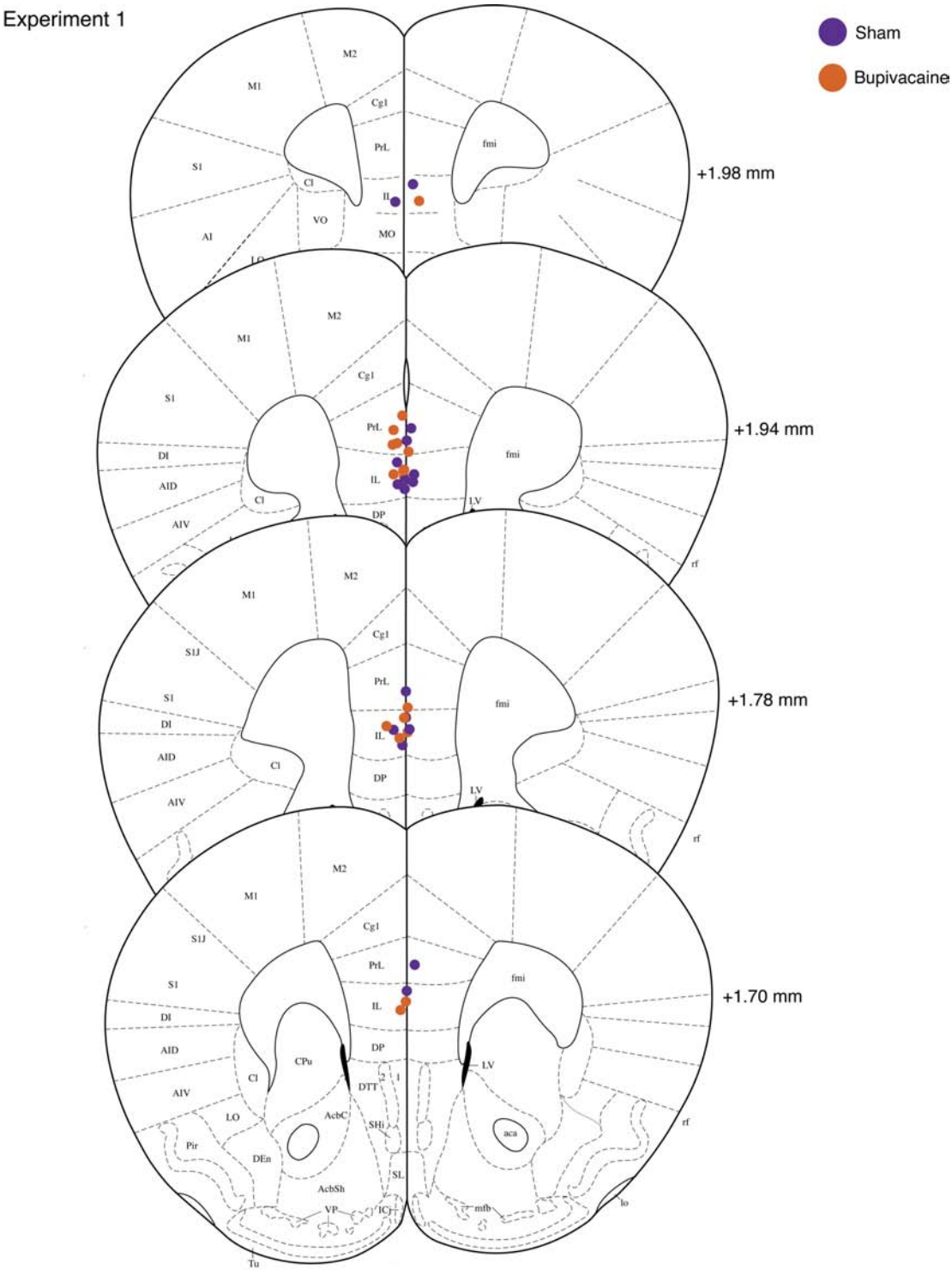


Figure 21. Injector-tip location for each subject in Experiment 1 across four stereotaxic levels (adapted from Paxinos & Franklin, 2001).

Figure 22.

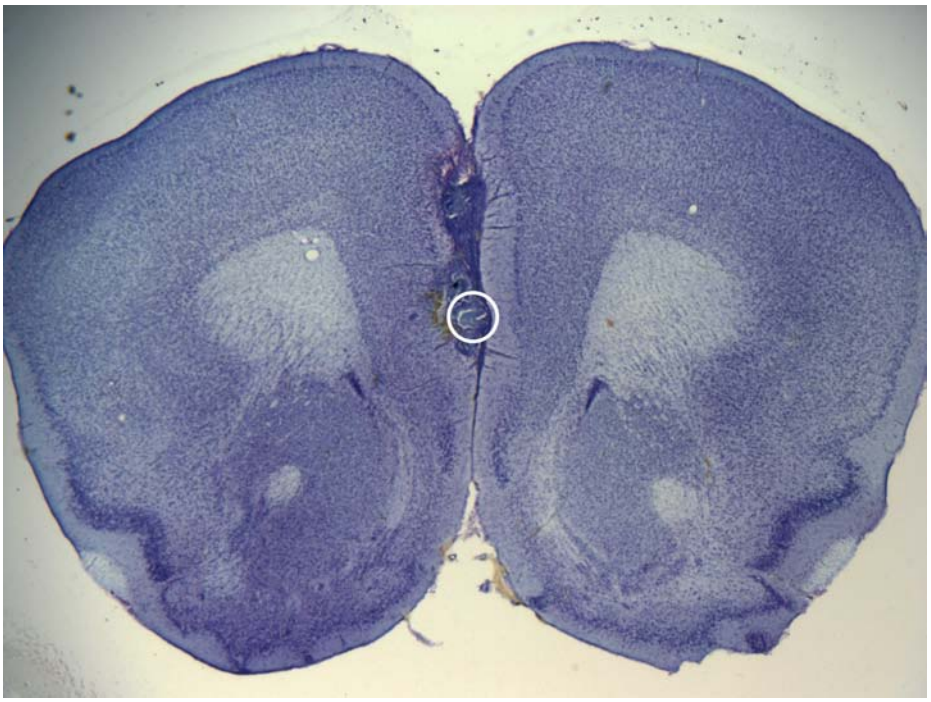


Figure 22. Representative photomicrograph of the cannula and injector track from a subject in Experiment 1. White circle depicts the intended mPFC stereotaxic target for the injector tip (AP: +1.8mm, ML: 0.0mm, DV: -2.5mm) determined from Paxinos & Franklin, (2001).

Figure 23.

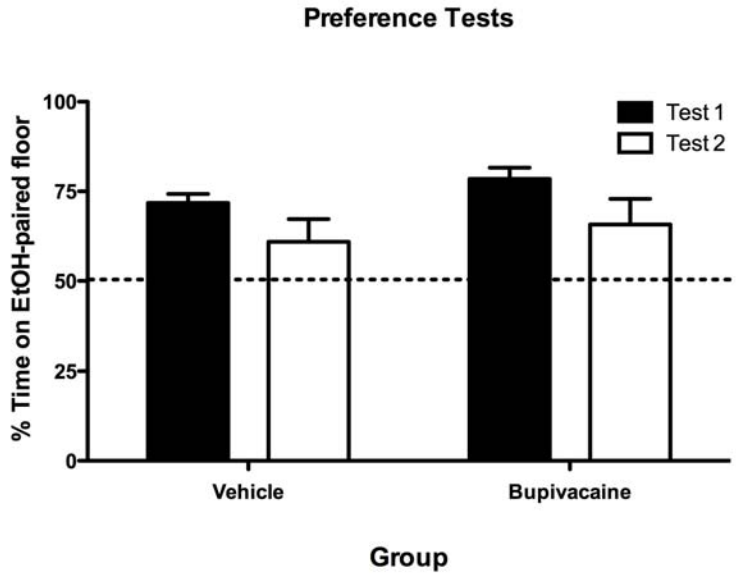


Figure 23. Preference Test data (expressed as Percent Time on EtOH-Paired Floor) before (Test 1) and after (Test 2) extinction. Data are expressed as mean (± SEM).

Table 5. Preference Test Data including Conditioning Subgroup (expressed as Time on Grid Floor)

Exp.	Group	Conditioning Subgroup	*n*	Test 1: Time on Grid Floor (sec/min)	Post hoc tests	Test 2: Time on Grid Floor (sec/min)	Post hoc tests
1	Vehicle	G+	7	41.8 ± 2.3	] *	27.3 ± 5.3	
		G-	9	16.0 ± 2.1		16.3 ± 4.2	
	Bupivacaine	G+	9	47.7 ± 2.4	] *	36.6 ± 5.7	] *
		G-	5	13.9 ± 3.0		15.4 ± 6.5	
2	No Sx	G+	10	45.6 ± 1.5	] *	30.4 ± 8.2	
		G-	8	18.6 ± 2.5		30.9 ± 9.2	
	Vehicle	G+	9	49.5 ± 1.9	] *	40.8 ± 5.7	
		G-	5	15.1 ± 1.4		15.6 ± 8.5	
	Bupivacaine	G+	8	47.1 ± 2.0	] *	33.3 ± 8.5	
		G-	9	13.0 ± 1.9		14.9 ± 4.5	

Significant place preference (*) as determined by Bonferroni-corrected post-hoc comparisons of G+ and G- subgroups ($p < .05$).

interaction, separate Group x Conditioning Subgroup ANOVAs were performed for each test. These analyses yielded significant main effects of Conditioning Subgroup on Test 1 [$F(1,26) = 141.8$, $p < .001$] and Test 2 [$F(1,26) = 8.6$, $p < .01$], suggesting that together, the Vehicle and Bupivacaine groups showed significant preference on both tests. Although there were no significant Group x Conditioning Subgroup interactions for either test, comparisons of G+ and G- Conditioning Subgroups were performed in order to assess preference for the two groups on each test as described in Chapters 2 and 3. On Test 1, the comparison between the G+ and G- subgroups yielded significant differences for both the Vehicle and Bupivacaine groups (p's $<.001$) confirming significant place preference for both groups (Table 5). However, on Test 2, a significant difference was only detected in the Bupivacaine group ($p < .05$). Therefore, although this analysis may have hinted at a possible effect of Bupivacaine on extinction, it was not in agreement with the initial analysis and therefore the results remained inconclusive.

Because of the incomplete extinction in the Vehicle group, it remained unknown if inactivation of the mPFC with Bupivacaine is able to impair extinction. In order further investigate these findings, Experiment 2 involved three procedural changes intended to increase the extinction exhibited by the Vehicle group. First, cannula implantation surgery was performed prior to conditioning. This manipulation was intended to eliminate any potential effects of the time interval between Test 1 and extinction (9 days). Previous reports have

suggested that inserting a time gap between conditioning and extinction can alter extinction (e.g., Myers et al., 2006). Second, extinction was extended by an extra trial for a total of four days of extinction to increase the amount of nonreinforced exposure to the cues in hopes of further reducing the preference in the Vehicle group. Finally, an additional control group that did not receive surgery was included (No Sx group) in order to assess the effects of the handling portion of the microinjection procedure on extinction learning. Although it is unclear how stressful handling (i.e., scruffing for 2-3 mins) could influence extinction, previous reports have shown that this type of handling did effect expression of EtOH-induced conditioned place aversion (Bechtholt et al., 2004).

Experiment 2

A total of 16 animals were removed from analysis (8 showed less than 50% preference on Test 1 and an additional 8 were removed for not meeting histological criteria).

Histology

Similar to Experiment 1, histological analysis revealed that for the most part injector tracks were aimed at the target region between the PL and IL subregions of the mPFC (Figure 24). There was no difference in the location of injector tips for the Bupivacaine and Vehicle groups (Figure 24). A total of 8 animals were removed from the study because of injector tips that were located too lateral, ventral, and/or rostral from the target.

Figure 24.

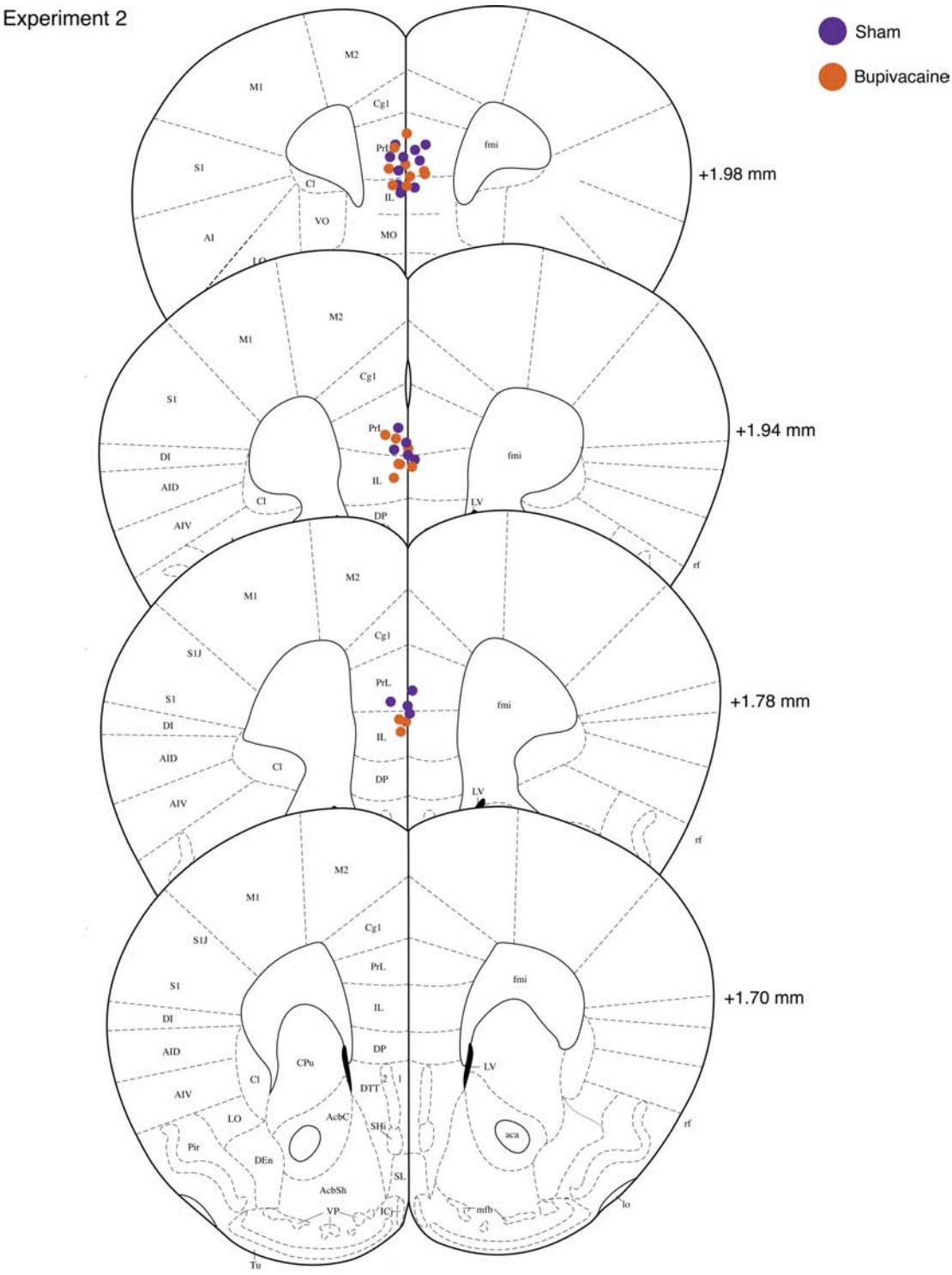


Figure 24. Injector-tip location for each subject in Experiment 2 across four stereotaxic levels (adapted from Paxinos & Franklin, 2001).

Conditioned Place Preference

Analysis of the place preference from Tests 1 and 2 revealed that only the No Sx group exhibited significant extinction (Figure 25). Specifically, the initial 2-way ANOVA revealed a main effect of Test [$F(1,40) = 9.3$, $p < .005$] but not Group, and no significant Group x Test interaction ($p's > .05$). In order to assess the within-subject extinction for each Group, separate paired t-tests were performed as previously described. The results of the separate paired t-tests showed a significant decrease in preference for only the No Sx group [$t(12) = 2.5$ $p < .05$]. These data suggest that the No Sx, but not Vehicle or Bupivacaine, group significantly extinguished. Inclusion of Conditioning Subgroup (Table 5) in the analysis yielded a significant Condition x Test [$F(1,37) = 8.3$, $p < .01$] interaction but no Group x Conditioning Subgroup x Test interaction ($p > .05$). As in Experiment 1 and previous experiments, separate Group x Conditioning Subgroup ANOVAs were performed for each test, although these were not statistically justified. These analyses revealed a significant main effect of Conditioning Subgroup on Test 1 [$F(1,37) = 371.1$, $p < .001$] and Test 2 [$F(1,37) = 5.6$, $p < .05$]. Subsequent analysis of Test 1 revealed significant differences between G+ and G- subgroups in all three groups ($p's < .001$). On Test 2, Conditioning Subgroup-comparisons yielded trends towards, but no, significant differences for the Vehicle ($p = .08$) and Bupivacaine ($p = 0.17$) groups. Although these analyses suggested that the No Sx group showed normal extinction of EtOH-CPP, as in Experiment 1, comparisons between the Bupivacaine and

Figure 25.

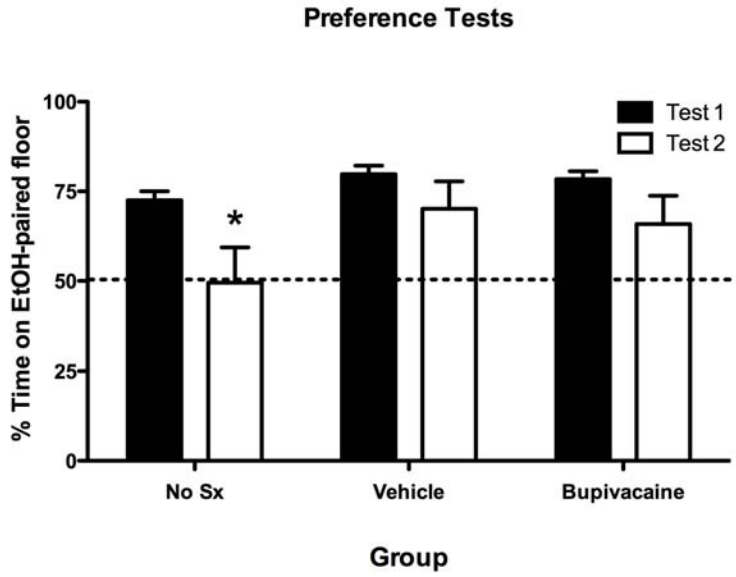


Figure 25. Preference Test data (expressed as Percent Time on EtOH-Paired Floor) before (Test 1) and after (Test 2) extinction. Significant reduction in place preference (∗) as determined by Bonferroni-corrected post-hoc comparisons of Tests 1 and 2 for each group ($p < .05$). Data are expressed as mean (± SEM).

Vehicle groups did not convincingly show 1) significant extinction in the Vehicle group or 2) significant differences in extinction of the Bupivacaine and Vehicle groups.

Therefore, despite increasing the number of extinction trials and changing the timing of surgery, the results of Experiment 2 are similar to those in Experiment 1. Together, these experiments suggest some component of the microinjection procedure may have interfered with extinction of EtOH-CPP. Use of the intra-mPFC microinjection procedure may therefore not be suitable to examine intra-mPFC manipulations in mice.

DISCUSSION

The current set of experiments was performed in order to 1) identify the optimal parameters for extinction-specific intra-mPFC microinjections and 2) determine the effects of temporary mPFC inactivation on extinction of EtOH-CPP. Although it appeared that intra-mPFC bupivacaine may have impaired extinction of EtOH-CPP (i.e., the Bupivacaine group did not show a significant reduction in preference following extinction, Figure 23), clear assessment of these effects were complicated by the findings that the control (Vehicle) groups did not show complete extinction. In both Experiments 1 and 2, the Vehicle groups showed incomplete extinction when compared to the No Sx group or to previous findings of the many extinction studies from our laboratory (e.g., Groblewski et al., 2011). The reasons underlying the lack of extinction in the Vehicle groups remain

unclear, however, it is apparent that this effect is consistent across multiple parametric variations of the extinction procedure.

The most parsimonious explanation for these findings is that the microinjection procedure, regardless of the compound being injected, caused tissue damage to an area of the brain shown to be necessary for extinction of EtOH-CPP (*see Chapter 5)*. The tissue damage that resulted from the multiple microinjections in Experiments 1 and 2 may have been sufficient to effectively result in a partial or complete lesion of portions of the mPFC. Post-mortem histological examination suggests that the tissue damage (i.e., cell proliferation) caused by implantation of the cannula, repeated insertion of the injector, and/or injection of solution (vehicle or bupivacaine) is substantial and limited primarily to Layers I-III of the mPFC (Figure 26). Importantly, because these cannulae were located on the sagital midline, tissue damage to these layers of the cortex was bilateral. Retrograde tracing experiments have shown that Layers II/III of the mPFC contain dense populations of projection neurons that terminate in both the nucleus accumbens (NAc) and basolateral amygdala complex (BLC) (Cassell et al., 1989; Miller & Marshall, 2004). Interestingly, activity in these mPFC projection neurons is altered by exposure to drug-paired cues that are capable of eliciting approach behavior (Miller & Marshall, 2004, 2005b). Therefore, it is conceivable that the damage caused by the microinjection procedure resulted in altered mPFC regulation of the NAc and BLC, thereby impairing extinction of EtOH-CPP. Of course, further examination of the effects of the microinjection

Figure 26.

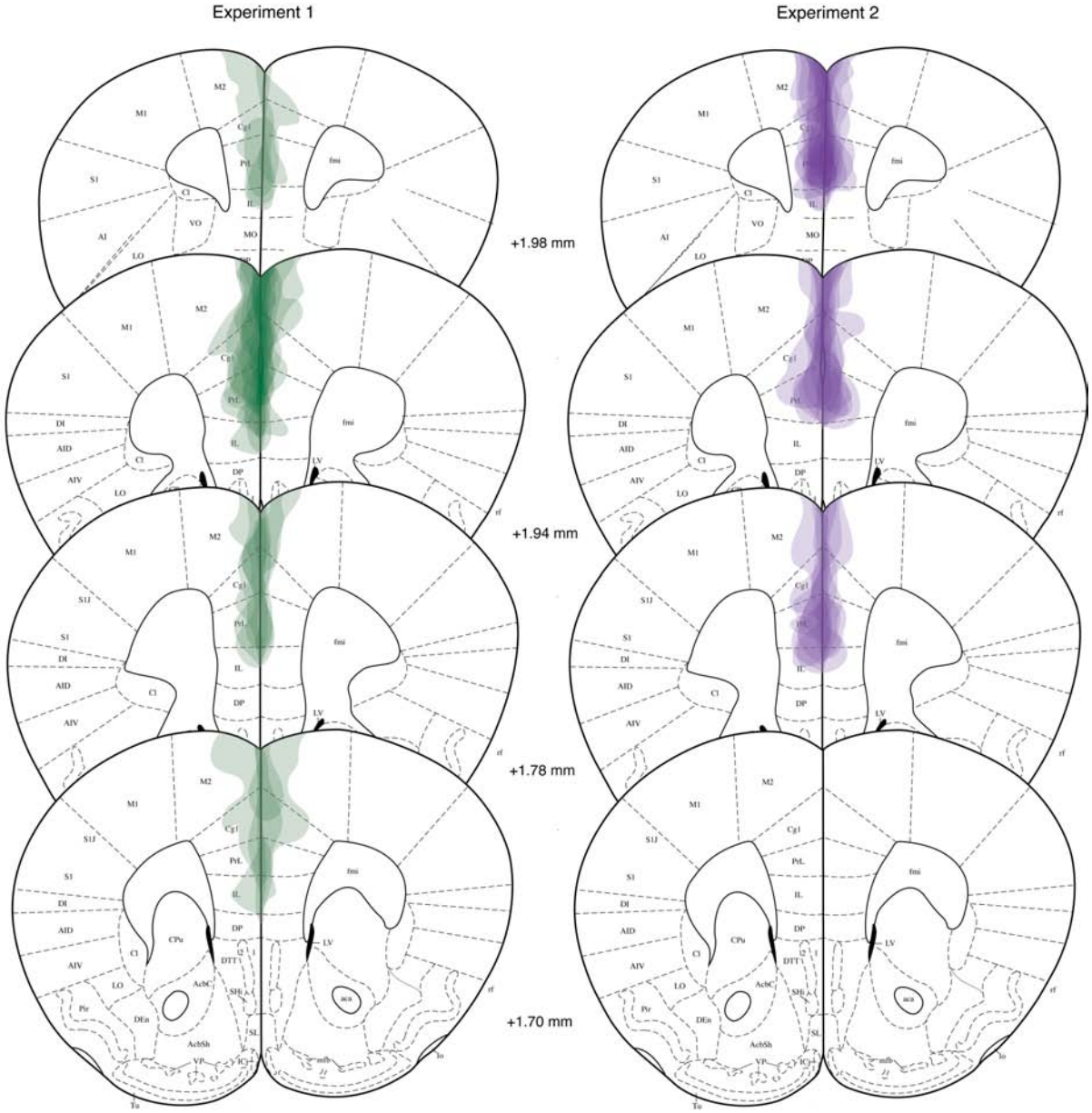


Figure 26. Tissue damage cause by implantation of the cannula and the microinjection procedure across four stereotaxic levels (adapted from Paxinos & Franklin, 2001) for each subject, represented at 15% opacity in order to show overlap.

procedure on these specific projections within the mPFC, as well as their projection targets, is required to confirm this suggestion.

Most studies examining the effects of intra-mPFC pharmacological manipulations on extinction have involved conditioned fear procedures that require only a single injection prior to a single extinction session (e.g., Fontanez-Nuin et al., 2011). Unlike extinction of conditioned fear, however, extinction of EtOH-CPP requires multiple extinction trials over the course of multiple days. Specifically, previous studies from our laboratory have shown that a minimum of 3 days of 30-min exposures to both the CS- and CS+ cues is required for complete extinction of EtOH-CPP (Groblewski et al., 2011). In order to minimize the number of microinjections administered in the current study, Experiment 1 consisted of this 3-day extinction procedure. However, because the Vehicle group exhibited incomplete extinction, Experiment 2 included an additional day of extinction. Regardless of whether the animals received 3 or 4 days of extinction, however, the Vehicle groups in both experiments failed to completely extinguish on Test 2. These findings suggest that the damage caused by intra-mPFC injections even when the number of extinction trials (and injections) is minimized, was sufficient enough to impair extinction. Therefore, as currently performed, the microinjection procedure is not adequately suited to examine the effects of intra-mPFC pharmacological manipulations on extinction of EtOH-CPP in mice. Further parametric studies that aim to reduce the damage caused by the microinjection procedure and/or reduce the number of required microinjections

are essential for future explorations of the different receptor systems and signaling pathways within the mPFC that are involved in extinction of CPP in mice.

7

GENERAL DISCUSSION: THE NEUROCHEMISTRY AND NEUROCIRCUITRY UNDERLYING EXTINCTION OF ETHANOL-SEEKING BEHAVIOR

Introduction

The previous chapters have described and discussed a number of studies intended to elucidate the neurochemistry and neurocircuitry underlying extinction of ethanol-seeking behavior in mice. These studies utilized a wide range of scientific approaches in order to identify the mechanisms that are responsible for extinction of EtOH-CPP. For the discussion of these results, these studies will be separated into three groups. The first group involved systemic approaches to understanding the involvement of NMDA receptors (Chapter 2) and ERK signaling (Chapter 3) in extinction of EtOH-CPP. The second group included the experiments described in Chapters 4 and 5 that demonstrated a role for the mPFC in extinction of EtOH-CPP using immunohistochemistry and lesion approaches. The final group attempted to assess the effect of pharmacological inactivation of the mPFC on extinction of EtOH-CPP (Chapter 6).

NMDA- and ERK-independence of EtOH-CPP extinction

The experiments of Chapters 2 and 3 report findings suggesting that extinction of EtOH-CPP may have relied on NMDA- and ERK-independent mechanisms. Most interestingly, these findings are in contrast to the published studies showing effects of both DCS and SL327 on extinction of CPPs induced by other drugs of abuse including cocaine (e.g., Botreau et al., 2006; Valjent et al., 2006). Therefore, the current results suggest that unlike with other drugs of abuse, extinction of EtOH-CPP, and perhaps other components of EtOH-related

learning, are not susceptible to manipulations of NMDA-receptor, or ERK, signaling.

A simplified interpretation of associative EtOH-related learning suggests that it depends on the ability of a subject to 1) detect and process the direct unconditioned stimulus properties of EtOH and 2) associate these stimulus properties with the contiguously presented cues and/or contexts. In the case of EtOH-CPP, for example, an animal learns to associate the rewarding effects of EtOH with the tactile floor cue following repeated, contiguous presentations (Cunningham et al., 2011). When these cues are presented in the absence of EtOH during an expression test or extinction trial, this novel post-acquisition EtOH-related learning relies on processing the indirect, or conditioned, rewarding effects of EtOH in the presence of the previously EtOH-paired cues.

The major findings reported in Chapter 2 were that the NMDA-receptor partial agonist DCS did not facilitate extinction of EtOH-CPP. DCS did, however, impair subsequent reconditioning. The effects on reconditioning were not due to a nonspecific effect of chronic DCS exposure on learning because DCS pre-exposure before initial conditioning did not impair development of EtOH-CPP. Although not specifically tested, one interpretation of these results was that reconditioning might have uncovered a potential extinction-facilitating effect of DCS (Groblewski et al., 2009).

The lack of an effect of DCS on extinction behavior *per se*, however, is in contrast to studies that have reported extinction-facilitating effects of DCS using

cocaine-induced CPP (Botreau et al., 2006, Paolone et al., 2008; Thanos et al., 2009). Together, these findings suggest that unlike with EtOH, some component of cocaine-related learning that occurs during extinction is susceptible to NMDA-receptor manipulations. It is unclear from these studies, however, whether DCS is directly acting to alter the conditioned reward and/or general learning component of extinction.

The conceptual mechanism underlying the effects of DCS on extinction is thought to rely on the ability of DCS to facilitate NMDA-receptor signaling via the glycine binding site (Norberg et al., 2008). Because NMDA-receptor activation requires binding at the glutamate and glycine binding sites, DCS effectively acts by increasing the likelihood that the glycine site is bound, thereby increasing the frequency and/or likelihood of channel opening and subsequent calcium influx (*for review see* Hofmann, 2007). As a partial agonist, DCS is able to indirectly modulate NMDA receptor signaling without causing the cellular toxicity seen with direct NMDA agonists (Deupree et al., 1996).

A large body of work has shown that extinction of a number of associative behaviors is blocked by NMDA-receptor antagonists (*for reviews see* Myers & Carlezon, 2010; Quirk & Mueller, 2008). As such, it has been established that extinction learning, like acquisition learning, involves NMDA-receptor signaling. In theory then, facilitation of NMDA-receptor signaling would result in stronger or more rapid extinction learning—an effect that would be desirable when treating humans suffering from PTSD and other associative anxiety disorders (Kaplan et

al., 2011). In fact, this has been shown to be the case with extinction of a number of behaviors, both in pre-clinical and clinical trials (e.g., Kushner et al., 2007; Ressler et al., 2004). However, there have also been reports of instances when extinction learning was not facilitated by DCS (e.g., Guastella et al., 2007; Tomilenko & Dubrovina, 2007; Woods & Bouton, 2006). Pre-clinically, the effects of DCS appear to be sensitive to a number of experimental factors including subject species and strain (Sunyer et al., 2008), subject anxiety (Tomilenko & Dubrovina 2007), cue- and unconditioned stimulus-modality (Weber et al., 2007), as well as the amount of initial training and subsequent within-session extinction (Bouton et al., 2008). Although not systematically tested in this work, given that these experiments all involved one strain of mice (DBA/2J) that were conditioned with one set of cues (distinct tactile floors) and did not exhibit significant amounts of within-session extinction (Groblewski et al., 2009), it is possible that any or all of these factors may have contributed to the discrepant findings. The current set of studies also suggests that the effects of DCS on extinction of drug-seeking behavior may be sensitive to the unconditioned stimulus used in conditioning, in this case EtOH. Specifically, it may be that cocaine's conditioned rewarding effects require NMDA receptor activity whereas EtOH's do not and that unlike extinction of conditioned fear, extinction of CPP is not sensitive to the extinction-facilitating effects of DCS.

It is interesting to note that both Matsuda et al. (2010) and Yan & Lu (2005) have reported that the effects of DCS on extinction of conditioned fear

were blocked by co-administration of MEK inhibitors, including SL327. These studies suggest that DCS facilitates extinction of fear by enhancing NMDA-receptor transmission, which in turn activates the intracellular ERK-signaling cascade. Therefore the results of Chapter 2 suggest that extinction of EtOH-CPP (including possibly the conditioned rewarding effects of EtOH) is not altered by manipulations of ERK-dependent mechanisms, including systemic administration of DCS.

The results discussed in Chapter 3 further support the suggestion that unlike with extinction of CPPs induced by other drugs of abuse, extinction of EtOH-CPP does not rely on ERK-dependent mechanisms. In fact, these experiments showed that no phase of EtOH-CPP was affected by significant (40-50%) inhibition of ERK-signaling in the brain. Because these experiments were the first of their kind to systematically examine the effects of multiple doses of SL327 on extinction of CPP, the lack of an effect on extinction of EtOH-CPP seemed plausible in light of a recent report showing no effect of SL327 on extinction of conditioned fear (Matsuda et al., 2010). On the other hand, the results of the acquisition experiment were somewhat surprising as they were in disagreement with other studies showing that SL327 impaired acquisition of cocaine-, THC-, and MDMA-induced CPPs (Salzmann et al., 2003; Valjent et al., 2000, 2001). Additionally, the finding that SL327 failed to prevent EtOH-induced sensitization did not agree with previous reports showing that both cocaine- and amphetamine-induced sensitization depended on intact ERK-signaling (Valjent et

al., 2006). Thus it appears that the lack of an effect of SL327 on all of these phases of EtOH-CPP may be due to the unique properties of the stimulus effects of EtOH.

Analysis of the current extinction findings strongly supports the results of Chapter 2 suggesting that extinction of EtOH-CPP is not altered by manipulations of ERK signaling. In conjunction with the data from the expression studies, it appears that the conditioned rewarding effects of EtOH (or the retrieval of these effects upon exposure to the previously EtOH-paired cues) are also ERK-independent. Similarly, the acquisition experiment suggests that the direct rewarding effects of EtOH are also not dependent on ERK signaling, as SL327 did not alter acquisition of CPP when it was co-administered with EtOH during conditioning. These assertions are further supported by the findings that EtOH did not significantly activate ERK signaling in the current experiments (*see* Figure 13) and in other previous studies (e.g., Neasta et al., in press). Therefore the experiments described in Chapters 2 and 3 suggest that both the direct rewarding effects of EtOH as assessed through acquisition, as well as the conditioned rewarding effects of EtOH as assessed through expression and extinction, do not involve ERK-dependent pathways.

Although these assertions are somewhat speculative, it is possible that extinction of EtOH-CPP, which requires processing of the indirect rewarding effects of EtOH, may rely more heavily on receptor systems other than NMDA. Instead of being primarily dependent on NMDA-receptor function, the conditioned

rewarding effects of EtOH may involve other systems such as opioid receptors. It is known that EtOH has direct actions on the opioid-receptor system by causing changes in endogenous opioid release and opioid receptor function and location (*for review see* Oswald & Wand, 2004). Additionally, naltrexone (an opioid receptor antagonist) blocks reinstatement of EtOH-SA induced by exposure to an EtOH-paired cue (Ciccocioppo et al., 2002). Furthermore, it has been reported that tolerance induced by the opioid-receptor agonist morphine and subsequent naloxone-precipitated withdrawal occurred independently of ERK-signaling (Mouledous et al., 2007). Thus, EtOH's actions on the opioid system may play a key role in the post-acquisition phases of EtOH-CPP and may do so independently of the ERK cascade. Interestingly, in the only two reports of positive effects of pharmacological manipulations of extinction of EtOH-CPP, Cunningham et al. (1995, 1998) showed that naloxone enhanced extinction. Therefore, it currently appears that the opioid-receptor system may be a good target for altering extinction of EtOH-CPP. Of course, future studies that assess the ERK-dependence of naloxone's modulation of EtOH-CPP extinction are required in order to further investigate this hypothesis.

The findings of the aforementioned studies are most important when considering the broader, clinical implications of extinction-specific systemic pharmacological manipulations. As initially described in Chapter 1 of this dissertation, experimental extinction and reinstatement in the laboratory are thought to model the rehabilitation phase of a number of associative disorders,

including SUDs (Kaplan et al., 2011; Shaham et al., 2003). Because a large portion of drug-seeking behaviors rely on associative learning (Duka et al., 2010), rehabilitation treatment for these disorders can often include some type of exposure therapy (*for review see* Drummond et al., 1990). Similar to experimental extinction, the purpose of exposure therapy is to weaken the control that the previously associated cues exert over a patient's behavior (Kaplan et al., 2011). For example, in the case of arachnophobia, behavioral therapy may include explicit exposure to spiders in the absence of any negative consequence (i.e., in a nonreinforced manner). In the case of addiction, rehabilitation may include exposure to previously alcohol-paired cues or contexts without contingent presentation of alcohol (Drummond & Glautier, 1994). It is clear from these examples that methods for facilitating this extinction-type learning could be a way to aid the recovery process in hopes of preventing the occurrence of relapse triggered by cue-exposure (Kaplan et al., 2011). It is for these reasons that systemically administered "cognitive enhancers" such as DCS have recently begun to receive significant attention for their potential therapeutic effects (e.g., *for review see* Hofmann, 2007). It is also for these reasons that a more basic understanding of the shared and distinct mechanisms that are involved in extinction of different learned behaviors is important.

Previously reported pharmacological manipulations, as well as the data presented in Chapters 2 and 3 of this dissertation, strongly suggest that extinction of drug-related memories may be different from extinction of fearful

memories. Furthermore, within a single model of drug-seeking behavior such as CPP, it is possible that components of EtOH-related learning, including the direct and conditioned rewarding effects of EtOH, are very different from those involved with other drugs of abuse. Therefore, behavioral and pharmacological therapies for treatments of a patient suffering from arachnophobia, for example, may require a different set of parameters from those designed for rehabilitating an alcoholic. It is plausible that targeting NMDA receptors with a partial agonist such as DCS may facilitate rehabilitation of phobic patients (*for review see* Ressler et al., 2004) while targeting conditioned rewarding effects via the opioid-receptor system may be more effective in aiding treatment of a recovering alcoholic. Interestingly, the opioid-receptor antagonist naltrexone remains one of the most effective pharmacotherapies that are currently used to help treat alcohol dependence (Anton et al., 2003). Treatments intended to aid rehabilitation continue to include those that target the general learning aspects of extinction and rehabilitation (e.g., "cognitive enhancers" such as DCS) as well as those that target the direct and/or conditioned rewarding effects of a drug (e.g., disulfiram). Development and characterization of pharmacotherapies using animal models will continue to contribute to the ongoing development of successful treatments of a diverse range of associative disorders including phobias, PTSD, and SUDs (*for review see* Kantak & Nic Dhonnchadha, 2011). Because the window of opportunity to treat all of these patients is often finite, and preventing relapse is

critical, it is imperative to continue characterizations of the receptor- and signaling-systems that are involved in extinction.

A role for the mPFC in extinction of EtOH-CPP

Although the studies described in Chapters 2 and 3 provided insight into the receptor systems and signaling pathways that are (and are not) involved in extinction of EtOH-CPP, the neurocircuitry remains unknown. The studies described in Chapters 4 and 5 involved a two-step approach to first identify, and subsequently manipulate, the brain regions that are involved in regulating extinction of EtOH-CPP.

Using IHC analysis of pCREB as a marker of neuronal activation (Moore et al., 1996), the experiment in Chapter 4 showed that the PL and IL subregions of the mPFC exhibited significant activation following a brief exposure to an EtOH-paired cue capable of eliciting approach behavior. Interestingly, following extinction of the EtOH-cue contingency, these regions were no longer activated when compared to Paired and Unpaired controls. These findings are in agreement with a previous SA study that reported extinction-sensitive cue-induced activation of the mPFC (Zavala et al., 2007). Together, these correlative findings suggested that the mPFC is capable of dynamic responses to cue exposure that are sensitive to extinction.

In addition to the mPFC, cue-induced changes in pCREB were also assessed in other brain regions including the NAc and BLA—areas that have

been shown to be involved in expression of EtOH-CPP (Gremel & Cunningham, 2008). It is therefore somewhat surprising that IHC analysis revealed no changes in pCREB levels in these areas following cue exposure.

One possible explanation for this apparent discrepancy is that, in contrast to the study by Gremel & Cunningham, exposure to the EtOH-paired cue in the current experiment was passive and did not occur during an actual preference test. Specifically, the NAc and BLA may be more involved in the actual expression of preference behavior whereas the mPFC may be required for initial assessment of cue valence and subsequent regulation of activity in these subcortical regions. In an IHC study involving cocaine-CPP, Bernardi et al. (2009) reported significant activation of the BLA only after a preference test during which both the CS+ and CS- cues were presented. Interestingly, the BLA was not significantly activated following exposure to either CS+ or CS- cues alone. Additionally, Hill et al. (2007) showed no significant activation of the NAc or BLA following a 5-min exposure to an EtOH-paired cue, further supporting the assertion that these areas are not stimulated following passive exposure to a drug-paired cue. Therefore, it appears that with CPP expression, the NAc and BLA may be activated when approach behavior is performed (e.g., during a preference test) but are not stimulated simply by cue exposure.

Another possible explanation for the lack of cue-induced activation in the NAc and BLA could involve the length of cue exposure. Specifically, in contrast to an actual preference test (15-60 mins) the cue exposure in the current

experiment was brief (5 mins). Therefore, it is plausible that cue-induced activation of the NAc and BLA is delayed and occurs only after initial mPFC activation. Delayed and sustained activity within these regions may then be required for maintaining the actual approach response that is exhibited during a preference test. Longer duration cue-exposures may therefore provide a method to detect changes in NAc and BLA activation (see Bernardi et al., 2009; Miller & Marshall, 2004). The brief, passive cue exposure used in the current experiment may not have been long enough to observe activation of the downstream targets of the mPFC, which include the NAc and BLA (Vertes, 2004). In order to determine whether cue configuration and/or cue exposure duration would affect activation of the NAc and BLA, further IHC studies involving additional cue configurations (e.g., a preference test with both cues present) that occur over longer durations (e.g., 30 mins) are required.

Because the IHC data of Chapter 4 were strictly correlative, the experiments of Chapter 5 were intended to determine a causal role of the mPFC in extinction behavior. In order to accomplish this, animals were given electrolytic lesions that encompassed the regions of the mPFC that showed significant cue-induced activation in Chapter 4. Thus, animals were given permanent lesions of both the PL and IL subregions of the mPFC following conditioning, but before extinction, of EtOH-CPP. The data revealed that a group with lesions of the mPFC showed significantly impaired extinction when compared to sham-lesioned animals (Sham group) or animals that did not receive surgery (No Surgery

group). These data suggest that the mPFC is involved in extinction, but not necessarily expression, of EtOH-CPP. Specifically, animals in the Lesion group continued to express significant place preference after surgery and extinction. In contrast, the Sham group showed significant preference only before extinction. If the mPFC were required for initiation and/or maintenance of CPP expression, the lesion group would have showed impaired place preference following surgery, regardless of whether extinction training was administered. Thus it appears that the mPFC is involved in extinction and not necessarily required for expression of EtOH-CPP.

Together with the IHC data in Chapter 4, these results suggest that the mPFC may be selectively activated at the onset of extinction, a time when the reward prediction error is greatest. Because the brief cue exposure was preceded by only a saline injection, the 5-min trial was effectively a short extinction trial. As in extinction, nonreinforced presentation of the CS+ created a large prediction error in that the Paired No-Extinction animals may have still expected EtOH's effects in the presence of the cue. This discrepancy between reward expectation and actual outcome may have resulted in the observed mPFC recruitment as the mPFC has been implicated in assessment of reward salience and prediction error encoding (*for review see* Rushworth & Behrens, 2008). On the other hand, in the Paired-Extinction group, the prediction error that occurred during the cue exposure was presumably minimal because of the repeated nonreinforced CS+ exposures previously experienced during extinction.

Therefore, the increase in mPFC-pCREB in the Paired-No Extinction group could have represented a prediction error-induced initiation of extinction learning, and not simply a cue-induced initiation of approach behavior.

One caveat to this interpretation, however, is that the IHC data in Chapter 4 did not actually identify the type of pCREB-containing cells within the mPFC. For example, if the mPFC cells that were activated by cue-exposure were inhibitory interneurons, it is possible that an increase in pCREB in these cells actually resulted in a net decrease of mPFC output. In fact, Miller & Marshall (2004) reported that exposure to a cocaine-paired cue resulted in an increase in activity of GABA-ergic PL neurons, thereby reducing overall PL inhibition of the NAc and BLA. The mPFC lesions described in Chapter 5, which were large and nonspecific enough to effectively destroy any cells within this region, would have effectively decreased overall mPFC output. If, as Miller & Marshall (2004) hypothesized, decreased PL output is necessary for expression of CPP, then it is possible that the mPFC lesions actually caused an expression of CPP that was resistant to extinction. This lesion-induced persistence of CPP may have been caused by an inability of the mPFC to process the inhibitory association and/or inhibit the actual approach behavior, during extinction. This suggestion is supported by reports showing the involvement of the mPFC in the formation of inhibitory associations (Rhodes & Killcross, 2007) and expression of inhibitory extinction learning (Ovari & Leri, 2008).

Future studies designed to assess the differential effects of mPFC lesions on both expression and extinction of EtOH-CPP could utilize a choice-extinction procedure. The choice-extinction procedure consists of repeated expression tests that reduce place preference by extinguishing the actual approach behavior (e.g., Groblewski et al., 2009; Botreau et al., 2006). This procedure would allow for the combined assessment of the effect of mPFC lesions on initial expression and subsequent extinction of EtOH-CPP. One disadvantage of this procedure is that choice-extinction of EtOH-CPP occurs slowly and does not typically extinguish the preference to a level as low as that seen with forced-extinction (see Groblewski et al., 2009, 2011). This procedure therefore presents some difficulties in detecting the effects of manipulations that are expected to impair extinction as the control group's preference decays slowly. Therefore, choice-extinction is more suitable for assessing manipulations that are expected to facilitate extinction. It is for these reasons that the choice-extinction procedure was utilized to examine the extinction-facilitating effects of DCS whereas the forced-extinction procedure was used to assess the extinction-impairing effects of SL327 and mPFC lesions.

Taken together, the results of these experiments showed that the mPFC plays a crucial role in extinction of EtOH-CPP. The IHC results of Chapter 4 first identified the mPFC as a dynamic region that is activated by exposure to an EtOH-paired cue—an effect that diminishes following extinction. This experiment was unable, however, to determine whether the mPFC was necessary for

expression and/or extinction. In order to do so, Chapter 5 then went on to identify a causal role of the mPFC in extinction of EtOH-CPP using electrolytic lesions administered after conditioning but before extinction. The results revealed that animals that had received mPFC lesions showed impaired extinction of EtOH-CPP. Therefore, it is possible that the mPFC region is integral to processing the cue-elicited prediction error that occurs during extinction, and based on this error, the mPFC subsequently controls subcortical regions necessary for expression and extinction of EtOH-CPP.

Assessment of an extinction-specific intra-mPFC microinjection procedure

The studies described up to this point have included systemic pharmacological and neuroanatomical manipulations in an attempt to further characterize extinction of EtOH-CPP. These findings suggest that extinction of EtOH-CPP relies on mPFC circuitry and that it occurs in an ERK-independent manner. However, there are currently no published studies that have examined the effects of intra-mPFC pharmacological manipulations on extinction of EtOH-CPP in mice. Therefore, the final set of experiments in this dissertation was designed to identify a microinjection procedure that allowed for extinction-specific intra-mPFC injections. In order to confirm, and expand upon, the lesion findings of Chapter 5, Chapter 6 preliminarily examined the effects of pharmacological inactivation of the mPFC on extinction. Injections of the sodium-channel blocker, bupivacaine, act by temporarily preventing neuronal activity (i.e., changes in

membrane potential due to intracellular depolarization) at the injection site (Catterall & Mackie, 2006). It was hypothesized that injections of bupivacaine prior to extinction trials would cause temporary effects comparable to those caused by the electrolytic lesions in Chapter 5, which in turn would similarly impair extinction of EtOH-CPP. A cursory analysis of the results of the two experiments in Chapter 6 suggested that similar to permanent lesions, temporary inactivation of the mPFC may have impaired extinction. However, accurate assessment of the effects of intra-mPFC bupivacaine was obstructed by the abnormal extinction exhibited by the vehicle-injected control group. In fact, the vehicle-injected group showed incomplete extinction despite an increase in the number of extinction trials (Chapter 6, Experiment 2).

Although there are a number of possible reasons for why the vehicle-injected group showed incomplete extinction, the most parsimonious explanation is that the microinjection procedure also caused damage to the tissue, thereby preventing normal function of the mPFC. As shown in Chapter 5, lesion-induced damage to the mPFC prevented normal extinction of EtOH-CPP. In order to minimize this potential damage, Experiment 1 was initially designed to include the absolute minimum number of extinction trials that would sufficiently eliminate place preference. Previous parametric studies (Chapter 3, Figure 8) indicated that 3, 30-min trials were required to eliminate expression of place preference. Minimizing the number of extinction trials also served to limit the required number of microinjections (and therefore the number of times that the injector tip is

lowered into the mPFC). However, because the vehicle-injected group did not show normal levels of extinction in Experiment 1, Experiment 2 required extending the number of trials to 4 in hopes of increasing the extinction shown by control animals. This manipulation did not change the outcome, however, in that the vehicle-injected group continued to show persistent preference. Thus, despite using the absolute minimum number of trials required to extinguish EtOH-CPP, the microinjections appeared to cause too much tissue damage. Furthermore, this damage was severe enough to prevent extinction despite increasing the number of extinction trials.

It is unclear at this time, whether it will be possible to employ an extinction-specific microinjection procedure for pharmacological manipulations of the mPFC in mice. Previous studies involving intra-mPFC microinjections have utilized rats (e.g., Hsu & Packard, 2008; Hugues et al., 2004) and were therefore able to insert bilateral cannulae into the mPFC with presumably less damage. It is possible that utilizing bilateral, angled cannulae in mice may be more optimal in that it spares the tissue of the dorsal subregions of the mPFC including the PL and anterior cingulate. Specifically, because in the current set of experiments a single, midline cannula was implanted, the tissue damage was primarily located bilaterally in the medial layers (Layers I-III) of the dorsal and ventral mPFC (Chapter 6, Figures 22 & 26). These medial layers have been shown to contain afferents from other cortical areas, projecting neurons, and inhibitory interneurons that project to pyramidal cells in the lateral layers (Yamamura et al.,

2009). Therefore, it is difficult to speculate how damage to the more lateral layers (Layer IV) of the mPFC caused by bilateral, angled injections would affect extinction learning.

It has been reported that exposure to cocaine-paired cues caused significant changes in activity of PL projection neurons in Layer V, as well as inhibitory interneurons of the medial layers within the PL (Miller & Marshall, 2004). Therefore, it appears that both the medial and lateral layers of the mPFC are important to drug-paired cue-induced approach behavior and damage to either or both of these areas could have significant consequences on mPFC function and connectivity. Unfortunately, as our parametric studies have shown, extinction of EtOH-CPP requires multiple trials (at least 3) of at least 30 mins in length (Groblewski et al., 2011). Because of the requirement for multiple trials over multiple days, extinction-specific microinjection procedures inherently require multiple injections. As these microinjections appear to result in tissue damage even when limited to the absolute minimum number, it may very well be that it is not possible to use this procedure. Further parametric studies are certainly required in order to identify a procedure that could allow for multiple intra-mPFC microinjections prior to extinction trials without causing behavioral-changing tissue damage. Studies may include altering the angle of the cannulae and/or identifying methods of decreasing the overall number of trials (and microinjections) required. Interestingly, it may be possible to utilize an abbreviated version of this procedure (i.e., fewer trials) if the goal is to achieve

limited or no extinction in the vehicle-injected group. An example of such an experiment would be one where the experimental pharmacological manipulation is expected to enhance extinction.

Summary and Conclusions

The findings described in this dissertation provide insight into the receptor and signaling systems, as well as the neurocircuitry, involved in extinction of EtOH-seeking behavior in mice. The systemic studies showed that extinction of EtOH-CPP is not altered by pharmacological manipulations of NMDA receptors with DCS or the ERK-signaling cascade with SL327. These findings put forward the possibility that the mechanisms underlying EtOH-paired cue-induced approach behavior (including the direct and conditioned rewarding effects of EtOH) may be different than those involved with other drugs of abuse (Groblewski et al., 2011). On the other hand, the IHC and lesion studies suggest that, as with CPPs induced by other drugs of abuse, extinction of EtOH-CPP involves mPFC-mediated neurocircuitry. However, further manipulation of this mPFC circuitry may be difficult as preliminary experiments involving intra-mPFC microinjections were confounded by inadequate extinction in the control groups.

In a broader sense, these studies support the notion that treatments aimed at strengthening rehabilitation and preventing relapse may require unique approaches depending on the drug of abuse. The pharmacological components of these approaches, which include targeting 1) the direct and conditioned

rewarding effects of drugs as well as 2) the general learning of aspects of extinction, will also need to be tailored to each patient's specific disorder(s). Furthermore, the current experiments suggest that administration of drugs that target the general learning component of rehabilitation such as the "cognitive-enhancers" that target the NMDA-receptor system (i.e., DCS) may not be ideal for aiding treatment of alcoholism despite their effectiveness with other disorders (e.g., Ressler et al., 2004).

In contrast, these studies have further strengthened the view that extinction of both alcohol- and drug-seeking behaviors involves mPFC-circuitry. In fact, the mPFC has been reported to play an integral role in extinction and reinstatement induced by a variety of environmental triggers (*for reviews see* Peters et al., 2009; Shaham et al., 2003). Interestingly, it has been reported that both alcohol and cocaine-cues significantly activate areas of the prefrontal cortex in human alcoholics and cocaine addicts, respectively (George et al., 2001; Wexler et al., 2001). Thus, while the systemic studies suggest that EtOH-related learning possesses unique characteristics, the lesion study exemplifies a common mechanism that is shared by a number of drugs of abuse. Taken together with previously published studies, the current experiments suggest that extinction of drug-seeking behavior may also possess neurocircuits that are similar to those involved in extinction of other associative memories such as conditioned fear. Treatments aimed specifically at mPFC mechanisms may

therefore be beneficial to patients recovering from a variety of associative disorders including SUDs and PTSD (e.g., Boggio et al., 2009).

In conclusion, these experiments are some of the first to systematically examine the neurochemistry and neurocircuitry underlying extinction of EtOH-CPP. As an animal model of cue-induced alcohol seeking, EtOH-CPP provides a unique method for understanding how environmental cues can control behavior following simple Pavlovian conditioning. Continued research into the acquisition and extinction of models such as CPP will help to further elucidate the shared and unique learning processes involved in the development and rehabilitation of drug- and alcohol-seeking behavior in humans (Kaplan et al., 2011). In the end these experiments, and others like it, were intended to identify specific pharmacological and neuroanatomical targets that, in the future, could lead to the development of better treatments for relapse prevention in patients recovering from SUDs.

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