THE ROLE OF DOPAMINE D1/5 RECEPTORS IN FEAR AND REWARD

By

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CERTIFICATE OF APPROVAL

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Chapter 1 and 5 has some sections adapted from Abraham, et al. (2014). I wrote and researched a majority of the review. Dr. Matt Lattal edited the review and guided the theoretical discussions in the review. Dr. Kim Neve edited the review and wrote the section concerning functional selectivity of dopamine agonists.

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Abstract

Extinction is a complex period of learning during which a predicted relationship between stimuli (i.e. conditioned stimulus-unconditioned stimulus association) is inhibited by presentation of the predictive stimulus alone (i.e. non-reinforced conditioned stimulus), and this learning can be impaired by blockade of dopamine receptor activity. Thus, examining the relationship between dopamine and extinction may be beneficial to understanding basic features of learning and memory. Furthermore, enhancing extinction may be beneficial at a clinical level, as impaired extinction is hypothesized to contribute to a number of diseases of learning and memory, such as posttraumatic stress disorder and substance use disorders. The overarching goal of this dissertation is to examine the consequence of altering dopamine signaling during acquisition and extinction of fear and reward to specify the contribution of dopamine signaling within these particular phases of learning.

In Chapter 2, dopamine activity is manipulated through the use of methylphenidate, a dopamine and norepinephrine transport blocker. I find that methylphenidate enhances contextual fear extinction when administered prior to or following an extinction session, and has no effect on fear acquisition. As methylphenidate may have actions through D1/5 receptors, I investigated the specific contribution of D1/5 receptors to fear extinction in Chapter 3.

Chapter 3 shows that the systemic activation of D1/5 receptors with SKF 81297 following extinction of either contextual or cued fear enhances consolidation of fear extinction. To assess the rewarding properties of SKF

81297, I utilized a conditioned place preference procedure. Pre-session, but not post-session, administration of SKF 81297 generates a conditioned place preference. I also found that post-session SKF 81297 would enhance the extinction of a cocaine conditioned place preference. These studies suggest there may be a common mechanism that underlies extinction of fear and reward within the dopamine signaling system.

Chapter 4 continues this investigation by testing whether D1 receptors are necessary for fear and reward learning in D1 receptor knockout mice. Chapter 4 shows that D1 receptor knockout enhances the expression of cued fear, but has no effect on contextual fear or cocaine conditioned place preference. Chapter 4 shows that the D1 receptor activation may not be necessary for learning fear or reward associations, although it may contribute to decreasing fear responses in certain conditions. Finally, Chapter 5 examines the neural substrates that underlie D1/5 receptor mediated extinction enhancements. I tested the effect of SKF 81297 microinjection into the infralimbic region of the prefrontal cortex, the nucleus accumbens core, dorsal hippocampus, and basolateral amygdala. I found no effect of SKF 81297 administration into these regions following extinction. Chapter 5 also examines the intracellular signaling pathways that may be involved with the observed fear extinction enhancements in Chapter 3 and finds that activation of PKA-coupled, but not PLC-coupled D1/5 receptors enhances fear extinction. Together, these experiments aim to specify the role of D1/5 receptors in fear and reward learning.

Chapter 1: Introduction

This chapter is adapted from the following publication:

Abraham AD, Neve KA, Lattal KM. (2014). Dopamine and extinction: A convergence of theory with fear and reward circuitry. *Neurobiology of Learning and Memory*, 108: 65-77.

General Introduction

Animal behavior is often allocated in a manner that maximizes rewarding experiences and minimizes aversive experiences. To achieve this goal, an animal must decipher complex environmental information to determine the particular stimuli that predict desired or undesired outcomes. Learning occurs as the animal assesses the conditions that led to a specific outcome and encodes these relationships for future retrieval. When confronted with similar conditions at a later period, the animal retrieves the memory of the previous experience and generates appropriate behaviors for the situation. As rewarding and aversive experiences frequently generate dissimilar or even opposing behavioral responses, these two types of learning and memory are often studied in isolation. However, there are indications that these two types of memory may interact in certain circumstances.

The distinction between an aversive and rewarding memory becomes less clear when examining conditions under which a predicted outcome does not occur. For example, consider a case in which a person regularly purchases a candy bar from a vending machine. The initial association would be considered rewarding, as the person receives an enjoyable treat each time they insert

money into the machine. However, if for some reason the vending machine does not produce a candy bar when the person inserts coins, this violates their expectation in a way that could be classified as aversive. The negative affect produced by the lack of a rewarding experience would lead the person to modify their behavior and expectations for the vending machine. The person may seek out alternative vending machines, or decrease the number of visits to the vending machine that does not consistently produce the expected outcome. In this case, it becomes apparent that rewarding and aversive experiences can interact in complex ways to modulate behavior. This relationship may also occur in aversive learning, as the lack of an expected aversive event may be considered a rewarding event in contrast to previous experiences. The study of these interactions could provide valuable information for understanding the principles of learning that guide flexible and sensitive responses to a dynamic environment. The central goal of this dissertation is to examine how dopamine, a neuromodulator primarily associated with reward, may guide behavior when expectancies are violated in aversive or reward learning.

Fear Extinction

A key insight from behavioral approaches to learning is that multiple theoretical processes contribute to the acquisition and extinction of learned behaviors. Over the years, theories have described effects on processing of the conditioned stimulus (CS), the unconditioned stimulus (US), the context in which learning occurs, and the associative connections that form among stimuli, responses, and outcomes. Learning relationships between stimuli allows an

animal to respond appropriately to cues associated with unconditioned stimuli (Rescorla, 1988). In Pavlovian fear conditioning, an animal receives several pairings of an initially neutral CS, such as an auditory tone or context, with an aversive US, such as footshock. If the CS is predictive of the occurrence of an US, a conditioned response (CR), such as freezing, is expressed following presentations of the CS.

The development of the fear memory involves three interrelated phases: acquisition, consolidation and retrieval (Abel and Lattal, 2001). The initial development of an association between the CS and US is termed fear acquisition. Following training in a fear conditioning paradigm, gene expression and protein synthesis gate the long-term storage of memory, in a process known as consolidation. When an animal is given a brief exposure to the CS, retrieval of the original CS-US association will initiate conditioned responding. However, in certain conditions, a shift in contingency can occur that interferes with previously learned CS-US associations.

When the outcome of the presentation of a CS becomes ambiguous, previously acquired contingencies must be updated to generate appropriate behavioral responses (Bouton, 2002). In fear conditioning paradigms, a contingency shift can be explored through the study of fear extinction. Initially, an animal learns that a CS reliably predicts a US during fear conditioning, but extinction of fear involves the presentation of the CS with no US. As the CS is repeatedly presented without the US during acquisition of fear extinction, an animal will decrease conditioned responding (e.g., freezing), and increase

exploratory behaviors. Similar to fear conditioning, fear extinction undergoes consolidation to enter into long-term storage and can be retrieved to inhibit fear behaviors (Myers and Davis, 2007). One objective of this dissertation is to examine how fear extinction consolidation and retrieval may be impacted by alterations in dopamine signaling.

Dopamine's Role in Learning

Neurobiological studies have revealed that dopamine signaling is involved in many of the different theoretical processes that underlie learning. For example, dopamine is involved in the circumstances that produce learning (e.g., prediction error, coding of stimulus salience); it mediates the content of that learning (e.g., hedonic value of associations); and it modulates the expression of learning in performance (e.g., response vigor, memory retrieval). Recent experiments examining the role of dopamine in acquisition and extinction of learned fear demonstrate that learning in aversive situations may be modulated by an engagement of appetitive systems (Pezze and Feldon, 2004), supporting long-held assumptions about appetitive–aversive interactions in learned behavior (Dickinson and Dearing, 1979; Dickinson and Pearce, 1977; Konorski, 1967).

Characterizing the role of dopamine in fear extinction may be particularly interesting because dopamine release within reward circuitry may alter the subjective value assigned to fearful stimuli, in addition to directly affecting memory consolidation (Horvitz, 2000; Pezze and Feldon, 2004; Redgrave et al., 1999; Salamone, 1994). There are multiple mechanisms through which dopamine may alter the establishment, maintenance, expression, and extinction

of fear. These include altering the conditions through which fear occurs (e.g., prediction error), altering the content of the association (e.g., attaching rewarding hedonic value to previously fearful stimuli), and altering the expression of the memory (e.g., changing the conditions under which conditioned fear occurs in behavior).

The varied role of dopamine in appetitive and aversive learning is not surprising given the distribution of dopamine receptors in the central nervous system (Mansour and Watson, 1995), where dopamine is found throughout regions important for aversive and reward memory. This creates a complicated pattern of regional activity that could have different behavioral outcomes depending on the behavioral experience. Dopaminergic innervation in the brain can be divided into four main pathways (Beaulieu and Gainetdinov, 2011). Generally, each of these projection pathways is regionally and functionally distinct, as dopamine plays different roles in guiding behavior in dopamine terminal regions. The nigrostriatal pathway connects the substantia nigra to the striatum and is important for guiding motivated motor responses. The tuberoinfundibular pathway connects dopamine neurons in the hypothalamus to the pituitary gland and induces hormone release (Reymond and Porter, 1985). The mesocortical pathway, emanating from the ventral tegmental area, connects dopamine neurons to cortical regions. The mesolimbic pathway connects the ventral tegmental area to the nucleus accumbens, amygdala, and hippocampus. Many of the regions innervated by the ventral tegmental area have been implicated in aspects of fear learning, and suggest a role for dopamine in fear

learning and extinction (Pezze and Feldon, 2004). A theoretical circuit for the involvement of dopamine in fear learning is shown in Figure 1. The activity of dopamine in these regions is described in further detail in the following section.

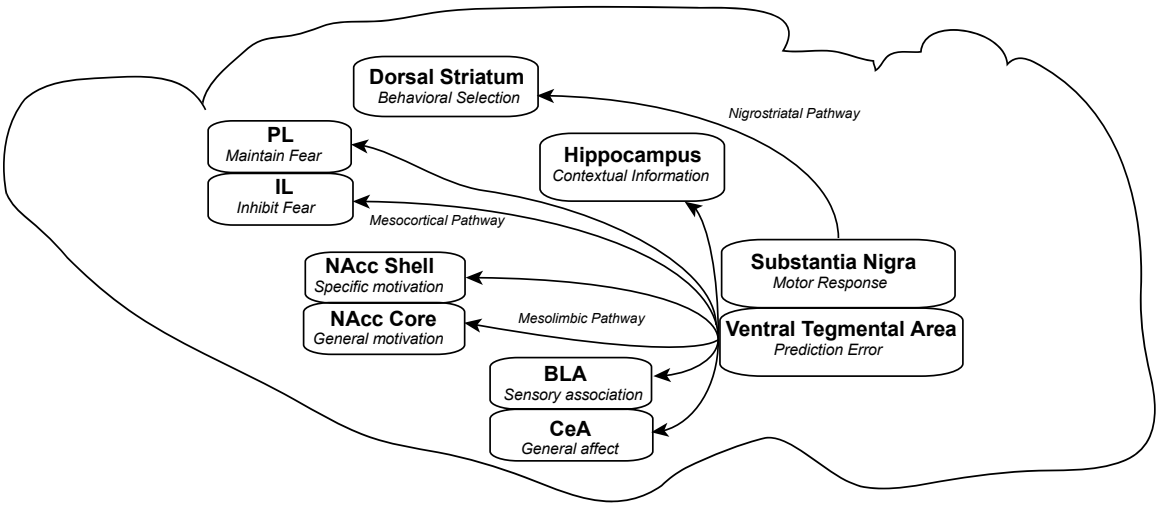


Figure 1. Dopamine circuitry in fear-related behaviors. Dopamine neurons in the ventral tegmental area (VTA) generate signals to encode discrepancy between expected and unexpected outcomes (prediction error). These signals are relayed to several regions that have reciprocal connections with the VTA, such as the amygdala, nucleus accumbens, prefrontal cortex, and hippocampus. These reciprocal connections allow for modification of signals arising from the VTA, leading to precise control of dopamine release within dopamine terminal regions. Within the amygdala, the basolateral amygdala (BLA) encodes CS-US associations, allowing for fear acquisition and retrieval. The BLA projects to the central amygdala (CeA) to generate fear responses and motivated behavior through motor circuitry. Dopamine receptor activity in the amygdala modulates the formation and retrieval of fear associations. Dopamine transmission in the nucleus accumbens core (NAcc Core) is important for encoding the general salience of environmental stimuli, and activity in the nucleus accumbens shell (Nacc Shell) encodes outcome-specific predictions to guide motivated behavior (Corbit and Balleine, 2011). In the prefrontal cortex, dopamine activity is important for working memory and fear extinction. In the infralimbic region of the prefrontal cortex (IL), dopamine D1 receptors are required for consolidation of fear extinction (Hikind and Maroun, 2008). Activation of D1 receptors in the prelimbic region of the prefrontal cortex (PL) blocks the expression of conditioned fear (Lauzon et al., 2013). Interactions between the hippocampus and VTA are important for relaying contextual information in fear and reward learning. The VTA provides a coordinating signal to generate particular patterns of activity in dopamine terminal regions based on environmental stimuli and prior experience. Dopamine neurons in the substantia nigra are involved in motor responses and project to the dorsal striatum, which selects and alters appropriate behavioral responses based on inputs from a variety of different regions. Together, activity in the substantia nigra, ventral tegmental area, and dopamine terminal regions allow for the generation of appropriate behavioral responses (e.g., freezing, approach, or active avoidance) in response to shifting external stimuli. Adapted from Abraham et al. (2014).

Neural Substrates of Fear Extinction

Dopamine signaling occurs through two main subfamilies of dopamine receptors. D1-like receptors, comprised of D1 and D5 dopamine receptors, activate the stimulatory G proteins Gαs and Gαolf. D2-like receptors, which include the D2, D3, and D4 receptors, activate the inhibitory G proteins Gαi and Gαo (Beaulieu and Gainetdinov, 2011). For simplicity, in this section, D1-like receptors and D2-like receptors are termed D1 receptors and D2 receptors, respectively. Although expression of dopamine receptors is well conserved in the basal ganglia across mammalian species including mouse, rat, guinea pig, cat, rhesus monkey, and human, some interspecies differences have been observed in the cerebellum, hippocampus, and cerebral cortex (Camps et al., 1990). Whereas there is little to no dopamine receptor expression in the cerebellum of primates, dopamine receptor expression has been observed in the cerebellum of rats, although midbrain dopamine neurons have not been shown to project into the cerebellum in rats. In the hippocampus, rodents show a slightly different distribution of dopamine receptors from primates, with higher densities of receptor expression in the dentate gyrus compared to CA1 or CA3 regions of the hippocampus. In primates, hippocampal dopamine receptor density appears to be higher in CA1 and CA3 regions of the hippocampus compared to the dentate gyrus. In the cerebral cortex, dopamine receptor expression is confined to deeper laminae in rodents, whereas primates and cats show dopamine receptor expression across all cortical layers (Camps et al., 1990).

In male CD rats (Charles River), the prefrontal cortex expresses D1 receptors in nonpyramidal neurons and D2 receptors in small pyramidal cells and large nonpyramidal neurons (Vincent et al., 1995). Dopamine is likely to be involved in modulating PFC activity in fear extinction, as shown by microdialysis experiments demonstrating that PFC dopamine is increased following extinction (Hugues et al., 2007) and conversely, lesion experiments showing that PFC dopamine depletion decreases retention of fear extinction (Fernandez Espejo, 2003; Morrow et al., 1999). The particular contribution of each dopamine receptor in the PFC has been more thoroughly explored through antagonist studies.

D1 antagonist studies show that the prefrontal cortical D1 receptors in the infralimbic region are critical to consolidation of fear extinction (Hikind and Maroun, 2008). When given following an extinction session, antagonist-treated animals maintained freezing during the following test day compared to vehicle-treated animals, demonstrating that infralimbic D1 receptors are required for consolidation of an extinction memory. Evidence for the involvement of other dopamine subtypes comes from Mueller et al. (2010), who tested the role of PFC D2 receptors in fear extinction. Mueller et al. (2010) showed that a pre-extinction infralimbic administration of a D2 receptor antagonist led to no difference in acquisition of fear extinction, but increased fear on the following test day. These studies indicate the involvement of D1 and D2 receptors in fear extinction consolidation, although some behavioral effects may also be related to retrieval of the fear memory.

Prelimbic and dorsal infralimbic PFC D1 and D2 receptors are also important for retrieval of a conditioned fear memory, as nonspecific blockade with cis-flupenthixol or activation with amphetamine led to decreased freezing during an extinction session (Pezze et al., 2003). Amphetamine treatment within the PFC during extinction led to decreased freezing on following test days, suggesting that activation of dopamine and noradrenergic receptors in the PFC may lead to enhanced consolidation of extinction. In summary, the activity of dopamine in the prefrontal cortex appears to be critical for the consolidation and retrieval of fear extinction. An additional level of complexity in the mesocorticolimbic circuit comes from observations that the PFC can fine-tune dopamine neuron activity in the ventral tegmental area through interactions with the basolateral amygdala and the ventral subiculum of the hippocampus (Patton et al., 2013).

Whereas the prefrontal cortex encodes consolidation of fear extinction, the acquisition of fear extinction has been linked to activity in the basolateral amygdala (Quirk and Mueller, 2008). There are several models for how the amygdala modulates learning. Research on aversive learning has generally followed a serial processing model for the amygdala, with associative information being integrated in the basolateral amygdala and sent to the central amygdala and downstream targets. Other models suggest parallel processing, in which the different nuclei process different aspects of the association (Balleine and Killcross, 2006; Corbit and Balleine, 2005; Holland and Gallagher, 1999). In the context of aversive learning, the basolateral amygdala (BLA) integrates particular

sensory stimuli with nociceptive unconditioned stimuli and the central amygdala encodes general affective or attentional states to guide motivated behavior. Acting together, these regions can generate appropriate fear responses based on specific sensory stimuli and general affective states. The wide range of associations that can be generated through increased activity in the amygdala indicates that examination of both terminal region and network activity from dopamine neurons is important to distinguish how appropriate and distinct behavioral responses are generated from common signaling mechanisms in appetitive and aversive situations.

The localization of dopamine receptors in the amygdala has important functional implications for the differences in information encoded by the BLA and central amygdala. In rats, the BLA expresses D1 receptors, and the central amygdala primarily expresses D2 receptors (Weiner et al., 1991). The intercalated cell mass, a group of cells associated with extinction (Busti et al., 2011), expresses D1 receptors highly (Mansour and Watson, 1995). Pharmacological manipulations of the D1 receptor in the amygdala generally have been consistent with the idea that the D1 receptor is involved in aversive learning. Intra-amygdalar D1 agonism enhances fear conditioning and intra-amygdalar D1 antagonism impairs both first- and second-order fear conditioning (Guarraci et al., 1999; Lamont and Kokkinidis, 1998; Nader and LeDoux, 1999). Further, antagonism of the D1 receptor in the BLA immediately before, but not immediately after, an extinction session slows the rate of extinction over days (Hikind and Maroun, 2008), consistent with the idea that acquisition, but not

consolidation of extinction depends on the D1 receptor in the BLA. Examining the interaction of the amygdala with other targets of dopamine innervation, such as the nucleus accumbens may reveal the underlying principles of dopamine-related mechanisms guiding fear extinction.

Dopamine signaling in the amygdala is important for acquisition and extinction of fear, but those signals need to be integrated with responses in other systems to result in memory. Although the nucleus accumbens is generally thought of as a region that mediates reward-related processes, there is growing evidence for its involvement in aversive memory regulation. Studies examining the combined action of the nucleus accumbens and the BLA, for example, suggest that concurrent activation of dopamine signals in the nucleus accumbens and BLA contributes to long-term memory formation (LaLumiere et al., 2005). This indicates that the nucleus accumbens and amygdala could be regions that integrate dopamine signaling to generate appropriate behavioral responses to new contingencies. In an inhibitory avoidance task, modulation of consolidation is directly influenced by activation of dopamine in both regions (LaLumiere et al., 2005). Similarly, restoration of dopamine in both the BLA and nucleus accumbens in dopamine deficient mice is sufficient for long term memory of fear conditioning (Fadok et al., 2010). Blocking D1/5 receptor signaling in the nucleus accumbens alone is sufficient to impair fear extinction, as found by microinjection of the non-selective dopamine receptor antagonist haloperidol into the nucleus accumbens (Holtzman-Assif et al., 2010). Using fast-scan cyclic voltammetry, Badrinarayan et al. (2012) demonstrated that aversive memory retrieval and

extinction decreases dopamine release in the nucleus accumbens core and increases dopamine transmission in the nucleus accumbens shell, with these alterations temporally locked with the offset of an aversive stimulus. The distinct patterns of dopamine release in the accumbens core and shell provide evidence for the hypothesis that dissociable projections from the ventral tegmental area mediate the representation of aversive information across the brain.

Pezze et al. (2001) suggested that the nucleus accumbens core and shell encode different aspects of fear conditioning by separately examining the effects of contextual and auditory conditioning. The authors demonstrate that during non-reinforced presentations of tone there is increased extracellular dopamine in the nucleus accumbens shell and non-reinforced presentation of context leads to increased extracellular dopamine in the core. Bradfield and McNally (2010) expanded on these findings by showing that accumbens shell lesions impair learning about less predictive stimuli in a compound conditioning procedure, consistent with the idea that the nucleus accumbens shell is involved in gating learning that occurs with relation to stimuli that vary in predictive value for an expected aversive outcome. Although nucleus accumbens activity is important for aspects of fear extinction, information from the accumbens, amygdala, and prefrontal cortex are integrated by the hippocampus to generate contextual representations during fear extinction (Gill and Grace, 2006; Bouton et al., 2006).

One of the most theoretically important findings in the behavioral study of extinction is that the learning that occurs during extinction is context specific (Bouton, 2004). Thomas et al. (2003) demonstrated fear renewal when animals

were tested in a different context from where they received extinction training. The phenomenon of spontaneous recovery, where fear responding returns upon a delay following extinction training, indicates that extinction is also subject to a temporal context (Rescorla, 2004). The nature of the context may be encoded through external stimuli (e.g., floors, odors, or shape of a testing chamber) or internal cues (e.g., motivational state, drug state, mood, recent events, or time) (Todd et al., 2014). The context dependency of extinction, as shown by renewal and spontaneous recovery studies, may be conceptualized as a mechanism that allows the animal to decipher the predictive value of an ambiguous signal (Bouton, 2004). Accounts of contextual control of extinction may be clarified through the analysis and manipulation of the neural substrates of extinction, and there are many suggestions that this effect is mediated by the hippocampus (e.g., Corcoran and Maren, 2001; but see Campese and Delamater, 2013). Because the hippocampus is thought to be important for developing representations of space and context, a disruption of hippocampal function at the time of extinction or testing may disrupt the development or retrieval of contextual associations with extinction.

Most of what is known about the role of hippocampal D1 receptors and fear comes from studies of fear acquisition. D1 receptor antagonists or agonists in the dorsal hippocampus impair or promote, respectively, initial inhibitory avoidance learning and long-term memory (Rossato et al., 2009). Interestingly, administration of a dopamine receptor agonist into the dorsal hippocampus within 12 h following training enhances subsequent fear expression, suggesting a role

for dopamine in a long-term consolidation mechanism that has not yet been thoroughly described (Rossato et al., 2009). Post-session activation of dorsal hippocampal D1 receptors with a partial agonist (SKF 38393) also enhances extinction of contextual fear (Fiorenza et al., 2012). Together, the studies presented here indicate that activity of dopamine receptors within dopamine terminal regions contributes to fear extinction. To understand the input received by these regions, the following section describes dopamine neuron activity in the ventral tegmental area.

The Ventral Tegmental Area & Prediction Error

The predominant neurobehavioral theory for dopamine neuron activity in the ventral tegmental area is a prediction error model (Schultz, 2006). Prediction error is a discrepancy between expected and actual outcomes and this discrepancy is a fundamental element of various models of associative learning (e.g., Mackintosh, 1974; Rescorla and Wagner, 1972). Dopamine neurons fire tonically under baseline conditions, but increase burst firing when a reward occurs that is greater than what is predicted or inhibit tonic firing when an expected reward is omitted. Over several pairings of a CS with a rewarding US, dopamine neurons begin to respond primarily to the CS. This suggests that instead of only encoding the hedonic value of a US, dopamine neuron firing signals expectations corresponding to previously neutral CSs. The ability to convey these predictions supports findings that dopamine conveys information to multiple target regions that are involved in different aspects of memory formation.

Theories to explain the nature of information encoded by the ventral tegmental area (VTA) have been restructured by observations that subpopulations of neurons in the VTA react differently to rewarding or aversive experiences. Dopamine neurons in the dorsal VTA are excited by reward or reward-associated stimuli, whereas dopamine neurons in the ventral VTA show high levels of bursting in response to footshock (Brischoux et al., 2009). The high levels of bursting could be important for the encoding of aversive stimuli and stimuli associated with the aversive experience, or it could signal the appropriate behavioral responses to aversive contingencies. This suggests that the prediction error model may be applicable for both aversive and appetitive tasks, and the distinct projection sites of dopamine neurons from the VTA provide a mechanism by which the subjective value of aversive or appetitive information is distinguished in dopamine terminal regions.

Lammel et al. (2011) demonstrated that prefrontocortical-projecting dopamine neurons in the posterior ventromedial portion of the VTA respond to aversive experiences by significant increases in the AMPA receptor (AMPAR) mediated to NMDA receptor (NMDAR) mediated excitatory postsynaptic current (EPSC) ratio seen at the synapse between VTA and PFC. An injection of formalin into the hindpaw of a mouse (an aversive experience), leads to increases in the AMPAR/NMDAR EPSC ratio in neurons that project to the prefrontal cortex and the lateral shell of the nucleus accumbens. In contrast to neurons excited by aversive events, dopamine neurons that are inhibited by aversive events also encode the level of conditioned fear, as the duration of

inhibitory pauses of tonic dopamine firing is related to the acute fear evoked by a CS previously paired with shock (Mileykovskiy and Morales, 2011). Together, these findings indicate that dopamine neurons show projection-specific responsivity to aversive contingencies signaled by the CS.

The offset of an aversive stimulus (Brischoux et al., 2009) has been shown to increase firing in particular dopamine neurons, suggesting that the lack of an expected aversive event may trigger dopamine release. Although this finding has not been thoroughly characterized, it has important implications for suggesting that the absence of a predicted aversive event may lead to increased firing in a subset of VTA neurons that have yet to be identified as belonging within the aversive/rewarding continuum. This also suggests a mechanism by which the VTA may encode the prediction error present during an extinction session. Whereas appetitive stimuli lead to increased dorsal VTA dopamine neuron firing and the absence of a reward leads to decreased dopamine neuron firing, an aversive stimulus may reduce firing in the dorsal VTA and the lack of an aversive US may lead to the increased activity of these dopamine neurons. These effects may also be reversed in the ventral VTA, with increased activity to an aversive stimulus and reduced firing in response to the absence of an aversive stimulus. The activity of dopamine neurons in the VTA in response to the absence and presence of predicted appetitive and aversive events provides a neurobiological basis for theoretical models of aversive-appetitive interactions proposed by Konorski (1967) and Dickinson (Dickinson and Dearing, 1979; Dickinson and Pearce, 1977). In combination, these studies suggest that the

acquisition of aversive learning requires coordinated action in subpopulations of VTA neurons, but other brain regions may maintain the long-term storage of these experiences. Modulating the activity of dopamine receptors in VTA terminal regions could provide clues as to how dopamine neuron activity is translated into behavioral and associative outcomes.

Dopamine Terminal Regions and Fear Extinction

The study of dopamine signaling in fear extinction provides an arena to examine interactions between appetitive and aversive learning. The ventral tegmental area provides aversive or appetitive information through separate circuits (Lammel et al., 2011) that alter behavioral responses to conditioned stimuli. Pan et al. (2008) showed evidence for the development of inhibitory activity on dopamine neurons responsive to rewarding stimuli following extinction, as well as the evidence for dopamine neurons excited by an extinction contingency, reinforcing theories that incorporate new learning, inhibitory activity, and forgetting as components of extinction. However, these studies do not incorporate how dopamine release translates into appropriate behavioral and associative outcomes during fear extinction. The overarching hypothesis of the studies in this dissertation is that increased dopamine activity during fear extinction can lead to enhancements of extinction.

One method to manipulate dopamine signaling in fear extinction is through the use of dopamine and norepinephrine transport (DAT; NET) blockers. Pharmacological agents that inhibit the reuptake of dopamine and norepinephrine are generally classified as psychostimulants, and have been

investigated for their potency in enhancing cognition as well as their abuse potential (Wood et al., 2013). Methylphendiate (Ritalin) enhances performance in reward-related tasks at low doses (Tye, et al. 2010), and increases catecholamine neurotransmission in the prefrontal cortex (Berridge et al., 2006). Many studies have found that subregions of the prefrontal cortex are important for the consolidation and retrieval of fear extinction (Quirk and Mueller, 2008) and increased post-traumatic stress disorder (PTSD) severity is correlated with decreased prefrontocortical activity (Shin et al., 2006). Patients with PTSD are able to acquire fear extinction normally in a laboratory setting, but recall of fear extinction is impaired (Milad et al., 2009). These observations indicate that strengthening consolidation and retention of extinction in the PFC through pharmacological intervention may be sufficient for lessening PTSD symptoms. Based on the characterization of methylphenidate as a cognitive enhancer with specific actions in the prefrontal cortex, I hypothesized that methylphenidate would enhance consolidation of fear extinction. In Chapter 2, I examine the effect of methylphenidate on the acquisition and consolidation of fear extinction in C57BL/6J (B6) mice. B6 mice have been well characterized in many studies that examine fear learning (Lattal and Maughan, 2012), and are commonly used as background strains for genetic manipulations, as seen in Chapter 4.

These broad approaches to increasing dopamine signaling may be informative for pharmacological interventions in human fear disorders, but there is evidence that methylphenidate's cognition enhancing properties are derived through signaling at the D1/5 receptor. Methylphenidate induces greater

inhibition of NET than DAT (Markowitz et al., 2006), but blockade of D1/5 receptors with SCH 23390 disrupts methylphenidate-mediated enhancements of working memory (Tye et al., 2012; Arnsten and Dudley, 2005). Increasing activity of D1-like receptors alone may be sufficient for enhancing fear extinction, although there is considerable inconsistency in the literature about the particular contributions of dopamine receptor subfamilies to fear learning.

D1/5 Receptors and Fear Extinction

D1/5 receptor antagonist and knockout studies have demonstrated impairments in acquisition and extinction of fear following loss of D1/5 receptor signaling, suggesting that dopamine signaling at D1/5 receptors directly contributes to the acquisition of fear learning (El-Ghundi et al., 2001; Fadok et al., 2009; Greba and Kokkinidis, 2000; Inoue et al., 2000). These effects may be mediated by activation of dopamine neurons through NMDA receptors (Zweifel et al., 2009; Zweifel et al., 2011) and they are time-limited, with post-session injections of a D1/5 receptor antagonist 30-min following fear conditioning having no effect (Inoue et al., 2000). These studies indicate that impaired signaling at the D1/5 receptor decreases fear learning, but there are few studies examining the effect of D1/5 activation on fear learning.

Although most studies examining the relationship between dopamine signaling and extinction have utilized loss-of-function approaches (e.g., antagonist or knockout studies), the approach of my experiments is to enhance learning through pharmacological activation of dopamine receptors in C57BL/6J mice. This approach allows for a dissection of how artificially increasing D1/5

receptor signaling alters learning that occurs in dopamine terminal regions, rather than blocking the signal arising from dopamine neurons. Although loss-of-function approaches provide value in understanding the neurobiology of fear extinction, clinical applications of these findings require identification of targets that enhance learning. To this end, I utilized a D1/5 receptor agonist, SKF 81297, and examined the effects of D1-like receptor activation on fear extinction in Chapter 3. I hypothesized that D1-like receptor activation would enhance fear extinction based on evidence that D1/5 receptor blockade impairs fear extinction (Hikind and Maroun, 2008).

Many of the studies that have investigated this question previously have primarily utilized pre-extinction session injections. In general, pre-session injections of any substance that alters dopamine release or dopamine binding are difficult to evaluate because of the activating or sedating effects that these agents have on locomotor activity. Post-session injections are not without their own complications (Cunningham et al., 1998), but they do allow the animal to learn the extinction contingencies without being affected by activating or sedating effects of the drugs. Differences in pre- and post-session effects are evident in studies that have examined the effects of psychostimulants on extinction.

Methamphetamine administered prior to an extinction session impairs extinction of conditioned fear, assessed through conditioned suppression (Miczek and Luttinger, 1978; but see null effects in Carmack et al., 2010; Mueller et al., 2009), and in agreement with these findings, Borowski and Kokkinidis (1998) demonstrated that a pre-extinction administration of cocaine, amphetamine, or a

dopamine D1/5 partial agonist (SKF 38393) impairs extinction of potentiated startle. However, L-Dopa, a dopamine precursor, administered immediately following a fear extinction session enhances the retention of fear extinction (Haaker et al., 2013). One of the challenges for the use of cognitive enhancers in human fear disorders is that broad enhancement of learning may reinforce fear and disrupt fear extinction if drug administration is not carefully linked with decreased fear responses (Bolkan and Lattal, 2014).

In a clinical setting, the use of post-session drug can overcome this challenge by allowing a therapist to administer the drug under conditions when behavioral therapy has been successful. Many of the experiments presented in the following chapters examine the effect of post-session administration of direct or indirect dopamine agonists, during consolidation of fear extinction. To determine whether targeting D1/5 receptor activation would increase fear expression, I examined the effect of post-conditioning administration of SKF 81297 in Chapter 3. If fear learning is enhanced, targeting D1/5 receptors in therapeutic settings may have disruptive consequences on fear extinction in humans. However, if SKF 81297 specifically enhances fear extinction and impairs or has no effect on fear conditioning, activating D1/5 receptors could be a strong therapeutic target for decreasing symptoms of PTSD.

Based on findings that the ventral tegmental area signals both fear and reward learning (Matsumoto and Hikosaka, 2009), one question of particular interest is whether similar mechanisms control fear and reward extinction in the dopamine system, as this might provide a valuable pharmacological target for

altering long established behaviors, such as those seen in substance abuse or post-traumatic stress disorder (Peters et al., 2009). It is possible that enhancing dopamine signaling during fear extinction can switch the ventral tegmental area signaling from aversive to appetitive circuits, essentially counter-conditioning an aversive stimulus to become a neutral or rewarding stimulus. Behavioral studies examining the effects of counterconditioning stimuli have suggested that there is a direct inhibitory interaction between appetitive and aversive tasks (Bouton, 1993; Nasser and McNally, 2012) that may mirror the development of inhibitory learning required for fear extinction (Bouton and Peck, 1992; Peck and Bouton, 1990). Thus, I initially examined the rewarding properties of SKF 81297 with conditioned place preference, then analyzed whether administration of SKF 81297 could enhance the extinction of a cocaine conditioned place preference (Chapter 3).

The conditioned place preference experiments allow us to determine whether D1/5 receptor agonism enhances fear extinction through counterconditioning mechanisms or if activation of D1/5 receptors is an underlying feature of extinguishing Pavlovian associations. If SKF 81297 impairs extinction of a cocaine conditioned place preference, it is possible that the rewarding properties of the dopamine agonist are inducing a competition between appetitive and aversive representations during fear extinction. However, if SKF 81297 enhances extinction of a cocaine conditioned place preference, it would demonstrate that D1/5 receptor activity is involved in the learning that

occurs during extinction of Pavlovian associations, regardless of the hedonic value of the initial associations.

The experiments in Chapter 3 allow for a dissection of the contribution of D1/5 receptors in fear and reward learning, but specifying whether the effects are due to D1 or D5 receptor activity is not currently feasible through pharmacological manipulations (Waddington et al., 2005). Instead, genetic inactivation of the D1 receptor gene, *drd1a*, can determine whether D1 receptors are necessary for fear and reward learning (Chapter 4). The D1 knockout (D1 KO) mice were backcrossed to B6 mice for several generations (Heusner et al., 2008), allowing comparison of the D1 KO behavior in Chapter 4 to the other experiments presented in this dissertation. Based on El-Ghundi et al. (2001), I hypothesized that D1 KO would impair fear extinction, but not fear conditioning. Based on Miner et al. (1995), I hypothesized that cocaine conditioned place preference would be retained in D1 KO mice. I also tested the effect of a D1/5 receptor agonist (SKF 81297) in D1 KO mice during consolidation of fear extinction to isolate D1 and D5 receptor contributions to the fear extinction effects presented in Chapter 3. Together, these studies provide an analysis of the behavioral consequences of systemic manipulation of D1-like receptors during Pavlovian conditioning and extinction. Chapter 5 continues this investigation by examining the intracellular signaling cascades and brain regions that may be involved in modulating fear extinction through D1/5 receptors.

The classical view of dopamine receptor activity has focused on intracellular signaling through adenylate cyclase and cAMP activity, both of which

are recognized to play central roles in learning and initiate protein kinase A (PKA) signaling. There is evidence, however, that in addition to modulating cAMP activity, D1-like receptors operate through activation of phospholipase C (PLC) and PLC activity has been implicated in the formation of fearful memories (Waddington et al., 2005; Buckley and Caldwell, 2004; Ouyang et al., 2012). To distinguish intracellular signaling pathways involved in enhancing fear extinction, in Chapter 5, I systemically administered the biased agonists SKF 83959 and SKF 83822 following fear extinction. Biased agonism, or functional selectivity, describes a pharmacological phenomenon by which ligand-specific receptor conformations induce different signaling pathways through the same receptor system (Urban et al., 2007).

Considerable data support the classification of the D1 receptor ligand SKF 83959 as a very weak partial agonist or antagonist for stimulation of adenylate cyclase but a full agonist for stimulation of PLC (Arnt et al., 1992 and Jin et al., 2003) and SKF 83822 as a full-efficacy agonist for adenylate cyclase with little or no efficacy for PLC (Rashid et al., 2007 and Undie et al., 1994). Further evidence that SKF 83959 and SKF 83822 are reciprocal biased agonists was provided by the report that SKF 83959 is capable of activating the D1/D2 heteromer to stimulate PLC, whereas SKF 83822 is inactive at the heteromer but activates the D1 receptor homomer to stimulate adenylate cyclase (Rashid et al., 2007). Based on evidence indicating that cAMP/PKA activity is important for the expression of memory in the prefrontal cortex and hippocampus (Lauzon et al., 2013; Gao et al., 2006), I hypothesized that SKF 83822, a D1-PKA pathway

selective agonist, would enhance fear extinction. However, SKF 83959, a D1-PLC pathway selective agonist, may also affect fear extinction due to a report that amygdalar D1 receptors are not linked to adenylate cyclase (Leonard et al., 2003). Comparing the effects of these biased agonists in the fear extinction procedure allow us to determine the cell signaling pathways involved in enhancing extinction and could specify some of the regions involved in D1/5 receptor-mediated modulation of extinction.

Examining the contribution of particular brain regions to the observed systemic effects requires the use of targeted microinjections of SKF 81297. Studies presented in Chapter 5 examine the effect of regional activation of D1/5 receptors following fear extinction within the infralimbic region of the prefrontal cortex, basolateral amygdala, nucleus accumbens core, and dorsal hippocampus. Initially, I hypothesized that activating D1/5 receptors in the infralimbic region of the prefrontal cortex would be sufficient to enhance fear extinction. However, the basolateral amygdala, nucleus accumbens core and hippocampus express D1/5 receptors as well, suggesting that the extinction effects of systemic SKF 81297 could arise from activity in these regions.

Research Questions

The central question of this dissertation is to identify the effect of activating D1/5 receptors on fear extinction. There are several possible ways that activating D1/5 receptors can interact with learning, and as discussed previously, Rescorla (1988) describes three primary issues to consider when examining learning: the circumstances that produce learning, the content of learning, and the impact of

learning on behavior. In the following chapters, the circumstances that produce learning are measured by pairing direct or indirect dopamine agonists with Pavlovian conditioning and extinction sessions. These allow for a distinction between modulatory effects of dopamine receptor activation on all learning compared to the possibility that D1/5 receptor activation is particularly involved in enhancing extinction. Additionally, testing both pre- and post-extinction manipulations of dopamine agonists separates effects on fear extinction acquisition from effects on consolidation of fear extinction. The parallel study of D1 KO mice (Chapter 4) examines whether D1 receptor activation is necessary for learning and extinguishing Pavlovian associations. Chapter 5 examines the neurobiological contributions that may underlie D1-like receptor modulation of fear extinction. In sum, these manipulations permit a clearer description of the circumstances under which D1/5 receptor activation will lead to enhancements or impairments of learning.

To determine the content of learning, there are several different approaches that are utilized in the following chapters. The content of extinction learning involves both inhibitory and excitatory components and research on enhancing extinction is interested in distinguishing enhancement effects on inhibitory representations, such as those generated by the extinction context to inhibit the original association, from enhancement effects on excitatory representations, such as those generated when a CS signals no US or a new US (Bouton, 2004). Inhibitory learning can be assessed through examining the contextual specificity of extinction enhancements and measuring fear renewal

(Chapter 3) provides one measure of whether dopamine receptor activation leads to context dependent extinction enhancements. To identify the contribution of excitatory learning in extinction following D1/5 receptor activation, I assessed the rewarding properties of the drug through conditioned place preference. This measure could indicate whether a new CS-US association is developed when fear extinction is paired with a dopamine agonist, and tests the hypothesis that D1/5 receptor activation enhances excitatory components of extinction learning.

Finally, the impact of learning on behavior is measured through assessing behavior on test days following extinction training. Additional tests examining D1-like receptor effects on locomotion and fear behavior following the omission of an extinction training session in combination with a D1/5 receptor agonist allow for the separation of simple performance effects from interactions of D1/5 receptor activation with extinction learning. Together, the studies in this dissertation describe the behavioral mechanisms by which D1/5 receptor activity modulates extinction learning in both rewarding and aversive tasks.

Chapter 2: Methylphenidate Enhances Contextual Fear Extinction

This chapter is adapted from the following publication:

Abraham A.D., Cunningham C. L., Lattal K. M. (2012) Methylphenidate enhances extinction of contextual fear. *Learning and Memory,* 19(2): 67-72.

Introduction:

As discussed in the previous chapter, pharmacological approaches that target prefrontal function may be useful for treatments that are designed to promote extinction (Quirk et al., 2006). Several experiments have shown that psychostimulants can alter prefrontal cortex function. For example, acute cocaine or amphetamine administration increases extracellular availability of dopamine in the mPFC (Sorg and Kalivas, 1993; Mazei et al., 2002). Although there is some evidence for fear extinction enhancements with intra-mPFC infusions of amphetamine (Pezze et al., 2003), the literature is generally mixed, with no effect (Mueller et al., 2009; Carmack et al., 2010) or even extinction-impairing effects of systemic administration of amphetamine or cocaine (Miczek and Luttinger, 1978; Borowski and Kokkinidis, 1998). These studies have examined psychostimulant effects on extinction following pre-session injections, which result in large locomotor effects that may interfere with the animal's ability to retrieve or express fear memory.

Methylphenidate hydrochloride (MPH) is a dopamine (DA) and norepinephrine (NE) transporter blocker that has been approved for clinical use in attention deficit hyperactivity disorder (ADHD) and narcolepsy under the trade name Ritalin. Deficits in prefrontocortical functioning are thought to underlie

ADHD and may be related to decreased catecholamine terminals in the prefrontal cortex of ADHD adults (Arnsten and Dudley, 2005). Berridge et al. (2006) demonstrated that low doses of MPH (0.25–2.0 mg/kg) preferentially increased DA and NE extracellular levels in the prefrontal cortex (compared to anterior cingulate cortex) and improved spatial working memory in the delayed alternation task in Sprague-Dawley rats. Zheng et al. (2008) found that a single post-training session infusion of MPH into the anterior cingulate or the basolateral amygdala augmented fear memory consolidation in a step through inhibitory avoidance task but did not see effects of systemically injected MPH (0.5 mg/kg and 5 mg/kg). Acute administration of MPH in rats has been shown to facilitate cortico-amygdalar plasticity following a cue–reward learning task (Tye et al., 2010). Because the prefrontal cortex is important for fear extinction (Myers and Davis, 2007), there is a strong likelihood that MPH may enhance the development and persistence of extinction through activation of dopamine and norepinephrine receptors in the prefrontal cortex.

The following experiments examine the effects of acute systemic MPH administration in contextual fear extinction before, immediately after, or 4 h after a single longer (12-min) extinction session or following a relatively shorter (3-min) extinction session. The persistence of MPH effects was tested for 3 d after the first extinction session. The use of pre- and post-session injections allows us to specifically examine effects of MPH on acquisition and consolidation of the learning that occurs during extinction. To rule out simple performance effects, the effect of pre- or post-session administration of methylphenidate is measured in a

locomotor chamber. To determine methylphenidate's effect on consolidation of fear learning, I tested the effect of a post-conditioning administration of methylphenidate. Together, these experiments describe the effect of systemic methylphenidate on fear behaviors.

Materials and Methods:

Subjects

Male C57BL/6 mice (n = 256) ranging from 7 to 11 wk of age (28 ± 5 g) were used in these experiments. Animals were purchased from Jackson Laboratory (Bar Harbor, ME) and given at least 7 d in the vivarium prior to experimental use. For 2 d before conditioning, mice were handled and given a 0.2-mL saline (0.9% NaCl) injection each day to habituate to injections and handling. Animals were housed four to a cage. Polycarbonate cages were held in a Thoren rack, and animals were given access to food and water ad libitum. Vivarium and experiment room temperatures were maintained at 22°C ± 1°C, and subjects were maintained on a 12-h light–dark cycle (lights on 0600 h–1800 h). Animals were moved from the vivarium to the experiment room 60 min before the start of an experiment, and experiments were conducted between 900 and 1700 h. All experimental procedures were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) "Principles of Laboratory Animal Care" (NIH Publication No. 86-23, revised 1985).

Drugs

Methylphenidate HCl (Sigma-Aldrich) was dissolved in saline (0.9% NaCl) at concentrations of 2.5, 5, 10, 20, and 40 mg/kg. Drug was administered intraperitoneally (i.p.) in a volume of 10 mL/kg. Doses were selected based on previous studies indicating MPH enhancement of cognitive function and fear memory (Arnsten and Dudley, 2005; Nolley and Kelley, 2007; Zheng et al., 2008).

Apparatus

Fear conditioning.

Four Coulbourn Instruments mouse-conditioning chambers (H10-11M-TC) were used. Chambers were housed in sound and light-attenuating cabinets with a fan providing 65 dB of background noise. A Plexiglas cylinder, 21.5 cm in diameter and 23 cm in height, was placed on a grid floor in the chamber. The grid floor consisted of stainless steel rods, 3.2 mm in diameter, spaced 6.4 mm apart. Scrambled shock (2 sec, 0.35 mA) was delivered to the grid floor by a computer controlled shock generator (Coulbourn H13-15). Above the Plexiglas cylinder, an automated infrared activity monitor (Coulbourn H24-61) recorded activity in Graphic State 3.01 software.

Locomotor Activity.

Four sound and light-attenuating cabinets contained clear acrylic cages (30 cm X 15 cm X 15 cm) with hole floors consisting of perforated stainless steel sheets with 6.4 mm diameter round holes on 9.5 mm staggered centers. Total activity in the box was assessed by Ethovision 5.0.216 computer software

(Noldus, Leesburg, VA) that recorded activity via a ceiling mounted camera. Ethovision sampling rate was set at 29.97 samples per second. Locomotor activity was measured by total distance moved in cm, and reported as distance moved (cm) per min.

Behavioral procedures

Contextual fear conditioning procedure

On Day 1 (acquisition), subjects received a 12-min exposure to the context with four unsignaled shocks, delivered at 2.5, 5, 9, and 11.5 min. In all experiments, groups were matched following acquisition to ensure equal terminal freezing levels across MPH dose assignments. On Day 2, mice received a 12-min (Experiments 1, 2, and 3) or a 3-min (Experiment 4) nonreinforced exposure to the context (Ext). In all post-session injection experiments (Experiments 2, 3, and 4), groups were matched within experiments to ensure equal levels of terminal freezing before MPH injections. Animals with higher freezing response during the final 3-min block compared with the initial 3-min block on Day 2 were excluded from data analysis due to their failure to extinguish fear responding in Experiments 2 and 3. Appendix A shows data analysis from Experiment 2 with all animals included. On Days 3–5 (Test Days 1–3), animals received 12-min nonreinforced exposures to the context on each day.

Experiment 1a: Pre-extinction administration of MPH

This experiment tested the effects of acute MPH given before an extinction session to determine whether MPH could enhance acquisition and retention of extinction. On Day 1, animals (n = 8 per group) received an injection

of saline, followed by acquisition training. On Day 2, animals received either MPH (2.5, 5, 10 mg/kg) or saline, followed immediately by a 12-min extinction session (Fig. 2a). On Days 3–5, animals received an injection of saline, followed by additional 12-min extinction sessions.

Experiment 1b: Locomotor effects of pre-session administration of MPH

Activity was assessed over 2 days, with animals (n=4 per group) receiving a saline injection and being placed into the locomotor chamber on Day 1 for 30 min followed by animals receiving methylphenidate (0, 2.5, 5, 10 & 20 mg/kg) and being placed into the locomotor chamber on Day 2 for 30 min (Fig. 2b). Animals had been exposed to drug (methylphenidate or sodium butyrate) and fear conditioning approximately 20 days prior to locomotor activity tests, but were matched for initial locomotor activity and prior experience across doses. A one-way ANOVA tested statistical differences between groups on each day.

Experiment 2a: Post-extinction administration of MPH

This experiment examined the effect of acute MPH administered immediately after an extinction session. The post-session administration allowed animals to be given drug or saline under common conditions during a period of memory consolidation (Abel and Lattal, 2001). On all days of conditioning and testing, animals received injections post-session. On Day 1, animals received an acquisition session with administration of saline. On Day 2, animals received either MPH or saline immediately following extinction. On Days 3, 4, and 5, animals received injections of saline following 12-min nonreinforced sessions (Fig. 3a). The doses of MPH that were given to the animals following extinction

were distributed across three separate replications. Experiment 2a.1 consisted of doses 0, 2.5, 5, and 10 mg/kg (n = 8 per group). Experiment 2a.2 consisted of doses 0, 10, and 20 mg/kg (n = 16 per group). Experiment 2a.3 consisted of doses 0, 20, and 40 mg/kg (n = 8 per group). Six animals were removed from both the saline and 10 mg/kg groups, and four animals were removed from the 20 mg/kg group because they failed to meet extinction criterion.

Experiment 2b: Locomotor effects of post-session administration of MPH

Activity was assessed over 5 days, with animals (n=4 per group) receiving saline following a 12-min context exposure on all days except day 2, when they received methylphenidate (0, 2.5, 5, 10, 20, 40 mg/kg). Animals were drug naïve, but had received fear conditioning approximately 20 days prior to locomotor testing. Animals were placed in a plexiglas cylinder, 21.5 cm in diameter and 23 cm in height placed on the locomotor chamber floor to measure locomotor activity within an identical space as used in fear conditioning studies. A one-way ANOVA tested for statistical differences on each day, in addition to a repeated measures ANOVA that tested for statistical differences across days 3-5 to match fear conditioning statistical protocol. Data is shown in Figure 3b.

Experiment 3: Four-hour delayed administration of MPH

In this experiment, the end of the extinction session and the administration of MPH were separated by 4 h. The temporal separation tested whether drug administration, unpaired from extinction training, would affect freezing response on the following days (Burgos-Robles et al. 2007). The animals (n = 12 per group) still received saline injections on all days following exposure to the context

in order to ensure equivalence to treatment conditions in Experiment 2. However, in this experiment, following the extinction session, all animals received an injection of saline and 4 h later received either MPH (20 or 40 mg/kg) or saline (Fig. 4). In accordance with exclusion criteria, one animal was removed from both saline and 40 mg/kg MPH groups, and two animals were removed from the 20 mg/kg MPH group for final analysis.

Experiment 4: Brief extinction followed by administration of MPH

This experiment tested whether MPH can facilitate extinction under conditions of reduced within-session extinction. A brief extinction period should ensure that animals only undergo partial extinction and may increase the detection of MPH enhancements. Animals (n = 8 per group) received injections of saline on Days 1, 3, 4, and 5. On Day 2, animals received either MPH (10, 20 mg/kg) or saline following a brief (3-min) extinction period (Fig. 5).

Experiment 5: Fear conditioning followed by administration of MPH

This experiment tested whether MPH can facilitate or impair consolidation of fear conditioning. Testing the effect of MPH on fear conditioning can determine whether MPH administration broadly enhances consolidation of fear learning or if the observed effects in these studies were specific to fear extinction. On Day 1, animals (n=8 per group) received an acquisition session immediately followed by administration of saline or MPH (20 mg/kg). On Days 2-4, animals received injections of saline following 12-min nonreinforced sessions (Fig. 6).

Data analysis

Fear memory expression was determined by freezing response within the context. Freezing was defined as an episode of at least 3 sec of inactivity. Total freezing time was divided by 12 min to calculate percentage of time freezing in each day. Data analyses were performed with SPSS version 17.0. Data for Acquisition, Extinction, and Brief Extinction were analyzed using analysis of variance (ANOVA). Test Days 1–3 were analyzed using Repeated Measures ANOVA with Day as a within-subjects factor and Dose as a between-subjects factor for Experiments. When sphericity could not be assumed for data (evaluated by Mauchly's sphericity test), reported degrees of freedom reflect a Greenhouse-Geisser estimate. All post hoc comparisons of Repeated Measures ANOVA data were performed using a Dunnett's test. For all statistical tests, the α was set to 0.05.

Results:

Experiment 1: Pre-extinction session administration of MPH reduces freezing during extinction and testing.

Experiment 1a examined the effect of MPH (0, 2.5, 5, or 10 mg/kg) on extinction acquisition through the use of a single pre-session administration immediately before extinction. Figure 2a shows that pre-session administration of MPH caused a dose-dependent reduction in freezing during the extinction session (Ext in Fig. 2a). A one-way between-subjects ANOVA found significant effects of MPH on freezing ($F(3,28) = 12.7$, $P < 0.0005$). Post hoc comparisons

with a Dunnett's test showed that saline was significantly different from 5 mg/kg ($P = 0.001$) and 10 mg/kg ($P < 0.0005$) but not from 2.5 mg/kg ($P = 0.305$). This effect was likely due to the locomotor effects of MPH, because doses of 10 and 20 mg/kg MPH showed significant differences in locomotor activity compared with saline (Figure 2b).

Test day analyses indicated that the effects of MPH persisted to the first drug-free test, when methylphenidate-treated (10 mg/kg) animals froze less than saline-treated animals (Test 1 in Fig. 2a). This effect did not persist to Tests 2 and 3. These effects were confirmed by a Dose × Test Day ANOVA that found no main effect of Dose ($F(3,28) = 0.6$, $P = 0.633$) but did find a significant main effect of Test Day ($F(2,56) = 7.5$, $P = 0.001$), as well as a reliable interaction for Dose × Test Day ($F(5,43) = 2.5$, $P = 0.049$). Post hoc analyses of individual test days did not show significant effects for doses, but there was a trend ($F(3,28) = 2.4$, $P = 0.085$) on Test Day 1 that was not seen on other test days. A Dunnett's test confirmed that there was a significant effect of MPH at 10 mg/kg compared with saline on Test Day 1 ($P = 0.048$) when administered before an extinction session.

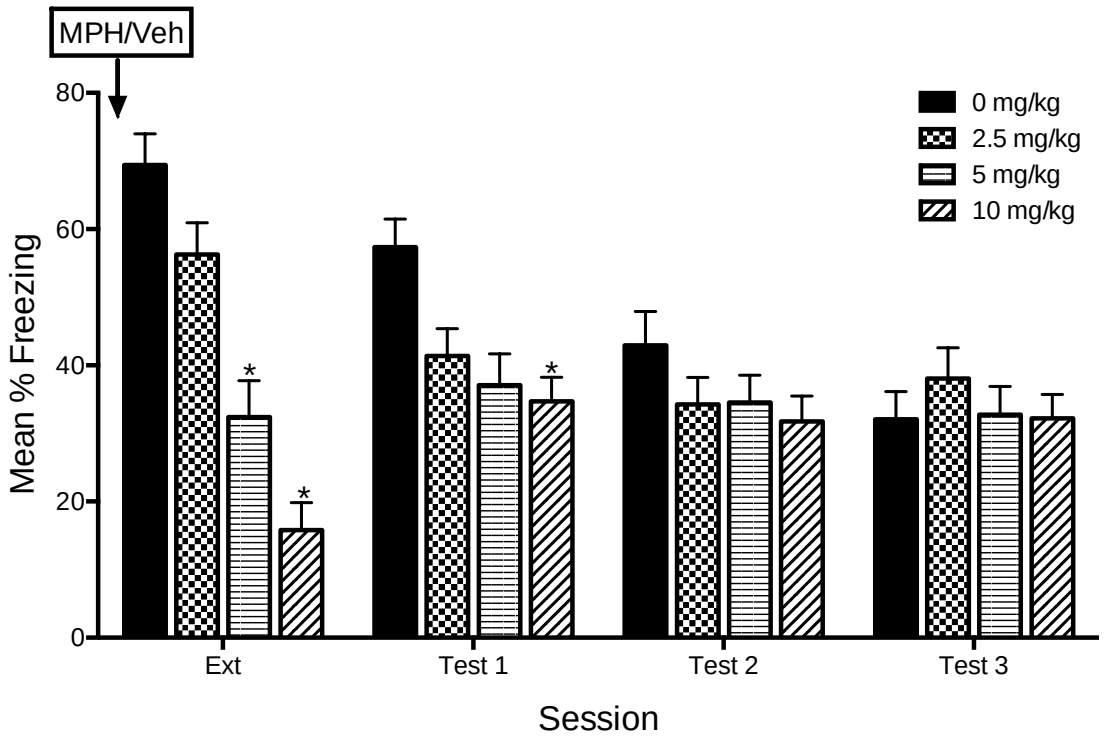


Figure 2a. Effects of MPH administered immediately before a 12-min extinction session. (Experiment 1a). Mice that received MPH showed differences in freezing response during the Extinction session (5 and 10 mg/kg) and during Test Day 1 (10 mg/kg). Error bars indicate SEM. (*) P < 0.05 significant difference compared with saline (Dunnett's test).

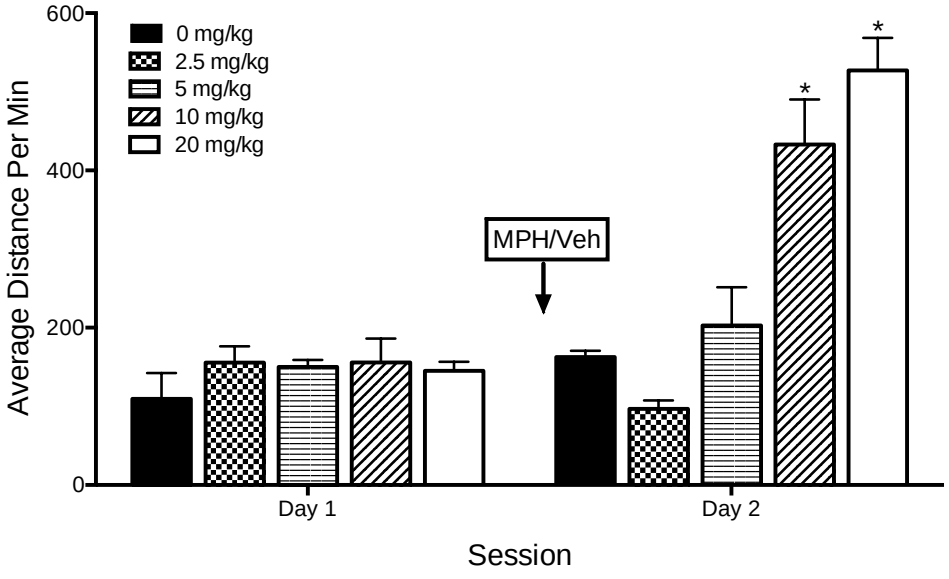


Figure 2b. Pre-session methylphenidate (10 & 20 mg/kg) increases spontaneous locomotor activity. (Experiment 1b). There were no differences between doses on Day 1, but a one-way ANOVA confirmed an effect of dose on Day 2 ($F(4,15)=22.8$, $p<.0005$). Post-hoc Dunnett's test confirmed that 10 and 20 mg/kg were significantly different from saline with $p < .001$ for 10 mg/kg and $p < .0005$ for 20 mg/kg.

Experiment 2: Post-extinction administration of MPH dose-dependently enhances retention of extinction.

Experiment 2a tested the effects of MPH (0, 2.5, 5, 10, 20, or 40 mg/kg) on extinction consolidation using a single post-session administration of MPH immediately following Extinction. Figure 3a indicates that there was a persistent effect of methylphenidate administration that caused lower freezing across test days. Animals that received 20 mg/kg MPH showed reliably lower freezing compared with the saline group, while animals that received other MPH doses did not show reliable differences across test days. Statistical analysis confirmed a significant main effect of Dose ($F(5,82) = 3.5$, $P = 0.006$) and Test Day ($F(2,130) = 17.0$, $P < 0.0005$). There was no significant Dose × Test Day interaction ($F(8,130) = 0.607$, $P < 0.770$). Post hoc analysis with Dunnett's test showed an overall significant difference between saline and 20 mg/kg ($P = 0.007$).

To assess potential nonspecific effects of these doses on locomotion, I conducted another experiment that was identical to Experiment 2, except that there was no shock in Day 1 (i.e., post-session injections following Day 2 and subsequent testing on Days 3–5). I found no significant effects of these doses on locomotor behavior during any of the sessions (Fig. 3b). Thus, although there are clear acute locomotor effects of pre-session MPH (Fig.2b), these effects do not persist to further test sessions with post-session MPH, indicating that the effects on freezing in Figure 3a are likely not due to simple persistent effects on activity.

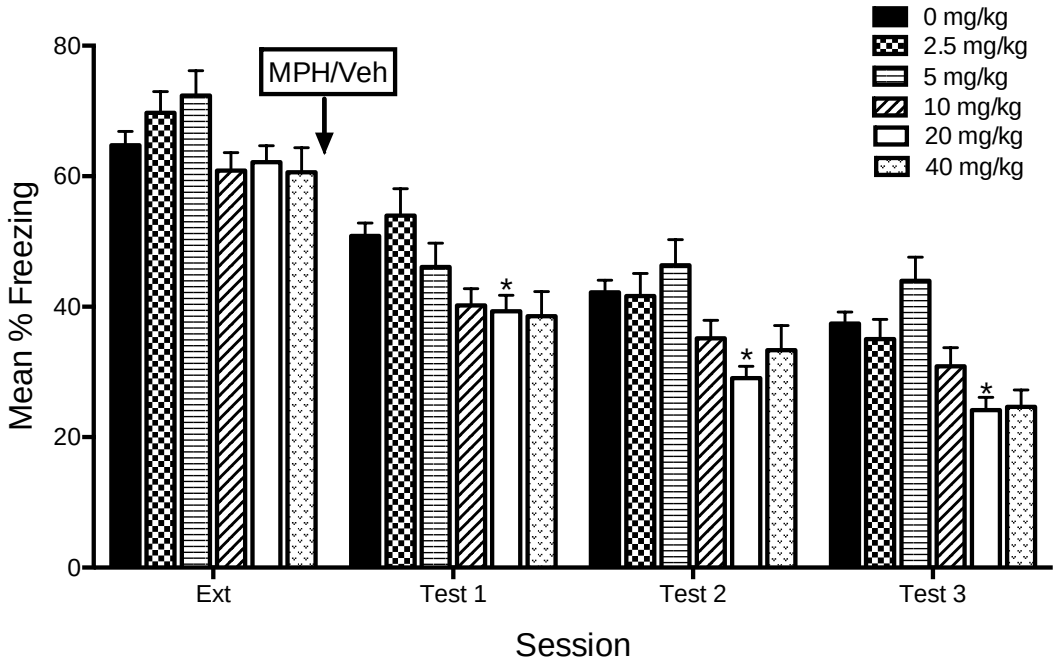


Figure 3a. Effects of MPH administered immediately following a 12-min extinction session. (Experiment 2a). Between-session effects of MPH administered immediately after a 12-min extinction session. Error bars indicate SEM. Repeated measures ANOVA revealed differences between MPH (20 mg/kg) and saline across Tests 1-3. (*) $P < 0.05$ significant difference compared with saline (Dunnett's test).

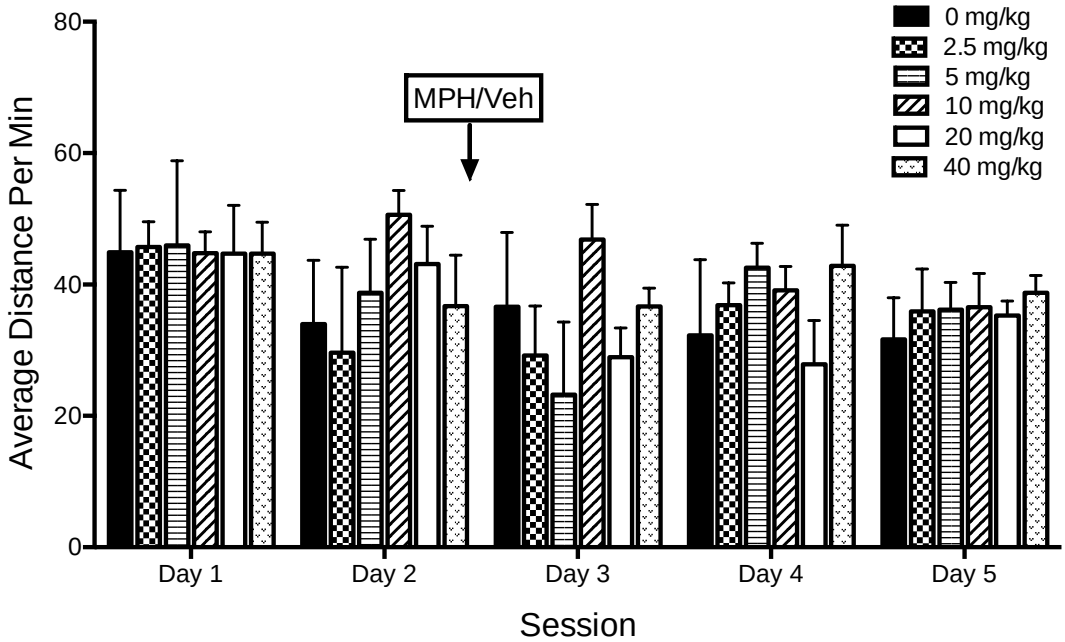


Figure 3b. Post-session MPH has no effect on locomotor behavior. (Experiment 2b). One-way ANOVAs for each day confirmed that there were no dose effects, as well as a repeated measures ANOVA for Session 3-5 confirmed no dose effect.

Experiment 3: Four-hour delayed administration of MPH following an extinction session has no effect on retention of extinction.

To test whether the extinction enhancement effects seen in Experiments 1 and 2 were due to the temporal proximity of the injection to the extinction session, animals were administered a single dose of MPH 4 h following an extinction session (Figure 4). If MPH targets extinction memory consolidation, then a 4-h post-session injection should have little effect. Animals were returned to their home cage during the 4-h interval before being administered methylphenidate (0, 20, or 40 mg/kg). Although Figure 4 visually suggests a dose-dependent effect of MPH, there was no significant effect of MPH treatment on freezing during test days. Statistical analysis confirmed that there was no main effect of Dose ($F(2,29) = 0.7$, $P = 0.503$) but there was a significant effect of Test Day ($F(2,58) = 23.4$, $P < 0.0005$). There was no Dose × Test Day interaction ($F(4,58) = 1.341$, $P = 0.266$).

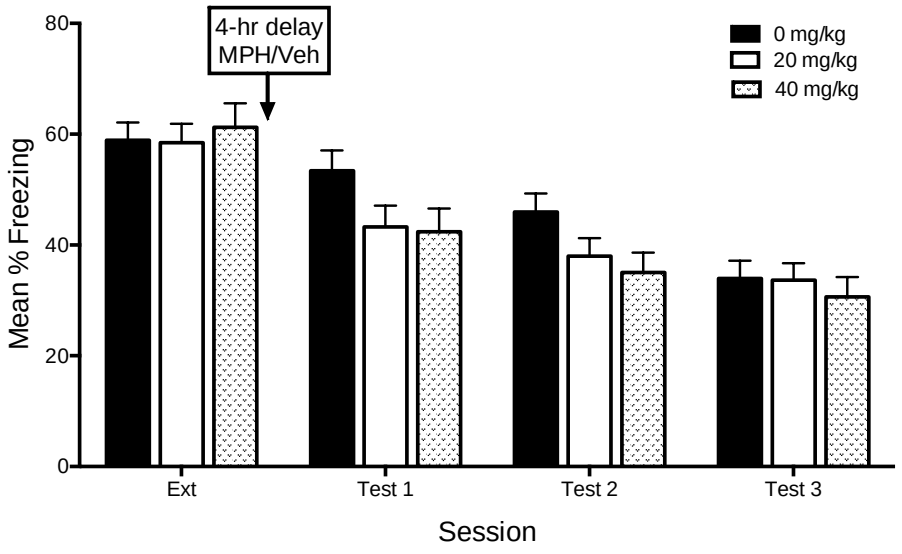


Figure 4. Effects of MPH administered 4 h after a 12-min extinction session. (Experiment 3). Mice that received methylphenidate (0, 20, and 40 mg/kg) 4 h following extinction did not show significant differences in freezing response during test days. Error bars indicate SEM.

Experiment 4: Brief extinction immediately followed by administration of MPH has no effect on extinction retention

This experiment examined the effects of MPH (0, 10, 20 mg/kg) on extinction under conditions of reduced within-session extinction. Figure 5 shows no effect of MPH on freezing during test days. Statistical analysis confirmed that there was no main effect of Dose ($F(2,21) = 0.416$, $P = 0.665$), but there was a significant effect of test days ($F(2,42) = 20.7$, $P < 0.0005$) and no Dose × Test Day interaction ($F(4,42) = 0.924$, $P = 0.459$).

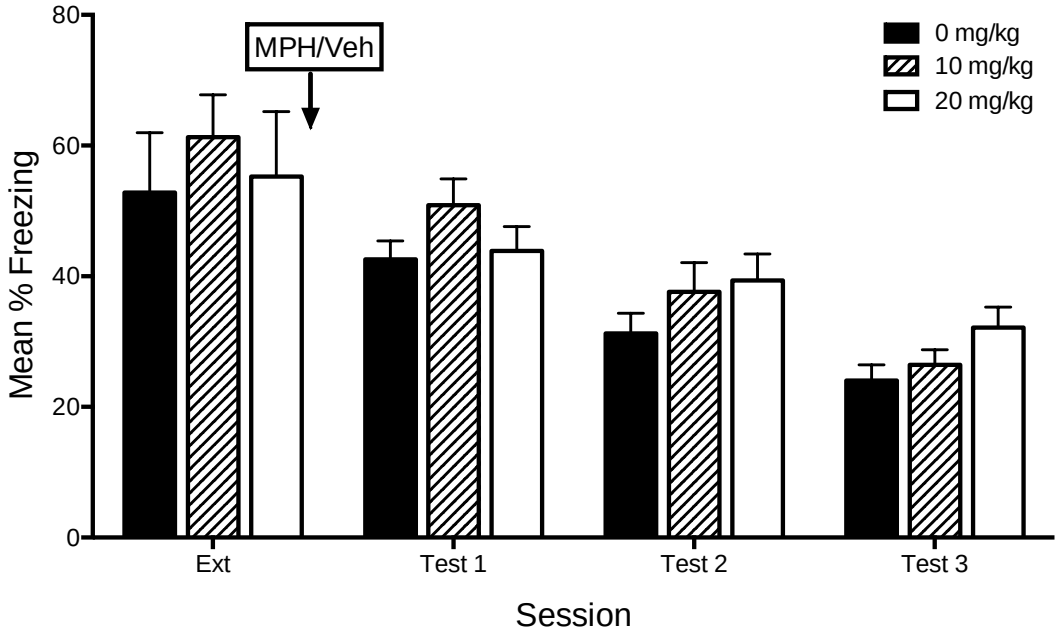


Figure 5. Effects of MPH administered immediately after a 3-min extinction session. (Experiment 4). Mice that received methylphenidate (0, 10, and 20 mg/kg) following brief (3-min) extinction did not show significant differences in freezing response during test days. Error bars indicate SEM.

Experiment 5: Fear conditioning immediately followed by administration of MPH has no effect on fear learning.

This experiment examined the effects of MPH (20 mg/kg) on consolidation of fear conditioning. Figure 6 shows no effect of MPH on freezing during test

days. Statistical analysis confirmed that there was no main effect of Dose ($F(1,14) = 0.027$, $P = 0.872$), but there was a significant effect of test days ($F(2,28) = 3.439$, $P = 0.046$) and no Dose × Test Day interaction ($F(2,28) = 0.457$, $P = 0.638$).

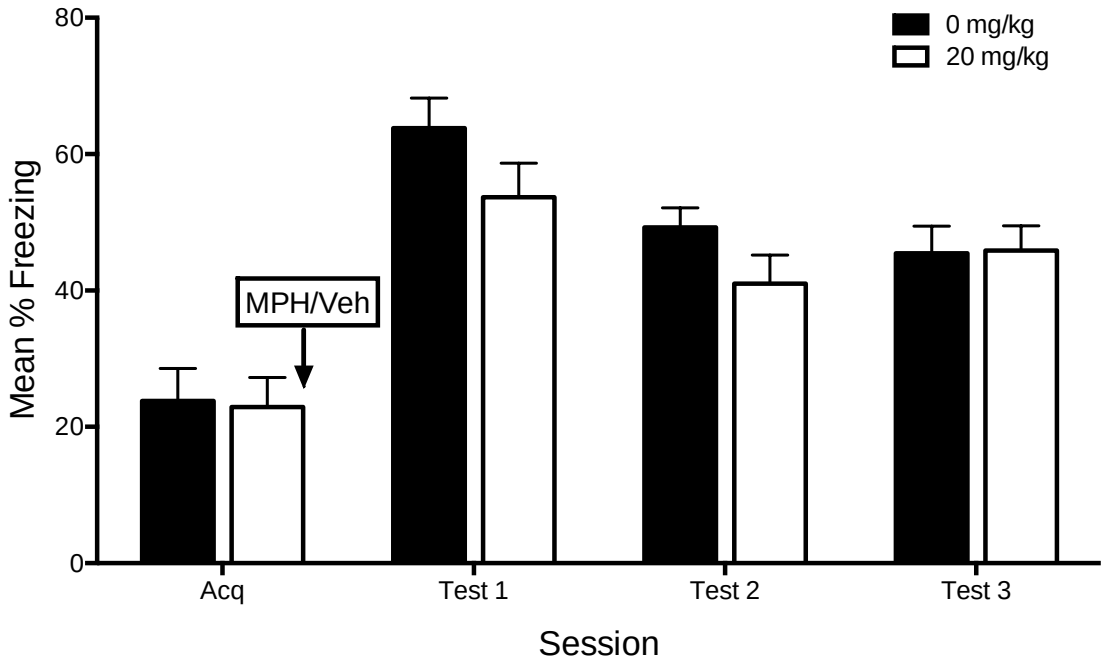


Figure 6. Effects of MPH administered immediately after a 12-min fear conditioning session. (Experiment 5). Mice that received methylphenidate (20 mg/kg) following fear conditioning did not show significant differences in freezing response during test days. Error bars indicate SEM.

Discussion:

These experiments demonstrate that extinction can be enhanced by MPH under certain conditions. Pre-session injections of MPH (10 mg/kg) promoted extinction on Test Day 1, but this effect was complicated by locomotor activation during extinction (Experiment 1). Experiment 2 showed that a single post-extinction session administration of 20 mg/kg MPH caused extinction enhancement on subsequent test days. The extinction enhancements were not

observed when MPH was administered 4 h following extinction (Experiment 3). No extinction or fear memory enhancement occurred when the animal received a brief extinction trial followed by MPH administration (Experiment 4). Administration of MPH following fear conditioning had no effect on fear expression of following test days (Experiment 5). These results are consistent with literature demonstrating the importance of dopaminergic and noradrenergic signaling for extinction learning (Bernardi and Lattal, 2010; Holtzmann-Assif et al., 2010).

Previous studies that have examined DAT and NET blockers such as cocaine or amphetamine have generally used a pre-session administration paradigm to determine whether these drugs may have effects on fear acquisition or extinction (e.g., Pezze and Feldon, 2004). One challenge in interpreting results of experiments with pre-session injections is disentangling the contribution of locomotor effects from effects on learning. Another challenge is that the interoceptive state induced by these drugs may impair attention, which would create a non-specific deficit in extinction learning. Administering MPH (10 mg/kg) immediately preceding an extinction session (Experiment 1) enhanced extinction on the following day, but the effects of MPH (10 mg/kg) on locomotor performance (Fig. 1b) during the extinction session complicate interpretation of the long-term effects. It is possible, for example, that the animal may superstitiously learn that increased locomotor activity prevents the occurrence of shock and would engage this behavior upon return to the context (Skinner, 1948). Thus, there are several interpretational challenges associated with pre-

session administration of MPH. However, when I assessed the effect of a pre-extinction administration of methamphetamine (Appendix A), I found no relationship between locomotor activation with methamphetamine during extinction and fear behavior on following test days. The methamphetamine study suggests that the observed extinction enhancement in Experiment 1 is specific to the pharmacological properties of methylphenidate, rather than all psychostimulant drugs.

By administering MPH immediately after extinction, I avoided some of the issues associated with pre-session injections. This approach ensures that all groups experience extinction under common conditions, and this treatment resulted in differences during test days after extinction. The persistence of the attenuation of freezing after a single injection is particularly notable, because MPH is generally prescribed as a chronic treatment. The changes in fear behavior here cannot be attributed to alterations in the acquisition of extinction or interference of fear memory retrieval and are most likely related to the effects of MPH on consolidation of extinction. It is unlikely that the effect is simply due to locomotor activation (Fig. 3b), and previous research has demonstrated that MPH's locomotor-activating effects do not persist to days when the drug is absent (Gaytan et al., 1997). The extinction enhancement effect did not occur when injections followed extinction by 4 h (Experiment 3) or when extinction sessions were short (Experiment 4). The absence of an effect with brief extinction trials may suggest that some within-session extinction is required for MPH to have enhancing effects (Weber et al., 2007). Future experiments could

clarify these effects by using targeted microinjections of MPH into the prefrontal cortex (PFC), amygdala, or hippocampus, because these are regions that may differentially mediate aspects of fear retrieval and extinction (Maren and Quirk, 2004; Busti et al., 2011).

Many studies that have examined MPH have been interested in the drug's effects on attention while MPH is active within the animal (Arnsten and Dudley, 2005; Berridge et al., 2006; Tye et al., 2010). Most of these studies have used rat models rather than mice, which show different pharmacokinetic properties with MPH (Faraj et al., 1974), such as longer times for drug metabolism in rats compared with mice. Comparisons between mouse dosing and human dosing are difficult to quantify due to the differences in affinity that are shown by mouse DAT (inhibition constant (KI): 0.26 nM) and human DAT (KI: 0.06 nM) to MPH, with MPH inhibiting human DAT fourfold more potently than mouse DAT (Wu and Gu, 1999). Mouse NET (KI: 0.17 nM) and human NET (KI: 0.10 nM) show similar affinities to MPH (Han and Gu, 2006). In attention or learning tasks with drug present, a locomotor effect from MPH would be detrimental to measuring behavior. However, in the context of enhancing extinction consolidation in mice, it is possible that higher doses of MPH may be necessary to create sufficiently large or persistent signaling changes from baseline levels for detectable differences in learning. Future studies could test human subjects undergoing exposure therapy while receiving MPH at clinically prescribed doses ranging up to 1.5 mg/kg (Arnsten and Dudley, 2005), which may provide comparable extinction enhancements in humans. Although the dosing is quite different

between mice and humans, the mechanism of action for extinction enhancements is likely to be closely related.

Activation of the prefrontal cortex by MPH (Berridge et al., 2006) is one candidate for the mediation of the extinction enhancements reported in this study. The infralimbic region of the prefrontal cortex is thought to modulate consolidation and expression of fear extinction (Vidal-Gonzalez et al., 2006; Burgos-Robles et al., 2007), and Marsteller et al. (2002) demonstrated that when MPH is paired with a mild stressor, there is a large increase in dopamine release in the medial prefrontal cortex compared with MPH administered with no stressor or 2 h following a stressor. The interaction between stress-induced norepinephrine release and MPH is less clear (Marsteller et al., 2002), although previous studies in our laboratory have shown the importance of adrenergic receptors to fear and drug memory consolidation following extinction (Bernardi et al., 2009; Bernardi and Lattal, 2010). These previous studies, in combination with the present study, suggest that MPH is likely to be specifically involved with enhancing consolidation of an extinction memory through dopaminergic and noradrenergic mechanisms when administered immediately following extinction.

An alternative explanation for the effects seen with post-extinction MPH may be related to counterconditioning of the fearful context. Using an aversive-to-appetitive transfer procedure, Bouton and Peck (1992) showed that cues previously associated with an aversive outcome can be trained to predict a rewarding outcome. Bouton (1993) proposed that the counterconditioning effect was not due to incompatible behavioral responses, but rather a central

interaction between a rewarding memory and an aversive memory. MPH is known to induce conditioned place preference (CPP) (Nolley and Kelley, 2007) at the dose range (20 mg/kg) that was effective for enhancing extinction in this study, indicating that an interaction between reward networks and the fear extinction circuit may allow for extinction enhancement. Because fear and drug extinction circuitry overlap in the prefrontal cortex (Peters et al., 2009), MPH's rewarding properties and unique pharmacology (Volkow et al.,1995; Berridge et al., 2006) could be quite useful for behavioral therapies involving extinction learning.

In these experiments, it appears that dopamine and norepinephrine transporter blockade following an extinction session caused enhanced consolidation. These findings have implications for disorders such as ADHD and PTSD. As discussed by Johansen et al. (2002), ADHD is associated with dysfunctional dopaminergic signaling, along with failures in reinforcement and extinction learning. Treatment with MPH may have several different beneficial effects on behavior, including restoration of extinction processes that are deficient in ADHD, and weakening of PTSD symptoms in veterans (Houlihan, 2010). This chapter demonstrates that MPH treatment in combination with fear extinction will enhance extinction retention and suggests a possible pharmacological treatment that could be useful in combination with exposure therapy for patients with fear disorders.

The effects of MPH on fear extinction may be beneficial in a clinical setting, but the broad activation of dopamine and norepinephrine receptors may

have undesirable side effects on learning in particular conditions. The following chapter examines the contribution of dopamine D1/5 receptors to fear extinction to specify whether one particular receptor system could generate similar extinction enhancements to those observed in this chapter.

Chapter 3: Activation of D1/5 receptors: A common mechanism for enhancing extinction of fear and reward-related behaviors

Introduction

In Chapter 2, I examined one method to explore the contribution of dopamine signaling in fear extinction through altering dopamine receptor activity in dopamine terminal regions. I found that methylphenidate (Ritalin; MPH), a DAT and NET transport blocker, enhances fear extinction. However, the effects observed in my MPH studies were sensitive to the learning that occurred during extinction, necessitating the use of an extinction criterion to identify whether MPH could enhance extinction. The extinction criterion allowed me to specifically examine learning only within animals that acquired extinction and decreased fear responses within an extinction session. This is a reasonable method to identify possible effects of an extinction-enhancing drug, but the enhancement effect may be weak if tested within human patient populations due to the difficulty of accurately assessing the extinction learning that occurs within a session. Another factor that suggests that the observed MPH enhancements were relatively weak was that significant effects only emerged over repeated testing, rather than being immediately identifiable within the first test session. Finally, the effect of methylphenidate on noradrenergic signaling (Han and Gu, 2006) confounds the possibility of determining the specific contribution of dopamine signaling in fear extinction. These caveats led to my interest in identifying dopamine receptors activated by methylphenidate that could modulate fear extinction on the first test day without requiring the use of an extinction criterion.

One possible target for pharmacological manipulations to enhance extinction comes from studies by Arnsten and Dudley (2005), who demonstrated that MPH actions on working memory are mediated in part by D1/5 receptors, and Hikind and Maroun (2008), who showed that blockade of infralimbic cortical D1/5 receptors impairs consolidation of extinction. These studies suggest that pharmacologically activating D1/5 receptors could have beneficial effects on extinction, although a previous study with the partial agonist SKF 38393 demonstrated impairments of extinction with a pre-session administration (Borowski and Kokkinidis,1998). The classification of SKF 38393 as a partial agonist comes from studies examining the efficacy of adenylate cyclase stimulation in striatal cells by dopamine D1/5 agonists (Andersen and Jansen, 1990). Compared to dopamine, SKF 38393 stimulates adenylate cyclase activity at approximately 45% efficacy, while SKF 81297, a full agonist, stimulates adenylate cyclase activity at approximately 88% efficacy. The differing pharmacological properties of SKF 38393 and SKF 81297 may have an impact on extinction learning, and suggests that previous characterizations using SKF 38393 may not fully capture the impact of D1/5 receptor activation on fear behaviors. These findings led to my interest in examining whether the use of a full D1/5 agonist, SKF 81297, could modulate extinction learning.

Post-extinction administration of L-dopa, a dopamine precursor, impairs renewal of fear in mice and humans, suggesting that targeting dopamine receptors could be beneficial in combination with behavioral treatments for post-traumatic stress disorder (Haaker et al., 2013). The goal of the studies presented

in this chapter is to specify the contribution of dopamine D1/5 receptor activation to fear learning. To this end, I examined the effect of SKF 81297 on the acquisition, consolidation, and renewal of fear extinction. To determine whether SKF 81297 broadly enhances fear learning, I measured the effect of SKF 81297 on consolidation of fear conditioning. To examine the content of learning produced by SKF 81297, I tested the rewarding properties of SKF 81297 with conditioned place preference and the impact of SKF 81297 on cocaine conditioned place preference extinction. Together, these experiments test whether activation of D1/5 receptors can enhance consolidation of extinction in both aversive and rewarding tasks.

Materials and Methods

Subjects

Male C57BL/6 mice (n = 318) ranging from 7 to 11 wk of age (28 ± 5 g) were used in these experiments. Animals were purchased from Jackson Laboratory (Bar Harbor, ME) and given at least 7 d in the vivarium prior to experimental use. Animals were housed four to a cage. Polycarbonate cages were held in a Thoren rack, and animals were given access to food and water ad libitum. Vivarium and experiment room temperatures were maintained at 22°C ± 1°C, and subjects were maintained on a 12-h light–dark cycle (lights on 0600 h–1800 h). Animals were moved from the vivarium to the experiment room 60 min before the start of an experiment, and experiments were conducted between 900 and 1700 h. All experimental procedures were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance

with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985).

Drugs

SKF 81297 (Tocris Bioscience, Bristol, UK) was dissolved in saline (0.9% NaCl) at concentrations of 1, 3, and 10 mg/kg. Gentle heating was used to dissolve SKF 81297 at 10 mg/kg in saline. Cocaine (20 mg/kg) was dissolved in saline. Both drugs were administered intraperitoneally (i.p.) in a volume of 10 mL/kg. SKF 81297 doses were selected based on doses previously used in rat studies examining cocaine-induced locomotor activity (Chausmer and Katz, 2002), conditioned place preference (Abrahams et al., 1998), and locomotion (Diaz Heijtz and Castellanos, 2006). Cocaine doses were selected based on previous work within the Lattal laboratory (Raybuck et al., 2013).

Apparatus

Fear conditioning.

Four Coulbourn Instruments (Whitehall, PA) mouse-conditioning chambers (H10-11M-TC) were used. Chambers were housed in sound and light-attenuating cabinets with a house light and a fan providing 65 dB of background noise. A Plexiglas cylinder, 21.5 cm in diameter and 23 cm in height, was placed on a grid floor in the chamber. The grid floor consisted of stainless steel rods, 3.2 mm in diameter, spaced 6.4 mm apart. Scrambled shock (2 sec, 0.35 mA) was delivered to the grid floor by a computer controlled shock generator (Coulbourn H13-15). For cued conditioning, an 85 dB noise CS was administered through a sound generator (Coulbourn A12-33). Above the Plexiglas cylinder, an

automated infrared activity monitor (Coulbourn H24-61) recorded activity in Graphic State 3.01 software. Contextual fear conditioning studies were conducted in these chambers, and fear conditioning and context testing occurred in these chambers for cued fear studies (Context A). Testing for cued conditioning was conducted in a separate room in rectangular conditioning chambers (Med-Associates, St. Albans, VT) with white Plexiglas floors, situated in sound attenuating cubicles with a house light and ventilation fan providing 65 dB of background noise (Context B). CS was generated with ANL-926 (Med) and administered through speakers mounted on the left wall of the chambers, controlled by an IBM-PC running MED-PC 4 (Med). Data in Context B were hand scored for analysis. Contexts were cleaned with 95% ethanol following each session.

Conditioned Place Preference.

Eight sound and light-attenuating cabinets contained clear acrylic cages (30 cm X 15 cm X 15 cm) with two distinct removable floor types (grid or hole) as interchangeable halves. A grid floor consisted of 2.3-mm stainless steel rods mounted 6.4 mm apart in an acrylic frame. A hole floor consisted of perforated 16 gauge stainless steel sheets with 6.4-mm diameter round holes on 9.5 mm staggered centers on an acrylic frame (Cunningham et al., 2006). A clear acrylic divider confined animals to one half of the apparatus during conditioning and extinction, and was removed during test days. Position in the box (left/right side) and general activity were assessed by EthoVision XT 5 software (Noldus,

Leesburg, VA) that records and analyzes the position of the center point of the mouse within the apparatus via a ceiling mounted camera.

Locomotor Activity.

Eight sound and light-attenuating cabinets contained a Plexiglas cylinder (21.5 cm in diameter and 23 cm in height) placed on the floor of the chamber. Total activity in the box was assessed by Ethovision 5.0.216 computer software (Noldus, Leesburg, VA) that recorded activity via a ceiling mounted camera. Ethovision sampling rate was set at 29.97 samples per second.

Behavioral procedures

Contextual fear conditioning

On Day 1, subjects received a 12-min exposure to the context with four unsignaled shocks, delivered at 2.5, 5, 9, and 11.5 min. In all experiments, groups were matched following acquisition to ensure equal terminal freezing levels across SKF 81297 dose assignments. On Day 2, mice received a 12-min nonreinforced exposure to the context (Ext). In all post-extinction injection experiments, groups were matched following extinction to ensure equal levels of terminal freezing before SKF 81297 administration. On Test Day, mice received a 12-min nonreinforced exposure to the context.

Experiment 6a: Pre-extinction effect of SKF 81297

This experiment tested the effects of acute SKF 81297 given before a contextual extinction session to determine whether SKF 81297 could enhance acquisition and retention of extinction. On Day 1, animals (n = 8 per group) received an injection of saline, followed by acquisition training. On Day 2,

animals received either SKF 81297 (1, 3, or 10 mg/kg) or saline, followed immediately by a 12-min extinction session (Ext in Fig. 7a). On Day 3, animals received an injection of saline, followed by additional 12-min extinction sessions (Test Day).

Experiment 6b: Locomotor effects of pre-session SKF 81297

Activity was assessed over 3 days, with animals (n=8 per group) receiving a saline injection and being placed into the locomotor chamber on Day 1 for 12 min. Animals were matched for locomotor activity on Day 1 then received SKF (10 mg/kg) or saline immediately prior to being placed into the locomotor chamber on Day 2 for 12 min. On Day 3, animals received an injection of saline and were immediately placed into the locomotor chamber (Figure 7b).

Experiment 7a: Post-extinction effect of SKF 81297

This experiment examined the effect of acute SKF 81297 administered immediately after a contextual extinction session. Similar to MPH studies, the post-session administration allowed animals to be given drug or saline under common conditions during a period of memory consolidation (Abel and Lattal, 2001). On all days of conditioning and testing, animals received injections post-session. On Day 1, animals (n =12 per group) received an acquisition session with administration of saline. On Day 2, animals received either SKF 81297 (1, 3, or 10 mg/kg) or saline immediately following extinction (Ext in Fig. 8a). On Day 3, animals were returned to the context for a 12-min nonreinforced test session (Test Day).

Experiment 7b: Locomotor effects of post-session SKF 81297

Activity was assessed over 3 days. On Day 1, mice (n=8 per group) received saline following a 12-min context exposure. On Day 2, mice received a 12-min context exposure followed by SKF 81297 (10 mg/kg) or saline. On Day 3, mice received a 12-min context exposure (Fig. 8b).

Experiment 8: Four-hour delayed administration of SKF 81297

In this experiment, the end of the extinction session and the administration of SKF 81297 were separated by 4 h. Rossato et al. (2009) proposed the possibility of a consolidation mechanism modulated by dopamine signaling that is temporally separate from the training session, and I tested whether SKF 81297 could enhance extinction when given 4 hours following an extinction session. The animals (n = 7-8 per group) still received saline injections on all days following exposure to the context in order to ensure equivalence to treatment conditions in Experiment 2. However, in this experiment, following the extinction session (Ext in Fig. 9), all animals received an injection of saline and 4 h later received either SKF (1, 3, or 10 mg/kg) or saline. Animals were returned to the context the following day for a 12-min nonreinforced exposure (Test Day).

Experiment 9: SKF 81297 administration with or without extinction

This experiment was similar to the procedure of Experiment 7a, but added two groups that received SKF 81297 or saline without receiving a context exposure on Day 2. These control groups allow for a determination of whether extinction is required for SKF 81297 to inhibit fear responding when tested in the context. Extinction and Test Day were separated by seven days to measure

whether SKF 81297 effects persisted several days following drug administration. On Day 1, all mice (n = 11-12 per group) received an acquisition session followed by injection of saline. Groups were matched for freezing during acquisition. On Day 2, half of the animals remained in the vivarium and received saline or SKF 81297 (10 mg/kg), while the other half received an extinction session followed by saline or SKF 81297 (10 mg/kg) (Ext in Fig. 10). Seven days later, all groups received a nonreinforced exposure to the context for 12-min (Test Day).

Experiment 10: Post-session SKF 81297 effect on fear conditioning

This experiment tested whether D1/5 receptor agonism enhances consolidation of contextual fear learning, or if enhancement effects are specific to fear extinction. A brief (3-min) fear conditioning procedure was used to reduce a possible ceiling effect on freezing during the test day. On Day 1, animals (n = 8 per group) received an acquisition session (Acq in Fig. 11) immediately followed by administration of saline or SKF 81297 (10 mg/kg). On Day 2, animals received a 12-min nonreinforced session (Test Day).

Experiment 11: Post-session SKF 81297 after non-reinforced pre-exposure

This experiment tested the impact of pre-exposure to the training context paired with post-session SKF 81297. If SKF 81297 impaired fear conditioning the following day, it would indicate that development of an association that inhibits fear learning. An alternate possibility, based on the prediction error model, would be that fear conditioning could be enhanced if SKF 81297 increases the discrepancy between what is expected (no shock) and what is received (shock).

On Day 1, subjects (n = 8 per group) received a nonreinforced 12-min exposure to the contextual fear conditioning chamber. One box was removed from analysis in this study due to equipment malfunction, leading to n = 6 mice per group. Immediately following the 12-min session, mice received SKF 81297 (10 mg/kg) or saline (Pre in Fig. 12). On Day 2, mice received a 12-min exposure to the context with four unsignaled shocks, delivered at 2.5, 5, 9, and 11.5 min and the session was followed by a saline injection (Acq). On Day 3, mice received a non-reinforced 12-min exposure to the context (Ext).

Experiment 12: Post-extinction SKF 81297 effect on cued fear and renewal

The previous experiments presented here have examined the effect of SKF 81297 on contextual fear learning, but the use of a discrete auditory cue during conditioning allows for a determination of whether SKF 81297 effects are context dependent. On Day 1, subjects (n = 8 per group) received a 6.5-min exposure to Context A with a 30 second noise CS co-terminating with shock presented at 2 and 4 min. On Day 2, mice received a 6-min nonreinforced exposure to context A. Groups were matched for acquisition and contextual freezing. On Day 3, mice were placed in Context B for 15 min with 3-min CS presentations at 3 and 9 min (Ext in Fig. 13). Groups were matched to ensure equal levels of terminal freezing during Day 3 before post-extinction SKF 81297 (10 mg/kg) administration. On Days 4-7, subjects were placed in Context B for 15-min with 3-min CS presentations at 3 and 9 min (Test 1-4), followed by saline injections. On Day 8, to measure fear renewal, mice were returned to Context A for a 15-min session with 3-min CS presentations at 3 and 9 min (Ren.). Twenty-

one days later, to measure retention of fear, mice were returned to Context A for a 15-min session with 3-min CS presentations at 3 and 9 min (Ret.). Freezing during CS-on periods is shown in Figure 13a and freezing during CS-off periods is shown in Figure 13b.

Experiment 13: SKF 81297 generation of conditioned place preference

This experiment tests the rewarding properties of pre- or post-session SKF 81297 using conditioned place preference. An unbiased conditioned place preference (CPP) apparatus was used for this experiment (Cunningham et al., 2006). Prior to conditioning, mice (n = 16 per group) were habituated (pre-test) to the CPP apparatus by receiving a saline injection followed by a 5-min exposure to the conditioning chamber with grid and hole floors. Animals were matched following pre-test to ensure no bias to hole or grid floors, and then assigned to counterbalanced groups that received SKF 81297 (10 mg/kg) or saline immediately before or after exposure to a grid or hole floor. During conditioning, animals were confined to one half of the CPP apparatus with grid or hole floor for 15-min. Mice received one pairing of SKF 81297 (pre or post-session) and one pairing of saline (pre or post-session), counterbalanced for order and floor type, on alternating days. 24 hours following the last conditioning session, mice were given a 15-min exposure to the CPP apparatus with both floors to assess preference for the drug-paired side (Test Day in Fig. 14).

Experiment 14: Post-extinction SKF 81297 modulation of cocaine conditioned place preference

This experiment tests whether SKF 81297 can enhance extinction of a cocaine conditioned place preference. Similar to SKF 81297 CPP procedure, an unbiased CPP apparatus was used and mice were habituated (pre-test) to the conditioning chamber. Animals (n =24 per group) were matched following pre-test to ensure no bias to hole or grid floors, and then assigned to counterbalanced groups that received cocaine (20 mg/kg) or saline immediate before exposure to a grid or hole floor. During conditioning, animals were confined to one half of the CPP apparatus with grid or hole floor for 15-min. Mice received two pairings of cocaine and two pairings of saline, counterbalanced for order and floor type, on alternating days. To minimize the possibility of a side preference, floor placement was alternated such that animals received a pairing of cocaine or saline on both right and left sides of the chamber. 24 h following the last conditioning session, mice were given a 5-min exposure to the CPP apparatus with both floors to assess preference for the drug-paired side (Preference Test). A 5-min, rather than 15-min, test session was used to reduce within-session extinction. 72 h later, mice were placed in the conditioning apparatus for 15-min, confined to the drug-paired side, for an extinction session. Immediately following extinction, animals received SKF 81297 (10 mg/kg) or saline. Twenty-four hours following extinction, mice received a 15-min exposure to the conditioning apparatus with both floors to assess preference for cocaine-paired side (Post-Ext Test in Fig. 15).

Data analysis

Fear memory expression was determined by freezing response within the context. Freezing was defined as an episode of at least 3 sec of inactivity. Total freezing time was divided by 12 min to calculate percentage of time freezing in each day. For Experiment 12, all data are hand-scored due to the lack of an automated measurement system in Context B. Locomotor activity was measured in Ethovision by total distance moved in cm, and reported as distance moved (cm) per min. Conditioned place preference (Experiments 13 and 14) was measured in Ethovision by time (s) spent on drug-paired floor (CS+) or vehicle-paired floor (CS-) per minute of test session, which was used to calculate percentage of time spent on CS+ floor. Conditioned place preference was also examined by analyzing time (s) spent on grid floor per minute in animals that received drug-conditioning on the grid floor compared to animals that received drug-conditioning on the hole floor (Cunnningham et al., 2006). These analyses are presented in Appendix A for Experiments 13 and 14. Data analyses were performed with SPSS version 22.0 for ANOVA and Prism 6 for t-tests. Data in Experiments 6a, 7a, and 8 were analyzed using a one-way between subjects analysis of variance (ANOVA) on each day. Experiment 9 was analyzed with a two-way ANOVA, with drug treatment and extinction status as between-subjects factors. All post hoc comparisons of ANOVA data were performed using a Dunnett's test. T-test comparisons are reported for Experiments 7b, 8b, and 10-14. For median split analysis in Experiment 14, data was analyzed with a t-test. For all statistical tests, the α was set to 0.05.

Results

Experiment 6: Pre-extinction administration of SKF 81297 reduces freezing during extinction and test day.

Experiment 6a tested the effect of SKF 81297 on acquisition of extinction by administering SKF 81297 (1, 3, or 10 mg/kg) or saline immediately before an extinction session. Figure 7a shows that pre-extinction administration of SKF 81297 caused a dose-dependent reduction in freezing during the extinction session (Ext in Fig. 7a). A one-way between-subjects ANOVA found significant effects of SKF 81297 on freezing ($F(3,28) = 26.153$, $p < 0.0005$) during Extinction. Post hoc comparisons with a Dunnett's test showed that saline was significantly different from 1 mg/kg ($P < 0.0005$), 3 mg/kg ($p < 0.0005$) and 10 mg/kg ($p < 0.0005$). Test Day analysis indicated that animals treated with SKF 81297 (10 mg/kg) showed less fear responding than saline-treated animals. This effect was confirmed by ANOVA ($F(3,28) = 6.094$, $p = 0.003$) and post-hoc Dunnett's test showed that saline was significantly different from 10 mg/kg SKF 81297 ($p = 0.003$), but not 1 mg/kg ($p = 0.999$) or 3 mg/kg ($p = 0.214$).

Although freezing decreased with SKF 81297 administration on Extinction and Test Day, this effect could be driven by persistent locomotor activation or context-driven behavioral sensitization (Berridge and Robinson, 1993). To identify locomotor contributions to the observed extinction enhancement effects, a separate experiment assessed the effect of pre-session SKF 81297 administration. The locomotor experiment was similar to Experiment 6a, but there was no shock on Day 1 (Experiment 6b). Figure 7b shows significantly increased

locomotor activity in pre-session SKF 81297 treated animals on Day 2, (t (14) = 4.304, p = 0.0007) and this increased locomotor effect persisted (t (14) = 4.031, p = 0.0012) on the following test day (Day 3 in Fig. 7b). To overcome these locomotor confounds in measuring fear responding, I examined the effect of post-extinction administration of SKF 81297 in Experiment 7.

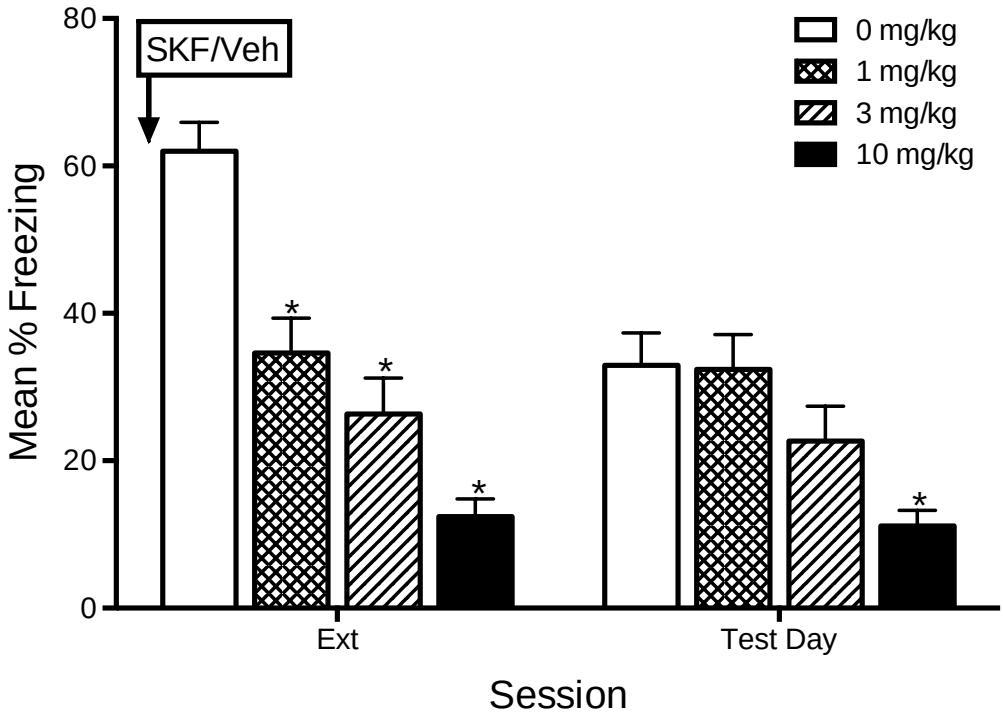


Figure 7a. Pre-extinction SKF 81297 (10 mg/kg) enhances extinction retention. (Experiment 6a). Mice that received SKF 81297 showed differences in freezing response during Extinction (1, 3, and 10 mg/kg) and Test Day (10 mg/kg). Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline (Dunnett's test).

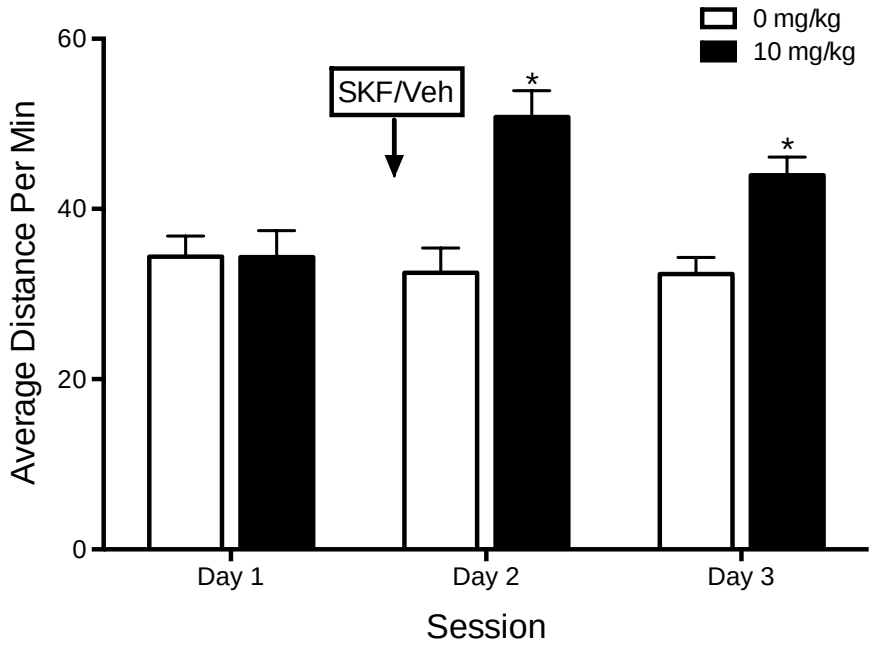


Figure7b. Pre-session SKF 81297 (10 mg/kg) increases locomotor activity. (Experiment 6b). Mice that received pre-session SKF 81297 (10 mg/kg) showed increased locomotor behavior on Day 2 and Day 3. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline.

Experiment 7: Post-extinction administration of SKF 81297 reduces freezing during test day.

Experiment 7a tested the effect of SKF 81297 on consolidation of extinction by administering SKF 81297 (1, 3, or 10 mg/kg) or saline immediately following an extinction session. Mice were matched for behavior during extinction, and then given SKF 81297 or saline. Figure 8a shows that post-extinction administration of SKF 81297 caused a reduction in freezing twenty-fours hours later during the test session (Test Day in Fig. 8a). A one-way between-subjects ANOVA found significant effects of SKF 81297 on freezing ($F(3,44) = 5.328$, $p = 0.0005$). Post hoc comparisons with a Dunnett's test showed that saline was significantly different from 10 mg/kg SKF 81297 ($p = .009$).

To determine whether locomotor activity alterations contributed to the extinction enhancement effect, a separate experiment assessed the effect of post-session SKF 81297 (10 mg/kg) on locomotor activity (Experiment 7b). The experimental procedure was similar to the fear conditioning procedure, except that mice received no shock on Day 1. Animals were matched for locomotor activity on Day 1 and 2, and then received SKF 81297 (10 mg/kg) or saline immediately after context exposure. Figure 8b shows no persistent locomotor activation induced by post-session SKF 81297 compared to saline ($t(14) = 1.256$, $p = 0.230$).

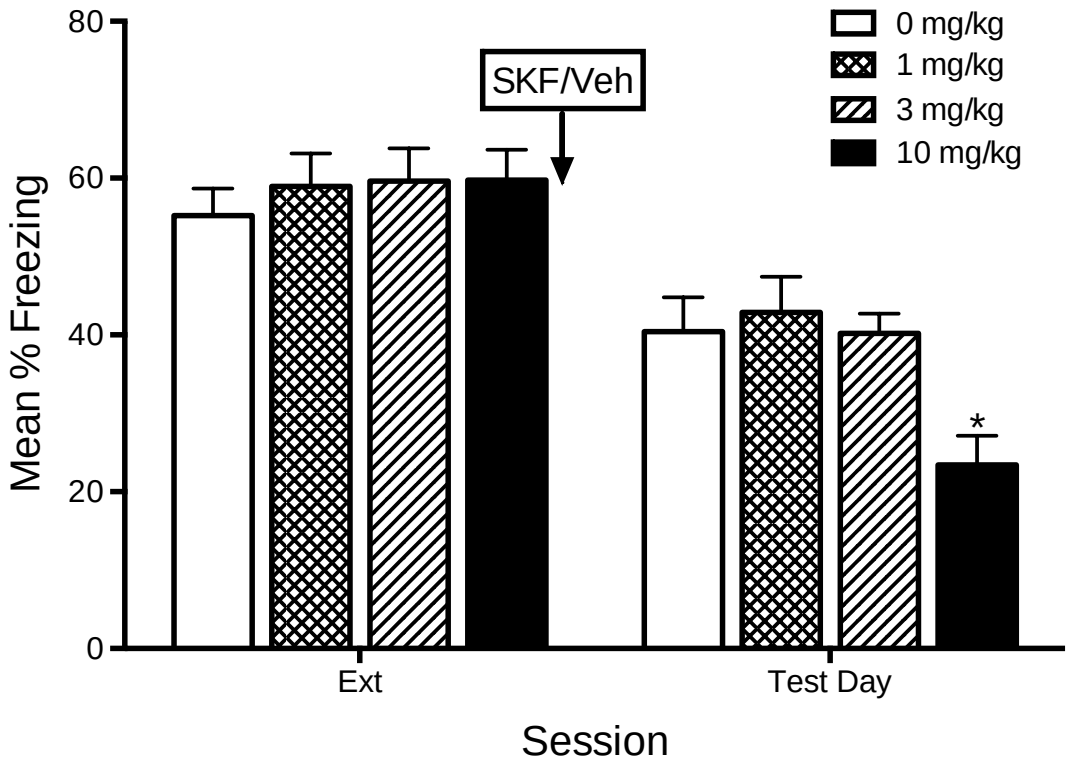


Figure 8a. Post-extinction SKF 81297 (10 mg/kg) enhances extinction retention. (Experiment 7a). Mice that received SKF 81297 (10 mg/kg) immediately following extinction showed significantly less freezing than saline treated animals during Test Day 1. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline (Dunnett's test)

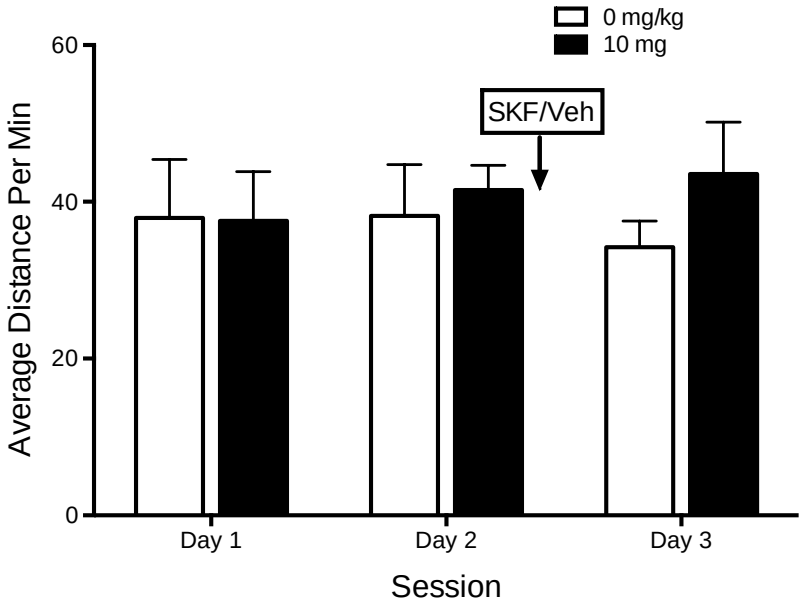


Figure 8b. Post-session SKF 81297 (10 mg/kg) had no effect on locomotor activity. (Experiment 7b). Mice that received post-session SKF 81297 (10 mg/kg) on Day 2 did not show a persistent increase of locomotor activity on Day 3. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline.

Experiment 8: Four hour delayed administration of SKF 81297 following extinction decreases freezing during test day.

A commonly used measure to determine whether a drug interacts with consolidation of a memory is to insert a four-hour separation between a learning experience and drug administration. This has been useful in investigating effects of drugs that impair early consolidation, such as anisomycin (Hernandez et al., 2002), but studies examining dopamine's role in consolidation of memory (Rossato et al., 2009) have indicated that dopamine signaling could be involved in a later consolidation mechanism that occurs several hours following the initial learning experience. Experiment 8 tests the effect of SKF 81297 on extinction retention when extinction training and drug administration are separated by a four-hour interval. To replicate the experimental procedures of Experiment 7, all

animals received injections of saline immediately following exposure to the fear conditioning chamber on each day, but on extinction day, animals also received either saline or SKF 81297 (1, 3, or 10 mg/kg) four hours following extinction training. Figure 9 shows that 4-hr delayed post-extinction administration of SKF 81297 (10 mg/kg) caused a reduction in freezing twenty-fours hours later during the test session (Test Day in Fig. 9). A one-way between-subjects ANOVA found significant effects of SKF 81297 on freezing ($F(3,27) = 3.438$, $p = 0.031$). Post hoc comparisons with a Dunnett's test showed that saline was significantly different from 10 mg/kg SKF 81297 ($p = .015$).

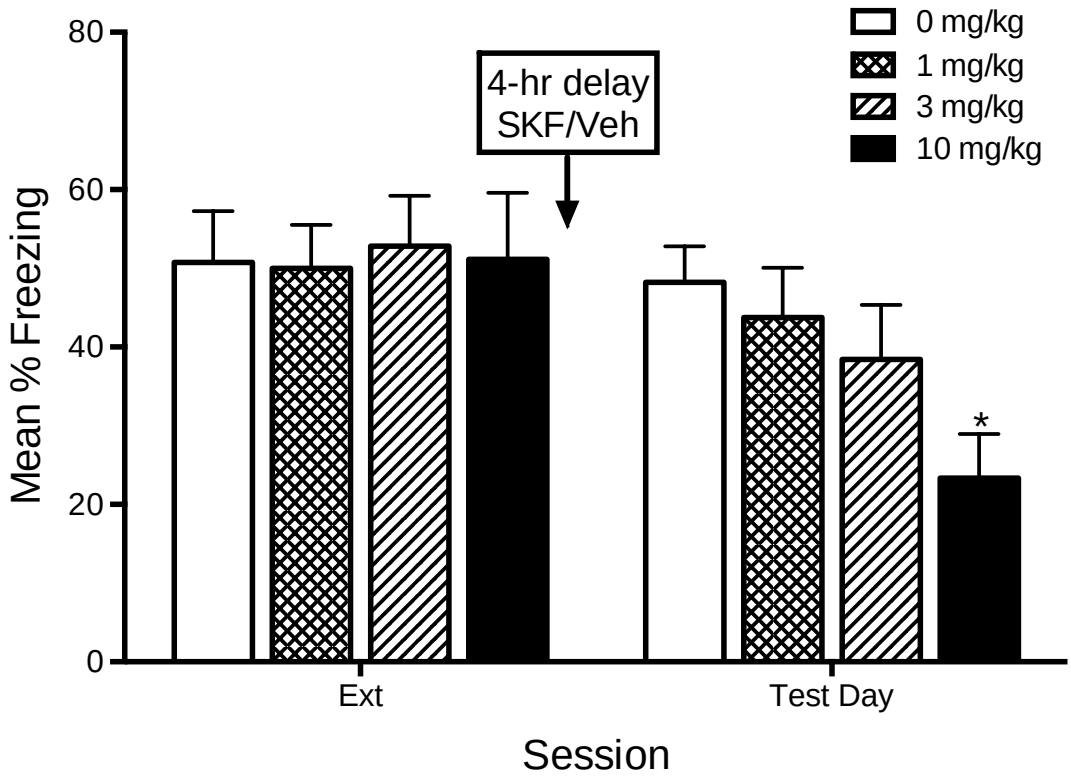


Figure 9. 4-hr delayed administration of SKF 81297 (10 mg/kg) enhances extinction retention. (Experiment 8). Mice that received SKF 81297 (10 mg/kg) four hours following extinction showed significantly less freezing than saline treated animals during Test Day 1. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline (Dunnett's test)

Experiment 9: Interaction between extinction and SKF 81297 administration.

This experiment examines the contribution of SKF 81297 administration on freezing behaviors in the absence of extinction. Animals were matched for freezing during fear conditioning, then assigned to either extinction or no-extinction groups. Animals receiving extinction were administered SKF 81297 (10 mg/kg) or saline immediately after extinction (SKF Ext; Sal Ext), while animals in the no-extinction group were given SKF 81297 or saline in the vivarium (SKF No Ext; Sal No Ext). Figure 10 shows that there were no significant differences between animals receiving extinction, and a two way ANOVA revealed a main effect of extinction ($F (1, 43) = 6.162$, $p = 0.017$). There was no main effect of drug administration ($F (1, 43) = 2.597$, $p = .114$), suggesting that SKF 81297 alone does not decrease freezing. Although there was a visual trend towards an interaction effect between drug treatment and extinction status (SKF Ext), there was no statistical interaction, indicating that the extinction effect may be weaker following a week delay, or that SKF/Ext animals reached floor levels of freezing, preventing detection of an interaction.

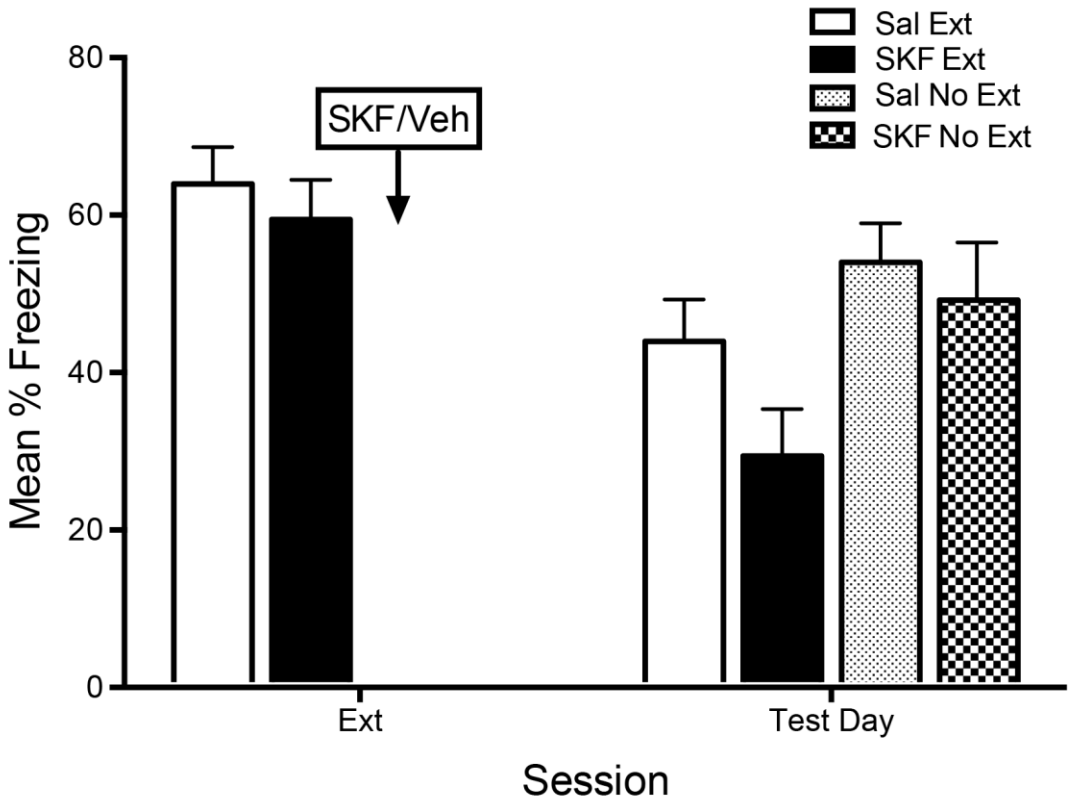


Figure 10. Interaction between SKF 81297 treatment and extinction status. (Experiment 9). Mice that received extinction showed significantly less freezing 7 days later than mice that did not receive extinction, likely driven by animals receiving SKF 81297 immediately following extinction. However, there was no significant interaction. Error bars indicate SEM.

Experiment 10: Post-session SKF 81297 impairs fear conditioning.

The previous experiments suggest that activation of D1/5 receptors with SKF 81297 enhances fear extinction, but some studies have demonstrated that D1/5 receptors are also involved in fear conditioning (Inoue et al., 2000). Experiment 10 tests whether activation of D1/5 receptors with SKF 81297 (10 mg/kg) during consolidation of fear conditioning alters fear expression on the following day. Animals were matched for freezing during fear conditioning, then received either saline or SKF 81297 immediately following fear conditioning. Figure 11 shows that post-conditioning administration of SKF 81297 (10 mg/kg)

decreases fear expression on the following day (Test Day in Fig. 11). A t-test comparison found significant effects of SKF 81297 (10 mg/kg) on freezing ($t(14) = 3.272$, $p = 0.006$).

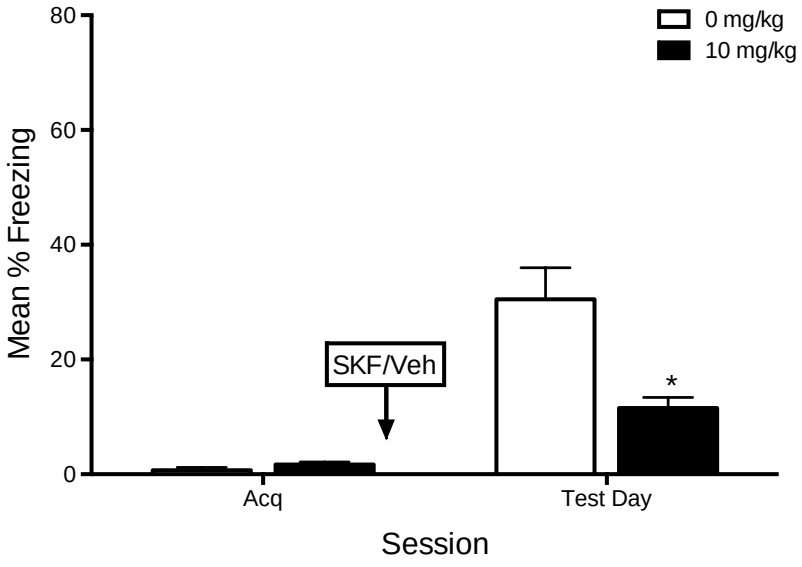


Figure 11. Consolidation of fear conditioning is impaired by SKF 81297. (Experiment 10). Mice that received SKF 81297 (10 mg/kg) following a 3-min contextual fear conditioning session showed significantly less freezing than saline treated animals during Test Day 1. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline.

Experiment 11: Non-reinforced pre-exposure and SKF 81297.

One hypothesis for the effects of SKF 81297 on fear extinction is that pairing the drug with context exposure leads to the enhancement of a CS-No US representation or the generation of a CS-New US association. If SKF 81297 acts as a separate US or enhances a CS-No US representation, it might be expected that there would be an inhibitory interaction between the two associations retrieved by context exposure on Acquisition or Test Day. Experiment 11 tests this hypothesis through the use of a non-reinforced pre-exposure to the conditioning context, where animals receive context exposure followed by SKF 81297 (10 mg/kg) or saline on Day 1 (Ctx in Figure 12). Animals received fear

conditioning (Acq) on Day 2 and an extinction session (Ext) on Day 3. There was no significant difference between groups during the pre-exposure day. Figure 12 shows that post-context exposure administration of SKF 81297 had a significant effect on acquisition of fear (t (10) = 3.035, p = 0.0126). When tested the following day (Ext), animals that had received SKF 81297 showed significantly less freezing than saline animals (t (10) = 4.638, p = 0.001).

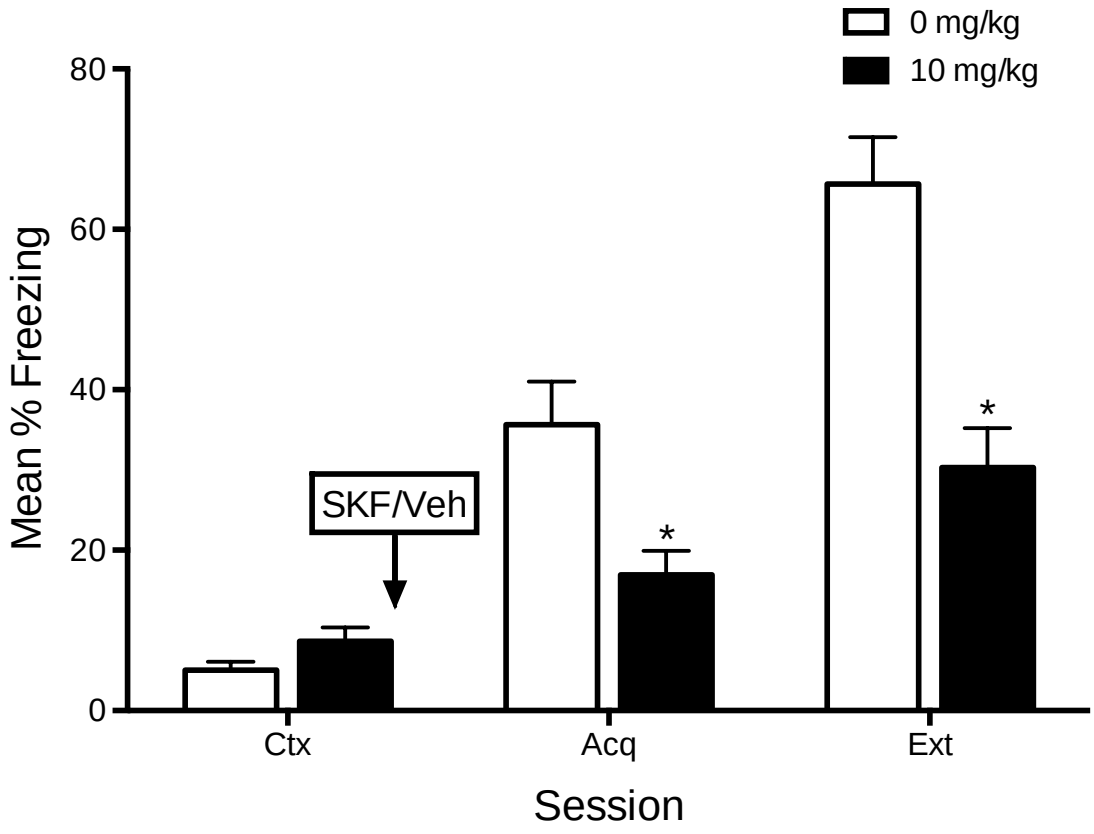


Figure 12. Context pre-exposure followed by SKF 81297 impairs fear acquisition. (Experiment 11). Mice that received post-session SKF 81297 (10 mg/kg) following a context exposure showed decreased freezing during acquisition (Acq) and extinction (Ext). Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline.

Experiment 12: Post-session SKF 81297 enhances cued fear extinction and impairs renewal.

The previous experiments demonstrate that SKF 81297 enhances fear extinction, but the contextual fear procedure does not easily allow for examination of fear renewal, as this requires a discrete cue to be extinguished in a separate context. Extinction learning is generally context specific, and examining fear renewal is useful for separating the contribution of contextual inhibition of fear from the learning associated with the CS. Experiment 12 tests the effect of SKF 81297 on extinction of cued fear, then measures renewal and spontaneous recovery of fear. There were no differences in acquisition or contextual extinction in Context A, and animals were matched for freezing during the first extinction session in Context B. Immediately following the extinction session, mice received either SKF 81297 (10 mg/kg) or saline, then were tested for several days until reaching equivalent levels of freezing. There were no significant differences between groups during Test Days on freezing during CS-off periods, but differences emerged during presentations of the auditory CS. Data in Figure 13a show freezing during CS-on periods. Figure 13b shows freezing during CS-off periods. On Test Day 1 in Figure 13a, SKF 81297 treated animals showed significantly less freezing than saline treated animals ($t(14) = 6.586$, $p < 0.0001$). This effect persisted to Test Day 2 ($t(14) = 6.144$, $p < 0.0001$) and Test Day 3 ($t(14) = 5.109$, $p = 0.0002$). By Test Day 4, both groups had extinguished freezing behaviors to equivalent levels ($t(14) = 1.509$, $p = 0.1536$).

The following day, animals were placed back in the conditioning context (Context A) to measure fear renewal. Over the whole renewal session, SKF treated animals showed significantly less freezing than saline treated animals ($t(14) = 2.511$, $p = 0.025$). However, these effects were dispersed between freezing during the CS-on period ($t(14) = 2.108$, $p = .0535$) (Ren. In Fig. 13a) and freezing during CS-off period ($t(14) = 2.598$, $p = .0211$) (Ren. In Fig 13b). When tested twenty-one days later, there were no differences between groups in long-term retention of fear across the whole session, or during the CS-on or CS-off periods (Ret. in Fig. 13).

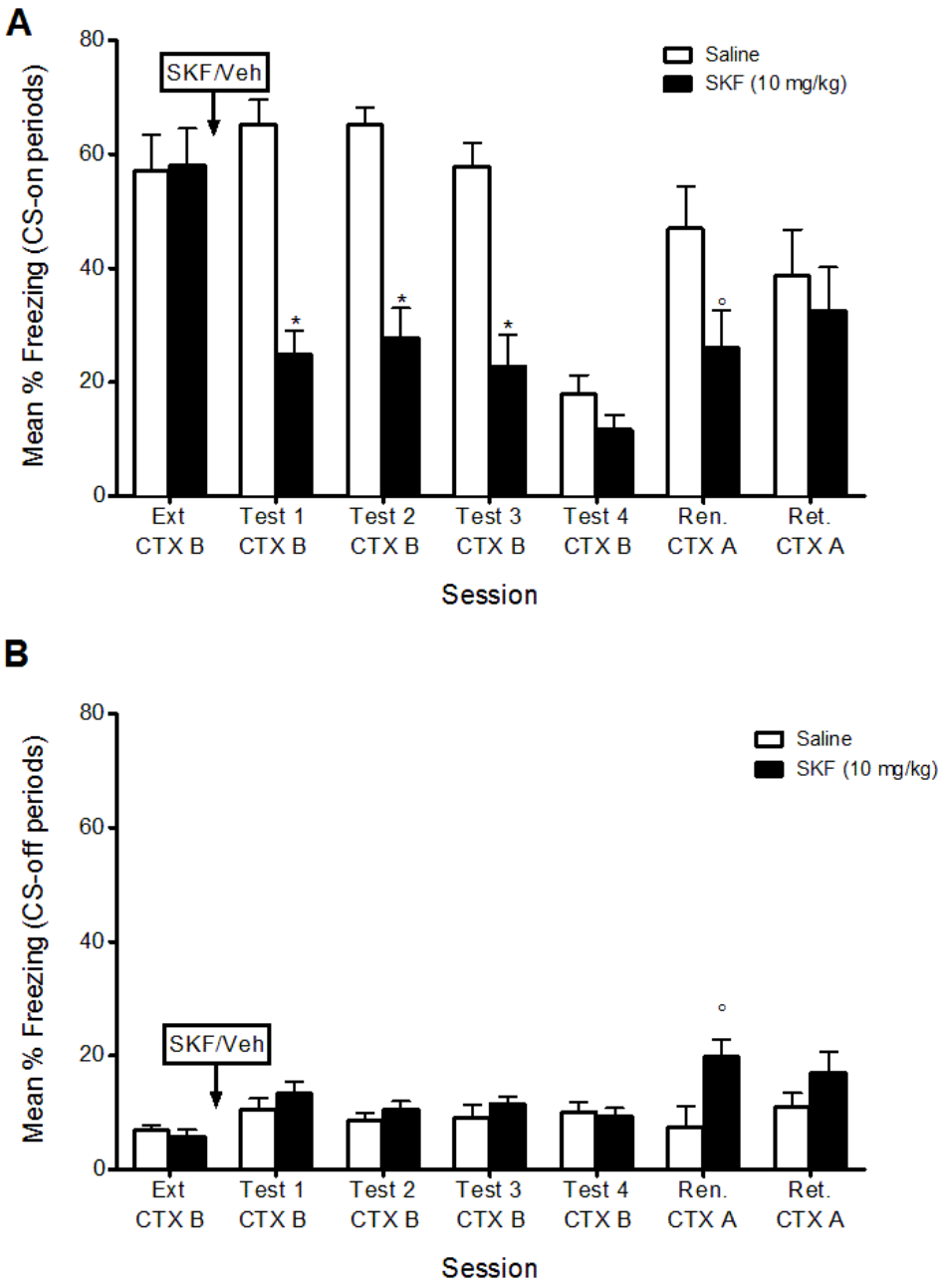


Figure 13. Post-session SKF 81297 (10 mg/kg) enhances extinction of cued fear and impairs fear renewal. (Experiment 12). Panel A shows freezing during CS-on periods and panel B shows freezing during CS-off periods. Mice that received post-session SKF 81297 (10 mg/kg) following cued extinction session showed decreased freezing during cue presentations (Panel A) on Test 1-3, and trended towards significant differences during cue presentations in renewal. Total freezing (CS-on and CS-off periods) during renewal (Ren.) session was significantly different for SKF 81297 treated animals ($p = 0.025$) compared to saline. There was no significant difference in long-term retention (Ret.). Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline. (°) $p = 0.0535$.

Experiment 13: Pre-session, but not post-session, SKF 81297 induces conditioned place preference.

Based on the hypothesis discussed in Experiment 12 that SKF 81297 may act as an unconditioned stimulus that enters into an associative link with the context or cue, I tested the rewarding properties of SKF 81297 in a conditioned place preference (CPP) procedure. To mirror timing of drug administration in fear extinction experiments, I compared acquisition of CPP with pre- or post-session administration of SKF 81297. Figure 14 shows that pre-session SKF 81297 was significantly different from post-session SKF 81297 ($t(30) = 2.962$, $p = 0.006$). To ensure that pre-session SKF 81297-treated mice generated conditioned place preference, grid/hole analysis for pre- and post-session SKF 81297 is provided in Appendix A. A previous experiment showed that pre-session administration of saline on both sides leads to approximately 50 % of time spent on both floors (Appendix A), confirming that the apparatus is unbiased and providing a baseline value to compare preference scores in this experiment (dashed line). Analysis of locomotor behavior during test showed that animals that received post-session SKF 81297 showed significantly less locomotor activity than pre-session SKF 81297 treated animals ($t(30) = 4.098$, $p = 0.0003$). Group mean and standard error of the mean for locomotor activity (cm/min) in pre-session SKF 81297 treated animals was 245.7 ± 8.0 and 204.1 ± 6.3 in post-session treated animals. In the experiment comparing pre-session SKF 81297 administration to saline (Appendix A), locomotor activity for saline-treated mice during preference testing was 210.0 ± 10.7 and 232.85 ± 7.2 for pre-session SKF 81297-treated mice, with

no significant difference between groups. Although direct statistical analysis between these experiments is precluded by slight procedural differences, the pre-session SKF 81297-treated mice appear to show slight elevations in activity during Preference Test compared to saline-treated mice and post-session SKF 81297-treated mice.

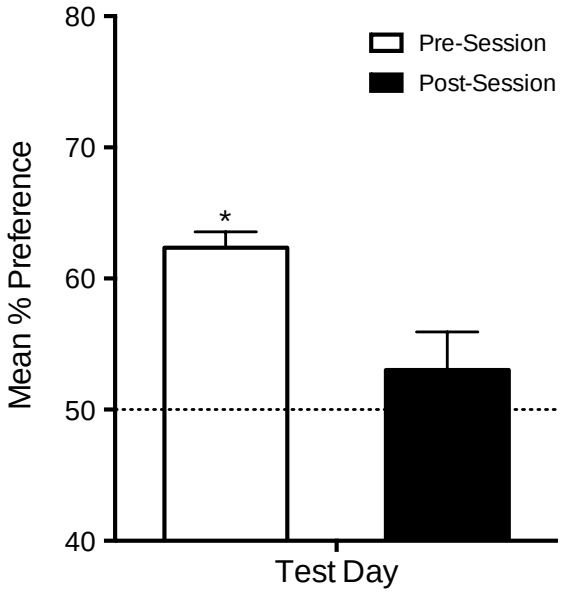


Figure 14. Pre-session SKF 81297 (10 mg/kg) induces conditioned place preference. (Experiment 13). Mice that received pre-session administration of SKF 81297 showed significantly more preference for drug-paired side than post-session treated animals. Error bars indicate SEM. (*) $p < 0.05$ significant difference.

Experiment 14: Post-session SKF 81297 enhances extinction of cocaine conditioned place preference.

Previous studies have indicated that antagonism of D1/5 receptors impairs consolidation of cocaine conditioned place preference extinction (Fricks-Gleason, Khalaj, & Marshall, 2012). Experiment 14 tested the effect of D1/5 receptor agonism with SKF 81297 (10 mg/kg) on extinction of a cocaine conditioned place preference. This experiment tests whether the observed extinction

enhancements are specific to aversive conditioning, or if extinction of rewarding Pavlovian associations operates through similar mechanisms. Mice were trained with cocaine for a conditioned place preference, then immediately following an extinction session, received SKF 81297 (10 mg/kg). Preference for cocaine-paired floor was tested prior to and following extinction. Figure 15A shows that there were no significant differences between groups during Preference Test in percent preference. Analysis of locomotor data showed no significant differences between groups during Preference Test. Group mean and standard error of the mean for locomotor activity during Preference Test in saline-treated mice was 325.8 ± 16.1 and 318.3 ± 7.6 in SKF 81297-treated mice. There was a non-significant trend towards decreased preference in animals that received SKF 81297 compared to saline ($t(46) = 1.730$, $p = 0.090$) during the post-extinction test (Post-Ext Test in Fig. 15A). Grid/hole analysis for Experiment 14 is provided in Appendix A. There were no significant differences in locomotor behavior during the Post-Extinction Test ($t(46) = 1.250$, $p = 0.2177$). Group mean and standard error of the mean for locomotor activity during Post-Ext Test in saline-treated mice was 261.5 ± 8.2 and 248.2 ± 6.8 in SKF 81297-treated mice.

The trend for decreased preference in SKF 81297-treated mice during Post-Extinction test led to examining whether initial preference had an impact on the effect of SKF 81297 on extinction. As preference testing occurred prior to extinction and manipulation with SKF 81297 or saline, there would be no effect of drug treatment group on initial preference. An extinction criterion was not used because animals were only given exposure to the drug-paired floor during

extinction, prohibiting an assessment of within session extinction. In order to assess high or low preference, a median preference value was calculated from all subjects (24 animals per group). Median preference was 64.92 % for all animals. Animals that fell below 65 % preference were characterized as low-preference (Fig. 15C), whereas animals showing above 65 % preference were labeled high-preference (Fig. 15B). For the saline-treated group, 10 mice were low-preference, and 14 were high-preference. For the SKF-treated group, 14 mice were low-preference, and 10 were high-preference.

Low preference animals (Fig 15C) showed a significant difference in Levene's test for equality of variance ($F = 6.965$, $p = 0.015$) during the Preference Test, leading to the adjustment of degrees of freedom in the t-test for Preference Test from 22 to 11.8. Levene's test was not significant for Post-Ext Test in low preference mice, or Preference Test or Post-Ext Test in high preference mice. There was no significant difference between saline- or SKF 81297-treated low preference animals during Preference Test ($t(11.8) = 1.837$, $p = 0.092$), or during Post-Ext Test ($t(22) = .574$, $p = 0.572$). However, low preference animals showed differences in locomotor activity during Preference Test ($t(22) = 2.184$, $p = 0.040$). Group mean with standard error shows that SKF 81297-treated mice showed significantly lower locomotor activity (321.0 ± 8.70) compared to saline-treated mice (364.6 ± 20.36). Low preference animals also showed a difference in locomotor activity during Post-Extinction Test ($t(22) = 2.436$, $p = 0.023$). Calculation of group mean with standard error of the mean for both groups indicated that SKF 81297-treated animals showed less locomotor

activity (250.5 ± 8.86) than saline-treated animals (286.8 ± 12.58). High preference animals showed no difference between saline- or SKF 81297-treated animals during Preference Test ($t(22) = 0.141$, $p = 0.889$), but SKF 81297-treated animals showed significantly less preference for the drug-paired floor during Post-Ext Test ($t(22) = 2.513$, $p = 0.020$).

There were no significant locomotor differences between SKF 81297- or saline-treated high preference animals during the Preference Test (Saline-treated mice: 298.1 ± 21.0; SKF 81297-treated mice: 314.4 ± 13.9) or Post-Extinction Test (Saline-treated mice: 243.4 ± 8.0; SKF 81297-treated mice: 244.9 ± 11.2). T-test comparison of locomotor activity between low preference mice and high preference mice indicated that low preference mice trended towards higher activity than high preference mice during Preference Test ($t(46) = 2.004$, $p = 0.051$). Group mean with standard error of the mean for low preference mice was 339.2 ± 10.6 for low preference mice and 304.9 ± 13.4 for high preference mice during Preference Test. During Post-Extinction Test, group mean with standard error of the mean for locomotor activity in low preference mice was 265.6 ± 8.1 and 244.0 ± 6.4 for high preference mice. Statistical analysis confirmed that low preference mice showed significantly higher locomotor activity during Post-Extinction Test than high preference mice ($t(46) = 2.087$, $p = 0.042$). The differences in locomotor activity between high and low preference mice may be driven by less exploration of the context by high-preference mice.

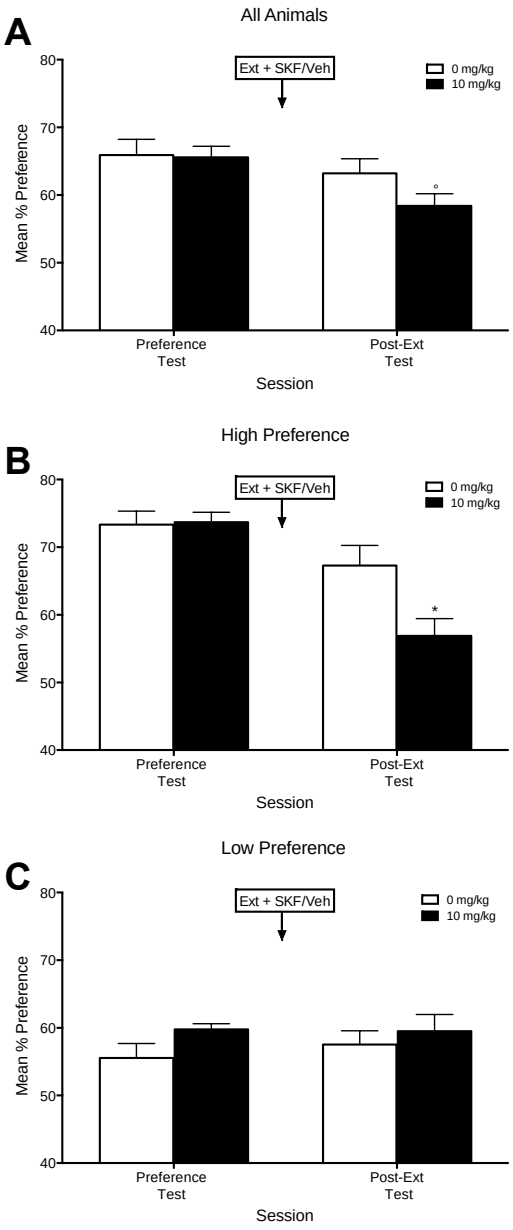


Figure 15. Post-extinction SKF 81297 decreases preference for cocaine-paired side in mice showing high preference. (Experiment 14). Panel A shows that mice that received post-extinction administration of SKF 81297 trended towards decreased preference for cocaine-paired side. Panel B shows that mice with high preference (over 65%) showed significantly decreased preference with post-extinction SKF 81297 compared to saline-treated mice. Panel C shows that mice with low preference showed no difference between groups. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with saline.

Discussion

The experiments in this chapter examine the effect of D1/5 receptor agonism on fear and reward learning. Experiments 6-8 generally demonstrate

that fear extinction is enhanced with SKF 81297 administration, although this effect may be bounded by a temporal context (Experiment 9 & 12). Fear conditioning is not enhanced (Experiment 10), suggesting that D1/5 receptor agonism does not broadly enhance all learning, and may be more specifically involved in interference paradigms in aversive learning (Experiments 6-9, 11, 12). Pre-session D1/5 receptor agonism can induce conditioned place preference (Experiment 13), while post-session D1/5 receptor agonism enhances extinction of cocaine conditioned place preference in high-preference animals (Experiment 14), demonstrating that dopamine D1/5 receptor agonism is involved in both reward conditioning and extinction. These studies reveal a nuanced role for D1/5 receptor signaling within aversive and reward learning paradigms.

The difference between tonic and phasic signals is important to consider when examining the effect of a dopamine agonist on behavior. As mentioned previously, L-dopa, a dopamine precursor can enhance extinction (Haaker et al. 2013). This study basically corroborates the findings in this chapter, but L-dopa administration is distinct from dopamine agonist activity because L-dopa is taken up by dopamine neurons, packaged into vesicles as dopamine, and facilitates both phasic and tonic dopaminergic signaling. In contrast, dopamine agonists mimic prolonged tonic stimulation of dopamine receptors, thereby possibly reducing the signaling power of phasic dopamine signals in altering behavior (Breitenstein et al. 2002). The tonic signaling at dopamine terminal regions is entrained by phasic dopamine release arising from dopamine neurons during learning and may be maintained by tonic dopamine release following CS-

presentation. As tonic signaling at dopamine terminal regions may serve in modifying CS-associated prediction signals, rather than US-associated prediction error (Suri and Schultz, 2001), these distinctions are important for examining the effect of SKF 81297 in behavior. Whereas agonist-induced tonic signaling in dopamine terminal regions may help to maintain CS-processing, the information about the US arising from phasic dopamine neuron activity may become weaker or disordered. This hypothesis is addressed in greater detail in the general discussion (Chapter 6).

Experiment 8 suggests that D1/5 receptor activation enhanced consolidation of fear extinction. The impact on consolidation is specific to extinction, as post-fear conditioning SKF 81297 impaired fear learning (Experiment 10). Experiment 11 also demonstrates that SKF 81297 administration following contextual exposure can lead to some interference of fear acquisition that emerges when the animal is tested the following day. It is possible that there may be some conditioned activity to the context generated by post-session SKF 81297 in Experiment 11, however Experiment 7b suggests that the fear conditioning impairment in Experiment 11 is unlikely to be driven solely by locomotor activity. The conditioned activity to the context may be inhibited by context pre-exposure in Experiment 7b, but this possibility was not directly tested in these studies. Together, Experiments 10 and 11 strengthen the hypothesis that D1/5 receptor activation specifically modulates learning related to the CS. In Experiment 10, the prediction signaled by the CS in the absence of shock (No US/New US) would likely impair consolidation of the previously formed CS-US

association or weaken fear expression to the CS. Similarly, Experiment 11 indicates that a prior learning experience with the CS can also block learning or expression of the CS-US association.

One confounding issue for determining whether the 4-h post-session administration was effective for separating the CS-presentation from drug administration is that drug administration occurred within the testing room. Procedural cues, such as the experimenter or test room could enter into an association with the drug and modify responding to the testing context on the following day. To remove these confounds, I tested the effect of the drug within a group of animals that did not receive extinction and remained in the vivarium during drug or saline administration in Experiment 9. I also inserted a one-week delay to measure whether the extinction effect persisted when the animals were not tested the day following drug administration. The goal of Experiment 9 was to identify whether there was an interaction between extinction status and drug treatment. One reason that I may not have been able to detect an interaction between extinction and drug treatment, as discussed before, is that there is a limited window within which to observe alterations in freezing. Although the use of B6 mice allows for a large span of behavior, there may not be sufficient space to detect these interaction effects. Based on all of the experiments presented in this dissertation, the lower limit for freezing behavior (with automated analysis and hand-scored data) during an extinction session in animals with a prior history of a 12-min fear conditioning session appears to be around 20-25 percent.

Another possibility for the lack of an interaction effect could be that prolonged tonic stimulation of D1/5 receptors may induce a particular neurobiological state, (such as receptor desensitization or internalization) that decreases expression of fear on following test, but this effect may not persist over the course of a week. Although I did not get an interaction, it is visually apparent that SKF 81297 administration alone did not alter freezing behavior. This, in combination with Experiment 7b and contextual freezing in cued fear tests, is important for providing evidence that the effects presented here cannot be explained solely through increases in locomotor activity.

Locomotor Activity and SKF 81297

The relationship between locomotor activity and SKF 81297 has been previously examined, and it appears that D1/5 receptor activation does not consistently induce locomotor activation. Chausmer and Katz (2002) demonstrated that SKF 81297 (10 mg/kg) administration in Swiss Webster mice had no effect on locomotion alone, and decreased locomotor activity in mice pre-treated with cocaine. Diaz Heijtz and Castellanos (2006) showed that Spontaneously Hypertensive Rats and Wistar Kyoto rats administered SKF 81297 (10 mg/kg) have biphasic locomotor responses to SKF 81297 administration, with initial decreases in locomotion during 0-20 min compared to saline, no difference from saline during 25-40 min, then locomotor stimulation at 45-60 min. These studies, in combination with the locomotor studies presented in this chapter, demonstrate the complexity of disentangling simple locomotor effects of SKF 81297 administration from effects on fear or reward behaviors.

As dopamine released from the substantia nigra is critical for motor behaviors and striatally driven behaviors (Andersson et al., 2006), it may be difficult to completely dissociate motoric components of dopamine neurotransmission from aversion or reward learning. The use of post-session administration of SKF 81297 allows for some separation between motor effects and learning effects of agonist administration. Another method to examine the role of locomotor activity on fear expression is through the use of cued fear conditioning (Experiment 12), which allows for freezing measurements during no-CS periods. I observed no difference in freezing behavior during CS-off periods in the extinction context, providing another indication that increased locomotor behavior is not sufficient for explaining the observed extinction enhancement effects. In addition to separating simple locomotor effects from fear behaviors, the use of cued fear allows for testing to occur in a context associated with post-session drug administration and non-drug associated contexts, which prevents possible contextual locomotor sensitization from obscuring fear responses.

SKF 81297 and Fear Renewal

To determine whether the effect of SKF 81297 on fear extinction is primarily linked to context-dependent mechanisms, as extinction is often context-specific, I tested the extinction of cued fear and renewal of fear. Experiment 12 demonstrates that cued fear extinction is enhanced by post-session SKF 81297. When returned to the conditioning context, renewal of fear is weaker in SKF 81297 treated animals, suggesting that the extinction enhancement is independent of the context. There was no difference in spontaneous recovery,

indicating that memory erasure had not occurred with SKF 81297 administration. Haaker et al. (2013) generated a similar finding of enhanced extinction and decreased fear renewal with L-dopa administration, and Experiment 12 suggests that dopamine agonism specifically modulates learning to the predictive CS, rather than increasing the inhibitory learning generated by the context during extinction. Together, Experiments 6-12 demonstrate that D1/5 receptor activation specifically enhances fear extinction, but as D1/5 receptor activation is generally associated with reward learning, the rewarding properties of SKF 81297 may contribute to this effect.

Rewarding Properties of SKF 81297

Testing the pre- and post-session effects of SKF 81297 on conditioned place preference (Experiment 13) provides a measure of how SKF 81297 modulates reward learning, and an indirect assessment of whether the fear extinction enhancements are due to a counterconditioning of the context within the aversive learning paradigm. The pre-session administration of SKF 81297 induces conditioned place preference (CPP), but post-session administration did not induce preference or aversion to the drug-paired floor.

It is possible that extended training with post-session SKF 81297 could eventually lead to a conditioned place preference, but the question of interest here is whether a single post-session administration of SKF 81297 induces reward learning such that the fear extinction effects could be explained as counterconditioning. One possibility that was not tested in these experiments is whether conditioned place preference would be generated by post-session SKF

81297 if the drug-paired floor was previously associated with an aversive stimulus. The pre-session CPP experiment could also simply suggest that SKF 81297 acts as a rewarding US, however based on the post-session experiment, this effect would not explain the enhancement of fear extinction. To test whether SKF 81297 is acting as a rewarding US or specifically modulating CS-prediction signals, I examined the effect of SKF 81297 on cocaine CPP.

SKF 81297 and Cocaine Conditioned Place Preference Extinction

Experiment 14 demonstrates that animals that strongly acquired cocaine CPP would show extinction enhancements with post-session SKF 81297. There was no effect in low preference animals, indicating that the effects of SKF 81297 on cocaine CPP extinction are dependent on the animal acquiring a strong association between the CS and US. If SKF 81297 were solely functioning as a rewarding US, it would be expected to possibly disrupt extinction, as the animal would learn that the drug-paired floor still indirectly signaled reward. However, if the CS-prediction signal is strengthened outside of the context, this could lead to inhibition of the previously formed CS-US association. This would lead to decreased preference for the drug-paired floor when tested the following day. Based on these experiments, it appears that a common mechanism underlies the extinction of both rewarding and aversive associations that is guided by D1/5 receptor activation.

Conclusions

In summary, this chapter demonstrates that dopamine D1/5 receptor activation is particularly important for the maintenance of CS-associated

prediction signals. This enhanced CS representation can inhibit previously formed CS-US associations in a manner that can appear as learning impairments, as seen in Experiment 10 and some studies examining the effect of D1/D2-like agonists on associative learning in humans (Breitenstein et al., 2006). However when an experimental paradigm is organized in such a way that favors the representation of the CS rather than the CS-US association (as in extinction), D1/5 receptor agonism may allow for an enhancement of learning. As most studies that have examined dopamine signaling in fear learning and extinction have primarily examined phasic signals (Brischoux et al., 2009; Badrinaryan et al., 2012), this chapter provides an examination of the role of prolonged tonic signaling in enhancing fear and reward extinction through modulation of CS-processing.

Chapter 4: The effect of D1 receptor knockout on fear and reward learning

Introduction

The use of a dopamine agonist allows for an examination of the impact of tonic dopamine D1/5 receptor signaling on behavior, but the role of phasic dopamine release in learning cannot be directly examined with a D1/5 receptor agonist. Under baseline conditions, D1-like receptors show lower affinity for dopamine than D2-like receptors, resulting in D1-like receptor sensitivity to conditions when there are large increases in dopamine availability in the synapse, such as during phasic dopamine release (Dryer et al., 2010). Phasic dopamine signals may be important for propagating US-based prediction errors to generate learning, and this has been indirectly verified using optogenetic stimulation of dopamine neurons to induce phasic firing, resulting in a conditioned place preference for the stimulation-paired side (Tsai et al., 2009). In agreement with these findings, the antagonism of D1/5 receptors leads to decreased cocaine conditioned place preference (Cervo and Samanin,1995), as well as impairment of fear conditioning (Inoue et al., 2000). These studies suggest that the loss of phasic signaling through D1-like receptors could have effects on fear and reward processing, although D1 receptor knockout studies have shown that cocaine conditioned place preference (Miner et al., 1995) and fear conditioning (El-Ghundi et al. 2001) can still be generated without D1 signaling.

Previous studies examining fear have not shown a consistent effect of D1 receptor knockout (D1 KO) on fear conditioning. For example, El-Ghundi et al.

(2001) demonstrated that expression of contextual fear is normal on the first test day in D1 KO, but extinction is impaired. In contrast, Ortiz et al. (2010) showed impaired cued fear conditioning and Fadok et al. (2009), showed impaired fear-potentiated startle in D1 KO mice. There are procedural variations within these studies that could lead to the different observed outcomes, such as the use of different types of fear conditioning procedures (contextual fear, cued fear, or fear-potentiated startle). Additionally, increased novelty-induced locomotion in D1 KO mice compared to wildtype (Karlsson et al., 2008) could interfere with freezing or potentiated startle measurements. To overcome this issue, Fadok et al. (2009) examined fear potentiated startle through a measurement of startle during CS-presentations compared to non-CS periods. However, Fadok et al. (2009) only detected a behavioral difference between D1 KO mice and wildtype (WT) mice following three alternating sessions of training and testing, and observed that WT mice showed significantly higher fear-potentiated startle than D1 KO mice on the second and third test. This suggests that D1 KO mice were insensitive to reconditioning, rather than showing impaired acquisition of fear.

Similar to Fadok et al. (2009), Ortiz et al. (2010) have also suggested that D1 KO mice show fear acquisition impairments in a cued fear conditioning procedure. Ortiz et al. (2010) utilized CS-on and CS-off periods to differentiate baseline freezing from CS induced freezing, but found significantly lower freezing in D1 KO mice during the pre-CS period. In spite of the decreased pre-CS freezing, D1 KO mice still showed elevated freezing during the CS-on period, indicating that D1 KO mice had acquired the CS-US contingency. Although Ortiz

et al. (2010) claim that D1 KO results in an impairment of fear conditioning, it appears that the effect is driven by a difference in behavioral expression (as seen by lower pre-CS freezing), rather than a loss of the learning itself. One procedural difference between Ortiz et al. (2010) and El-Ghundi et al. (2001) that could decrease expression of fear was the use of one- or two-shock training protocols. When given a one-shock protocol, as in Ortiz et al. (2010), D1 KO mice showed decreased freezing, but with two footshocks, El-Ghundi et al. (2001) did not observe differences between wildtype or D1 KO mice. In El-Ghundi et al. (2001), wildtype, heterozygote, and D1 KO mice showed equivalent levels of high freezing on the first test day, but in contrast to wildtype and heterozygote animals, freezing remained elevated in D1 KO mice following several test sessions, and this effect was described as delayed extinction. However, this effect may come from an enhanced acquisition of fear that could have been masked by ceiling effects when measuring freezing. From this mix of findings, it is difficult to assess whether acquisition or extinction of fear is impaired or enhanced in D1 KO mice.

This chapter aims to contribute to this literature by characterizing the behavior of D1 KO mice in fear conditioning and cocaine conditioned place preference to determine whether associative learning can be acquired and maintained without D1 receptors. Based on the studies described above, I tested the effect of both a two-shock and one-shock cued fear conditioning protocol to determine whether the strength of training impacts the expression of fear in D1 KO mice. The use of cued fear conditioning also allows for an examination of fear

responses during CS-off and CS-on periods, which could determine whether there are baseline differences in freezing that would impact the expression of fear in D1 KO mice. Additionally, I extend the previous literature by examining renewal of fear in D1 KO mice. Testing fear renewal indicates whether extinction learning persists outside of the extinction context in D1 KO mice or if extinction in D1 KO mice is strongly context-specific.

To assess whether D1 KO mice can learn context-specific reward associations, I measure cocaine conditioned place preference. Although Miner et al. (1995) found an equivalent cocaine conditioned place preference (CPP) in D1 KO mice compared to wildtype, their measurements utilized a biased apparatus, with preference value reported as a change from a preferred side to a drug-paired side. As discussed by Cunningham et al. (2006), the use of a biased procedure may result in conditioning through decreased aversion to the non-preferred floor or decreased anxiety in the non-preferred floor, rather than directly measuring the rewarding properties of the US. In the cocaine CPP experiment presented in this chapter, I utilize an unbiased apparatus to directly test whether the rewarding properties of cocaine leads to a conditioned place preference in D1 KO mice. Finally, I also characterize the effect of SKF 81297 in D1 KO mice to test whether the observed enhancements in fear extinction in Chapter 3 may be modulated by D5 receptors.

Materials and Methods

Subjects

Wildtype (male = 22, female = 20), heterozygote (m = 33, f = 17) and D1 receptor knockout mice (m = 16, f = 13) were used in these experiments ranging from three to eight months of age. D1 knockout mice (D1 KO; *Drd1a*$^{Cre/Cre}$) were generated by insertion of Cre recombinase at the initiation codon of the *Drd1a* gene locus, resulting in the deletion of the *Drd1a* gene (Heusner et al., 2008). Mice were generated on a 129/Sv background, but backcrossed to C57BL/6 for >10 generations prior to experimental manipulations. Animals were maintained in the laboratory of Dr. Kim Neve as heterozygote mice (D1 HET; *Drd1aCre/+)* and heterozygote pairs were bred to produce wildtype C57BL/6 mice and homozygous D1 KO mice. Genotypes were determined in the laboratory of Dr. Kim Neve through polymerase chain reaction (PCR) of tail snips using primers targeting *Drd1a.*

One to three months following weaning, mice were transferred to the Oregon Health & Science University vivarium and given at least 3 weeks in the vivarium prior to experimental use. D1 KO mice used in this study showed lower body weight (Appendix B) compared to wildtype or heterozygotes (KO: 17-20 g; HET: 24-30 g; WT: 25-30 g), which may be related to deficits in food pellet consumption in early life (Fadok et al. 2009). These differences in weight lead to the use of a special diet for cages housing D1 KO mice (DietGel Recovery; Clear H_20) until postnatal day 80 in my experiments. All D1 KO mice were placed on a regular laboratory diet (irradiated Pico Lab Rodent diet 5053) following postnatal

day 80 and experiments occurred at least 2 weeks following removal of the special diet. Wildtype and heterozygote mice (25-30 g) were fed regular laboratory diet throughout the entirety of experiments, although some wildtype and heterozygote mice had access to the diet gel in addition to standard lab diet during early life because genotypes were intermixed within home cages. Animals were housed two to five per cage. Polycarbonate cages were held in a Thoren rack, and animals were given access to food and water ad libitum. Vivarium and experiment room temperatures were maintained at 22°C ± 1°C, and subjects were maintained on a 12-h light–dark cycle (lights on 0600 h–1800 h). Animals were moved from the vivarium to the experiment room 60 min before the start of an experiment, and experiments were conducted between 900 and 1700 h. All experimental procedures were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985).

Drugs and Apparatus

These studies used the same chambers for fear conditioning and conditioned place preference described in Chapter 3. SKF 81297 (10 mg/kg) and cocaine (20 mg/kg) was dissolved and administered (intraperitoneal; i.p.) as described in Chapter 3.

Behavioral Procedures

Experiment 15a: One-shock cued fear conditioning in D1 knockout mice.

This experiment used a weak fear conditioning protocol (i.e., one CS-US pairing) to determine whether expression of fear in D1 knockout mice is decreased compared to other genotypes. The primary question for this experiment is whether a weak training protocol could impair acquisition of fear in D1 KO mice, as reported in Ortiz et al. (2010). On Day 1, naïve subjects (n = 2 male D1 KO, 8 male HET, 7 male WT, 4 female D1 KO, 4 female HET, 3 female WT) received a 6.5-min exposure to Context A with a 30 second noise CS (85 dB) co-terminating with shock (2 s, 0.35 mA) presented at 2 min (Acq in Fig. 16). Due to the variation of animal numbers within sex and genotype, male and female subjects were pooled within genotype for this analysis. On Day 2, mice received a 6-min nonreinforced exposure to context A (Ctx Ext in Fig. 16). On Days 3 and 4, subjects were placed in Context B for 15-min with 3-min CS presentations at 3 and 9 min (Test 1 and 2 in Fig.16).

Experiment 15b: Shock reactivity in D1 KO mice.

This experiment assesses whether shock reactivity was altered between genotypes. Animals (n= 3 male D1 KO, 5 male HET, 5 male WT, 3 female D1 KO, 4 female HET, 5 female WT) were given footshocks (2 s) increasing in strength (.05 mA intervals) from 0.05 mA to 0.25 mA with three administrations of each shock. Animals received 5 s between each shock administration. Following the third shock, animals received a 10 s interval prior to shock administration of a higher strength. Experimenter observations determined and recorded whether

mice jumped during shock administration. Jumping in response to shock was recorded as “yes” or “no” responses by the experimenter. Mice that jumped in response to at least one of three shocks administered (within each shock strength) were recorded as a “yes” response and classified as showing shock reactivity. Experimenter was blinded to the genotype of the animals, although knockout mice were visually identifiable based on consistently smaller body weights. Experiment was terminated at 0.25 mA (0.10 mA below conditioning strength) to prevent unnecessary stress to the animals, as all animals responded consistently (with jumping) to shock starting at 0.15 mA. Shock reactivity testing occurred >4 weeks after mice had been used in fear conditioning (Experiment 15 and 16) and cocaine conditioned place preference studies (Experiment 17).

Experiment 16: Cued fear and renewal in D1 knockout mice.

This experiment compares D1 knockout (D1 KO), heterozygotes (HET) and wildtype (WT) mice on acquisition, extinction and renewal of fear. On Day 1, naïve subjects (n = 7 male D1 KO, 8 male HET, 8 male WT, 7 female D1 KO, 8 female HET, 8 female WT) received a 6.5-min exposure to Context A with a 30 second noise CS (85 dB) co-terminating with shock (2 s, .35 mA) presented at 2 and 4 min (Acq in Fig. 17a). On Day 2, mice received a 6-min nonreinforced exposure to context A (Ctx Ext in Fig. 17a). Figure 17a shows freezing during CS-on periods and Figure 17b shows freezing during CS-off periods. On Days 3-8, subjects were placed in Context B for 15-min with 3-min CS presentations at 3 and 9 min (Test 1-6 in Fig.17). On Day 9, to measure fear renewal, mice were

returned to Context A for a 15-min session with 3-min CS presentations at 3 and 9 min (Ren. in Fig. 17).

Experiment 17: Cocaine conditioned place preference in D1 knockout mice.

This experiment tests whether D1 knockout mice can acquire a cocaine conditioned place preference. An unbiased CPP apparatus was used and mice were habituated (5-min pretest) to the conditioning chamber with both floors present. Animals (n = 5 male D1 KO, 7 male HET, 5 male WT, 9 female D1 KO, 10 female HET, 10 female WT) were balanced based on pretest data to ensure no bias to hole or grid floors, and then assigned to counterbalanced groups that received cocaine (20 mg/kg) or saline immediately before exposure to a grid or hole floor. All subjects had received fear conditioning >4 weeks prior to conditioned place preference experiment (from Experiments 15a and 16), and no bias was observed to hole or grid floors during the pre-conditioning test. During conditioning, animals were confined to one half of the CPP apparatus with grid or hole floor for 15-min. Mice received two pairings of pre-session cocaine (20 mg/kg i.p.) and two pairings of pre-session saline, counterbalanced for order and floor type, on alternating days. To minimize the possibility of a side preference, floor placement was alternated such that animals received a pairing of cocaine or saline on both right and left sides of the chamber. Twenty-four h following the last conditioning session, mice were given a 15-min exposure to the CPP apparatus with both floors to assess preference for the drug-paired side (Preference Test in Fig. 18a).

Experiment 18: Effect of SKF 81297 on contextual fear extinction in D1 knockout mice.

There are currently no pharmacological tools to distinguish between D1 and D5 receptors within the D1-like receptor subfamily. To determine whether the observed enhancements of fear extinction in Chapter 3 were due to activity of D5 receptors, I tested the effect of SKF 81297 on fear extinction in D1 knockout mice. On Day 1, naïve subjects received a 12-min exposure to the context with four unsignaled shocks (2s, .35 mA), delivered at 2.5, 5, 9, and 11.5 min. Groups were matched within genotypes following acquisition to ensure equal terminal freezing levels across SKF 81297 dose assignments (Acq in Fig. 19). For male SKF treated animals, there were n = 4 D1 KO, 9 HET, and 3 WT. For female SKF treated animals, there were n = 1 D1 KO, 2 HET, and 4 WT. For male saline treated animals, there were n = 3 D1 KO, 8 HET, 4 WT. For female saline treated animals, there were n = 1 D1 KO, 3 HET, and 5 WT. Sexes were pooled within genotype for statistical analysis. On Day 2, mice received a 12-min nonreinforced exposure to the context (Ext in Fig. 19). Groups were matched within genotypes following extinction to ensure equal levels of terminal freezing before SKF 81297 administration. On Test Day, mice received a 12-min nonreinforced exposure to the context.

Data analysis

Fear memory expression was determined by freezing response within the context. Freezing was defined as an episode of at least 3 sec of inactivity. For Experiment 15 and 16, all data are hand-scored due to the lack of an automated

measurement system in Context B. Freezing in Experiment 15a and 16 is reported as percentage of time freezing during the CS-on period. Locomotor data in Experiment 16 was measured by automated activity counts in Context A prior to CS presentation. For Experiment 18, total freezing time was divided by 12 min to calculate percentage of time freezing in each day. Locomotor activity was measured by total distance moved in cm, and reported as distance moved (cm) per min. Conditioned place preference in Experiment 17 was measured in Ethovision XT 5 (Noldus) by tracking the center point of the mouse for time (s) spent on drug-paired floor (CS+) or vehicle-paired floor (CS-) per minute of test session. Average time on CS+ floor was used to calculate percentage of time spent on CS+ floor over the whole session. Grid/hole (G+/G-) analysis in Experiment 17 examined time (s) spent on grid floor per min during Preference Test in animals that received cocaine-conditioning sessions on grid floor compared to animals that received cocaine-conditioning sessions on hole floor (Cunningham et al., 2006). Data analyses were performed with Prism 6 and SPSS Statistics 22 (IBM). Data in Experiment 15a were analyzed with a one-way analysis of variance (ANOVA) on each day. Data in Experiments 16 and 17 were analyzed using a two-way ANOVA on each day with genotype and sex as between-subjects factors. G+/G- data in Experiment 17 were analyzed using a three-way ANOVA, with genotype, sex and floor assignment as between-subjects factors. Pearson's correlation tests were used to determine whether locomotor behavior correlated with preference scores in Experiment 17. Data in Experiment 18 were analyzed using a two-way ANOVA on each day with genotype and drug

as between-subjects factors. Post hoc comparisons of ANOVA data were performed using a Dunnett's test (Experiments 15,16, and 18) or Bonferroni's test (Experiment 17).

Results

Experiment 15a: D1 knockout does not impair one-shock cued fear conditioning.

Ortiz et al. (2010) had suggested that a one-shock cued fear conditioning protocol leads to decreased fear expression in D1 KO mice. This study examines whether fear learning is impaired in D1 KO mice compared to wildtype or heterozygote mice following a single CS-US pairing. Due to the small number of knockout mice used in this study, animals were collapsed across sex for statistical power. Figure 16 shows there were no significant effects of genotype on Acquisition (Acq.), Contextual Extinction (Ctx Ext), Test 1, or Test 2. There were no differences in baseline freezing between groups during Test 1 or Test 2. This experiment demonstrates that fear learning is retained in D1 KO following a brief conditioning session.

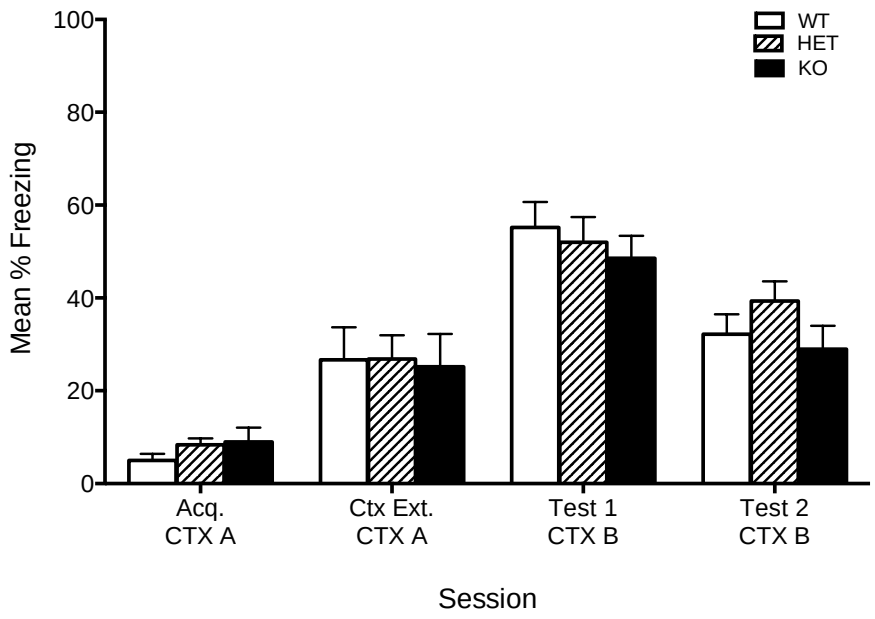


Figure 16. One shock cued fear conditioning is retained in D1 knockout mice (Experiment 15a). Mice did not show differences in freezing between genotypes in Acquisition (Acq.), Contextual Extinction (Ctx. Ext.), Test 1 or Test 2. Male and female wildtype (WT), heterozygote (HET), knockout (KO) were pooled within genotypes for statistical analysis.

Experiment 15b: D1 knockout does not alter shock reactivity.

This experiment addresses whether there may be differences in shock reactivity between genotypes that could affect fear learning. Table 1 shows percent of subjects jumping in response to shock. At 0.05 mA, there was no effect of genotype or sex, and no interaction between genotype and sex. At 0.10 mA there was no significant effect of sex, but there was a significant effect of genotype ($F(2,19) = 4.071$, $p = 0.0337$) and a significant interaction between sex and genotype ($F(2,19) = 4.071$, $p = 0.0337$). Post-hoc analysis confirmed that heterozygote mice showed greater shock reactivity than wildtype mice ($p = 0.0179$) to 0.10 mA shock. At 0.15 mA and above, there was no significant effect of genotype or sex and no interaction between sex and genotype. There were no genotype specific differences in shock reactivity that would affect fear

conditioning, as D1 KO, heterozygote and wildtype mice consistently responded to footshock starting at .15 mA, which was below the .35 mA footshock used for training in these studies.

Genotype	Shock Strength				
	0.05 mA	0.10 mA	0.15 mA	0.20 mA	0.25 mA
MWT	50%	100%	100%	100%	100%
MHET	33%	100%	100%	100%	100%
MKO	20%	100%	100%	100%	100%
FWT	0%	40%	100%	100%	100%
FHET	33%	100%	100%	100%	100%
FKO	20%	100%	100%	100%	100%

Table 1. Shock reactivity in D1 knockout mice (Experiment 15b). Table shows percent of subjects showing jumping responses at each shock strength. Male wildtype, heterozygote and knockout (MWT, MHET, MKO) showed consistent jumping responses starting at 0.10 mA. Female wildtype, heterozygote, and knockout (FWT, FHET, FKO) showed consistent jumping responses starting at 0.15 mA.

Experiment 16: D1 knockout does not impair cued fear conditioning.

Experiment 16 tested the effect of D1 knockout on fear conditioning, extinction and renewal. For fear conditioning in Experiment 16, mice received two tone-footshock pairings. Figure 17a shows that there was no significant effect of genotype on acquisition of fear. Figure 17a shows freezing during CS-on periods and Figure 17b shows freezing during CS-off periods. Data from acquisition (Acq in Fig. 17a) are presented as freezing during the whole session. To determine whether there were locomotor activity differences between genotypes during exposure to the context, mean activity counts were examined prior to CS-US presentation. There was no significant effect of genotype, but there was a main effect of sex ($F(1,40) = 7.337$, $p = 0.0099$) during the 2-min pre-CS presentation period and no interaction between sex and genotype. Group mean and standard error of the mean for locomotor activity in female mice (450 ± 13.2), and male

mice (500 ± 12.3) showed that female mice moved significantly less than male mice upon initial exposure to the context.

During Contextual Extinction (Ctx Ext in Fig. 17a), there was a main effect of sex ($F(1,40) = 6.167$, $p = 0.0173$), with female mice generally showing higher freezing to the conditioning context than male mice. There was no significant effect of genotype on freezing, and no interaction between genotype and sex during Contextual Extinction. The sex-specific increases in freezing in female mice compared to male mice did not persist to the following Test 1 in Context B (Test 1 in Fig 17a). However, there was a main effect of genotype during Test 1 ($F(2,40) = 3.628$, $p = 0.0357$), and post-hoc analysis revealed a trend indicating that the genotype effect was driven by increased freezing during the CS-on period in knockout mice compared to wildtype mice ($p = 0.053$). There was no main effect of sex or interaction between genotype and sex during Test 1. Analysis of the CS-off period during Test 1 (Fig. 17b) indicated no significant differences between genotype or sex, or interaction between genotype and sex, on baseline freezing in Context B. However, there was a trend towards a genotype effect on freezing ($F(2,40) = 2.860$, $p = 0.069$), and post-hoc analysis indicated that knockout mice showed significantly higher freezing during baseline periods compared to wildtype mice ($p = 0.046$). This suggests that the observed increases in freezing during CS-on periods in Test 1 may be driven by differences in baseline freezing, rather than an enhancement of fear acquisition in knockout mice.

During CS-on periods in Test 2, there was no significant effect of sex and no interaction between sex and genotype, but there was a main effect of genotype on freezing ($F(2,40) = 3.934$, $p = 0.0276$). A post-hoc analysis revealed that knockout mice showed significantly higher freezing compared to wildtype mice ($p = 0.038$). There were no significant effects of genotype or sex and no interaction between sex and genotype during the CS-off period in Test 2, indicating that fear expression differences on Test 2 during the CS-on period cannot be solely attributed to different baseline levels of freezing between genotypes. There were no significant differences in freezing between males and females or genotypes during CS-on or CS-off periods on any day from Test 3 to Test 6.

When returned to the conditioning context to test for renewal of fear, there was a main effect of genotype during CS-on periods ($F(2,40) = 4.202$, $p = .0221$). There was a non-significant trend for differences between wildtype and knockout mice ($p = 0.066$). There was no main effect of sex and no interaction effect during Renewal CS-on periods (Ren. in Fig. 17a). There were no main effects of sex or genotype on freezing during CS-off periods and no interaction between sex and genotype (Ren. in Fig. 17b). Analysis of the whole renewal session, with CS-on and CS-off periods combined, showed a significant main effect of genotype on freezing ($F(2,40) = 4.641$, $p = 0.0154$) and no significant effect of sex and no interaction between sex and genotype. Post-hoc analysis showed a non-significant trend of knockout mice freezing less than wildtype mice ($p = 0.0576$). Together, these data indicate that fear acquisition and expression is

retained in D1 knockout mice, and fear expression may be slightly enhanced in knockout mice during initial test days. There is an overall genotype effect of fear renewal, suggesting that knockout of the D1 receptor leads to decreases in fear renewal or that enhanced acquisition of fear decreases fear renewal.

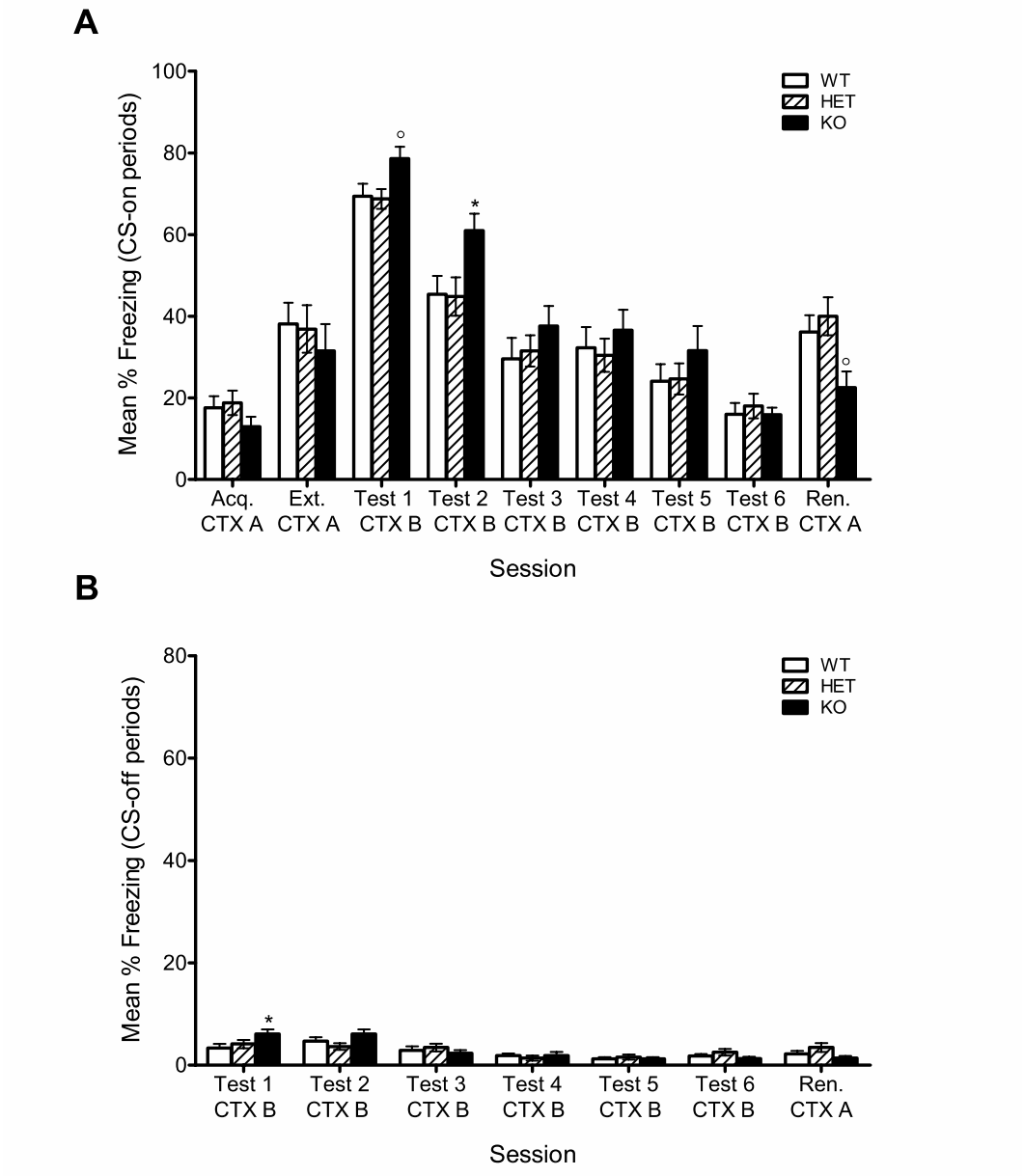


Figure 17. Cued fear conditioning is retained in D1 knockout mice. (Experiment 16). Panel A shows freezing during CS-on periods in wildtype (WT), heterozygote (HET), and knockout (KO) mice. Panel B shows freezing during CS-off periods in WT, HET, and KO mice. KO mice showed increased freezing in response to the CS on Test 1 (compared to WT) and on Test 2 (compared to WT). KO mice showed a trend (genotype effect: $p = 0.069$, but significant post-hoc : $p = 0.046$) towards increased freezing during CS-off periods on Test 1. KO mice showed a non-significant decrease ($p = 0.0576$) in freezing during CS-presentations compared to WT during Renewal. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared to WT. (°) $p =$ non-significant trend for KO compared to WT.

Experiment 17: D1 knockout effect on cocaine conditioned place preference.

This experiment examines the effect of D1 receptor knockout on the acquisition of cocaine conditioned place preference. All mice were matched following a pre-test to ensure no bias to grid or floor and given two conditioning sessions with cocaine and saline over four days. When tested for preference (Preference Test in Figure 18A), there was a main effect of genotype on preference ($F(2,40) = 3.618$, $p = 0.036$). There was no significant effect of sex and no interaction between sex and genotype during the preference test. Post-hoc analysis showed that the genotype effect was significant in heterozygote mice compared to knockout mice ($p = 0.028$), with heterozygotes showing greater preference than knockout mice.

To assess whether animals acquired a significant cocaine conditioned place preference, time spent on grid floor during the test day was compared between animals that received cocaine conditioning sessions on the grid floor compared to animals that were received cocaine conditioning sessions on the hole floor. A significant difference between grid-trained (G+) compared to hole-trained (G-) mice indicates that animals acquired a conditioned place preference. There was a significant effect of floor ($F(1,34) = 153.22$, $p < 0.0001$), but no significant effect of sex or genotype and no significant interactions between floor, sex or genotype. Figure 18B shows that all genotypes showed a preference for the cocaine-paired grid floor (G+) compared to saline-paired grid floor (G-).

Examination of locomotor behavior during pre-test (Fig. 18C) indicated that there was a main effect of sex ($F(1,40) = 10.96$, $p = 0.002$), with females showing less locomotor activity than males, and a main effect of genotype ($F(2,40) = 4.153$, $p = 0.0230$), with no interaction effect. Post hoc analysis confirmed that knockout mice showed significantly less locomotor activity than wildtype mice ($p = 0.004$) during pre-test. Assessment of locomotor behavior during cocaine-conditioning sessions showed that there was a significant effect of genotype ($F(2,40) = 12.64$, $p < 0.0001$) and no significant effect of sex and no interaction between sex and genotype. Post-hoc analysis confirmed that knockouts showed significantly less locomotor response to cocaine compared to wildtype mice ($p < 0.0001$). There was no significant effect of genotype or sex on locomotor behavior during both saline-conditioning days and no interaction between genotype and sex. During the preference test, there was no significant effect of sex and no interaction between sex and genotype, but there was a main effect of genotype ($F(2,40) = 4.806$, $p = 0.0135$). Post-hoc analysis confirmed that knockout mice showed significantly less locomotor activity compared to wildtype mice ($p = 0.001$). Although there were significant locomotor effects in knockout animals, there was no correlation across all animals between percent preference and locomotor activity during pre-test, cocaine or saline conditioning days, or preference test.

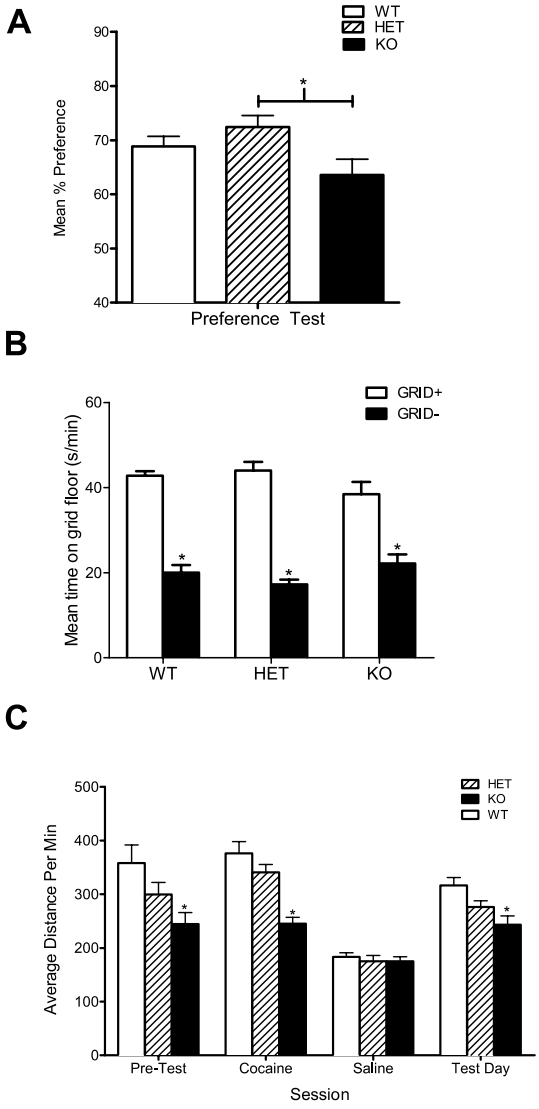


Figure 19. Cocaine conditioned place preference in D1 knockout mice. (Experiment 17). Panel A shows percent preference in male and female wildtype (WT), heterozygotes (HET) and knockouts (KO). Panel B shows grid+/grid- analysis of WT, HET and KO during preference test day. Panel C shows locomotor activity of WT, HET and KO during pre-test, training days, and preference test day. Knockout mice showed significantly less preference for cocaine-paired floor compared to heterozygote mice in Panel A. Grid+/grid- analysis did not indicate a main effect of genotype, but showed that all genotypes acquired a conditioned place preference when comparing animals that received cocaine on grid floor (grid+) against animals that received saline on grid floor (grid-) during training. During pre-test, cocaine conditioning sessions, and Test Day, knockout mice showed decreased locomotor activity compared to wildtype mice. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared to HET or WT.

Experiment 18: SKF 81297 effect on contextual fear extinction in D1 knockout.

One challenge for assessing the effect of activation of D1-like receptors (Chapter 3) is that there are currently no pharmacological tools to distinguish D1 from D5 receptors. As D1 knockout mice are expected to only express D5 receptors, this experiment tests whether activation of D5 receptors with SKF 81297 in D1 knockout mice will lead to an enhancement of contextual fear extinction. Sexes were pooled within genotypes for increased statistical power. During acquisition of contextual fear conditioning (Acq. In Fig. 19), there was a trend towards a genotype effect ($F(2,41) = 2.992$, $p = 0.0612$), likely driven by increased freezing in heterozygote mice. There was no interaction between drug-treatment and genotype during acquisition. Post-hoc analysis did not show any significant differences between genotypes or treatment groups. There were no significant effects of genotype or drug-treatment and no interaction between genotype and drug-treatment during Extinction or Test Day (Ext. and Test Day in Fig. 19). Due to the small number of knockout animals available in this study, there was a slight imbalance in freezing between SKF 81297 and saline treated knockout animals prior to drug treatment, although the effect was not statistically significant. The imbalance in freezing may prevent clear assessment of extinction learning between drug- and vehicle-treated knockout mice. Experiments in Chapter 3 had indicated that administration of SKF 81297 should enhance extinction in wildtype mice, but there was no effect of SKF 81297 administration in this study on any genotype.

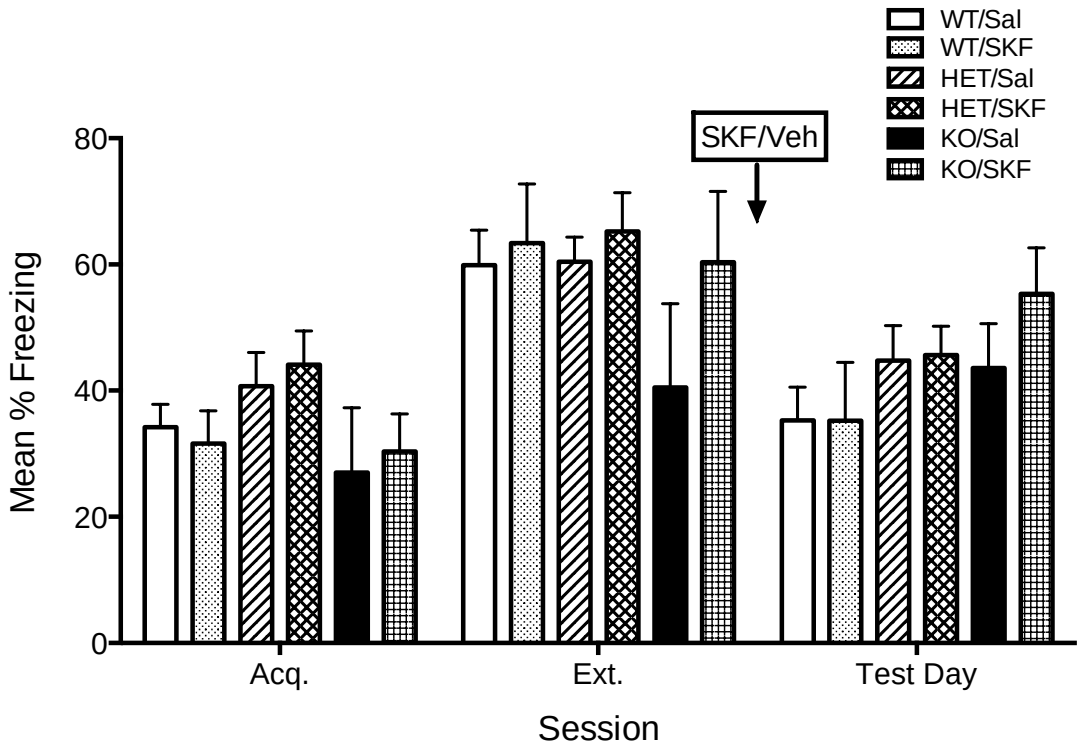


Figure 19. SKF effects on contextual fear extinction in D1 knockout mice. (Experiment 18). Mice were treated with SKF 81297 or saline following extinction. Drug treatment had no effect within any genotype.

Discussion

The goal of the experiments presented in this chapter was to examine the effect of D1 receptor knockout on Pavlovian fear and reward learning. Experiments 15, 16 and 18 demonstrate that both cued and contextual fear conditioning is retained in D1 KO mice. Experiment 17 shows that conditioned place preference is retained in D1 KO mice compared to wildtype mice. These experiments provide evidence that associative learning can be maintained in animals lacking the D1-receptor. The current data contradict some previous reports of impaired fear conditioning in D1 knockout mice, but there are a number

of findings within this chapter that support previous characterizations of the phenotype arising from D1 receptor knockout.

As discussed previously, D1 receptors are important in a number of motivated behaviors, and loss of this signaling system appears to have deleterious effects on body weight and locomotor behavior. As reported by Fadok et al. (2009) and Karlsson et al. (2008), D1 KO mice exhibit decreased food consumption in the absence of a palatable food replacement, and decreased body weight compared to heterozygote and wildtype mice. These effects on body weight were observed within the D1 KO mice used in these studies (data in Appendix B). Similar to Fadok et al. (2009), there was no difference in shock reactivity (Experiment 15b). However, in contrast to the report by Karlsson et al. (2008), the D1 KO mice in this study exhibit significantly decreased locomotor activity during exposure to a novel context. D1 KO mice showed decreased locomotor activity during an initial 5-min pre-test in Experiment 17. Decreased locomotor activity was also observed during the preference test (Experiment 17) in D1 KO mice. Karlsson et al. (2008) observed consistently increased locomotor activity over a two-hour open-field test, and the effects cannot be attributed to differences in genetic background as both studies used animals that were backcrossed for several generations to C57BL/6 mice. There could be an effect of the size of the chamber that is used to examine locomotor behavior, as the CPP chamber (30 cm X 15 cm X 15 cm) is smaller than the open-field chamber (40 cm X 35 cm X 35 cm) used in Karlsson et al. (2008). The exact cause for the differing locomotor effects of D1 knockout is unclear.

One key difference between the animals used in this study and other D1 KO mice is that the D1 KO mice in this study express Cre-recombinase in place of the D1 receptor. The effect of linking Cre-recombinase expression to the D1 receptor promoter is unknown, but Forni et al. (2006) demonstrated that increased Cre-expression in neuronal progenitors has toxic effects on neuron development. This effect only emerges when Cre-recombinase is present at high levels within the nucleus, and has little effect when Cre-recombinase is stored in the cytoplasm. This suggests that the D1 promoter driven expression of Cre-recombinase may not explain the differences in behavior between the studies in this chapter compared to previous studies of D1 KO mice. However, as Cre-recombinase may not translocate to the cytoplasm similar to D1 receptors, there may be effects of Cre-recombinase expression in the nucleus during development leading to behavioral changes.

The primary finding of the studies here is that fear conditioning is enhanced in D1 KO mice. When trained with two CS-US pairings, D1 KO mice trended towards increased freezing compared to wildtype during Test 1 in Fig. 17 and showed significantly higher freezing than wildtype during Test 2 in Fig. 17. This could be considered an enhancement of fear acquisition, but the general decreases in locomotor behavior and increased freezing during CS-off periods in Test 1 prevent the clear determination of whether the increases in freezing are conclusively showing enhanced acquisition of fear. One possibility for the observed acquisition of fear is that the general decrease in locomotion (as observed during the pre-test in conditioned place preference in Experiment 17)

could artificially inflate fear responses measured as freezing. However, there was no observed decrease in locomotor behavior between genotypes during the pre-CS period in acquisition, suggesting that general decreases in locomotor behavior alone would not be a likely explanation for the acquisition of fear. Additionally, there were no differences in freezing during the CS-off period in Test 2 and increased freezing during CS-on periods, indicating that knockout mice showed enhanced fear acquisition.

One finding in Experiment 16 suggesting that the increased expression of fear in D1 KO mice is related to learning, rather than differences in behavioral expression, is the decreased renewal of fear in D1 KO mice. If D1 KO mice showed consistently high levels of freezing across all tests, including renewal, it would be difficult to assess whether fear expression or learning is impacted. However, D1 KO mice only show increased freezing during Test 1 and Test 2, then show relatively equal or slightly higher levels of fear responding compared to other genotypes. When tested for fear renewal, the D1 KO animals trend towards less freezing. This effect may initially appear incongruent to the increased acquisition of fear, but a similar effect has been previously reported in literature examining the effect of retrieval on fear extinction. Leung and Westbrook (2008) tested the differential effect of extinction between a remotely extinguished CS that exhibits greater spontaneous recovery of fear compared to a recently extinguished CS. The extinction of the remote CS led to a deepened response loss to the CS (decreased fear) compared to the recently extinguished CS. This effect is attributed to the fact that there is a larger error between the

predicted outcome (shock) than the actual outcome (no shock) in animals expressing high fear compared to animals showing low fear. Similarly, in Experiment 16, the D1 KO mice showed greater fear during CS presentations, leading to stronger extinction, as revealed by decreased freezing during the renewal test.

To match the training protocol used by Ortiz et al. (2010), I also tested the effect of a single CS-US pairing on fear conditioning in Experiment 15a. Unlike Ortiz et al. (2010), I observed normal acquisition of fear across genotypes. This effect may be dependent on the longer test sessions used in my studies, as Ortiz et al. (2010) used 3-min test session (1-min pre-CS, 30 sec CS presentation, 1.5-min post-CS test), compared to the 15-min test session used here. Additionally, freezing behavior was closely linked with CS-presentations in my experiments, whereas combining CS-on and post-CS periods as in Ortiz et al. (2010) may negatively impact measurements of freezing.

Contextual fear (Experiment 18) also did not appear to be affected by D1 KO, in concordance with El Ghundi et al. (2001). However, there was no clear elevation of freezing in D1 KO animals during the Test, as reported by El-Ghundi et al. (2001). Procedural differences may be a factor, as El-Ghundi et al. (2001) measured behavior during repeated five-min extinction sessions, rather than the 12-min extinction sessions used here, which indicates that a brief extinction session may be ineffective in D1 KO mice when tested over several days. In summary, it does not appear that the D1 receptor is required for acquisition or extinction of fear, and instead, may modulate mechanisms that decrease fear

responding. As D1 receptors are closely associated with reward learning, the findings in Experiments 15 and 16 suggest that D1 receptors may be involved in fear learning as a safety/relief signal or opposing motivational system that inhibits expression of fear. To directly examine reward learning, I tested if D1 KO mice could acquire cocaine conditioned place preference.

Miner et al. (1995), using a biased apparatus, demonstrated that cocaine conditioned place preference is equivalent between wildtype, heterozygote, and D1 KO mice. Experiment 17 shows that D1 KO mice can acquire cocaine conditioned place preference in an unbiased apparatus. Although there was no difference in preference between heterozygotes and wildtypes, preference for the cocaine-paired floor is lower in D1 KO mice compared to heterozygote mice. This difference between heterozygote and D1 KO mice was not observed in Miner et al. (1995). This suggests that there may be some contribution of D1 receptors to cocaine conditioned place preference, but the absence of D1 receptors does not critically impair reward learning. However, the locomotor response to cocaine does appear to be strongly impacted by D1 receptor loss. There was no clear correlation between cocaine-induced locomotor behavior and cocaine conditioned place preference indicating a dissociation between the locomotor activating effects of cocaine from the rewarding aspects of cocaine. Cocaine conditioned place preference can be retained within dopamine deficient mice through serotonergic mechanisms (Hnasko et al., 2007), so there may be similar compensatory mechanisms that maintain reward learning in D1 KO mice. There may also be changes within the dopamine system following D1 knockout, such

as increased expression of D5 receptors to maintain normal behaviors. The examination of these compensatory changes following D1 receptor deletion is beyond the scope of this dissertation, but one possible reason for the discrepancies between the studies presented here and previous literature could be different mechanisms of compensating for the loss of D1 receptors between different D1 KO lines.

The increased freezing behavior of female mice during contextual extinction observed in Experiment 16 suggests that there could be some interaction between D1 receptor signaling and sex, although there was no genotype effect observed in contextual extinction. Rey et al. (2014) demonstrated that during low-estrogen phases in female mice, D1/5 receptor activation enhances fear extinction, whereas during high-estrogen phases, D1/5 receptor activation impairs fear extinction. These data suggest that there is a relationship between the estrous cycle and D1 receptor activation, but the current experiments did not measure estrous cycles during experiments to assess this possibility. However, the finding that D1/5 receptor activation enhances fear extinction in low estrogen females corresponds with findings that D1/5 receptor activation enhances extinction in male mice (Chapter 3).

In Chapter 3, I consistently observed that D1/5 receptor activation enhances fear extinction. However, in Experiment 18, there was no effect of SKF 81297 administration following contextual fear extinction in any genotype. This may be an issue of statistical power, as there were relatively few numbers of subjects used in these studies, although previous studies had found effects with

similar numbers of animals. Independently analyzing male and female mice within Experiment 18 is not possible because mice were balanced within genotype, and separating the groups by sex leads to imbalances in average fear responses during extinction prior to drug administration. Because of these caveats, this experiment does not conclusively demonstrate whether SKF 81297 mediated enhancements in extinction are specific to D1 or D5 receptors.

The overarching theoretical question from the experiments in this chapter is whether the loss of phasic dopamine signals through D1 receptors can impair learning. From the studies presented in this chapter, it appears that D1 receptor loss enhances fear acquisition, but does not impact conditioned place preference. Together, Chapters 3 and 4 assess the impact of tonic dopamine D1/5 receptor activation and phasic signaling in the network of dopamine terminal regions. The following chapter continues this investigation by examining specific brain regions within the dopamine circuit and intracellular signaling pathways that may be involved in fear extinction.

Chapter 5: Neural Substrates of D1/5 Receptor Mediated Enhancement of Fear Extinction

Introduction

Intracellular Signaling Cascades Involved in Fear Extinction Enhancement

Chapters 3 and 4 examined the effect of manipulating global activity of D1/5 receptors on fear and reward learning. However, the systemic approach is limited in its ability to describe particular neural substrates that may contribute to fear extinction enhancement. Specifying the intracellular signaling cascades induced by SKF 81297 is challenging because SKF 81297 can have downstream effects through multiple cascades, including the protein kinase A (PKA) pathway, and phospholipase C (PLC)-mediated calcium signaling (Lee et al. 2004). To distinguish the intracellular signaling cascades involved in the enhancement of fear extinction via D1/5 receptor activation, I utilized two functionally selective D1/5 receptor agonists in this chapter. As previously discussed, the biased agonist SKF 83822 selectively targets protein kinase A (PKA) -coupled D1/5 receptors and the biased agonist SKF 83959 selectively targets phospholipase C (PLC) -coupled D1/5 receptors (Fig. 20). There are distinct patterns of expression across the brain for PLC-coupled and PKA-coupled D1/5 receptors, and the differing actions of SKF 83959 and SKF 83822 could provide some insight into which brain regions are modulating the observed enhancement of extinction. For example, the hippocampus and amygdala appear to primarily contain PLC-coupled D1/5 receptors (Leonard et al., 2003; Jin et al., 2001), whereas the prefrontal cortex and striatum express both PLC- and PKA-coupled D1/5

receptors (Nishi et al., 2011). This suggests that there may be distinct networks of D1/5 receptor expressing brain regions that could be activated by SKF 83959 or SKF 83822. Comparing the effects of these two agonists specifies whether PKA or PLC coupled D1/5 receptor activation leads to extinction enhancements, as well as indirectly providing information about brain regions that mediate the enhancement of extinction. A more direct approach is also considered in this chapter through the use of targeted microinjections of SKF 81297 into brain regions associated with fear extinction.

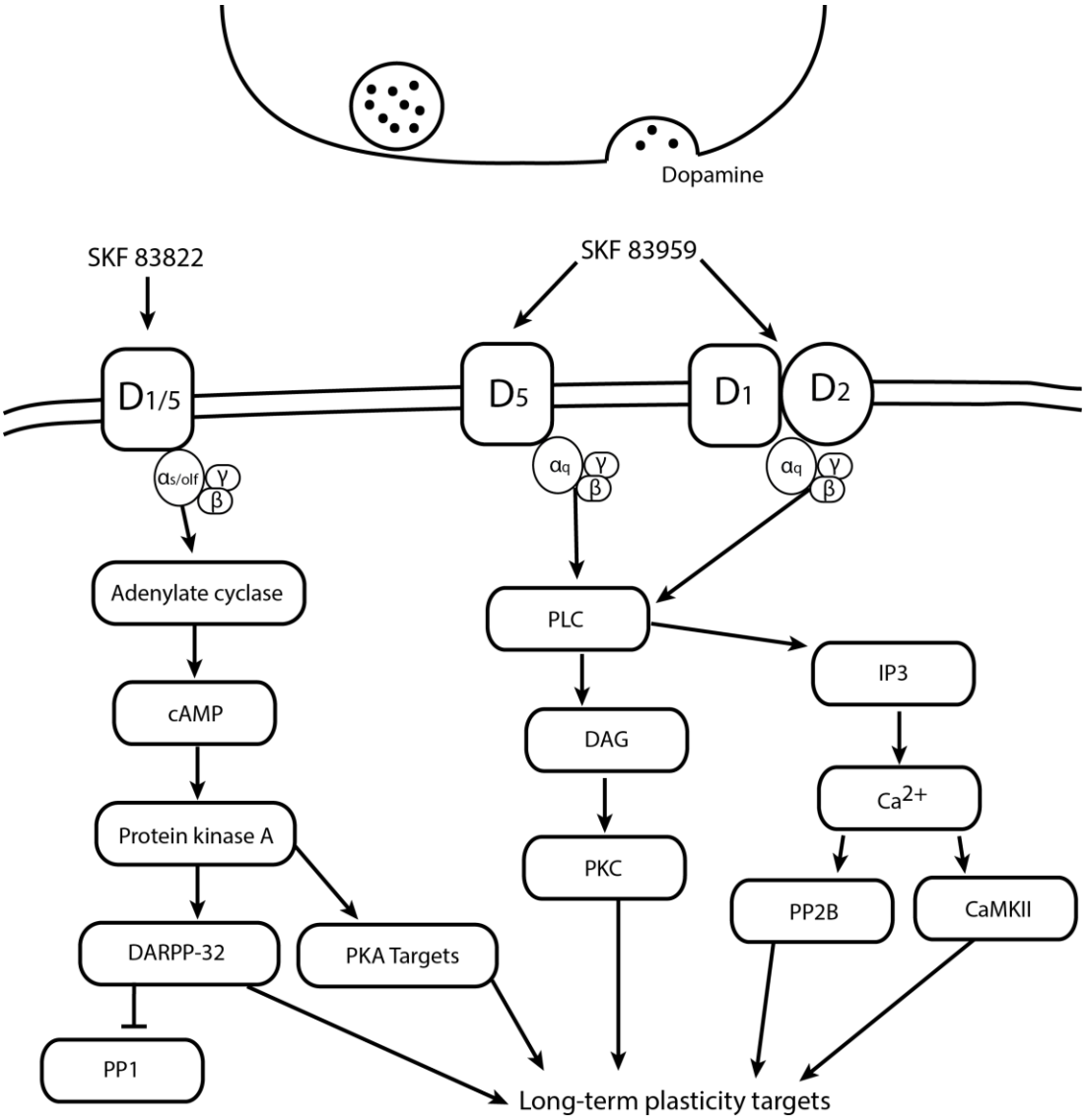


Figure 20. Dopamine receptor signaling pathways. Activation of Gαs/olf proteins coupled to D1-like receptors with SKF 83822 stimulates adenylate cyclase (Beaulieu and Gainetdinov, 2011). Adenylate cyclase induces production of cyclic adenosine monophosphate (cAMP), leading to activation of protein kinase A (PKA). PKA activates cellular signaling cascades necessary for long-term plasticity. PKA also induces phosphorylation of dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32), which inhibits protein phosphatase 1 (PP1). Inhibitory interactions between DARPP-32 and PP1 regulate neural plasticity through extracellular signal-regulated kinase (ERK) pathways (Valjent et al., 2005). The phospholipase C pathway can be activated with SKF 83959 by G proteins from D5 receptors, or G proteins from D1-D2 heteromers. These convergent pathways regulate the PLC-mediated cleavage of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG). DAG activity regulates protein kinase C, while IP3 binding to IP3 receptors in the endoplasmic reticulum increases intracellular calcium (Ca2+) levels. Increased Ca2+ levels lead to activation of protein phosphatase 2B (PP2B) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) signaling cascades that have been identified as regulators of long-term plasticity (Colbran, 2004). Expression of these different dopamine receptor types varies across neuronal populations innervated by dopamine neurons. (Adapted from Abraham, Neve, & Lattal, 2014).

Region specific modulation of extinction with SKF 81297

As many of the effects reported in Chapter 3 utilized post-session injections, the second goal of this chapter is to identify brain regions where post-session D1/5 receptor activation could lead to enhancement of fear extinction consolidation. One particular area of interest for this question is the infralimbic region of the prefrontal cortex (ILPFC), based on previous studies indicating that the ILPFC regulates consolidation of fear extinction (Quirk and Mueller, 2008). Hikind and Maroun (2008) demonstrated that post-session administration of a D1/5 receptor antagonist into the ILPFC impairs extinction consolidation. In addition to the prefrontal cortex, there are several other brain regions where D1/5 receptor activity is necessary for extinction learning. Blockade of D1/5 receptors in the basolateral amygdala (pre-session, but not post-session), nucleus accumbens (pre-session) or dorsal hippocampus (post-session) all lead to impairments in extinction (Hikind and Maroun, 2008; Holtzmann-Assif et al., 2010; Fiorenza, et al. 2012). Each of these regions controls different elements of learning in fear extinction, as discussed in the introduction. Determining whether activation of any of these regions could lead to fear extinction enhancement would specify the particular elements that lead to decreased fear responses.

Based on the theoretical circuit presented in the introduction (Fig. 1), there are several possibilities for processes that may be enhanced by targeted microinjections of SKF 81297. Activation of D1/5 receptors in the ILPFC could directly modulate signaling cascades that maintain inhibitory control of fear responses. In the dorsal hippocampus, D1/5 receptor activation could strengthen

or weaken the contextual elements involved in extinction learning and allow for decreased fear expression when tested later. Within the basolateral amygdala, activation of D1/5 receptors may alter retrieval of the original CS-US association or enhance CS-No US representations. In the nucleus accumbens, activating D1/5 receptors may strengthen prediction error signals associated with the unexpected absence of shock. There are clear theoretical reasons to examine each region of the fear extinction network, but only one study has shown a targeted effect of D1/5 receptor activation on fear extinction.

Fiorenza, et al. (2012) demonstrated that post-session SKF 38393 administration into the dorsal hippocampus, but not ventromedial prefrontal cortex (i.e., ILPFC), leads to decreased fear expression on the following test day. As SKF 38393 is characterized as a partial D1/5 receptor agonist (Andersen and Jansen, 1990), I was interested in examining the effect of a full D1/5 receptor agonist on fear extinction in the dorsal hippocampus and ILPFC, as well as the basolateral amygdala and nucleus accumbens core. To match the timing of drug administration in Chapter 3, this chapter examines the effect of post-extinction microinjection of SKF 81297. This approach allows for an examination of the individual contributions of these regions to fear extinction that may underlie the effects of systemic post-session SKF 81297.

Materials and Methods

Subjects

Male C57BL/6 mice (n = 181) ranging from 7 to 11 wk of age (28 ± 5 g) were used in these experiments. Animals were purchased from Jackson

Laboratory (Bar Harbor, ME) and given at least 7 d in the vivarium prior to experimental use. Animals were housed four to a cage. Polycarbonate cages were held in a Thoren rack, and animals were given access to food and water ad libitum. Vivarium and experiment room temperatures were maintained at 22°C ± 1°C, and subjects were maintained on a 12-h light–dark cycle (lights on 0600 h–1800 h). Animals were moved from the vivarium to the experiment room 60 min before the start of an experiment, and experiments were conducted between 900 and 1700 h. All experimental procedures were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985).

Drugs & Cannulation

SKF 83822 and SKF 83959 (Tocris) were dissolved in 10 % DMSO (dimethyl sulfoxide in distilled water) and administered subcutaneously. Drug dosing for SKF 83822 (0.1, 1, 2 mg/kg) and SKF 83959 (0.1, 1, 10 mg/kg) were estimated based on doses used by O’Sullivan et al. (2008). The 10 mg/kg dose used in SKF 83959 experiments has not been previously tested, but the dose was used to assess whether a high dose of SKF 83959 could impact fear extinction. For microinjection studies, SKF 81297 (Tocris) was dissolved in saline (0.9% NaCl) at concentrations of 0.1, 0.25, and 0.3 µg in 0.25 µL per side. Drug doses were similar to those used in the prefrontal cortex of male Sprague-Dawley rats by Sorg et al. (2001). Gentle heating was used to dissolve SKF 81297 mg/kg in saline. Cannulation surgery occurred as described in Raybuck

and Lattal (2011). Thirty minutes prior to surgery, mice received an intraperitoneal injection of 5 mg/kg Rimadyl (Pfizer) to facilitate post-operative recovery. Mice were anesthetized with isoflurane (2%-5%), and mounted in stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Scalp was scrubbed, then excised to expose the skull. Small holes were drilled for anterior (A/P) and lateral (M/L) coordinates then guide cannulae were lowered into place with the stereotaxic to match dorsal/ventral (D/V) coordinates. Guide cannulae were secured to the skull with dental cement (Ketac Cem Aplicap, 3M ESPE).

For cannulations in the infralimbic region of the prefrontal cortex (ILPFC), an angled injection was used to prevent disruption of the prelimbic region. A unilateral injection was used to minimize damage to the prefrontal cortex during infusions. Coordinates for microinjections describe the location of the guide cannulae, and the length of the injector cannulae projection past the guide cannulae indicates the injection site. Mice were mounted on the stereotax, and rotated 30 degrees following leveling of the skull. For ILPFC microinjections, a hole was drilled at 1.7 mm A/P, 1.67 mm M/L, and a 7.0 mm, 26 gauge (ga) stainless steel guide cannula (Small Parts, Inc.) was lowered -1.43 mm D/V. Injector cannulae (32 ga) extended 1.0 mm below the cannulae (Stafford et al., 2012). For dorsal hippocampal injections, 26 ga cannulae guides (Plastics One, Inc., Roanoke, VA) were used (-1.7 mm A/P, ± 1.5 mm M/L). Guide cannula projected -1.5 mm D/V, and 33 ga cannulae projected .5 mm past the tip of the guide. For basolateral amygdala injections, 7 mm 26 ga cannulae were made from stainless steel tubing (Small Parts, Inc.) targeted at -1.46 mm A/P and ± 3.1

mm M/L. Guide cannulae was placed -1.8 mm D/V with injector cannulae (32 ga) projecting 4 mm past guide cannulae (Bolkan and Lattal, 2014). For nucleus accumbens core injections, 10 mm 26 ga stainless steel guide cannulae were aimed at 1.40 mm A/P, ± 1.26 mm M/L and -3.2 mm D/V. Injector cannulae (32 ga) extended 1 mm below guide cannulae for injection (Gremel and Cunningham, 2009).

Drug was administered unilaterally in the infralimbic region of the prefrontal cortex, and bilaterally in the dorsal hippocampus, nucleus accumbens, and basolateral amygdala. Drug was infused over 1 min at a rate of 0.25 µL per min. Injection cannulae were left in place for 30 s following infusion to ensure complete diffusion from injector tip.

Histology

After completion of experiments, mice were euthanized with carbon dioxide then brains were removed and post-fixed in 4% paraformaldehyde in a laboratory refrigerator (10° C). Brains were sectioned (60 µm) on a cryostat and mounted on gelatin-coated slides. Slides were stained with cresyl violet and cannulae tracks were evaluated under a light microscope for correct placements.

Apparatus

Chapter 3 describes the contextual fear conditioning apparatus.

Behavioral procedures

Contextual fear conditioning

On Day 1, subjects received a 12-min exposure to the context with four unsignaled shocks (0.35 mA, 2s), delivered at 2.5, 5, 9, and 11.5 min. In all

experiments, groups were matched following acquisition to ensure equal terminal freezing levels across dose assignments. On Day 2, mice received a 12-min nonreinforced exposure to the context (Ext). In all post-extinction injection experiments, groups were matched following extinction to ensure equal levels of terminal freezing before drug administration. On Test Day, mice received a 12-min nonreinforced exposure to the context.

Experiment 19: Effect of SKF 83959, a PLC-coupled D1/5 receptor biased agonist, on contextual fear extinction.

This experiment tests whether activation of PLC-coupled D1/5 receptors enhances fear extinction consolidation. The procedure matched Experiment 7a in Chapter 3. On all days of conditioning and testing, animals received post-session injections. On Day 1, animals (n = 8 per group) received an acquisition session with administration of vehicle. On Day 2, animals received either SKF 83959 (0.1, 1, or 10 mg/kg) or vehicle immediately following extinction (Ext in Fig. 21). On Day 3, animals were returned to the context for a 12-min nonreinforced test session (Test Day).

Experiment 20: Effect of SKF 83822, a PKA-coupled D1/5 receptor biased agonist, on contextual fear extinction.

This experiment tests whether activation of PKA-coupled D1/5 receptors enhances fear extinction consolidation. On all days of conditioning and testing, animals received post-session injections. On Day 1, animals (n = 8 per group) received an acquisition session with administration of vehicle (10 % DMSO). On Day 2, animals received either SKF 83822 (0.1, 1, or 2 mg/kg) or vehicle

immediately following extinction (Ext in Fig. 22). On Day 3, animals were returned to the context for a 12-min nonreinforced test session (Test Day).

Experiment 21: Targeted microinjections of SKF 81297 following contextual fear extinction.

This experiment tests the effect of brain region specific activation of D1/5 receptors on fear extinction (Fig. 23). In all animals, microinjections occurred immediately following fear extinction. For unilateral injections into the infralimbic region of the prefrontal cortex, mice received either saline (n = 16) or 0.1 µg/ 0.25 µL SKF 81297 (n =15) following fear extinction. Following verification of unilateral placements, saline animals (n = 9) and 0.1 µg/ .25 µL SKF 81297 (n = 7) animals were compared for freezing during Acquisition, Extinction and Test Day.

In animals receiving microinjections into the nucleus accumbens core, the concentration of SKF 81297 was matched to the concentration of systemically injected SKF 81297. This led to a concentration of 0.25 µg/ 0.25 µL. Although dosing between systemic administration and intracranial administration may be difficult to compare, similar doses (0.30 µg) have been used by Sorg et al. (2001) in the prefrontal cortex of Sprague-Dawley rats. Animals were matched during extinction and received either saline (n = 10) or 0.25 µg/ 0.25 µL SKF 81297 (n = 11). Bilateral placements were verified and saline-treated animals (n = 9) were compared to SKF 81297-treated animals (n = 7) for fear responding during each day. Unilateral placements were verified for 2 SKF 81297-treated animals and 1 saline-treated mouse. Animals showing bilateral cannula marks were separately analyzed, then pooled with animals showing unilateral cannula marks for analysis

in nucleus accumbens core, dorsal hippocampus, and basolateral amygdala studies shown in Figure 23.

In animals receiving microinjections into the dorsal hippocampus or basolateral amygdala, the concentration of SKF 81297 was 0.30 µg/ 0.25 µL. The high dose was used to determine whether there could be any impact of SKF 81297 on fear responding. In the basolateral amygdala study, there were n = 8 saline-treated and n = 8 SKF 81297-treated mice prior to placement verification. Following verification of bilateral placements, there were n = 6 saline-treated animals and n = 2 SKF 81297-treated animals that were compared for fear responses on each day. There were n = 4 SKF 81297-treated mice and n = 1 saline-treated mouse that showed unilateral placements in the basolateral amygdala. In the dorsal hippocampus study, there were n = 17 saline-treated mice and n = 18 SKF 81297-treated mice. Following verification of bilateral placements, there were n = 9 saline-treated animals and n = 12 SKF 81297-treated animals that were compared for fear responding on each day. There were n = 4 saline-treated animals and n = 4 SKF 81297-treated animals showing unilateral placements in the dorsal hippocampus study.

Data Analysis

Fear memory expression was determined by freezing response within the context. Freezing was defined as an episode of at least 3 sec of inactivity. Total freezing time was divided by 12 min to calculate percentage of time freezing in each day. Data analyses were performed with Prism 6. Data during Extinction

and Test Day were analyzed with a one-way between subjects ANOVA. All post hoc comparisons of ANOVA data were performed using a Dunnett's test.

Results

Experiment 19: Post-extinction administration of a PLC-coupled D1/5 receptor agonist, SKF 83959, does not affect fear extinction.

This experiment tests whether activation of PLC-coupled D1/5 receptors can enhance the extinction of fear. Animals were matched for freezing during acquisition and extinction. Figure 21 shows that there were no differences in freezing during Extinction or Test Day between drug-treated or vehicle-treated mice. Statistical analysis of Test Day showed no difference between drug-treated and vehicle-treated mice ($F(3,28) = 0.8069$, $p = 0.5007$). This experiment indicates that activation of PLC-coupled D1/5 receptors is not sufficient to enhance extinction.

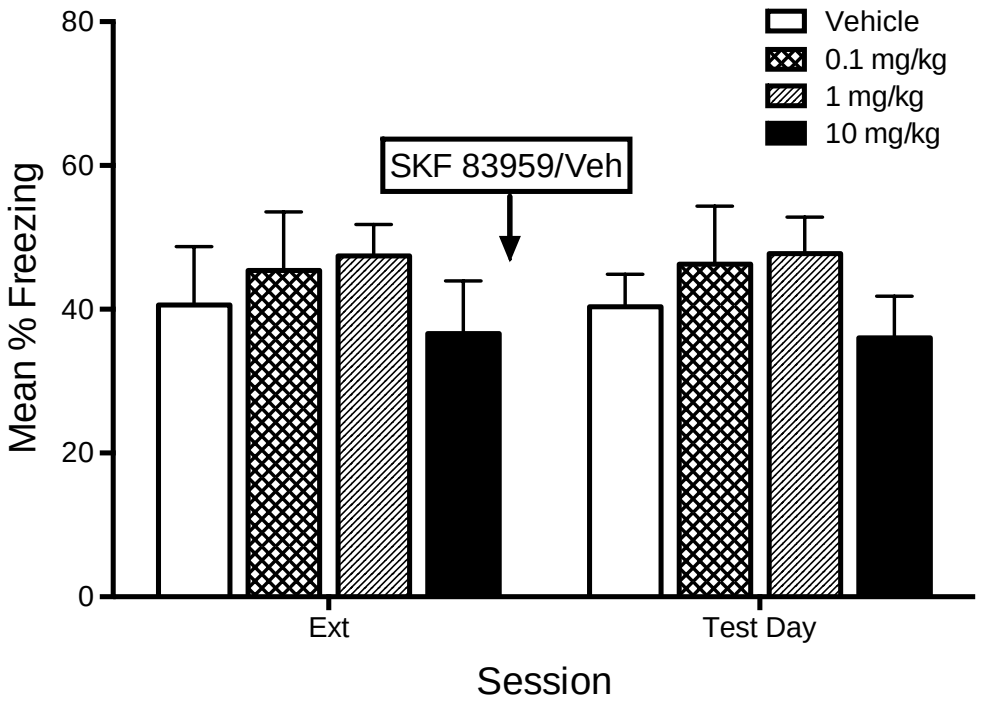


Figure 21. Post-extinction SKF 83959 (PLC coupled-D1/5 receptor biased agonist) has no effect on extinction retention. (Experiment 19). Mice that received SKF 83959 immediately following extinction showed no difference in freezing compared to vehicle-treated mice during Test Day. Error bars indicate SEM.

Experiment 20: Post-extinction administration of a PKA-coupled D1/5 receptor agonist, SKF 83822, enhances fear extinction.

This experiment tests whether activation of PKA-coupled D1/5 receptors can enhance the extinction of fear. Animals were matched for freezing during acquisition and extinction. Figure 22 shows that there were no differences in freezing during Extinction, but during Test Day, mice that had received SKF 83822 decreased fear responding compared to vehicle-treated mice. Statistical analysis of Test Day showed a significant effect of drug treatment ($F(3,28) = 4.857$, $p = 0.0076$). Post-hoc analysis showed that mice receiving 1 mg/kg SKF 83822 were significantly different from vehicle-treated mice ($p = 0.0397$). This

experiment demonstrates that fear extinction can be enhanced by activation of PKA-coupled D1/5 receptors.

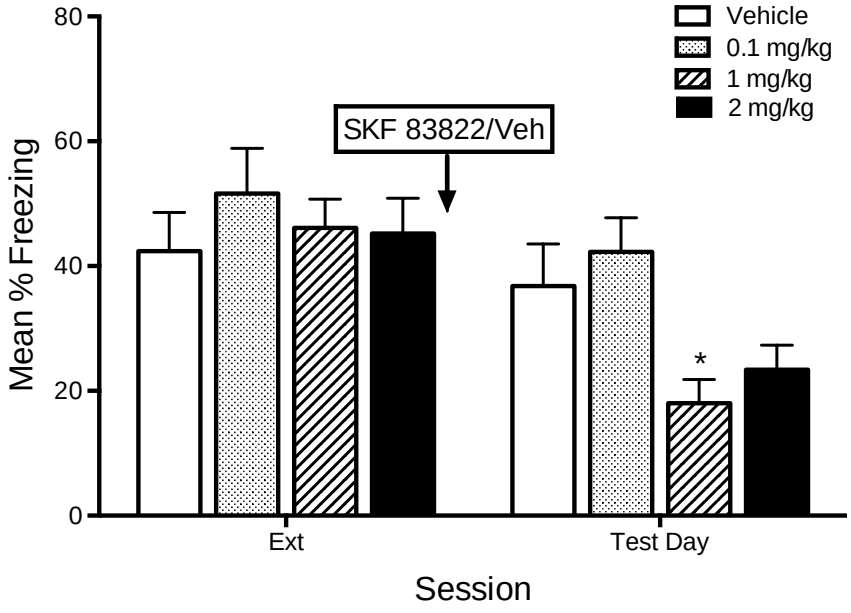


Figure 22. Post-extinction SKF 83822 (PKA coupled-D1/5 receptor biased agonist) decrease freezing. (Experiment 20). Mice that received SKF 83822 (1 mg/kg) immediately following extinction showed a significant difference in freezing compared to vehicle-treated mice during Test Day. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with vehicle (Dunnett's test).

Experiment 21: Targeted microinjections of SKF 81297 following contextual fear extinction has no effect on fear responses.

This experiment examines the effect of SKF 81297 microinjection into the infralimbic region of the prefrontal cortex (ILPFC), nucleus accumbens core (NAcc), basolateral amygdala (BLA), or dorsal hippocampus (DH). All animals were matched for freezing during acquisition and extinction. Statistical analysis verified that there were no differences in acquisition or extinction in saline-treated animals compared to SKF 81297-treated animals within ILPFC (Fig. 23A), NAcc (Fig. 23B), DH (Fig. 23C), or BLA (Fig. 23D) group with bilateral and unilateral placements pooled. Independent analysis of bilateral placements in Nacc, DH

and BLA showed no differences between saline- and SKF 81297-treated mice during acquisition or extinction. Due to the low number of unilateral placements, the effect of unilateral injections in Nacc and BLA was not independently assessed. Figure 23 shows that there were no significant differences between saline- or drug-treated animals during Test Day in any of the brain regions tested with bilateral and unilateral placements pooled. There was a non-significant trend ($t(27) = 1.927$, $p = .0645$) towards increased freezing in mice treated with SKF 81297 in the dorsal hippocampus (Test Day in Fig. 23C). This effect was not observed when mice showing unilateral cannula marks were excluded from the statistical analysis. Statistical analysis of bilateral hippocampal injections showed no significant effect ($t(19) = 1.640$, $p = 0.1175$) between groups. There was no effect of unilateral hippocampal injections when analyzed independently. There was no effect of bilateral microinjection of SKF 81297 in the basolateral amygdala or nucleus accumbens compared to saline-treated animals during Test Day. Cannula placements are shown in Appendix C. Together, these experiments show that activation of D1/5 receptors within the brain regions tested may not be sufficient to enhance extinction.

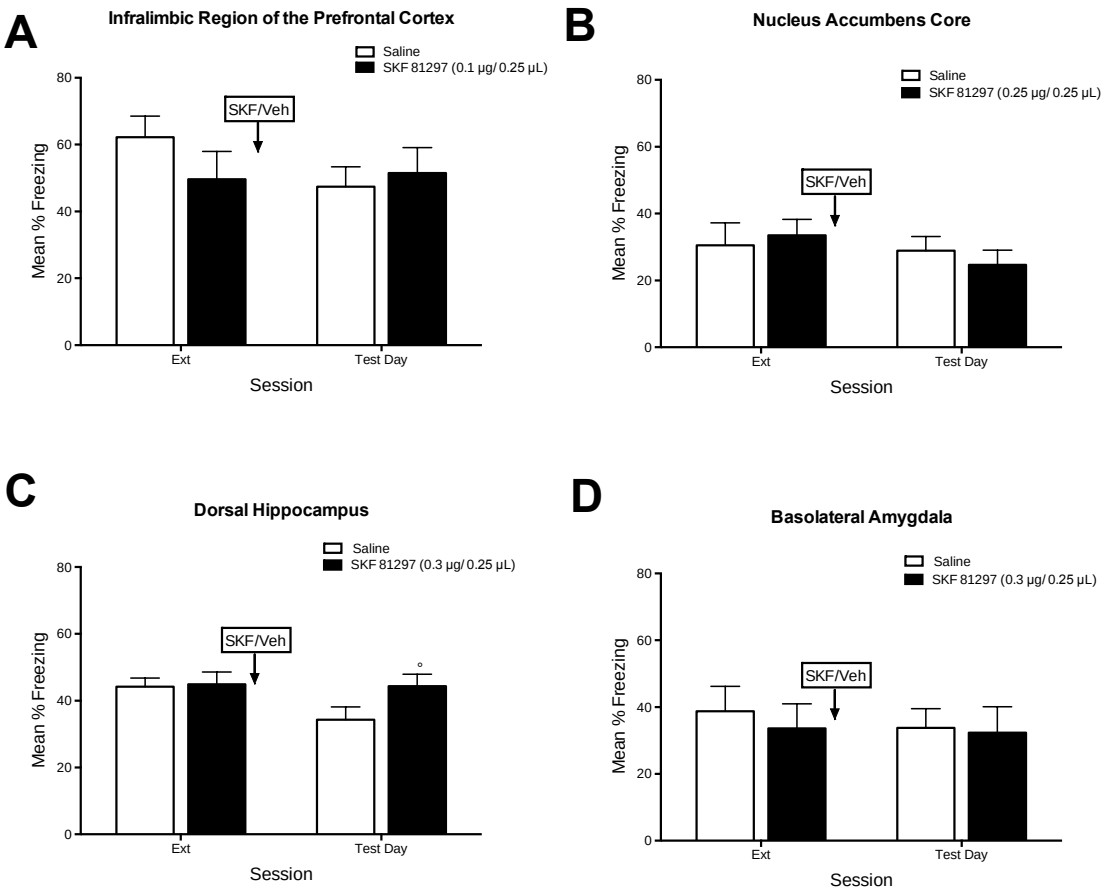


Figure 23. Post-extinction microinjection of SKF 81297 has no effect on extinction retention. (Experiment 21). Microinjection of SKF 81297 into the infralimbic region of the prefrontal cortex (Panel A), nucleus accumbens core (Panel B), dorsal hippocampus (Panel C), or basolateral amygdala (Panel D) has no effect on fear expression during Test Day. Data presented includes animals with unilateral tracks in panels B, C, and D. There was a non-significant trend ($p = 0.0645$) in panel C (°) towards increased freezing in mice treated with SKF 81297 in the dorsal hippocampus, but the effect was not observed when animals with confirmed bilateral cannula tracks were analyzed separately. Error bars indicate SEM.

Discussion

The experiments in this chapter examine the neural substrates that may underlie the systemic D1/5 agonist mediated extinction enhancement observed in Chapter 3. Experiment 21 examines the effect of targeted microinjections of SKF 81297 into the infralimbic region of the prefrontal cortex (ILPFC), dorsal

hippocampus (DH), nucleus accumbens core (Nacc core), and basolateral amygdala (BLA), and finds no effect of D1/5 receptor activation within a particular brain region on retention of fear extinction. One possibility for the lack of an effect on fear extinction in Experiment 21 is that activation of dopamine receptors in other regions, such as the prelimbic region of the prefrontal cortex, dorsal striatum, substantia nigra or ventral tegmental area could replicate the extinction enhancement effect. Technical limitations prevented the infusion of SKF 81297 into the nucleus accumbens shell in mice, as infusions would be likely to spread into nucleus accumbens core. While these regions may be involved in fear extinction, most examinations (e.g. Hikind and Maroun, 2008) of the role of dopamine signaling in fear extinction have focused on the regions tested in Experiment 21. An additional concern is that there may be disruptions in extinction induced by cannula placement and microinjection (Groblewski and Cunningham, 2012), however Experiment 21 only used one microinjection, compared to several microinjections used in Groblewski and Cunningham (2012). These issues may have prevented the detection of an extinction enhancement effect in Experiment 21.

Fiorenza et al. (2012) demonstrated that D1/5 receptor activation in the dorsal hippocampus with a partial agonist (SKF 38393) can lead to fear extinction enhancements, but this effect was not observed in my experiments. There is no clear explanation for this discrepancy, as both studies examined contextual fear extinction, but the differing affinities of dopamine agonist drugs (Andersen and Jansen, 1990) may contribute to the inconsistencies between my study and

Fiorenza et al. (2012). Additionally, Fiorenza et al. (2012) used a weaker conditioning session (2 shocks) and a longer extinction session (20 min) than my experiments, which may lead to a different pattern of brain activity that is acted upon by a post-session D1/5 receptor agonist in the dorsal hippocampus. Similar to the studies here, Fiorenza et al. (2012) found no effect of SKF 38393 administration into the ventromedial prefrontal cortex or basolateral amygdala.

The lack of an effect in the prefrontal cortex is surprising given the evidence that indicates that D1/5 receptors are required for consolidation of extinction (Hikind and Maroun, 2008). However, many studies have shown that the prefrontal cortex is sensitive to dopamine concentration fluctuations, and supranormal levels of dopamine receptor activation may have disruptive effects on learning and memory (Arnsten, 2011). There was no evidence of disrupted extinction in Experiment 21 when comparing saline and drug-treated animals, indicating that the concentration of SKF 81297 did not impair extinction. In addition to sensitivity to dopamine concentration, the prefrontal cortex, as well as many other regions receiving dopamine input, have reciprocal inputs to dopamine neurons, which allows fine-tuning of dopamine neuron activation or inactivation. For example, activation of ILPFC neurons with NMDA leads to inhibition of ventral tegmental area (VTA) dopamine neuron spontaneous activity, whereas inactivation of the ILPFC with tetrodotoxin leads to increased spontaneous activity within VTA dopamine neurons (Patton et al., 2013). These mechanisms of reciprocal signaling could lead to a pattern of activity along a network of brain regions that would oppose the specific activity increases within

one particular dopamine terminal region. However, increasing the activity of the whole network of dopamine terminal regions or in a few regions may overcome the inhibition that would be generated by one feedback loop.

In agreement with this hypothesis, Fadok et al. (2010) observed that restoration of dopamine signaling in dopamine-deficient mice within the basolateral amygdala and nucleus accumbens together was required for restoration of long-term memory of fear-potentiated startle. When dopamine signaling was restored in the basolateral amygdala, there was a brief effect on short-term memory (within ten min of training) but the effect had dissipated by the following day. When dopamine signaling was restored in the nucleus accumbens alone, there was no effect. However, when dopamine signaling in the nucleus accumbens and basolateral amygdala was restored together, there was an enhancement of long-term memory (tested 24 hours later). Fadok et al. (2010) indicates that multiple VTA dopamine targets may have to coordinate activity to observe enhanced long term-memory. This suggestion was indirectly tested in this chapter through the use of SKF 83822 and SKF 83959.

The contrasting effects of SKF 83822 and SKF 83959 can be informative for both intracellular signaling pathways and networks of dopamine terminal regions that could be involved in fear extinction enhancement. At the intracellular level, Experiments 19 and 20 demonstrate that activating PKA-coupled, but not PLC-coupled D1/5 receptors can enhance fear extinction. Global inhibition of PKA activity has been shown to enhance extinction (Isiegas et al., 2006), but in Experiment 20, increased activation of PKA-coupled D1/5 receptors led to

enhanced extinction. This suggests that a majority of PKA-coupled metabotropic receptors are involved in the increased expression of fear, but D1/5 receptor coupled PKA activity may act within particular neurons groups that maintain or strengthen extinction learning. Jin et al. (2001) reported that cortical areas show high expression of Gαs proteins (associated with PKA signaling) that are responsive to dopamine, whereas the hippocampus express primarily dopamine receptors coupled to Gαq (associated with PLC signaling) proteins. Similarly, Leonard et al. (2003) demonstrated that D1-receptor signaling in the amygdala is not linked with adenylate cyclase (and PKA) pathways.

The distinct patterns of PKA vs. PLC coupled D1/5 receptor expression (Nishi et al., 2011) indicate that SKF 83822 (PKA-coupled D1/5R agonist) would activate portions of the cortex and striatum, whereas SKF 83959 (PLC-coupled D1/5R agonist) may primarily activate the amygdala and hippocampus. Previous behavioral evidence also supports this suggestion, as SKF 83959 acts similar to a D1/5 receptor antagonist when administered into the prefrontal cortex, inhibiting SKF 81297 mediated locomotor behaviors (Cools et al., 2002). Thus, it appears that SKF 83822 may specifically engage the cortical networks involved in fear extinction through activation of PKA-coupled D1/5 receptors, whereas SKF 83959 does not directly impact fear extinction.

One caveat in interpreting the effects of SKF 83959 compared to SKF 83822 comes from Chun et al. (2013), who examined the contribution of D1-D2 heteromers to the PLC-mediated intracellular calcium increases observed with SKF 83959. Chun et al. (2013) showed that SKF 83959 would elicit intracellular

calcium increases in D1 and D2 co-expressing HEK (Human Embryonic Kidney) 293 cells with high levels Gαq proteins present, without the presence of a D1-D2 heteromer. Although the D1-D2 heteromer has been considered the site of SKF 83959 action, the Chun et al. (2013) study suggests that SKF 83959 may alter intracellular calcium mobilization through other dopamine receptor mechanisms. Additionally, Chun et al. (2013) indicated that SKF 83959 has high affinity for a number of other G-protein coupled receptors, such as the 5HT2C serotonin receptor and the α2C adrenergic receptor, and the serotonin transporter. These issues could impact the interpretation of comparing SKF 83822 and SKF 83959 as reciprocal biased D1/5 receptor agonists, although a majority of evidence supports the characterization of SKF 83822 as a PKA-coupled D1/5 receptor agonist and SKF 83959 as a PLC-coupled D1/5 receptor agonist (O'Sullivan et al. 2008). Future studies could examine the signaling mechanisms underlying SKF 83959-mediated increases in calcium signaling in neurons and test whether PLC-coupled D1/5 receptors are involved in other aspects of fear learning, such as fear conditioning. Distinguishing the learning supported by these two different biased agonists could help clarify some of the inconsistencies that have been reported through analysis of D1/5 receptor signaling in fear learning.

One such inconsistency, as described previously, is the difference between Fiorenza et al. (2012) and the null effect in dorsal hippocampus injections with SKF 81297 in Experiment 21. Although SKF 38393 is described as a partial agonist, this classification has arisen from findings that SKF 38393 stimulates cyclic AMP activity at lower levels than dopamine in striatal cells

(Andersen and Jansen, 1990). However, Jin et al. (2001) showed that SKF 38393 primarily binds to Gαs in the frontal cortex and Gαq in the hippocampus, suggesting that SKF 38393 is likely to stimulate both PKA and PLC-coupled dopamine D1/5 receptors in a complex interplay. It is possible that many dopamine receptor agonists show differential selectivity between these two signaling pathways, leading to particular patterns of brain activation that may support or inhibit the learning of interest. The following general discussion aims to specify the components of learning that are modulated by D1/5 receptor activation based on the results presented in Experiments 1-21.

Chapter 6: General Discussion

Overview

The overarching goal of this dissertation was to assess the role of dopamine D1/5 receptor signaling in fear extinction. To this end, I studied different aspects of fear learning that may be impacted by activation or deletion of dopamine D1/5 receptors. As dopamine is often studied within the context of reward learning, my experiments also included examination of reward learning and extinction to allow for a broader understanding of the particular elements of learning that are modulated by dopamine D1/5 receptors. Finally, I investigated the neural substrates that are involved in D1/5 receptor modulation of extinction. Together, these studies aim to clarify how D1/5 receptor activation impacts associative learning.

Summary of Major Findings

In Chapter 2, I examined the impact of methylphenidate, a dopamine and norepinephrine transport blocker, on contextual fear extinction and fear conditioning. I found that pre- and post-session methylphenidate enhances fear extinction (Experiment 1 & 2), but only under certain conditions. The effect was not present when methylphenidate was administered four hours following an extinction experience (Experiment 3) and could not enhance extinction if the animal did not strongly extinguish within a session (Experiment 4). Additionally, there was no effect on fear conditioning (Experiment 5), indicating that the enhancement was particular to extinction, rather than broadly enhancing all learning. As methylphenidate is thought to act in part through dopamine D1/5

receptors, I examined whether activation of D1/5 receptors would generate a similar effect.

In Chapter 3, I found that SKF 81297, a D1/5 receptor agonist, would enhance fear extinction. When given prior to or following an extinction session, SKF 81297 generally enhanced contextual and cued fear extinction (Experiments 6-9, 12). Unlike methylphenidate, SKF 81297 impaired fear conditioning (Experiments 10 & 11). Although SKF 81297 can have some locomotor effects, the learning effects do not appear to be driven by simple behavioral activation (Experiment 7). Renewal of fear was also reduced by SKF 81297, indicating that the enhancement of cued fear extinction is context-independent. Long-term retention of fear was not affected by SKF 81297 (Experiment 12). While these studies strongly implicate D1/5 receptor activation in enhancing fear extinction, the mechanism that drives this learning may be driven in part by the rewarding effects of SKF 81297. To assess this hypothesis, I examined the effect of SKF 81297 on reward learning. I found that pre-session, but not post-session SKF 81297 induced conditioned place preference (Experiment 13) and post-session SKF 81297 enhanced extinction of cocaine conditioned place preference in high-preference animals (Experiment 14). By examining aversive and reward learning in parallel, these experiments provide a broader understanding of how D1/5 receptor activation may impact associative learning.

In Chapter 4, I utilized D1 receptor knockout mice to examine whether D1 receptors were necessary for fear or reward learning. In contrast to previous studies, I found that D1 receptor knockout mice show enhanced fear acquisition

and decreased fear renewal (Experiment 16). D1 receptor knockout mice showed normal cocaine conditioned place preference (Experiment 17).

Chapter 5 provides evidence that PKA- (and not PLC-) coupled D1/5 receptors are responsible for the observed fear extinction enhancements (Experiments 19 & 20). Activation of D1/5 receptors within the brain regions tested was not sufficient to enhance fear extinction in Experiment 21. In total, these experiments provide strong evidence that D1/5 receptor activation can enhance extinction learning. Examining the behavioral theories associated with dopamine may help to clarify the exact features of learning that are modulated by D1/5 receptors.

Dopamine Signaling and Prediction Error

A long held assumption about dopamine signaling arising from the ventral tegmental area has been that release of dopamine in mesocorticolimbic regions is synonymous with reward, but many studies have demonstrated that dopamine neurons are particularly sensitive to reward-related contingencies, rather than directly encoding the hedonic value of an unconditioned stimulus (Schultz and Dickinson, 2000). In addition to encoding reward contingencies, a smaller subpopulation of dopamine neurons also show increased activity to aversive stimuli (Matsumoto and Hikosaka, 2009). Dopamine neurons are fairly large, have a low maximal firing rate, and axons of adjacent dopamine neurons are electrically coupled for synchronous activity, allowing for tightly correlated signaling output within subpopulations of dopamine neurons (Glimcher, 2011). Along with these physiological properties of dopamine neurons, there is a wide

distribution of projection sites that receive dopamine neuron innervation, leading to this description of dopamine neuron activity by Glimcher (2011): “[Dopamine neurons] cannot say much, but what they say must be widely heard.”

The current theory for what is encoded by dopamine neuron activity has been described as a teaching signal arising from prediction error. Prediction error can be broadly described as a discrepancy between expected and actual outcomes. For instance, during initial conditioning, a reward will induce phasic increases in dopamine neuron signaling. However, if an animal is well trained that a particular tone accurately predicts the delivery of a reward, phasic dopamine neuron firing is primarily increased during tone presentation, with a small increase during delivery of reward. If an expected reward is omitted, then tonic dopamine neuron activity will be inhibited (Schultz and Dickinson, 2000). The increases and decreases in dopamine neuron activity are not equivalent, as a positive prediction error (outcome greater than expected) can increase dopamine firing from 3-5 Hz (baseline) to 20-30 Hz. Negative predictions (outcome smaller than expected) have a lesser impact on dopamine firing, as the shift in firing goes from 3-5 Hz to approximately 0 Hz (Glimcher, 2011), suggesting that negative predictions either occur in a non-linear manner (where the impact of decreased tonic signaling is much stronger than a similar positive increase in phasic firing) or that negative predictions may be represented more strongly in other neural signaling systems. In general, it is hypothesized that dopamine neuron activity is closely associated with positive reward-prediction signals. These observations correspond nicely with theoretical descriptions of

learning as described by Rescorla and Wagner (1972), which posits that learning is driven by the salience of the CS, the strength of the US, and discrepancies between expected and learned outcomes.

To clarify the components of prediction error, Suri and Schultz (2001), examined how the activity of dopamine neurons fit the temporal difference model of Pavlovian conditioning (Sutton and Barto, 1990). The temporal difference (TD) model describes reward predictive learning as a two-part process, with reinforcement prediction errors associated with delivery of the US, and reinforcement prediction signals associated with presentations of the CS. Reinforced prediction errors serve as teaching signals that strengthen future prediction signals about contingencies in the environment to guide appropriate behavioral responses. The goal of the TD model is to describe how these components work together to generate learning when a CS is predictive of the occurrence of a US in addition to incorporating intervals of time in which these events occur. While total dopamine neuron activation does not strongly distinguish between the reinforcement prediction errors and reinforcement prediction signals (as seen by increases of dopamine activity during both the predictive CS and delivery of the US), the projection targets of dopamine neurons may differentially encode either reinforcement prediction signals associated with the CS, or reinforcement prediction errors associated with the US. Suri and Schultz (2001) hypothesize that the limbic system encodes US-prediction errors, while cortical processing modulates CS-prediction signals. This distinction does not necessarily comply with recent observations that aversive and rewarding

signals are projection specific, but Lammel et al. (2011) examined primary unconditioned stimuli, rather than associative learning. An important observation described by Suri and Schultz (2001) is that tonic activity in dopamine neuron target regions, like the orbitofrontal cortex or putamen are critical for bridging the time gap between CS presentation and US delivery. There is a gradual increase in tonic activity in these regions following CS presentation that predict the occurrence of reward delivery, which implies that phasic dopamine release can lead to the training of a tonic anticipatory state in dopamine projection sites.

As discussed in Chapter 3, the activation of dopamine receptors with a D1/5 receptor agonist more closely resembles tonic activation of receptors than a phasic increase. Based on this characterization of dopamine agonists, it seems plausible that prolonged agonist presence following a learning experience would induce tonic activity in dopamine terminal regions that could modulate CS-prediction signals. One possible route to integrate these theories of dopamine agonist activity with the observed enhancements in fear extinction observed is through Wagner's sometimes-opponent-process or standard-operating-procedure (SOP) theory (1981).

Incorporating Prediction Error Models into Wagner's SOP theory

Wagner represented learning of a CS and US association as two separate nodes that can be activated into multiple processing states. The presentation of a CS along with a US leads to both reaching an A1 activation state, allowing an association to be generated between the stimuli. If the stimuli are not in the A1 activation state together, they will not enter into an association. Following

presentation of the CS and US together, both gradually degrade into an A2 activation state, likened to being at the margin of attention (Pearce and Bouton, 2001). After some time, the CS and US degrade into an inactive (I) state. During extinction, when the CS is presented alone, it is maintained in the A1 state, while the US association is retrieved to the A2 state. This mismatch leads to the formation of an inhibitory association between the CS and US, as the CS no longer strongly predicts the presence of a US. The tonic activation of dopamine terminal regions may lead to maintenance of the CS in an A1 state, or increase the inhibitory learning formed between the CS and US. In order to understand how the present experiments fit within Wagner's SOP theory, it may be helpful to extend the definition of the CS node to include the CS prediction signal, and the US node to include US prediction error.

Wagner's theory would predict that enhanced activation of the CS prediction node would either inhibit the previously formed CS-US association, or allow the CS to enter into a new association. Both of these possibilities could account for the decreased fear responding observed throughout the experiments in Chapter 3 following SKF 81297 administration. For example, in Experiment 7, following presentation of the context (CS) alone, the CS is in an A1 state and the US is in an A2 state. Under normal conditions, as in the saline group, fear extinction would occur, but when the CS component is strengthened by tonic D1/5 receptor activation, the inhibition of the US in an A2 state would be much stronger or the CS may enter into alternate associations that inhibit or compete with fear responding. A similar explanation could also describe the effect of SKF

81297 on fear conditioning, as the US-associated phasic dopamine signals may be masked by tonic D1/5 receptor stimulation. The CS would remain in an A1 state for a greater period of time, accruing inhibitory strength towards the CS-US association.

The effect may be weaker with methylphenidate (Chapter 2) because both tonic and phasic signaling would be prolonged by blockade of the dopamine transporter. In the case of CS-alone presentations (Experiments 1 & 2), methylphenidate could enhance the CS-no US representation. However, when US-associated phasic dopamine signals occur, as in fear conditioning (Experiment 5), the signals would still be conveyed, and the increase in tonic dopamine receptor activity may not be strong enough to inhibit learning of the CS-US association.

In Chapter 3, Experiments 6 and 7 conform to this proposed model, but the effect becomes less clear with the 4-h post-session study (Experiment 8). The CS is unlikely to be in an A1 state 4 h following administration of the drug, although administration of SKF 81297 may artificially induce the CS to return to the A1 state. These explanations may provide some value in considering how SKF 81297 may enhance fear extinction, but one other possibility is that SKF 81297 is acting as an alternative US (i.e., a rewarding stimulus) that inhibits the expression of fear. To test this possibility, I examined the effect of SKF 81297 on reward learning and extinction.

In Experiment 13, I assessed the rewarding properties of pre- and post-session SKF 81297 in conditioned place preference. As post-session SKF 81297

did not generate a conditioned place preference, it is difficult to claim that the effects observed in these studies can be solely attributed to a counterconditioning mechanism. However, the generation of a place preference with pre-session SKF 81297 indicates some amount of reward associated with administration of the D1/5 receptor agonist. These effects may be explained by the hypothesis that SKF 81297 administration specifically modulates CS-prediction signals. In the pre-session CPP experiment, the animal pairs the context with an agonist-induced reinforced prediction that the CS will predict a rewarding event. Although there may be a negative prediction that arrives when the expected US is not delivered, it may be obscured by the continuous tonic activation of D1/5 receptors or may not be strong enough to inhibit the CS-prediction signal association formed within dopamine terminal regions. In the post-session experiment, the CS-prediction signal does not explicitly become paired with the context, and may instead signal greater reward value in the home cage or other salient features of the environment. There is no previous US association that would be inhibited in this case, as in fear extinction, and exposure to the relatively neutral context during CPP acquisition may not generate a strong enough signal to predict that context exposure will lead to SKF 81297 administration.

As discussed in Chapter 3, the enhancement of cocaine conditioned place preference extinction (Experiment 14) provides evidence against the idea that the rewarding elements of SKF 81297 are mainly responsible for the fear extinction enhancements. Instead, the hypothesis that CS-prediction signals are

strengthened by D1/5 receptor activation is in agreement with the observed extinction enhancement effects in Experiment 14. If post-session SKF 81297 were acting as a rewarding US in Experiment 14, the decrease in preference for the drug-paired side would be unlikely, as the drug paired side would essentially receive another conditioning session. The combination of the Temporal Difference model of prediction error and Wagner's SOP theory provides a unifying interpretation of many of the effects observed in these studies.

Although it appears that D1/5 receptor activation is sufficient to enhance extinction, Chapter 4 indicates that D1 receptor activation is not a necessary feature for learning and extinguishing associations. Furthermore, Chapter 5 indicates that activating D1/5 receptors in the ILPFC, NAcc core, DH, or BLA (Experiment 21) is not sufficient to induce extinction enhancements, suggesting a requirement of coordinated activity between several regions for the CS-prediction signal to be strengthened. Experiments 19 and 20 provide an indication that the enhancements of extinction are likely to be upheld by coordinated activity between the prefrontal cortex and striatum, as these regions are activated more strongly by a PKA-coupled D1/5 receptor agonist than a PLC-coupled D1/5 receptor agonist (Nishi et al., 2011). Activation of the hippocampus and amygdala through PLC-coupled D1/5 receptors may not be effective because the prefrontal cortex may not be sufficiently engaged to enhance extinction.

PKA activity in the hippocampus, amygdala, nucleus accumbens and prefrontal cortex has been previously implicated in long-term memory formation (Arnsten et al., 2005). Each of these regions contributes to fear extinction, as

described in the General Introduction (Chapter 1), however decreased PKA activity throughout these regions has been linked to enhanced fear extinction (Isiegas et al., 2006). The demonstration that a PKA-coupled D1/5 receptor agonist will enhance extinction (Chapter 5) may be distinct from these previous reports due to the specificity of only activating neurons that express D1 or D5 receptors, among other receptors targeted by the agonist. Several different intracellular signaling pathways in these neurons through which activation of PKA may lead to memory formation have been identified. For example, decreased phosphorylation of cAMP response element-binding protein (CREB) by inhibition of PKA in the nucleus blocks long-term potentiation (LTP), which could block memory formation (Matsushita et al., 2001). Phosphorylation of cytosolic proteins by PKA, such as DARPP-32, has also been shown to regulate memory formation via inhibition of protein phosphatase-1 (PP-1) and enhancement of extracellular signal-regulated kinase (ERK) activity (Valjent et al. 2005). ERK activity mediates a variety of cellular processes involved in the formation and storage of memory, and indirect regulation of ERK by PKA may be involved in LTP induction (Sweatt, 2004). An additional level of complexity in PKA function emerges from PKA-mediated phosphorylation of AMPA and NMDA receptors (Abel and Nguyen, 2008), leading to alterations in LTP and long-term memory storage. Understanding the exact role of PKA in fear extinction may require differentiating the inhibitory and excitatory components of fear extinction, but Chapter 5 demonstrates that activation of PKA-coupled D1/5 receptors contributes to fear extinction enhancement.

Limitations and Clinical Applications

One issue that could have considerable impact on the clinical use of a D1/5 receptor agonist in patient populations is that PKA-coupled D1/5 receptor agonists induce seizure-like activity in C57BL/6 mice. Both SKF 81297 and SKF 83822 have been shown to induce behavioral seizures and seizure like activity in the hippocampus at the same doses tested in these studies (Gangarossa et al. 2011, O'Sullivan et al. 2008). It is not clear how these seizure-inducing effects may impact some of the learning enhancements observed in these studies, but such findings have generally prevented the use of direct dopamine D1/5 receptor agonists in humans. However, both L-dopa and methylphenidate have been widely used in humans and show relatively similar effects, suggesting that these could be used in place of a direct dopamine agonist. Haaker et al. (2013) directly demonstrated that L-dopa enhances fear extinction in humans and Houlihan (2010) reported some positive effects of methylphenidate in decreasing posttraumatic stress disorder symptoms in combat-exposed veterans. The studies presented here may provide some value in understanding the mechanism of action in these clinically relevant drugs, but direct application of these findings in humans may require the development of PKA-coupled D1/5 receptor agonists that do not induce seizure-like activity.

Future Directions

In summary, I have shown that D1/5 receptor activation enhances extinction of contextual and cued fear and conditioned place preference. From the studies presented in this dissertation, it appears that targeting dopamine

signaling could be particularly beneficial in diseases of learning and memory such as post-traumatic stress disorder and substance use disorders. Although there were fairly strong effects in these studies within a number of associative learning tasks, it is still unknown how widely these effects may transfer to other aversive paradigms or other drugs of abuse. Future studies could specify whether the seizure like effects of these drugs may impact fear expression and additional tests may be required to thoroughly characterize D1/5 receptor activation as specifically enhancing CS-prediction signals. One such experiment may involve training multiple CS-US associations then specifically extinguishing one CS with SKF 81297 while keeping one stimulus non-extinguished to measure whether SKF 81297 only decreases freezing to the extinguished CS. If freezing is decreased in response to other stimuli, it may suggest that SKF 81297 generalizes easily between stimuli or that SKF 81297 is altering a different component of learning or behavior than what has been proposed here. In spite of these caveats, there are several findings within this dissertation that provide evidence that altering dopamine signaling can modulate fear extinction.

Conclusions

To close this chapter, I return to the three questions (from Rescorla, 1988) presented at the end of Chapter 1. Through the experiments presented here, I have examined the behavioral and neurobiological circumstances that lead to enhanced extinction, such as pre- or post-session administration of direct and indirect dopamine agonists. It appears that activity of PKA-coupled D1 receptor activation may be the neurobiological circumstance for dopamine modulated

extinction enhancements. In examining the content of learning, I have differentiated between the rewarding elements of SKF 81297 from the learning or CS-processing elements activated by SKF 81297. The content of the learning also appears to be context-independent, suggesting strong potential for using dopamine-targeted treatments in human disorders. Finally, examining the impact of learning on behavior indicates that the learning effects are persistent and robust. Together, the studies presented in this dissertation show that examining the interactions between aversive and reward learning provides a great deal of value for investigating the basic principles that underlie learning and memory.

Appendix A: Supplemental Data for Chapters 2 and 3

A1. Effects of post-extinction methylphenidate (MPH) across test days.

In Chapter 2, an extinction criterion was used to examine whether methylphenidate specifically enhanced extinction in animals that showed decreased freezing across the extinction session. Here, I examine how inclusion of all animals in Experiment 2a impacts data analysis for animals receiving MPH immediately following extinction. Figure A1 shows Extinction (Ext), and Test 1-3 in all animals receiving MPH (2, 5, 10, 20, & 40 mg/kg) or saline. There was no difference between groups during extinction. With a repeated measures ANOVA using test days as a within subjects factor and dose as a between subjects factor, there is a significant effect of drug treatment on fear responses during test days. There was a significant effect of Test Day ($F(2, 196) < 0.0005$) and dose ($F(5,98) = 0.009$), and no interaction between dose and Test Day. Post-hoc analysis with Dunnett's test showed a significant difference between 20 mg/kg MPH and saline ($p = 0.026$). However, when each test day is examined independently with an ANOVA, there was no significant effect of drug treatment on Test 1 ($F(5,98) = 1.884$, $p = 0.104$). There was a significant effect of drug treatment on Test 2 ($F(5,98) = 2.439$, $p = 0.040$) and Test 3 ($F(5,98) = 2.722$, $p = 0.024$), but post-hoc analysis on each test day with Dunnet's test showed no significant effect of any dose of MPH. These issues led to the exclusion of non-extinguishing animals from this data set in Chapter 2 to determine whether MPH may have effects in animals that show decreases in freezing across the extinction session.

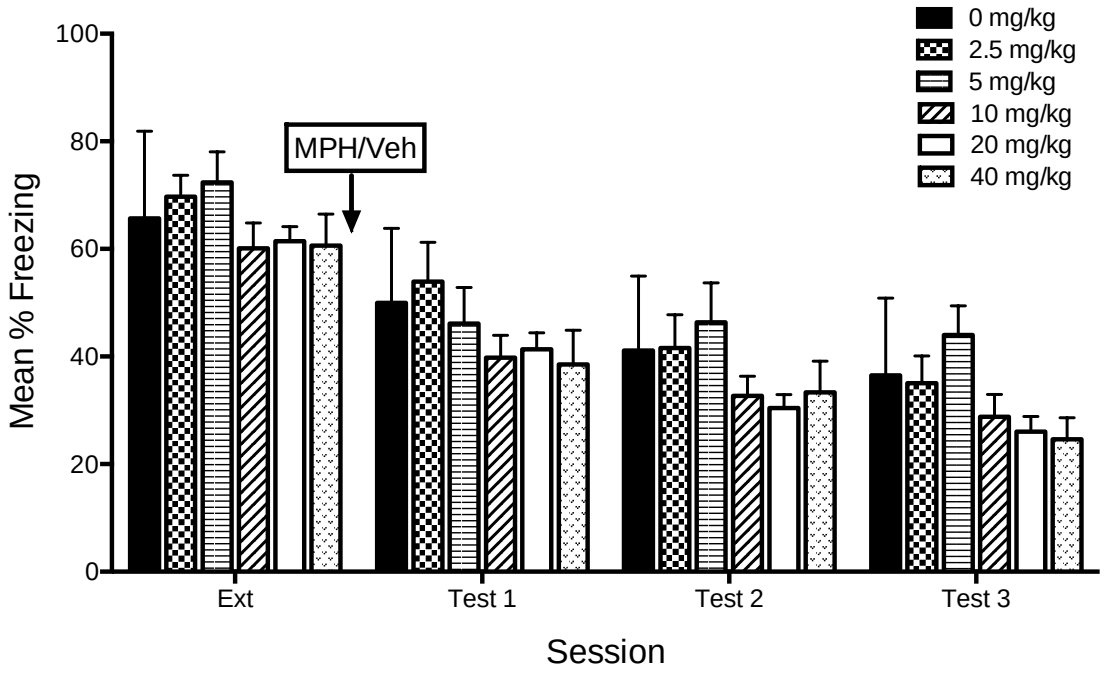


Figure A1. Post-extinction methylphenidate with all animals included. When analyzed across test days, there was an effect of 20 mg/kg methylphenidate. However, there was no effect of a single dose when test days were analyzed separately. Error bars indicate SEM.

A2. Effect of pre-extinction methamphetamine.

This experiment matches the procedure and apparatus for Experiment 1 (pre-session MPH), except a 2 mg/kg dose of methamphetamine was administered to C57BL/6 mice (n =8 per group) prior to the extinction session rather than methylphenidate. The 2 mg/kg dose of methamphetamine has been shown to increase locomotor activity (Zhao et al., 2014). This allows me to examine whether the fear extinction enhancement effects in Experiment 1 are specific to the pharmacological properties of methylphenidate, or if a different psychostimulant with locomotor activating effects could have similar effects on fear extinction. A two-tailed type 2 t-test of the data in figure A2 shows that pre-extinction methamphetamine significantly decreases freezing during the

extinction session (t (14) = 2.247, p = 0.0413), which is most likely due to the locomotor activating effects of methamphetamine. However, when tested the following days, there was no effect of drug-treatment on freezing compared to saline-treated mice. This experiment suggests that increased locomotor activation during an extinction session is not sufficient to decrease freezing on the following test days.

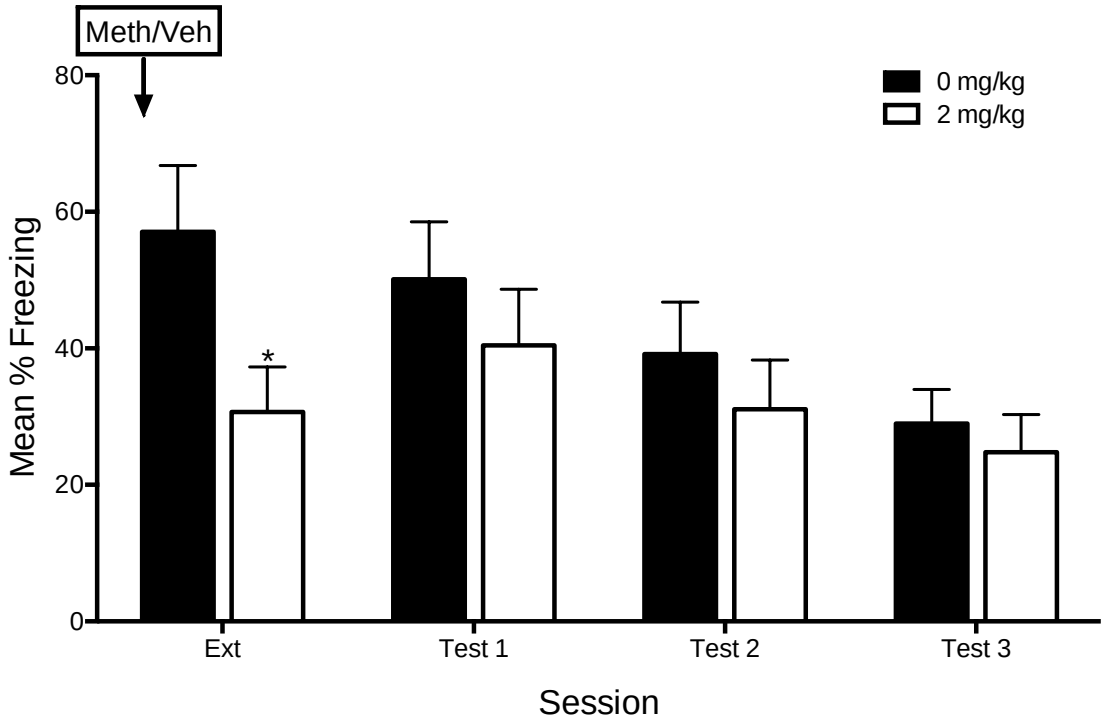


Figure A2. Pre-extinction methamphetamine does not affect freezing on subsequent test days. Although methamphetamine (2 mg/kg) significantly decreased freezing during the extinction session, the effect did not persist to following test days. Error bars indicate SEM. (*) $p < 0.05$ significant difference compared with vehicle.

A3. Analysis of SKF 81297 with extinction criterion.

In this section, I describe the effect of separately analyzing extinguishing and non-extinguishing animals that received post-session SKF 81297. I specifically examine the dose that was effective in enhancing fear extinction

when given immediately following extinction in Experiment 7 (10 mg/kg SKF 81297) compared to saline. Extinction criteria were identical to those used in MPH studies: animals that decreased freezing from the first 3-min block of the extinction session compared to the last 3-min block during a 12-min contextual fear extinction session were considered extinguishers, whereas animals that showed no decrease from the first 3-min block to the last 3-min block were considered non-extinguishers. All animals within the saline group (n = 12) were extinguishers. In the SKF 81297 animals, n = 5 were non-extinguishers and n = 7 were extinguishers. Analyzed with a two-tailed type 2 t-test, there were no differences between saline and extinguishers or non-extinguishers during acquisition or extinction. During the first test day following extinction, saline-treated animals were significantly different from extinguishers ($t(17) = 2.321$, $p = 0.0330$) and non-extinguishers ($t(15) = 2.328$, $p = 0.0343$). This indicates that the extinction enhancement effect of SKF 81297 in Experiment 7 may be more robust than the enhancements observed with methylphenidate in Experiment 2.

A4. Pre-session SKF 81297 compared against pre-session saline.

Experiment 13 discusses an additional experiment that compared pre-session SKF 81297 against pre-session saline. The experiment used the same apparatus and a similar procedure to that described in Experiment 13. Briefly, Prior to conditioning, mice (n = 8 per group) were habituated (pre-test) to the CPP apparatus by receiving a saline injection followed by a 5-min exposure to the conditioning chamber with grid and hole floors. Animals were matched following pre-test to ensure no bias to hole or grid floors, and then assigned to

counterbalanced groups that received SKF 81297 (10 mg/kg) or saline immediately before or after exposure to a grid or hole floor. During conditioning, animals were confined to one half of the CPP apparatus with grid or hole floor for 15-min. Mice received one pairing of SKF 81297 or saline (pre-session) and one pairing of saline (pre-session), counterbalanced for order and floor type, on alternating days. Twenty-four hours following the last conditioning session, mice were given a 15-min exposure to the CPP apparatus with both floors to assess preference for the drug-paired side. As shown in Figure A3, preference for the pre-session SKF 81297-paired side was higher than animals receiving pre-session saline on both sides, confirmed by a type 2 two-tailed t-test ($t(14) = 3.028$, $p = 0.009$). There was a trend towards a significant difference ($t(14) = 1.775$, $p = 0.098$) in locomotion during the preference test (mean values: locomotor activity for saline-treated mice during preference testing was 210.0 ± 10.7 and 232.85 ± 7.2 for pre-session SKF 81297-treated mice.

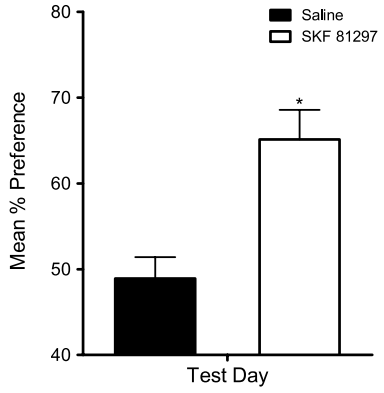


Figure A3. Pre-session SKF 81297 generates conditioned place preference. Administration of pre-session SKF 81297 induces a conditioned place preference when compared against saline. Error bars indicate SEM. (*) = $p < 0.05$ significant difference when compared against saline.

A5. Grid+/grid- analysis for Experiments 13: Pre- vs. Post-session SKF 81297 and conditioned place preference.

To ensure that pre-session SKF 81297-treated animals generated a conditioned place preference whereas post-session SKF 81297-treated animals did not, a grid+/grid- analysis is shown here. Using the grid+/grid- analysis allows for a comparison within drug-treatment groups that compares animals that received drug paired with the grid floor (grid +) against animals that received drug on the hole floor (grid -). For Experiment 13, a two-way ANOVA with drug-treatment timing (pre vs. post) and floor assignment (grid + vs. grid -) as factors was used. There was a significant effect of floor assignment ($F(1,28) = 23.577$, $p < 0.0005$), no effect of drug-treatment timing, and a significant interaction between floor assignment and drug-treatment timing ($F(1,28) = 8.682$, $p = 0.006$), indicating that there was a difference between drug treatment groups in floor preference. Figure A4 shows that pre-session SKF 81297-treated mice showed a preference for the grid+ floor whereas post-session SKF 81297-treated mice did not.

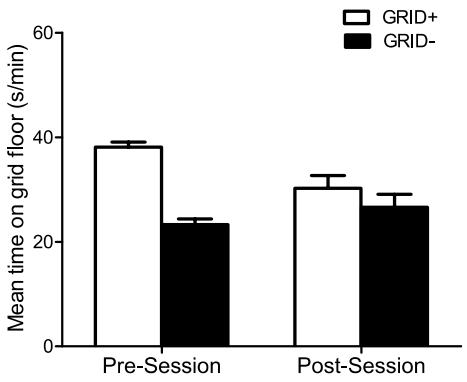


Figure A4. Animals that received pre-session SKF 81297 showed a significant preference for drug-paired floor during Preference Test. Two-way ANOVA confirmed that there was a significant interaction between preference for drug-paired floor and drug-timing, indicating that pre-session SKF 81297-treated animals generated a preference whereas post-session SKF 81297 treated animals did not show a preference for the drug-paired floor during Preference Test. Error bars indicate S.E.M.

A6. Grid+/grid- analysis for Experiment 14: Enhanced extinction of a cocaine conditioned place preference with post-session SKF 81297.

Grid+/grid- analysis for Experiment 14 used drug treatment (SKF vs. saline) and floor assignment (grid +/grid-) as factors in a two way ANOVA. There was a significant effect of floor during Preference Test ($F(1, 44) = 156.72$, $p < 0.0005$) and Post-Ext Test ($F(1,44) = 61.287$, $p < 0.0005$). There was no significant interaction between drug treatment and floor assignment, although there was a trend towards an effect during the Post-Ext Test ($F(1,44) = 3.032$, $p = 0.089$). Analyzing the high and low preference animals (as described in Chapter 3) separately showed that in high preference animals, during Preference Test, there was no effect of drug-treatment, a main effect of floor assignment ($F(1, 20) = 389.005$, $p < 0.0005$), and no interaction between drug-treatment and floor assignment. During Post-Ext Test in high preference animals, there was a main effect of floor assignment ($F(1,20) = 26.74$, $p < 0.0005$), no effect of drug

treatment, and an interaction between drug treatment and floor assignment ($F(1,20) = 5.481$, $p = 0.030$), indicating that animals receiving SKF 81297 after an extinction session showed decreased preference for the drug-paired floor compared to saline-treated animals. The grid+/grid- analysis reinforces the finding in Experiment 14 showing that SKF 81297 enhances extinction of a cocaine conditioned place preference in high preference animals.

In low preference animals, there was a main effect of floor during Preference Test ($F(1,20) = 86.505$, $p < 0.0005$) and Post-Ext Test ($F(1,20) = 30.985$, $p < 0.0005$). There was no effect of drug during Preference Test or Post-Ext Test. There was a significant interaction during Preference Test between drug treatment and floor assignment ($F(1,20) = 4.888$, $p = 0.039$) but not during Post-Ext Test. The interaction between drug treatment and floor assignment may be driven by higher preference for the drug-paired floor in SKF 81297-treated animals during Preference Test (prior to SKF 81297 administration). However, similar to the results reported in Experiment 14 with percent preference, there was no effect on extinction of cocaine conditioned place preference in low preference animals. Table A1 shows the mean time spent on grid floor and standard error of the mean for each group. Grid+/grid- assignments were counterbalanced for equal numbers in SKF 81297 and saline treated groups, but median split analysis led to some imbalances in grid+/grid- floor assignments. In high preference mice treated with saline, there were 8 grid+ and 6 grid- animals. In high preference SKF 81297-treated mice, there were 7 grid+ and 3 grid- animals. In low preference saline-treated mice, there were 4 grid+ and 6 grid-

animals. In low preference SKF 81297-treated mice, there were 6 grid+ animals and 9 grid- animals.

Table A1.	Preference Test		Post-Ext Test	
	Grid +	Grid -	Grid +	Grid -
Saline (all)	41.9 ± 1.7	22.8 ± 2.1	39.5 ± 1.7	23.6 ± 1.9
SKF 81297 (all)	41.8 ± 1.3	23.1 ± 3.6	36.2 ± 1.4	26.1 ± 5.4
Sal (low pref)	36.2 ± 1.0	28.6 ± 1.7	36.7 ± 2.5	26.9 ± 1.0
SKF (low pref)	37.2 ± 0.7	24.9 ± 0.5	38.6 ± 2.2	25.8 ± 1.9
Sal (high pref)	44.7 ± 1.8	17.0 ± 1.6	40.9 ± 2.3	20.3 ± 3.1
SKF (high pref)	45.1 ± 1.0	17.8 ± 0.9	34.5 ± 1.8	26.8 ± 3.5

Table A1. Grid+/grid- analysis for Experiment 14. Mean ± S.E.M. shown for each group. (all) indicates the inclusion of all subjects for mean grid+ and grid- values in saline- or SKF 81297-treated mice. (low pref) indicates saline or SKF 81297 subgroup included in analysis for low preference animals and (high pref) indicates saline or SKF 81297 subgroup included in analysis for high preference animals.

Appendix B: Supplemental Data for Chapter 4

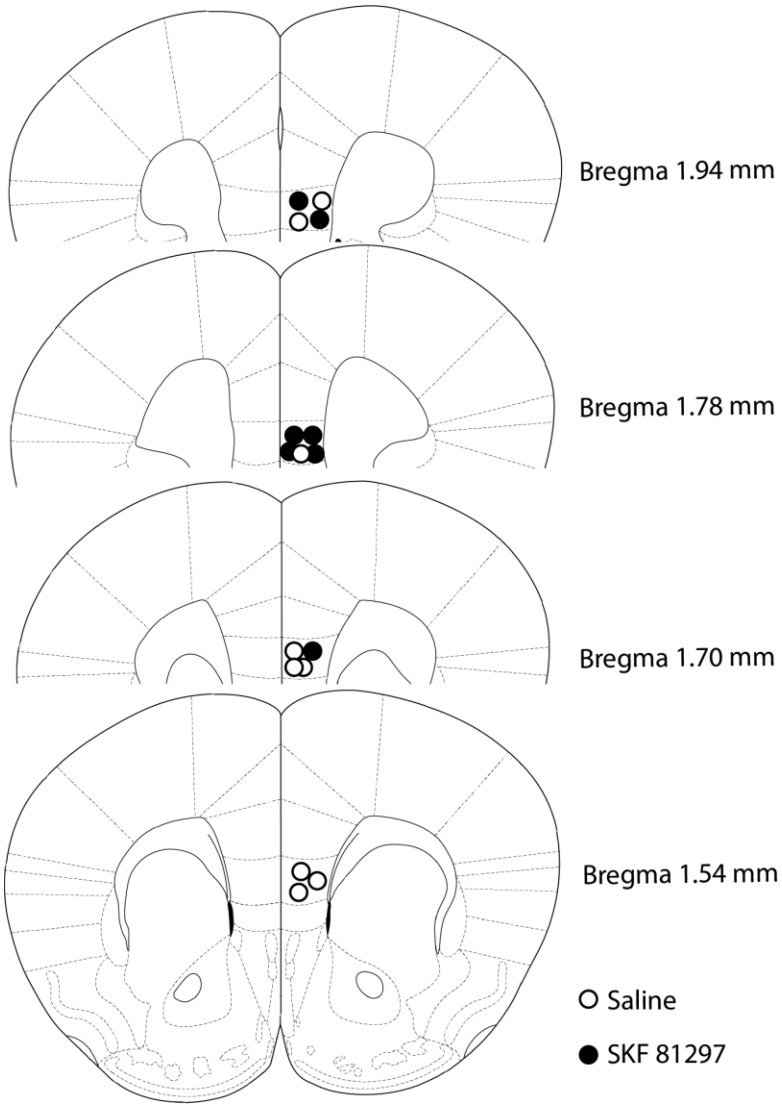
B1. Body weight averages across genotypes.

Genotype	Body weight (g) Mean ± SEM
MWT	29.5 ± 0.99
MHET	29.2 ± 0.61
MKO	21.1 ± 0.59
FWT	25.6 ± 1.27
FHET	24.2 ± 1.04
FKO	17.4 ± 0.70

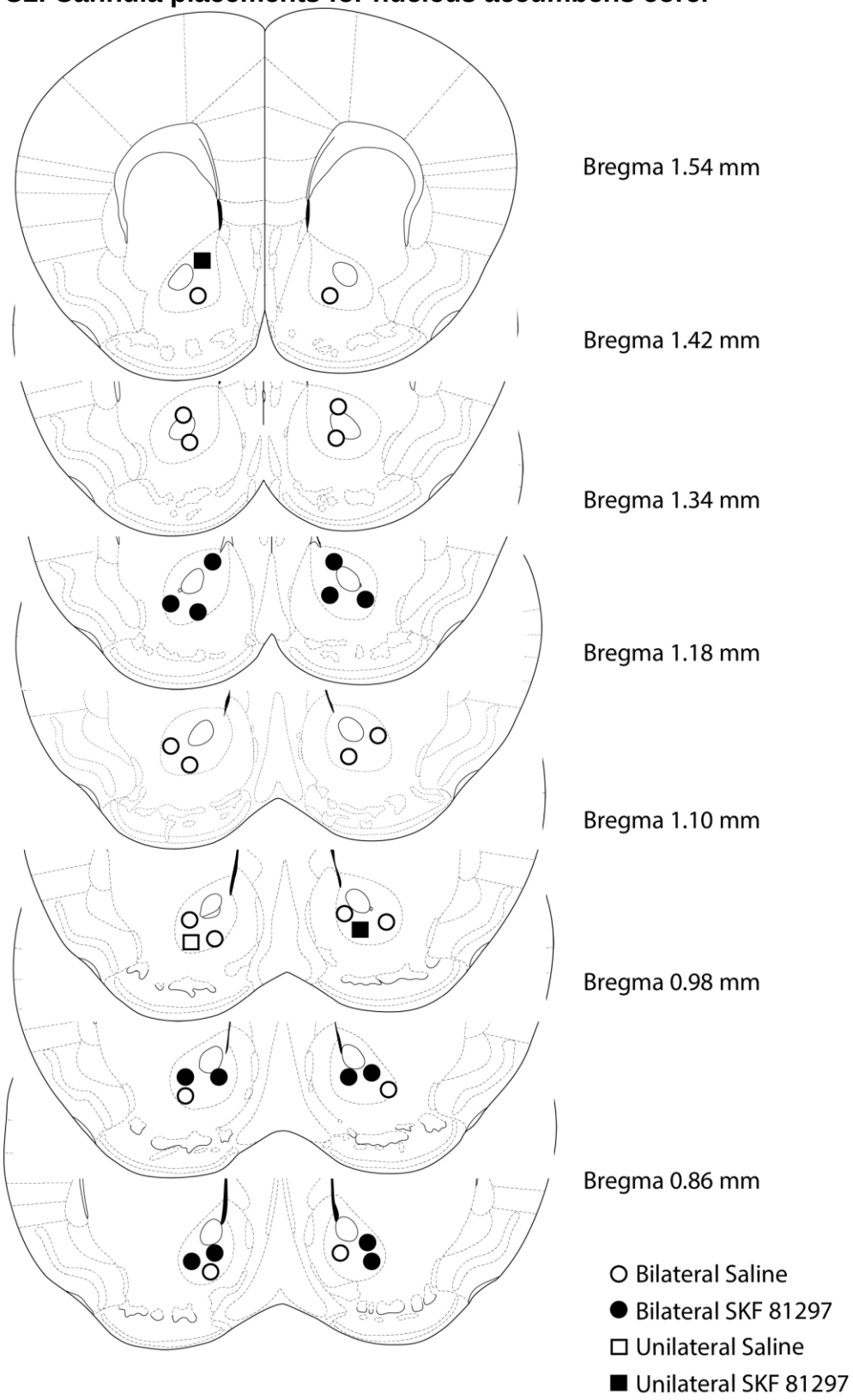
Appendix C: Supplemental Data for Chapter 5.

Cannula placements for Chapter 5. Only placements verified within the area of interest (hits) are shown. In the infralimbic region, unilateral placements are shown with open circles for saline and closed circles for SKF 81297. In dorsal hippocampus, nucleus accumbens core, and basolateral amygdala, open circles show bilateral saline placements and open squares show unilateral saline placements whereas closed circles show bilateral SKF 81297 placements and closed squares show unilateral SKF 81297 placements.

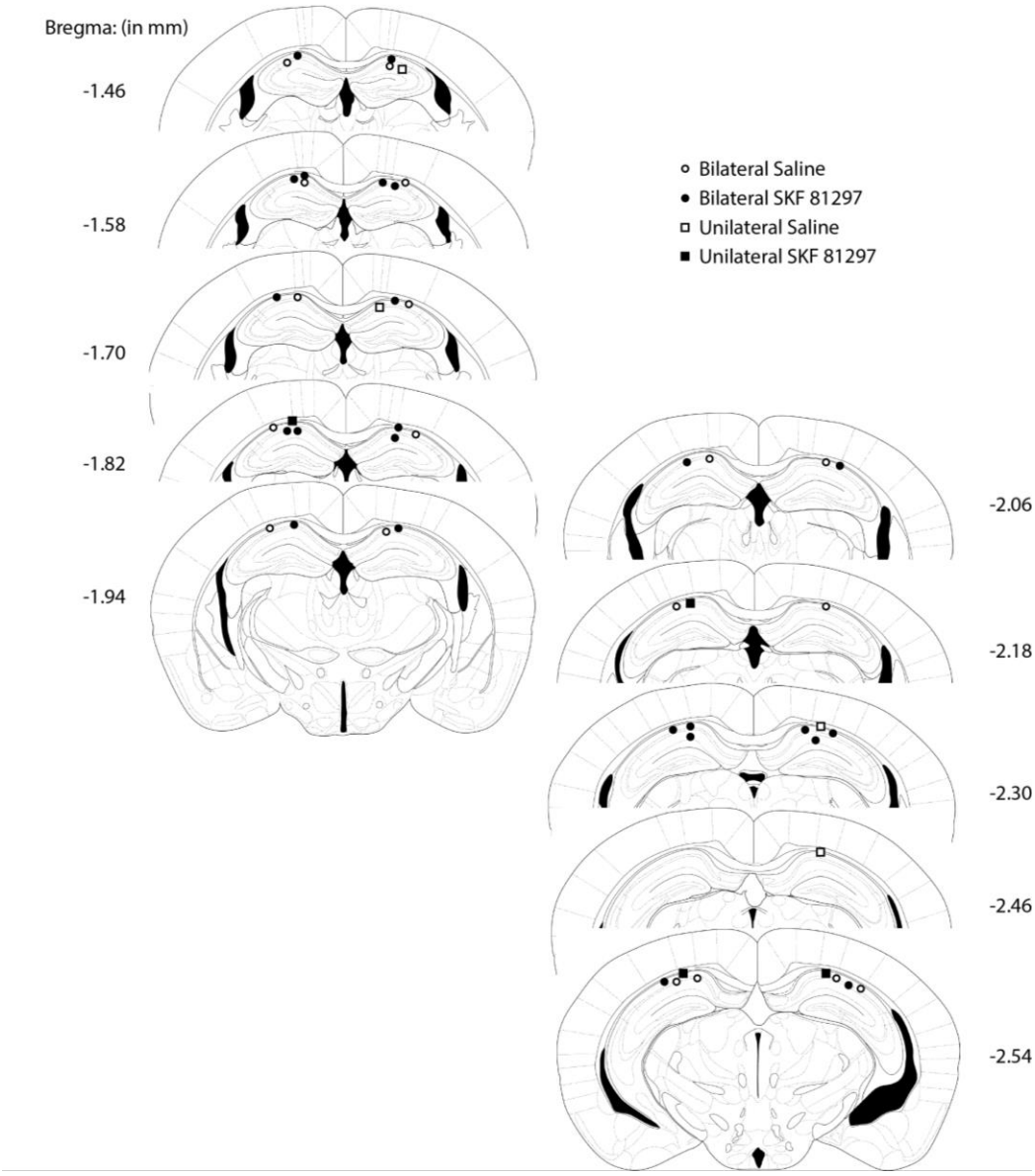
C1. Cannula placements for infralimbic region of the prefrontal cortex.



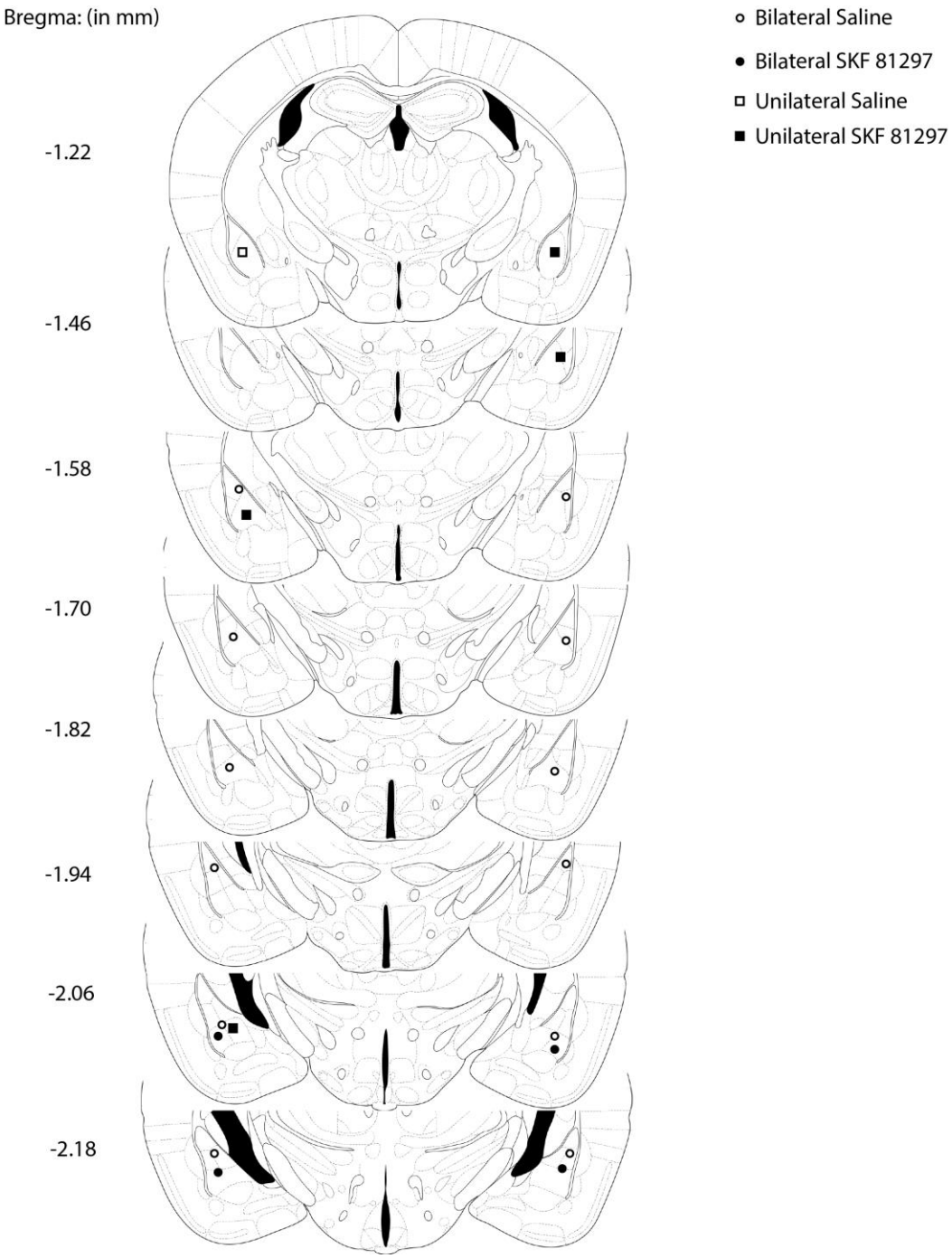
C2. Cannula placements for nucleus accumbens core.



C3. Cannula placements for dorsal hippocampus.



C4. Cannula placements for basolateral amygdala.



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