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THE USE OF OLFACTORY FEAR CONDITIONING IN RATS
TO INVESTIGATE THE EFFICACY OF
POSTTRAUMATIC STRESS DISORDER TREATMENTS

By

Ebony M. Glover

B.S. Spelman College, 2002
M.A. Emory University, 2005

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Abstract

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One of the cardinal symptoms of posttraumatic stress disorder (PTSD) is the presence of intrusive and persistent memories of the traumatic event. Disrupting fear memories in the aftermath of a traumatic experience may be a useful approach for mitigating these symptoms. A major challenge to this approach is a number of inconsistent findings in the literature concerning the efficacy of various post-training treatments in disrupting long-term fear memories. In general, investigators using hippocampal-dependent associative fear models found that certain post-training treatments disrupted fear memories. However, investigators using hippocampal-independent models found that post-training treatments had no effect on fear memories. This dissertation project used a rat model of olfactory-mediated fear-potentiated startle to examine the clinical efficacy of two FDA approved drugs, propranolol and rapamycin, as well as a novel extinction procedure that could be implemented in the aftermath of a traumatic event to prevent or remedy PTSD. Experiments were designed to examine the central hypothesis that hippocampal-dependent fear memories are vulnerable to disruption by post-training manipulations, whereas hippocampal-independent memories are not. The major findings of this project are that hippocampal-independent forms of Pavlovian fear memories are extremely persistent in the face of various post-training manipulations shown to disrupt hippocampal-dependent fear memories. However, when the conditioning procedure is modified to engage the hippocampus, then Pavlovian fear conditioned memories become susceptible to disruption. In addition, results suggest that while treatments given soon after a traumatic experience may have limited effectiveness, treatments given soon after the retrieval of traumatic memories have clinical promise. The present findings underscore the importance of using diverse animal models in translational research.

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In loving memory of Deon Latelle Glover

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Table of Contents

Page

Acknowledgements……………………………………………………………….ii

Table of Contents…………………………………………………………………v

List of Figures……………………………………………………………………vi

Chapter 1: General introduction…………………………………………………...1

Chapter 2: Differing effects of electroconvulsive shock on consolidation
and reconsolidation of olfactory fear memory……………………………………2

Chapter 3: Effects of immediate and delayed extinction on inhibition and recovery
of olfactory fear memory…………………………………………………………3

Chapter 4: Dose and time-dependent effects of systemic propranolol on consolidation
and reconsolidation of delay, trace, and context fear memories…………………..4

Chapter 5: Differing effects of systemic rapamycin on consolidation and
reconsolidation of context versus cued fear memories……………………………5

Chapter 6: General discussion……………………………………………………6

List of Figures

1. ECS disrupts the consolidation of trace but not delay fear memory…………………..30

2. Olfactory fear conditioning shows normal extinction………………………………...59

3. Extinguised olfactory fear memories show spontaneous recovery……………………60

4. Immediate post-acquisition extinction shows less renewal than delayed extinction….61

5. Immediate post-recall extinction shows less renewal then delayed extinction………..62

6. Propranolol (1, 3, or 10 mg/kg) had no effect on the consolidation of delay fear memory……………………………………………………………………………110

7. High dose of propranolol (10 mg/kg) had no effect on the reconsolidation of delay fear conditioning………………………………………………………………………111

8. Low dose (1 mg/kg) of propranolol disrupted the reconsolidation of delay fear memory, but intermediate (5 mg/kg) and high (10 mg/kg) doses did not……….......112

9. Low dose (1 mg/kg) or propranolol disrupted the consolidation of delay fear memory when administered 15 min before but not immediately before training……………..113

10. Propranolol given 15 min before training did not impair acquisition………………114

11. High dose (10 mg/kg) of propranolol had no effect on the consolidation of delay or trace fear memory…………………………………………………………………..115

12. High dose (10 mg/kg) of propranolol disrupted the reconsolidation of trace but not delay fear memory…………………………………………………………………116

13. Low (1 mg/kg) and high (10 mg/kg) doses of propranolol disrupted the consolidation of context fear memory. Low (1 mg/kg), but not high (10 mg/kg) dose of propranolol disrupted the consolidation of trace fear memory…………………………………117

14. Systemic rapamycin disrupted the consolidation of context but not

delay fear memory……………………………………………………………………….145

15. Systemic rapamycin disrupted the reconsolidation of context but not cued fear memory……………………………………………………………………………..146

16. Single-trial olfactory fear conditioning activated phosphorylation of p70s6K in the amgydala, which was prevented by post-training systemic rapamycin administration………………………………………………………………………147

CHAPTER 1

General introduction

Posttraumatic stress disorder (PTSD) is a potentially debilitating anxiety disorder that may develop following exposure to a traumatic event. It is estimated that in the general population, the lifetime rate of DSM-IV PTSD ranges from 8% to 12%, making it a major public health problem (Kessler et al., 1995). Hence, finding treatments to forestall or remedy PTSD is of vital importance in mental health research.

There are three main symptom types that characterize PTSD: (1) persistent and intrusive recollections of the traumatic event, (2) hyperactivity and increased arousal, usually in response to trauma reminders, and (3) avoidance of sensory cues associated with the traumatic event (Layton & Krikorian, 2002). Because the dominant features of PTSD reflect memory-related mechanisms, researchers have focused on the neural basis of fear-motivated learning and memory to explain the biological underpinnings of this disorder. It is generally believed that traumatic memories form when individuals contemporaneously experience neutral stimuli with highly aversive stimuli in their environment. Many behavioral scientists attribute this to associative learning, a phenomenon that has been extensively modeled in animals (Davis, 1990; Kolb & Mutalipassi, 1982; Rasmusson & Charney, 1997; Rescorla & Wagner, 1972). The most widely used behavioral models for investigating fear-related associative learning are Pavlovian fear conditioning and inhibitory avoidance training.

Animal Models of Fear Learning

In simple Pavlovian fear conditioning, an animal is exposed to a neutral conditioned stimulus (CS), which could be presented in the form of a discrete cue, such

as an odor, light, or tone (cued fear conditioning), or a distinctive environment (context fear conditioning), that overlaps in time with an aversive unconditioned stimulus (US), such as a footshock. Consequently, animals show a species-specific response, such as increased startle or freezing behavior, to the CS due to its prior association with the US. The CS may also elicit autonomic (i.e., changes in heart rate or blood pressure) and endocrine (i.e., hormone release) responses. Thus, memory is inferred from a quantifiable behavioral or physiological change observed in the presence versus the absence of the CS. There are many variants of Pavlovian fear conditioning which differ in experimental arrangement of stimulus contingencies (Pearce & Bouton, 2001; Rescorla & Wagner, 1972; Rescorla, 1988).

In a typical inhibitory avoidance task, an animal is placed in a lit compartment of an alley and allowed to enter an adjacent dark compartment where upon entry it is given a footshock. Consequently, the animal demonstrates an inhibition of its innate tendency to enter the dark place. Memory is inferred from this latency to step into this dark compartment due to its association with the footshock. Like Pavlovian fear conditioning, there are numerous types of avoidance paradigms which mostly differ in the nature of the response measured (Archer & Nilsson, 1989; Mackintosh, 1988; Rescorla & Solomon, 1967). Common measures involve active avoidance, where the subject takes an overt action to prevent the occurrence of an aversive stimulus, and passive responses, where the subject abstains from behavioral action in order to prevent an aversive outcome.

It is generally believed inhibitory avoidance training engages both Pavlovian and instrumental processes. This idea, proposed by Mowrer (1947), is called the two-process theory of avoidance. The theoretical position behind this idea is that an instrumental

training procedure cannot be arranged in the absence of Pavlovian conditioning (Kimble, 1961; Rescorla & Solomon, 1967). For example, because US delivery must always occur in a particular context, it is unavoidable that contextual cues will become classically conditioned. Mowrer believed that instrumental avoidance learning required that a Pavlovian conditioning process occurred first. This elicits a fear state, which serves to drive the instrumental escape response. On the other hand, it is thought that Pavlovian fear conditioning can occur independent of instrumental learning (Mowrer, 1947).

Pavlovian conditioning and inhibitory avoidance training are extremely powerful animal models for studying the mechanisms of fear learning and memory. These tasks allow for stringent experimental control over the delivery of aversive stimuli, and the fear responses can be easily quantified. Both forms of learning can be rapidly acquired in a single trial, allowing the precise moment of learning to be pinpointed to the presentation of the US. What's more, memory for a single training experience can be extremely persistent, and retention can be observed throughout the lifetime of an organism (e.g., Gale et al., 2004).

In the clinical presentation of PTSD, stimuli associated with the trauma can trigger vivid recollections of the traumatic event, and evoke intense fear responses well after the threat has passed. These fear responses closely resemble those observed in Pavlovian fear conditioning (i.e., increased arousal and hyper-reactivity to trauma-related cues) and inhibitory avoidance training (i.e., avoidance of cues associated with the traumatic event). Hence, animal models of fear conditioning reflect many of the key features that characterize PTSD.

While fear memory and the conditioned fear response are highly adaptive survival mechanisms, which serve to optimize an organism's response to potential life-threatening dangers in the environment, unwanted fear memories in PTSD produces maladaptive and potentially debilitating fear- and anxiety-related behaviors that interfere with patients' quality of life and well-being (Bonne et al., 2004). Thus, there is much pre-clinical and clinical research focused on understanding the neural mechanisms involved in the reduction of conditioned fear, and finding tools in which to inhibit or eliminate the expression of conditioned fear memories. The overall goal of this project was to use an animal model of fear conditioning to explore the efficacy of certain clinically relevant tools to eliminate unwanted fear memories in humans.

Over the past several decades, there has been tremendous growth in the use of animal models of fear conditioning to study the neurobiological basis of PTSD, and also to explore clinically useful strategies to mitigate debilitating memory-related PTSD symptoms. This research has generated two important ideas: (1) at the heart of PTSD pathology is the formation of tremendously strong and persistent emotional memories that, through fear conditioning, acquire the ability to evoke intense fear- and anxiety-related behaviors that are highly resistant to extinction, (2) useful strategies to treat PTSD involve diminishing the capacity for trauma-related stimuli to evoke a fear response. Prevailing approaches in both pre-clinical and clinical research settings involve employing behavioral and/or pharmacological techniques to disrupt fear memory in the aftermath of a traumatic experience (Garakani et al., 2006). These endeavors have been catapulted by the enormous progress made in the past thirty years in elucidating the neurobiological underpinnings of fear conditioning.

Neural Basis of Associative Fear Conditioning

Brain lesion, pharmacological, and electrophysiological studies have identified the amygdala, a collection of neurons located deep within the temporal lobes, as the critical neuroanatomical region responsible for fear memory processing (Amorapanth, LeDoux, & Nader, 2000; Davis, 2000; Nader, Majidishad, Amorapanth, & LeDoux, 2001; Pitkänen, Savander, & Ledoux, 1997). In Pavlovian fear conditioning, sensory information about the CS and US converge in the lateral nucleus of the amygdala (LA) (Romanski et al., 1993, but see, Killcross, Robbins, & Everitt, 1997). The LA projects to the central nucleus of the amygdala (CE), which is the primary output nucleus of the amygdala fear circuitry. The CE, in turn sends heavy projections to various hypothalamic and brainstem areas, which mediate an array of fear-related responses (e.g., potentiated startle, freezing, bradycardia, hypoalgesia) (Davis, 1993, 2000; LeDoux et al., 1988; Kapp et al., 1979).

Compared to Pavlovian fear conditioning, much less is known about the neural circuitry underlying inhibitory avoidance. Nevertheless, there is considerable evidence showing a critical role of the hippocampus as well as the amygdala in mediating inhibitory avoidance learning (e.g., Roozendaal et al., 1999; Izquierdo et al., 1997). There is also much neuroanatomical and electrophysiological evidence to suggest that these two anatomical regions interact during this and other forms of fear-motivated associative learning (e.g., Paré, Collins, & Pelletier, 2002; Ikegaya, Saito, & Abe, 1995). Inputs from the amygdala facilitate information processing and memory-related changes in the hippocampus (Akirav & Richter-Levin, 1999, 2002; Majak & Pitkanen, 2003). Hence,

studying amygdala and hippocampus interaction might shed light on the neural basis of inhibitory avoidance and other hippocampal-dependent forms of fear-motivated memories (Abe, 2001; Richter-Levin & Akirav, 2003).

Consolidation theory

This dissertation project examines the idea that disrupting fear memory formation in the aftermath of a traumatic experience may be a useful approach for mitigating PTSD symptoms. This idea is based on the theory of memory consolidation, which maintains that newly acquired memories are initially fragile, but stabilize over time into lasting traces that are resistant to disruption (McGaugh, 2005; Dudai, 2004). This theory predicts that if the consolidation process is somehow perturbed before the memory is fully stabilized, then, theoretically, the memory should be permanently eliminated.

The term, consolidation, has acquired two conceptually different meanings in the literature. One meaning refers to a well-described phenomenon by which hippocampal-dependent memories are reorganized over time into hippocampal-independent memories (Eichenbaum & Cohen, 2001; Dash, Hebert, & Runyan, 2004). This process, often termed systems consolidation, is not addressed in this project, and therefore is not considered further. The term, consolidation, also describes a cascade of cellular and molecular events that underlie post-acquisition stabilization of initially labile short-term memories into stable long-term memories (Dudai, 2004; Kandel, 2001). Typically called cellular or molecular consolidation, this process involves protein synthesis dependent memories that transform over time into protein synthesis independent memories. It also describes a process by which certain memories are initially dependent on

neuromodulators and over time become resistant to neuromodulatory influences (McGaugh, 2005).

Reconsolidation Hypothesis

In 1968, Misanin and colleagues reported a seminal experiment where they fear conditioned rats in an avoidance task, and 24 hours later – when the fear memory was presumably consolidated – administered electroconvulsive shock (ECS) immediately following a 2-s presentation of the CS. On subsequent retention tests, these rats showed amnesia for the avoidance task, while another group of rats that got ECS alone (without the CS reminder presentation) showed normal avoidance memory. This phenomenon has since been demonstrated with a variety of amnesic agents in many different animal species (Tronson & Taylor, 2007). These findings challenged the notion that once memories are consolidated, they are no longer vulnerable to interference.

It is now thought that the act of retrieving a memory can induce a process called reconsolidation whereby stable memories temporarily return to a labile state, and become susceptible to modification or disruption. Importantly, many of the same pharmacological agents that disrupt fear memory consolidation (i.e., inhibitors of protein synthesis, kinase activity, transcription factors and translation activators) have also been found to disrupt reconsolidation of fear memories when given soon after recall (e.g., Nader, Schafe & LeDoux, 2000; Parsons et al., 2006). These pre-clinical findings gave rise to the idea that reconsolidation blockade may be a clinically useful approach for eliminating remote fear memories in PTSD (Diergaarde et al., 2008).

Pharmacological Intervention in PTSD Treatment

Currently the main treatment approach for PTSD is psychological intervention, such as cognitive behavioral therapy (Mendes et al., 2008). However, research on the biology of emotional memories and conditioned responses is paving the way for pharmacological intervention as a preventive strategy for new trauma victims (consolidation blockade) or a treatment strategy for existing PTSD patients (reconsolidation blockage). It is critical that researchers find effective drugs that can be administered to humans safely and ethically. There is a substantial body of evidence characterizing a number of drugs with various mechanisms of action that effectively target and inhibit critical fear memory-related cellular processes. The overwhelming majority of these studies administered drugs locally into certain brain regions. This is a powerful experimental technique that has provided tremendous insight into cellular and molecular underpinnings of fear memory consolidation. However, in order for these drugs to be clinically useful, they must effectively disrupt fear memory when given the systemic route.

Nevertheless, there are very few pre-clinical studies that examined the effect of systemic drug treatment on fear memory consolidation for Pavlovian fear conditioning. Of the handful of published studies that took this approach, many have reported a failure to demonstrate an amnesic effect of post-training systemic drug administration. For example, Thomson and Sutherland (2005) found that post-training systemic administration of lipopolysaccharide, a proinflammatory cytokine, disrupted the consolidation of context fear but not cued fear. Most notable is the apparent resistance of Pavlovian fear conditioning to post-training systemic administration of adrenergic

antagonists (Debiec & LeDoux, 2004; Grillon et al., 2004; Lee et al., 2001). In spite of a wealth of evidence showing that systemic administration of adrenergic drugs and hormones can modulate the consolidation of inhibitory avoidance training, it has yet to be demonstrated that systemic administration of adrenergic antagonists effects the consolidation of Pavlovian fear conditioning. Nevertheless, propranolol has received widespread attention as a possible prophylactic agent to prevent unwanted memories PTSD.

Historically, eletroconvulsive shock (ECS) was used to characterize the consolidation of associative fear conditioning. However, depending on whether inhibitory avoidance or Pavlovian fear conditioning tasks were employed, researchers arrived at different conclusions about the effectiveness of post-training ECS in disrupting fear memory consolidation. Immediate post-training delivery of ECS consistently disrupted inhibitory avoidance retention (e.g., Boggan & Schlesinger, 1974; Sara et al., 1975). However, it did not impair Pavlovian conditioned autonomic and behavior responses (Chorover & Schiller, 1966; Mendoza & Adams, 1968; Hine & Paolino, 1970; Springer, 1975; Yaginuma & Iwahara, 1971, but see Caul & Barrett, 1972).

Taken together, evidence shows that when stimuli are presented under conditions that are suitable for instrumental avoidance learning, the resultant fear memory is susceptible to certain post-training influences. Likewise, when a contextual cue is used as a CS in Pavlovian fear conditioning, then that fear memory can also become susceptible to certain post-training manipulations. On the other hand, when a discrete cue is used in Pavlovian fear conditioning, the fear memory is resistant to various systemic post-training manipulation. These are important observations in light of the fact that several

investigators are using pre-clinical studies that exclusively utilized inhibitory avoidance measures as a rationale for the clinical use of post-trauma administration of certain drugs to prevent the development of debilitating fear memories in PTSD. However, the evidence shows that while certain behavioral indicators of fear memory are vulnerable to disruption (i.e., avoidance of a place, heightened freezing or startle response in a distinct context), other behaviors appear to be resistant to disruption (heighted freezing or startle in the presence of a discrete cue). Therefore, the clinical approach of giving pharmacological treatments in the aftermath of a traumatic event might have limited effectiveness in disrupting unwanted fear memories associated with the trauma.

The current thesis presents a set of experiments in which a rat model of olfactory-mediated fear-potentiated startle was used to explore the efficacy of various clinically relevant tools in disrupting the consolidation and reconsolidation of different types Pavlovian fear memory tasks. Chapter 2 explores the memory disruptive potential of ECS, a historically significant post-training manipulation. In Chapter 3, a novel behavioral technique of fear reduction, whereby extinction is delivered soon after fear acquisition, or fear reactivation is examined. In Chapter 4, the fear memory-disruptive potential of the FDA approved drug, propranolol, is investigated. Finally in Chapter 5, the memory-disruptive effects of a clinically promising and FDA approved drug, called rapamycin, are tested on this model. Together, these experiments have important implications for the clinical approach of disrupting persistent fear memories in PTSD.

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CHAPTER 2

Differing effects of electroconvulsive shock on consolidation and reconsolidation of olfactory fear memory

Abstract

Post-training electroconvulsive shock treatment (ECS) blocks the consolidation of inhibitory avoidance learning (McGaugh & Dawson, 1971). However, evidence suggests that this effect of ECS is not wholly amnesic. When latency to enter a shock compartment was measured, a retention deficit was apparent. However, under the same conditions, animals showed signs of retention when such responses as heart-rate suppression, urination, and defecation were measured (Bueno et al., 1993; Chorover & Schiller, 1966; Hine & Paolino, 1970). The present study tested the hypothesis that ECS disrupts hippocampal-dependent fear memory, but not hippocampal-independent fear memory. We tested the effect of post-training ECS treatment on two Pavlovian fear conditioning tasks that differ in hippocampal involvement. Rats were presented with a single odor-shock pairing which either overlapped and co-terminated in time (delay fear conditioning – hippocampal independent) or was separated by a 15-s trace interval (trace fear conditioning – hippocampal dependent). Within 30-s after the footshock, rats were either given a 0.5 s, 40 mA ECS, or no ECS. When tested 24 hrs later, trace conditioned rats showed significantly less startle to the odor CS than delay conditioned rats. These findings suggest that ECS selective disrupts hippocampal-dependent forms of fear memories, but is ineffective in disrupting hippocampal-independent fear memories.

Introduction

This project explores the idea that disrupting fear memory formation in the aftermath of a traumatic experience might be a useful approach for mitigating persistent fear memories in posttraumatic stress disorder (PTSD). This idea is rooted in consolidation theory, which holds that newly acquired memories are labile and take time to stabilize into stronger traces that are more resistant to disruption (McGaugh, 2005; Dudai, 2004). If this consolidation process is somehow perturbed before the memory is fully stabilized, then, theoretically, the memory should be permanently disrupted. However, depending on the behavioral model employed, researchers have arrived at different conclusions concerning the efficacy of various post-training manipulations in disrupting fear memory consolidation (e.g., Debiec & Ledoux, 2004; Lee et al., 200l).

The historical foundation of consolidation research was almost exclusively built upon studies that used electroconvulsive shock (ECS) treatment as an amnesic agent to access the consolidation gradient of instrumental avoidance training in rats. A fascinating set of findings came out of these studies, which concurred that ECS treatment was not wholly amnesic. Immediate post-training delivery of ECS appeared to disrupt retention when the latency to enter a shock compartment was measured. However, under the same conditions, animals showed signs of retention when classically conditioned behavioral (Bueno et al., 1993) and autonomic responses, such as heart-rate suppression, urination, and defecation were measured (Chorover & Schiller, 1966; Mendoza & Adams, 1968; Hine & Paolino, 1970; Springer, 1975; Yaginuma & Iwahara, 1971, but see Caul & Barrett, 1972).

Early researchers speculated that ECS differentially affects Pavlovian vs. instrumental conditioned memories (Bueno et al., 1993; Yaginuma & Iwahara, 1971). This idea was supported by the findings of Bueno et al. (1993), who trained rats on inhibitory avoidance as well as Pavlovian conditioning to a tone. Freezing to the tone was measured as well as latency to enter the shock compartment. The results show that ECS rats had shorter latencies, but conditioned freezing was not affected. They concluded that the amnesic effects of ECS depend on the nature of the task.

Others have offered that the differential vulnerabilities of conditioned responses to ECS is a function of higher threshold to disruption for lower order automatic fear memories compared to higher-order cognitive fear memories (Springer, 1975). Both inhibitory avoidance learning and Pavlovian fear conditioning depend on the amygdala, a subcortical, phylogenically old brain system that detects specific danger cues and mediates rapid and reflexive fear memory responses. Unlike Pavlovian fear conditioning to a simple cue, inhibitory avoidance training recruits the hippocampus, which coordinates more complex forms of memories that involve contextual and temporal cues. It is possible that the critical determinant of fear memory's susceptibility to post-training ECS is the brain regions involved in encoding the fear memory. Specifically, hippocampal-dependent forms of fear memories are susceptible to ECS influences, but hippocampal-independent fear memories are not. In support of this idea, Squire, Cohen, & Zouzounis (1983) showed in humans that ECS disrupted hippocampal-dependent declarative memory, but not hippocampal-independent skill learning. Similarly, Vakil et al. (2000) demonstrated in humans that ECS disrupted declarative memories but left nondeclarative perceptural priming and skill learning memories intact.

The present study uses a rat model of Pavlovian fear conditioning to examine the efficacy of ECS in disrupting hippocampal-dependent versus hippocampal-independent forms of fear memories. Our laboratory has developed an olfactory-mediated fear-potentiated startle task that is amygdala-dependent (Walker, Paschall, & Davis, 2005), and produces robust, long-lasting fear memories after a single training trial (Glover, Paschall, & Davis unpublished results; Paschall & Davis, 2002). The current study utilizes this paradigm to compare the effects of ECS on the consolidation of two different kinds of Pavlovian fear conditioning tasks, which are either hippocampal-independent or amenable to hippocampal influence. In a typical Pavlovian fear conditioning task, the CS and US overlap in time, and this protocol is called delay fear conditioning. It has been established that the hippocampus is not necessary for delay fear conditioning when a discrete, simple cue is used as the CS (e.g., Bast et al., 2003; Selden et al., 1991; Kim & Fanselow, 1992). In trace fear conditioning, the CS and US are separated by a time interval (typically on the order of seconds). There is a considerable literature showing hippocampal as well as amygdala involvement in trace fear conditioning (e.g., McEchron et al., 1998; Quinn et al., 2002). It is hypothesized that ECS will disrupt the consolidation of trace, but not delay fear conditioning.

Methods

Subjects

Male Sprague-Dawley rats (N = 31) (Charles River, Raleigh, NC), weighing between 300 and 350 grams at the time of testing, were group housed four to a cage, and maintained on a 12:12 hour light / dark cycle with food and water available ad libitum. All behavioral procedures took place during animals’ light cycle.

Apparatus

Rats were trained and tested in two identical 8 x 15 x 15 cm Plexiglas and wire mesh cages as previously described by Cassella and Davis (1986). Background noise (60 dB wideband) and startle stimuli (50-ms white-noise bursts; rise decay, 5-ms) were delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5-cm from the front of each cage. Sound-level measurements were made with a Brüel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (type 4176) located 7-cm from the center of the speaker, which approximates the distance of the rat's ear from the speaker during testing. Startle response amplitudes were quantified using an Endevco (San Juan Capistrano, CA) 2217E accelerometer. Cage movement produced by the rat's startle response resulted in displacement of the accelerometer, the output of which was integrated, producing a voltage output proportional to the velocity of cage movement. This signal was amplified by an Endevco model 104 amplifier and digitized on a scale of 0–2510 units by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 (Apple Computers, Cupertino, CA) computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 300-ms after onset of the startle-eliciting noise burst.

Olfactory Apparatus

The olfactory fear conditionined apparatus has been described in detail elsewhere (Paschall & Davis, 2002). In brief, a continuous flow of air was delivered from a compressed-air cylinder at a rate of 1.0 L/min through a small port (1.3-mm lumen diameter) positioned just above a 12.5-mm diameter opening in the top of each cage. For

delivery of the olfactory stimulus, a computer-controlled solenoid (Model H15-03; Coulbourn Instruments, Allentown, PA) was opened for 4-s, thereby diverting clean air from the compressed-air cylinder into and through a sealed 135-cm^3 glass jar containing 20 ml of 5% (vol/vol) amyl acetate (i.e., the odorant) in propylene glycol solution. The inlet and outlet ports of the glass jar were positioned above the solution such that clean air from the tank mixed with the amyl acetate-containing vapor. The output was then mixed in a 3:5 ratio with clean air before flowing into the cage.

The chamber was actively exhausted into the building's ventilation system at a rate of 0.0114m^3/s. Thus, a volume of air equal to the chamber's total volume was vented every 25-s. Previous results with fear-conditioned rats indicate that with theses procedures startle amplitude returns to baseline levels within 30-s of solenoid closure (Paschall & Davis, 2002). Cages were cleaned daily with warm tap water and 95% alcohol, and were air dried overnight. The unconditioned stimulus was a 0.5-s 0.4 mA scrambled shock delivered through the four floor bars as described by Walker and Davis (1997). The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads, Newton, CT).

Behavioral Procedures

Acclimation session. On each of two consecutive days, rats were placed in the startle chamber and after a 5-min acclimation period, received 30 presentations of startle stimuli (95 dB noise burst) separated by a 30-s intertrial interval. Rats were removed from the chamber immediately after the last startle stimulus presentation. Their mean startle amplitudes were calculated, and marked as their pre-training startle baseline. Rats were

then divided into treatment groups according to their startle baselines, such that the mean startle amplitudes were balanced across groups.

Fear conditioning. The next day, rats were returned to the same startle chambers in which they were matched. After 5-min of acclimation, rats received a single odor-shock pairing that overlapped and co-terminated (delay fear conditioning, n = 14) or was separated by a 15-s trace interval (trace fear conditioning, n = 17). The CS was a discrete presentation of a 5% amyl acetate odorant and the US was 0.5-s footshock (0.4mA). Immediately after footshock, rats were given ECS. Current (40 mA, 0.5-s duration) was delivered by a shock generator through electrodes attached to both ears via alligator clips.

Fear-potentiated startle test. Seven days after training, rats were returned to the startle cages, and after 5-min, were presented with 30 startle stimuli (leaders). Thirty seconds after the final leader stimulus, rats received 30 startle-eliciting noise bursts presented alone (noise-alone trial) and 10 noise bursts presented 3.2-s after onset of the 4-s odor (odor-noise trials). The two trial types were presented in a manner such that after each odor-noise trial, three noise-alone trials were presented at a 30-s ITI, so that noise-alone trials occurred 30, 60, and 90-s after each odor.

Statistical Analyses

Percent fear-potentiated startle was calculated as: [(CS-noise minus noise-alone trials) / (noise-alone trials)] × 100. A two-way ANOVA was used to compare Treatment (ECS vs. No ECS) and Condition (Delay vs. Trace fear). A significance level of $p < 0.05$ was taken for all results.

Results

Figure 1 shows the mean percent potentiation of the acoustic startle response for delay fear-conditioned (n = 14) and trace fear-conditioned animals (n = 17) that either received immediate post-training ECS treatment (black) or no ECS treatment (stripes). A two-way ANOVA comparing Treatment (ECS vs. No ECS) and Condition (Delay vs. Trace fear) yielded a significant interaction, $F(1, 31) = 4.71$, $p < .05$, but no main effect of Treatment, $F(1, 31) = 3.77$, $p \geq .06$, and no main effect of Condition $F(1, 31) = .95$, $p > .05$. From these results, it is concluded that ECS disrupts the consolidation of trace fear conditioning, but has no impact on the consolidation of delay fear conditioning.

Discussion

It has been well documented that ECS, given immediately after training, disrupts long-term retention of inhibitory avoidance memory. A far less cited literature show that classically conditioned fear memories remain intact in the face of ECS-induced inhibitory avoidance retention deficits (Chorover & Schiller, 1966; Mendoza & Adams, 1968; Hine & Paolino, 1970; Springer, 1975; Yaginuma & Iwahara, 1971, but see Caul & Barrett, 1972). The present study investigates the hypothesis that hippocampal-dependent fear memories are vulnerable to ECS influence, but hippocampal-independent fear memories are not. In support of our hypothesis, the major finding of this study is that post-training ECS treatment disrupts long-term retention of trace fear conditioning, but has no effect on retention of delay fear conditioning.

The present findings are important because they underscore the problem of disproportionately using a single animal model in translational research. This is particularly relevant for fear memory research, where the majority of preclinical evidence informing pharmacological interventions in PTSD were almost exclusive obtained from

inhibitory avoidance models. However, the present findings cast doubt on the assumption that post-training manipulations are wholly effective in disrupting fear memory consolidation. This has wide implications for the clinical efficacy of various post-trauma treatments aimed at disrupting fear memory formation and forestalling PTSD. Further research is need to clarity factors that determine fear memory vulnerability to disruption.

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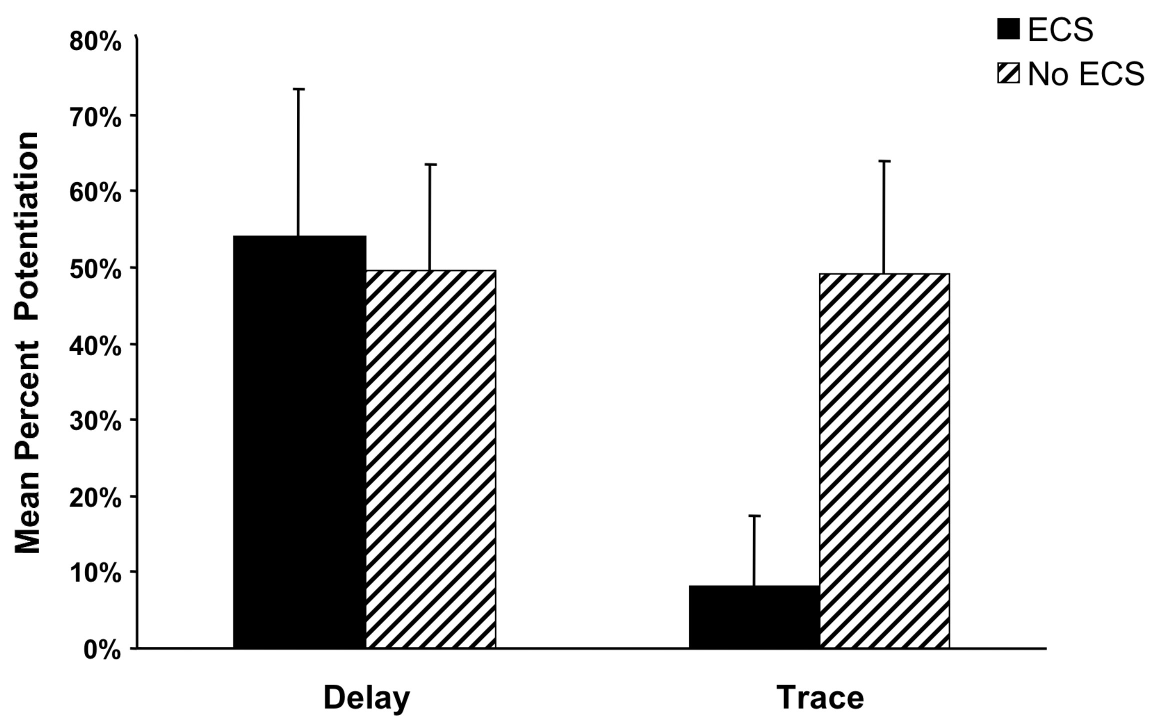
Figure Caption

***Figure 1.* ECS disrupts the consolidation of trace but not delay fear memory.** Mean percent potentiation of the acoustic startle response for delay fear-conditioned (n=14) and trace fear-conditioned animals (n=17) that received either immediate post-training ECS treatment or no ECS treatment. A two-way ANOVA comparing Treatment (ECS vs. No ECS) and Condition (Delay vs. Trace fear) yielded a significant interaction, $F(1, 31) = 4.71$, $p < .05$, but no main effect of Treatment, $F(1, 31) = 3.77$, $p > .05$, and nomain effect of Condition $F(1, 31) = .95$, $p > .05$.

Figure 1



CHAPTER 3

Effects of immediate and delayed extinction on inhibition and recovery of olfactory fear memory

Abstract

A major challenge to the clinical treatment of anxiety disorders is the reemergence of fear following extinction-based psychotherapies. In animal models of extinction, the recovery of fear responses can occur following unsignaled presentations of feared stimuli (reinstatement), by testing for fear in a context different from where extinction took place (renewal), or by the simple passage of time (spontaneous recovery). From these observations, it is generally believed that extinction involves new inhibitory learning rather than "unlearning" or memory erasure. However, recent findings from our laboratory show that extinction given soon after acquisition is resistant to recovery, and may be more akin to erasure. The current project attempts to replicate those findings, as well as test a novel strategy of delivering extinction immediately following the retrieval of day old fear memories. The rationale is that retrieval of remote fear memories will render them labile and susceptible to disruption by immediate extinction. Experiments were designed to test the effects of immediate and delayed extinction, when delivered post-acquisition and post-recall, on renewal of fear. The results are consistent with previous findings that immediate extinction results in less renewal than delayed extinction. Furthermore, results are consistent with recent findings showing that immediate post-recall extinction precludes fear memory recovery. Hence, the strategy of delivering extinction immediately after fear memory retrieval shows clinical promise.

Introduction

This project used an animal model of odor-mediated fear-potentiated startle to explore the therapeutic efficacy of an extinction procedure in eliminating long-term retention of conditioned fear. Characteristic symptoms of posttraumatic stress disorder (PTSD) include recurrent, intrusive memories of a traumatic experience, which may elicit intense fear responses well after the threat has passed. While fear memory and the conditioned fear response are highly adaptive survival mechanisms, unwanted fear memories in PTSD produce maladaptive and potentially debilitating behaviors that interfere with quality of life and well-being (Bonne et al., 2004). Hence, there is much preclinical and clinical research focused on understanding the neural mechanisms involved in the reduction of conditioned fear, and finding tools in which to inhibit or eliminate the expression of conditioned fear memories. The goal of the present project was to use an animal model of Pavlovian fear conditioning to explore the therapeutic efficacy of an extinction procedure in eliminating long-term retention of conditioned fear.

In experimental extinction, fear conditioned rats are given repeated nonreinforced presentations of the conditioned stimulus. This leads to a reduction in the amplitude and frequency of conditioned responding to the CS cue. Currently, the predominant clinical intervention for fear- and anxiety-related disorders involves extinction procedures (i.e., repeated exposure to feared stimuli in a safe setting). While extinction procedures effectively reduce behavioral expression of fear, a reemergence of fear-related behaviors often occurs, especially in animal models. In such models, recovery of fear responses can occur following unsignaled presentations of feared stimuli (reinstatement) (Rescorla & Heth, 1975; Bouton & Bolles, 1979a), by testing for fear in a context different from

where extinction took place (renewal) (Bouton & Bolles, 1979b), or by the simple passage of time (spontaneous recovery) (Pavlov, 1927; Robbins, 1990). Based on these observations, it is generally agreed that the mechanisms of extinction involve new inhibitory learning rather than "unlearning" or memory erasure. However, recent evidence suggests that under certain conditions, extinction mechanisms may be analogous to a cellular model of "unlearning", called synaptic depotentiation.

Depotentiation is mechanistically a reversal of long-term potentiation (LTP), a cellular model of learning. It is induced by application of low-frequency stimulation (LFS) to certain afferent pathways immediately after LTP induction. This weakens potentiated synapses, thereby returning them back to their baseline state. Depotentiation also counteracts molecular processes associated with LTP induction. Whereas LTP induces phosphorylation of several memory-associated intracellular signaling cascades and down-regulates phosphatases (Riedel, 1999; Malinow & Malenka, 2002), depotentiation induction dephosphorylates many of the same intracellular messengers and up-regulates protein phosphatases (Zhou & Poo, 2004).

Depotentiation can be induced in the amygdala by LFS of the external capsule (Lin, Lee, & Gean, 2003; Lin et al., 2005). *In vivo* LFS of the amygdala given 10-min after Pavlovian fear conditioning disrupts fear-potentiated startle, increases phosphatase activity, and reverses learning-induced phosphorylation of Akt and mitogen-activated protein kinase (MAPK) (Lin, Lee, & Gean, 2003). Importantly, behavioral extinction can produce similar phenomena (Cannich et al., 2004; Myers & Davis, 2006). A critical feature of the abovementioned *in vivo* and *in vitro* amygdala depotentiation experiments is that they were given immediately after LTP or behavioral learning. These findings led

our laboratory to hypothesize that extinction training given soon after learning will disrupt fear expression in a manner akin to depotentiation or "unlearning".

Myers and colleagues (2006) tested the idea that immediate extinction (given 10-min post-acquisition) will preclude reemergence of fear expression due to reinstatement, renewal, and spontaneous recovery, whereas delayed extinction (given 72-hr post-acquisition) will not. Rats were presented with 90 extinction trials (lights in the absence of footshock), either 10-min, 1-hr, 24-hrs, or 72-hrs after 15 light-shock conditioning. When extinction was given 24-72 hrs after acquisition, rats showed signs of reinstatement, renewal, and spontaneous recovery. On the other hand, when extinction was given 10-min to 1-hr after acquisition, rats exhibited little to no reinstatement, renewal, or spontaneous recovery. These findings suggest that unlike delayed extinction, which is associated with new inhibitory learning, immediate extinction is associated with a process akin to erasure or "unlearning". These very exciting findings have clear implications for optimizing behavioral intervention in PTSD.

A number of studies ensued, in varied attempts to verify the generality of these findings. Contrary to the findings of Myers et al. (2006), several different laboratories – using a variety of protocols in rats and human – observed a reemergence of fear when extinction was administered both shortly and long after fear acquisition (Alvarez et al., 2007; Chang & Maren, 2009; Kim et al., 2010; Maren & Chang, 2006; Norrholm et al., 2008; Schiller et al., 2008; Woods & Bouton, 2008). Moreover, many of these studies reported that immediate extinction was even *less* efficacious in suppressing long-term fear expression than delayed extinction (Cammarota et al., 2005; Chang & Maren 2009; Kim et al., 2010; Maren & Chang, 2006; Woods & Bouton, 2008). These findings are

perhaps consistent with growing evidence that cognitive-behavioral treatments, which are usually delivered weeks or months after a traumatic experience, are generally more effective in reducing PTSD incidence than psychological debriefing procedures, which are typically given within hours or days following trauma (Bisson et al., 1997; Gray & Litz, 2005; McNally, 2003, but see Campfield & Hills, 2001). Taken together, these studies challenge the therapeutic efficacy of immediate extinction in thwarting PTSD.

Our goal is to use animal models of fear conditioning to develop clinically useful tools to treat people with fear- and anxiety-related disorders such as PTSD. When given shortly after trauma, extinction may be clinically ineffective. However, preclinical and clinical research suggests that memory traces are susceptible to disruption whenever they are in an active state of retrieval. A number of post-recall manipulations, such as electroconvulsive shock (ECS) and various pharmacological treatments have been shown to persistently inhibit fear memory (e.g., Misanin et al., 1968; Nader et al., 2000; Monfils et al., 2009). Hence, in a grant proposal in 2007 we proposed a novel procedure of delivering extinction immediately following fear memory recall. The central hypothesis behind this proposed model is that reactivation of an old fear memory will render the memory trace labile and susceptible to disruption by immediate extinction. Since that time, two papers found exactly this (Monfils et al., 2009; Schiller et al., 2010).

The present study sought to replicate Monfils et al. (2009) by testing the efficacy of immediate and delayed extinction, when delivered post-acquisition and post-recall, in reducing fear memory expression and preventing recovery. To test these ideas, the present study employed an olfactory-mediated fear-potentiated startle paradigm, which produces robust, long-lasting fear memories after a single training trial (e.g., Paschall &

Davis, 2002). This is a powerful tool to study the effects of post-training manipulations because it allows one to precisely pinpoint when learning occurs, and administer the putative memory-disrupting treatment immediately thereafter. The utility of an olfactory-mediated fear conditioning paradigm as a means to induce rapid and robust fear learning is largely based on findings of dense monosynaptic and reciprocal connections between primary olfactory structures and subcortical limbic regions implicated in learning and memory (Otto et al., 2000). Importantly, compared to other sensory modalities, olfactory information has unique direct access to the amygdala, exclusive of thalamic relay. Indeed, Ressler et al. (2002), has demonstrated with *in situ* hybridization that the same set of genes expressed in the amygdala after 5 trials of odor-shock pairings was similarly expressed after 10 trials of light-shock pairings. Hence, olfactory stimuli are especially salient cues that require few associative pairings with aversive stimuli to induce robust fear learning.

While odor-guided fear conditioning has garnered considerable attention for its ecological relevance and capacity for robust learning in rodents (e.g, Otto et al., 1997; Paschall & Davis, 2002; Richardson et al., 1999), very few studies have examined extinction of olfactory fear conditioning to nonsocial/nonpheromonal odor cues (e.g., Cloutier et al., 2006; Fannes et al., 2008; Richardson et al., 2000; Yap & Richardson; 2005). Interestingly, one group repeatedly showed that olfactory-mediated fear-potentiated startle did not readily extinguish, even after extended extinction trials, leading these researchers to conclude that olfactory-mediated fear conditioning is resistant to extinction (Richardson et al., 1999; Richardson, Paxinos, & Lee, 2000; Richardson et al., 2002). If this were true, then it would have important implications for the use of

olfactory-mediated fear conditioning models as a translational approach for informing treatments for PTSD.

Clinicians have known for a long time that certain trauma-related smells can trigger disturbing memories and induce debilitating fear- and anxiety-related behaviors in PTSD patients (e.g., the smell of blood or diesel in combat veterans) (Vermetten & Bremner, 2003). For instance, in a recent Positron Tomographic Emission (PET) study, Vermetten and colleagues (2007) exposed combat veterans with PTSD and combat controls without PTSD to a diesel smell and found an increase in regional blood flow in the amygdala, among other brain regions, in PTSD patients but not in combat controls. Based on these and similar findings, several investigators have recommended the clinical application of odor cues in PTSD assessment (Hinton et al., 2004; Kline & Rausch, 1985; Vermetten & Bremner, 2003). If animal models of olfactory fear conditioning are proven to be resistant to extinction, it could be problematic for the clinical use of odor stimuli to assess and remedy PTSD symptoms. To examine the generality of the findings of Richardson and colleagues (1999, 2000, 2002), the present study examines the capacity for olfactory-mediated fear conditioning to undergo experimental extinction and its susceptibility to spontaneous recovery.

Methods

Animals

Male Sprague-Dawley rats (N=52) (Charles River, Raleigh, NC), weighing between 300 and 400 grams at the start of experimentation, were group housed four to a cage, and maintained on a 12:12 hour light / dark cycle with food and water available ad libitum. All behavioral procedures took place during animals' light cycle.

Apparatus

Rats were trained and tested in two identical 8 x 15 x 15 cm Plexiglas and wire mesh cages as previously described by Cassella and Davis (1986). Background noise (60 dB wideband) and startle stimuli (50 ms white-noise bursts; rise decay, 5 ms) were delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound-level measurements were made with a Brüel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (type 4176) located 7 cm from the center of the speaker, which approximates the distance of the rat's ear from the speaker during testing. Startle response amplitudes were quantified using an Endevco (San Juan Capistrano, CA) 2217E accelerometer. Cage movement produced by the rat's startle response resulted in displacement of the accelerometer, the output of which was integrated, producing a voltage output proportional to the velocity of cage movement. This signal was amplified by an Endevco model 104 amplifier and digitized on a scale of 0–2510 units by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 (Apple Computers, Cupertino, CA) computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 300 ms after onset of the startle-eliciting noise burst.

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delivery of the olfactory stimulus, a computer-controlled solenoid (Model H15-03; Coulbourn Instruments, Allentown, PA) was opened for 4s, thereby diverting clean air from the compressed-air cylinder into and through a sealed 135-cm^3 glass jar containing 20 ml of 5% (vol/vol) amyl acetate (i.e., the odorant) in propylene glycol solution. The inlet and outlet ports of the glass jar were positioned above the solution such that clean air from the tank mixed with the amyl acetate-containing vapor. The output was then mixed in a 3:5 ratio with clean air before flowing into the cage.

The chamber was actively exhausted into the building's ventilation system at a rate of 0.0114m^3/s. Thus, a volume of air equal to the chamber's total volume was vented every 25 s. Previous results with fear-conditioned rats indicate that with theses procedures startle amplitude returns to baseline levels within 30 s of solenoid closure (Paschall & Davis, 2002). Cages were cleaned daily with warm tap water and 95% alcohol, and were air dried overnight.

The unconditioned stimulus was a 0.5 s 0.4 mA scrambled shock delivered through the four floor bars as described by Walker and Davis (1997). The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads, Newton, CT).

Behavioral procedures

Matching. On each of two consecutive days, rats were placed in the startle chamber and after a 5-min acclimation period, received 30 presentations of startle stimuli (95 dB noise burst) separated by a 30-s intertrial interval. Rats were removed from the chamber immediately after the last startle stimulus presentation. Their mean startle amplitudes were calculated, and marked as their pre-training startle baseline. Rats were then divided

into treatment groups according to their startle baselines, such that the mean startle amplitudes were balanced across groups.

Training

Experiment 1. The next day, rats were returned to the same startle chambers in which they were matched. After five minutes of acclimation, rats received a series of 5 odor-shock pairings (4-s odor co-terminating with a 0.5-s footshock), with 4-min intertrial intervals. Seventy-two hours after training, rats were returned to the startle cages. After a 5-min acclimation period, one group of rats received 15 noise-alone trials (post-train baseline) followed by 30 odor-noise trials, which were intermixed with 30 noise-alone trials presented in a balanced mixed order with 30-s ISI (extinction group). This procedure allowed for within-session extinction testing. This procedure was repeated over 3 days such that the extinction group received a total of 90 odor-noise trials. A separate group of rats were returned to their home cages after fear acquisition where they remained until testing (no extinction group). All rats were tested for spontaneous recovery 21-days after the initial fear-potentiated startle test.

Experiment 2. Twenty-four hours after matching, rats were presented with a single odor-shock pairing then immediately returned to their home cages. Five mins later, some rats (10 min post-acquisition group) were returned to the conditioning chamber, and after a 5-min acclimation period, presented with 90 odor-alone extinction trials with a 30-s ISI. Another group of rats (72 hrs post-acquisition group) received similar extinction training 72-hrs after acquisition. For both 10 min and 72 hr extinction conditions, the acquisition and extinction contexts were either identical (AAA groups), or the extinction context was altered with sandpaper inserts over the shock bars, Velcro on the sides, and 2 metal-link

chains suspended from the top (ABA groups). These alterations have been previously shown to reliably produce discriminable context conditioning (McNish et al., 1997).

Experiment 3. Twenty-four hours after matching, rats were presented with a single odor-shock pairing then immediately returned to their home cages. Twenty-four hrs later, rats were returned to the conditioning chamber and after a 5-min acclimation period were presented with a single 4-s odor CS. Immediately thereafter, rats were returned to their home cages. One group of rats was returned to the conditioning chamber 5-min later and after a 5-min acclimation period, was presented with 90 extinction trials (10 min post-recall group). Another group was returned to the chambers 72 hrs after the recall trial and similarly delivered 90 odor presentations. Similar to Experiment 2, rats received fear conditioning and extinction training in the same context (AAA), or different context (ABA).

Testing

Twenty-four hours after extinction training, rats were returned to the startle cages, and after 5-min, were presented with 30 startle stimuli (leaders). Thirty seconds after the final leader stimulus, rats received 30 startle-eliciting noise bursts presented alone (noise-alone trial) and 10 noise bursts presented 3.2-s after onset of the 4-s odor (odor-noise trials). The two trial types were presented in a manner such that after each odor-noise trial, three noise-alone trials were presented at a 30-s ITI, so that noise-alone trials occurred 30, 60, and 90-s after each odor. All testing occurred in the same context as fear conditioning.

Statistical Analyses

The mean startle amplitude on noise-alone and odor-noise trials was calculated for each rat. Difference scores were calculated as: (odor-noise minus noise alone trials). Percent fear-potentiated startle was calculated as: [(odor-noise minus noise-alone trials) / (noise-alone trials)] × 100. All data were analyzed by independent sample's t-test, analysis of variance (ANOVA), or repeated measures followed by individual mean comparison using Tukey's post hoc tests. A significance level of $p < 0.05$ is taken for all results.

Results

Experiment 1

Extinction of olfactory fear memory and spontaneous recovery

This experiment addressed the issue of whether or not olfactory fear conditioning is resistant to extinction, as suggest by Richardson et al. (1999, 2000, 2002). There are major parametric differences between our olfactory-mediated fear-potentiated startle protocol and that of Richardson's group. Their training, extinction, and testing procedures involved soaking an odorant on a paper towel and placing it in a plastic specimen jar. This jar was manually placed underneath the startle chamber for CS-US presentations, and similarly removed between trials. Extinction training was delivered as a single presentation of continuous odor throughout the extinction session.

In our model, a 4-s odor is presented in a discrete manner, rather than as a diffuse cue. Throughout conditioning, extinction and testing, a continuous flow of filtered clean air is delivered into the chamber. During odor CS presentations, an odor solenoid valve opens, and the odor CS blends into the clean airstream for 4-s, after which the solenoid valve closes, and clean air continues to flow. Meanwhile, the chamber is actively

exhausted whereby a volume of air equal to the chamber's total volume is vented every 25-s. We consistently find with theses procedures that fear-potentiated startle amplitude returns to baseline levels within 30-s of solenoid closure (Glover & Davis, unpublished observations; Paschall & Davis, 2002). We believe that this procedure allows for a more stringent measure of rats' startle responding in the presence versus the absence of an odor CS, and hence, a better quantification of conditioned fear expression. We used this protocol to test the generality of the findings of Richardson and colleagues (1999, 2000, 2002).

The experimental procedure is shown schematically in Figure 1a. Figure 1b shows the mean startle amplitudes for rats that received extinction training (n=10, white) or no extinction training (n=10, black), measured during the 30 noise alone trials of the pre-training baseline startle test (pre-training baseline), the 30 noise alone trials which occurred at the beginning of the test session (post-training baseline), the 10 odor-noise test trials (odor-noise), and the 30 noise-alone test trials that were intermixed with odor-noise test trials (noise-alone). Rats that got extinction training startled considerably less in the presence of the odor CS, relative to rats that did not get extinction training. Our laboratory has previously observed an increase in baseline startle responding from the pre-training acclimation session to the post-training test session, which we believe reflects fear of the context where conditioning occurred (McNish et al., 1997; McNish et al., 2000). Interestingly, the present findings show that non-extinguished rats display appreciable context fear, but the extinguished rats do not (Figure 1b)

Figure 1c shows the mean startle amplitudes during the noise alone trials (dark gray), the odor-noise trials (light gray), and the difference between the two (stripes). An

independent-samples t-test comparing the difference scores of extinction (M = 0.29, SD = 0.33) versus no extinction groups (M = 0.85, SD = 0.74) groups yielded a significant difference between groups, $t(18) = 2.17$, $p < .05$.

In animal models of extinction, spontaneous recovery of fear responses can occur with the passage of time (Pavlov, 1927; Robbins, 1990). To test for this phenomenon in olfactory fear extinction, animals were given another fear-potentiated startle test 21-days following testing. Figure 2a shows a schematic representation of the entire experimental procedure. Figure 2b shows the mean difference scores (±SEM) between odor-noise trials and noise-alone trials of groups that either got extinction (n=10, white) or no extinction (n=10, black) and tested 24-hrs and 21-days later. A two-way ANOVA comparing Test Time (24-hr vs. 21-day) as the within subject factor and Treatment (extinction vs. no extinction) as the between subject factor yielded no main of effect of Test Time, $F(1,18) = .51$, $p > .05$, and no main effect of Treatment, $F(1,18) = 1.34$, $p > .05$, but a significant Test Time X Treatment interaction $F(1,18) = 3.34$, $p < .05$. While the no extinction group displayed a modest decrease in fear from the 24-hr test to the 21-day test, the extinction group showed considerably more fear at the 21-day test interval relative to the 24-day test, indicating spontaneous recovery.

Experiment 2

Effects of immediate versus delayed extinction on renewal of olfactory fear memory

This experiment tests the generality of the findings of Myers et al. (2006) that extinction given within 10-min of acquisition prevents renewal of fear, typically seen when testing for fear in a context different from where extinction took place. Figure 6a shows a schematic representation of the experimental procedure. Rats were trained with a

single odor-shock pairing, and were extinguished 10-min or 72-hrs later, either in the same (context A) or different (context B) context from which acquisition took place. Figure 6b shows the mean difference scores (±SEM) for rats that were extinguished either 10-min (n=10) or 72-hrs (n=10) post-acquisition and tested in the same context as where extinction occurred (AAA-white, n=5), or tested in a different context from extinction (ABA-gray, n=5).

Considering the means, it is apparent that the 10-min extinction group displayed comparable levels of fear as the 72-hr extinction group when trained, extinguished, and tested in the same context (AAA). However, the 10-min groups shows considerable less renewal of fear relative to the 72-hr group when tested in a different context from extinction (ABA). Nevertheless, these apparent differences did not reach statistical significance. A two-way ANOVA comparing Time point (10-min vs. 72-hr) and Condition (AAA vs. ABA) yielded no significant main effect of Time points, $F(1,20) = .03$, $p > .05$, no main effect of Condition, $F(1,20) = 3.81$, $p > .05$ and no Time point X Condition interaction $F(1,20) = .24$, $p > .05$. It is concluded that delayed extinction produces more renewal of fear than immediate extinction. However, this is not a robust phenomenon.

Experiment 3

Effects of immediate post-recall extinction versus delayed extinction on renewal of olfactory fear memory

This study compares the efficacy of extinction when delivered within 10-min after recall versus 72-hrs after recall on preventing renewal of fear memory. Figure 7a shows the protocol for this experiment. Figure 7b shows the mean difference scores (±SEM) of

rats that were extinguished either 10-min (n=12) or 72-hrs (n=12) post-recall and tested in the same context as where extinction occurred (AAA-white, n=6), or tested in a different context from where extinction occurred (ABA-gray, n=6). When extinguished and tested in the same context (AAA), rats that were extinguished 10-min after recall expressed similar levels of fear as rats that were extinguished 72-hrs after recall. On the other hand, when extinguished and tested in different contexts (ABA), rats in the 72-hr group showed slightly more renewal of fear than rats in the 10-min group. However, this difference was not robust and did not reach statistical significance. A two-way ANOVA comparing Time point (10-min vs. 72-hr) and Condition (AAA vs. ABA) yielded a significant main effect of Condition, $F(1,24) = 6.86$, $p < .05$, but no main effect of Time points, $F(1,24) = .77$, $p > .05$, and no Time point X Condition interaction $F(1,24) = 1.93$, $p > .05$.

Discussion

The present study uses the fear-potentiated startle model to examine extinction and recovery of olfactory conditioned fear memories. It has been previously shown that olfactory fear memories do not readily extinguish (Richardson et al., 1999, 2000, 2002). However, in each of those studies, the odor conditioned stimulus (CS) was presented as a diffuse, continuous, environmental cue, rather than as a discrete unit. Our experimental set-up is equipped for discrete 4-s odor presentations, permitting the measurement of startle in the presence versus the absence of an odor CS. The current study demonstrates that five-trial olfactory fear conditioning produces robust fear-potentiated startle in no-extinction controls. However, fear-potentiated startle was greatly diminished in rats that got 90 non-reinforced, discrete odor presentations 72-hrs after fear acquisition (Figure 1).

When tested again after a 21-day delay, fear-potentiated startle was significantly increased in the extinction group, and slightly decreased in no-extinction controls (Figure 2). The increase in startle from the 24-day test to 21-day test was not due to incubation of fear memory, because the extinction controls did not show an increase in fear memory over time. From these data, we conclude that olfactory fear conditioning shows normal extinction that is susceptible to spontaneous recovery over time. The findings by Richardson and collagues (1999, 2000, 2002) that olfactory fear memories are resistant to extinction might reflect limitations in their experimental protocol.

Spontaneous recovery, renewal, and reinstatement of extinguished fear present a major challenge to the clinical effectiveness of extinction procedures in treating anxiety disorders (Rodriguez et al., 1999). Hence, finding strategies to prevent the reemergence of fear after extinction is of vital importance to fear memory researchers. After confirming that olfactory fear memories undergo extinction and recovery, we set out to explore the efficacy of immediate extinction in preventing the recovery of extinguished fear memories. The present findings show that when extinction is given within 10-min of fear acquisition or retrieval, it inhibits fear memory expression to levels that are comparable to levels produced by 72-hr extinction. Importantly, while both 10-min and 72-hr extinction showed signs of renewal, the 72-hr extinction groups showed more renewal than the 10-min extinction groups. This pattern of results was evident in both post-acquisition and post-retrieval extinction treatment conditions. While these findings were not statistically significant, we conclude that they show promise and warrant further research.

The present results are consistent with Myers et al. (2006), who demonstrated that extinction given 10-min after fear acquisition produced less renewal than that of extinction given 72-days after training. On the other hand, the current results do not support the finding that immediate extinction is less efficacious than delayed extinction in preventing recovery (Cammarota et al., 2005; Chang & Maren 2009; Kim et al., 2010; Maren & Chang, 2006; Woods & Bouton, 2008). In an attempt to reconcile their discrepant findings, Maren and Chang (2006) found that they could replicate the findings of Myers et al. (2006) when they used weak (single-trial) conditioning as opposed to five-trial conditioning. The present finding that single-trial olfactory fear conditioning produced a similar pattern of results as Myers et al. (2006) suggest that there might be some merit to the idea that strength of conditioning is a determining factor in the efficacy of immediate extinction in preventing recovery. Further research is needed to identify other factors that may contribute to differential vulnerabilities of fear memory expression to immediate extinction.

Monfils and colleagues (2009) recently published a high profile paper demonstrating that extinction given 10-min and up to 1-hr after, but not 6-hrs after fear memory retrieval prevented spontaneous recovery, renewal, and reinstatement. They further demonstrated that fear memory retrieval by itself resulted in phosphorylation of the GlurR1 glutamate receptor, and immediate post-retrieval extinction reversed these learning-related changes. This evidence provides the strongest support to date for the idea that immediate post-recall extinction inhibits long-term fear expression via erasure mechanisms. The clinical promise of immediate post-recall extinction was further established by Schiller et al. (2010), who demonstrated in humans that extinction given

10-min, but not 6-hrs after fear memory recall prevented spontaneous recovery and reinstatement, even when measured up to a year later. Those findings, together with the present findings provide support for the clinical use of a novel behavioral technique to prevent the return of unwanted fear memories in PTSD.

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Figure Captions

Figure 1. **Olfactory fear conditioning shows normal extinction.** **(A)** Schematic representation of experimental protocol. **(B)** Mean startle amplitudes for rats that received extinction training (n=10, white) or no extinction training (n=10, black), measured during pre-training baseline testing, post-training baseline testing, odor-noise test trials, and noise-alone test trials. **(C)** Mean startle amplitudes during the noise alone trials (dark gray), the odor-noise trials (light gray), and the difference between the two (stripes). There was a significant difference among no extinction and extinction groups, * $p < .05$.

Figure 2. **Extinguished olfactory fear memories show spontaneous recovery. (A)** Schematic representation of experimental protocol. **(B)** Mean difference scores (±SEM) between odor-noise trials and noise-alone trials of groups that either got extinction (n=10, white) or no extinction (n=10, black) and tested 24-hrs and 21-days later. A significant Test Time X Treatment interaction, * $p < .05$ showed that the extinction group startled significantly considerably more at the 21-day test interval relative to the 24-day test, indicating spontaneous recovery.

Figure 3. **Immediate post-acquisition extinction shows less renewal than delayed extinction. (A)** Schematic representation of experimental design. **(B)** Mean difference scores (±SEM) for rats that were extinguished either 10-min (n=10) or 72-hrs (n=10) post-acquisition and tested in the same context as where extinction occurred (AAA-white, n=5), or tested in a different context from extinction (ABA-gray, n=5). The 10-min

extinction group showed similar fear inhibition as the 72-hr extinction group, but less renewal. These effects did not reach statistical significance.

Figure 4. **Immediate post-recall extinction shows less renewal than delayed post-recall extinction.** **(A)** Schematic representation of experimental design. **(B)** Mean difference scores (±SEM) of rats that were extinguished either 10-min (n=12) or 72-hrs (n=12) post-recall and tested in the same context as where extinction occurred (AAA-white, n=6), or tested in a different context from where extinction occurred (ABA-gray, n=6). When extinguished and tested in the same context (AAA), rats that were extinguished 10-min after recall expressed similar levels of fear as rats that were extinguished 72-hrs after recall. On the other hand, when extinguished and tested in different contexts (ABA), rats in the 72-hr group showed slightly more renewal of fear than rats in the 10-min group. This effect was not significant.

Figure 1.

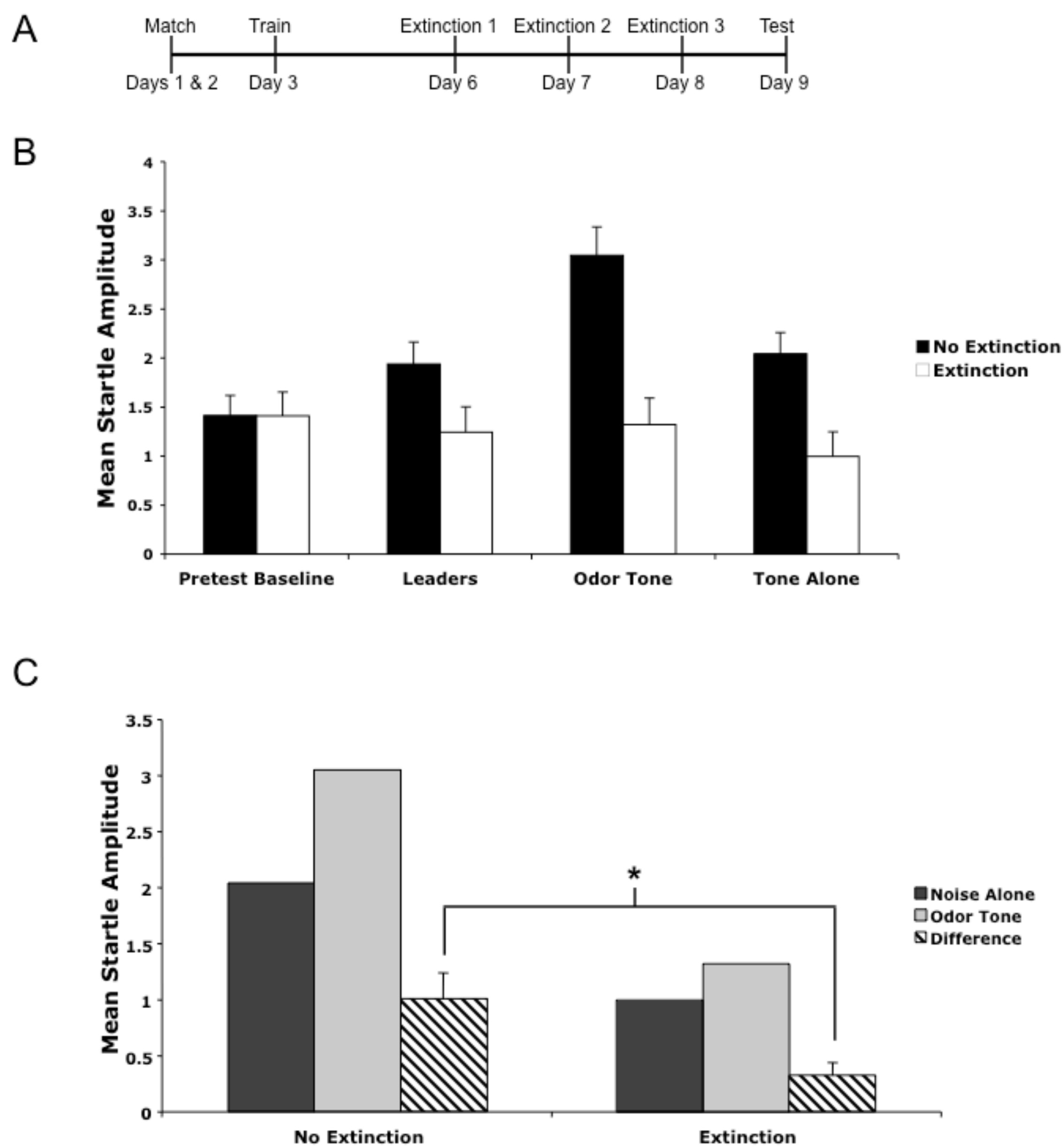

Figure 2.

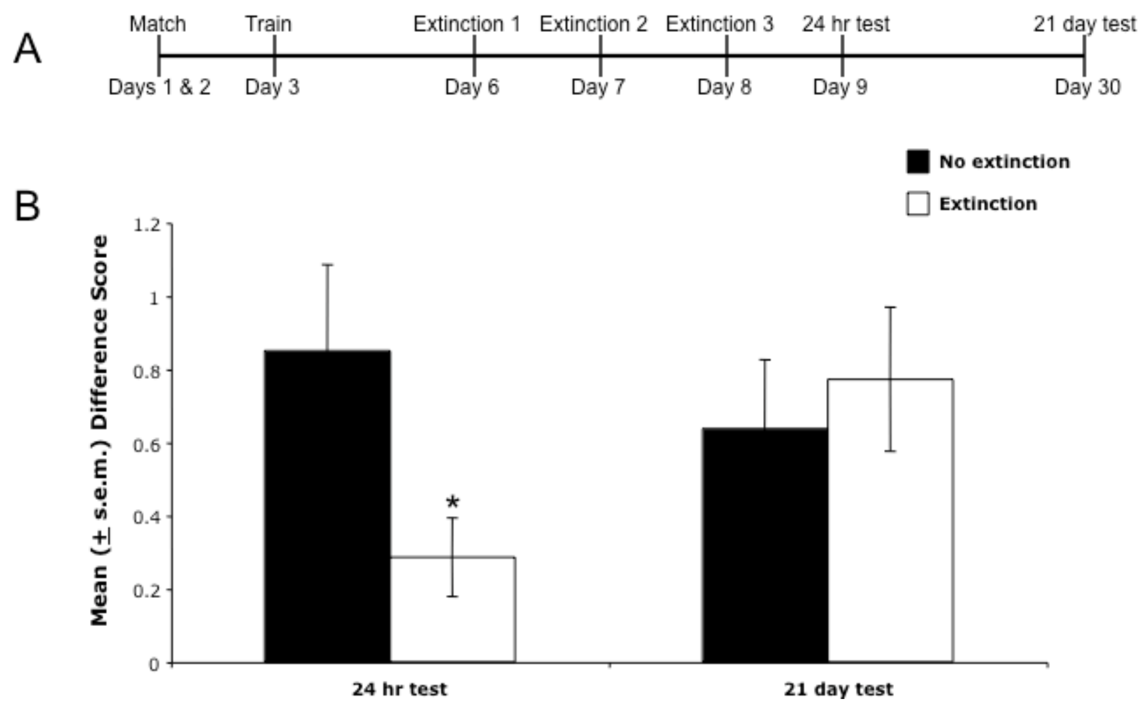

Figure 3.

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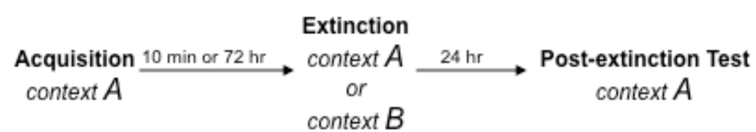

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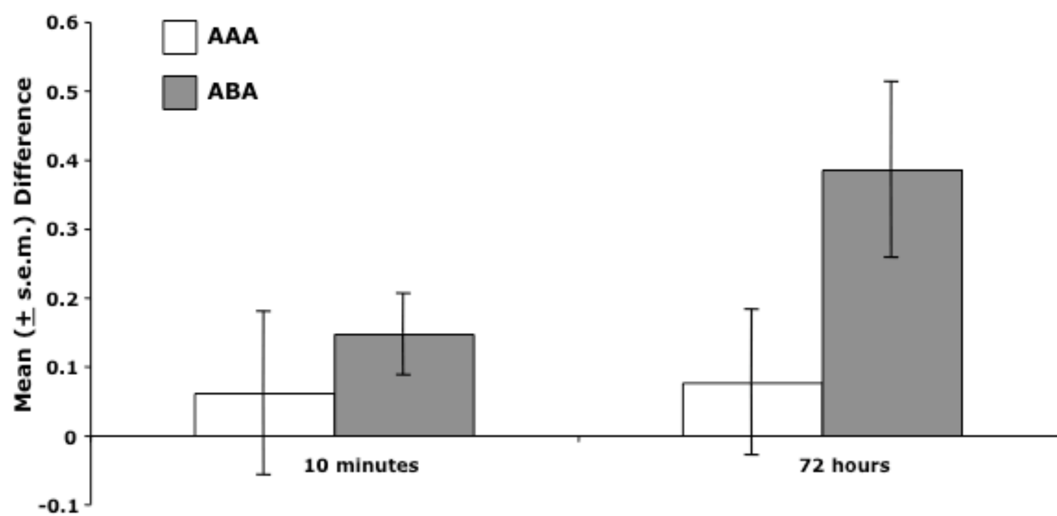

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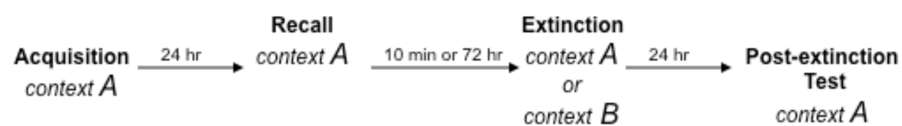

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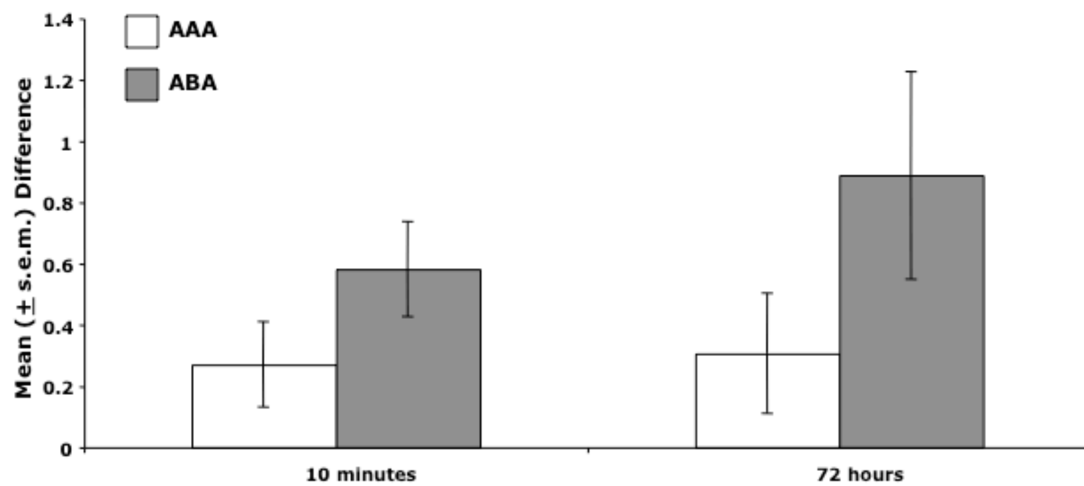

CHAPTER 4

Dose and time-dependent effects of systemic propranolol on consolidation and reconsolidation of delay, trace, and context fear memories

Abstract

Research demonstrating a critical role of norepinephrine in fear memory consolidation has led investigators to suggest that the β-adrenergic blocker, propranolol, might hold clinical promise in preventing unwanted memories in PTSD. However, despite ample preclinical evidence showing that propranolol disrupts consolidation of inhibitory avoidance, there is a small literature showing that propranolol does not block the consolidation of Pavlovian fear conditioning to a simple cue. However, propranolol effectively disrupted the reconsolidation of this memory. The current study tested the hypothesis that propranolol will disrupt the consolidation of Pavlovian fear conditioning tasks that are hippocampal-dependent (i.e., trace, and context fear), but not a Pavlovian conditioning task that is hippocampal-independent (i.e., delay fear conditioning). The effects of propranolol on the reconsolidation of these memories were also examined. It has been demonstrated that a low dose of systemically administered propranolol decreased spontaneous activity in the amygdala, whereas a high dose increased spontaneous activity. The present study tested the idea that low doses of propranolol will be more efficacious in disrupting fear retention than high doses. Taken together, the current findings generally supported these hypotheses.

Introduction

Persistent and intrusive fear memories (flashbacks) are one of the major symptoms of posttraumatic stress disorder (PTSD). A substantial body of evidence has implicated the adrenergic system in the pathophysiology of these symptoms (see O'Donnell, Hegadoren, & Coupland, 2004; Strawn & Geracioti, 2008). The current project uses a rat model of Pavlovian fear conditioning to explore the idea that administering the β-adrenergic blocker, propranolol, in the aftermath of a traumatic experience might be a useful approach to prevent fear memory formation and forestall PTSD.

It is widely held that endogenous stress hormones, epinephrine and norepinephrine (NE), are released in response to fear-provoking stimuli, and play a critical role in modulating the strength of emotional memories (McGaugh, 2005). Early evidence supporting this idea was provided by Gold and van Buskirk (1975), who demonstrated that immediate post-training, subcutaneous (s.c.) injections of epinephrine enhanced retention of inhibitory avoidance learning. However, when they injected epinephrine two hours after training, no effect on retention was found. Interestingly, this time-dependent effect of epinephrine was also dose-dependent, and took the course of an inverted-U dose-response curve. Thus, small doses had no effect, moderate doses had the greatest effect, and large doses seemed to impair retention. Furthermore, the intensity of footshock delivered during training predicted the optimal dose for enhancing retention. That is, a dose that had been shown to enhance memory of a low intensity footshock training experience, now actually impaired retention when a high intensity footshock was used (Gold & van Buskirk, 1978). These findings advanced the viewpoint that post-

training processes can amplify or dampen memory strength, that epinephrine may serve as an endogenous mediator of these processes, and that its actions are graded by the motivational significance of the training experience.

However, because epinephrine does not readily cross the blood brain barrier (Weil-Malherbe, Axelrod, & Tomchick, 1959), it was obvious that some other mechanism was mediating these effects in the central nervous system. It was later demonstrated that levels of NE were increased in the brain of rats given a memory-enhancing dose of epinephrine immediately after training (Gold & van Buskirk, 1978). It is now known that emotionally arousing experiences prompt the release of epinephrine from the adrenal medulla, and that peripheral epinephrine acts on β-adrenergic receptors located on vagal afferents to the nucleus of the solitary tract (NTS) in the brain stem. Furthermore, the NTS sends noradrenergic projections to forebrain structures thought to be involved in learning and memory processes (Clayton & Williams, 2000; Williams, Men, & Clayton, 2000; Williams et al., 1998). Thus, epinephrine may serve as a peripheral messenger of stimulus salience that initiates the release of NE in the brain, which modulates memories accordingly.

In support of this hypothesis, Jensen et al. (1977) found that intracerebroventricular (i.c.v.) administration of diethydithiocarbamate, a drug that decreases central norepinephrine levels, impaired inhibitory avoidance retention when administered immediately post-training and Haycock et al. (1977) reported that retention of inhibitory avoidance is enhanced by post-training i.c.v. administration of NE. These findings have since been substantiated by a great deal of evidence showing that immediate post-training administration of NE facilitates retention of inhibitory avoidance

learning, while β-adrenergic receptor antagonists impair retention (for review, see McIntyre et al., 2003; Ferry, Roozendaal, & McGaugh, 1999; McGaugh, 2004).

It is now widely held that NE acts in the amygdala to modulate the consolidation of certain fear memories. Immediate post-training infusion of NE into the amygdala enhances retention of inhibitory avoidance training, while lesions of the amygdala or intra-amygdala infusions of β-adrenergic receptor antagonists block this enhancement (see Liang, 1986; McGaugh, 2004; McIntyre et al., 2003). Also, NE is released in the amygdala by immobilization (Tanaka et al., 1991), tail pinch stress (Pacak et al., 1993), footshock (Galvez, Mesches & McGaugh, 1996) and by drugs known to enhance inhibitory avoidance retention (McIntyre et al., 2003). Research linking the amygdala – the brain region most implicated in fear-related memories – to the adrenergic system, has led investigators to suggest that adrenergic drugs may hold clinical promise in forestalling maladaptive memories in PTSD (Cahill, 1997).

Pitman (1989) postulated that a traumatic experience could over-stimulate endogenous stress hormonal systems, and precipitate a heightened release of epinephrine and norepinephine. This brings about an “over-consolidation” of memories, leading to the formation of extremely strong, potentially maladaptive fear memories, such as those apparent in PTSD. Thus, it follows that certain adrenergic antagonists, when administered in the aftermath of the trauma, might oppose noradrenergic influences on fear memory formation and reduce the formation PTSD.

This hypothesis was advanced by a seminal finding in humans that the β-adrenergic antagonist, propranolol, impaired long-term memory of an emotionally arousing story but did not affect memory of an emotionally neutral story (Cahill et al.,

1994). It was also shown PTSD patients show higher levels of sympathetic nervous system arousal during aversive conditioning and are “more conditionable” than trauma-exposed individuals who did not develop PTSD (Orr et al., 2000). Furthermore, clinical investigators have correlated prolonged adrenergic activation in the aftermath of a trauma with an increased risk for developing PTSD (Viava et al., 2003).

Propranolol is a centrally acting, nonselective β-adrenergic antagonist that has already received FDA approval for the treatment of various heart-related conditions. Researchers are currently examining propranolol in clinical trials to assess its efficacy at weakening early symptoms of extreme arousal and ultimately forestalling memory-related PTSD symptoms. In 2002, Pitman launched the first clinical study to directly test this hypothesis in traumatized people. In a pilot study to investigate the efficacy of propranolol in preventing PTSD, traumatized emergency room patients were administered 40 mg of propranolol or placebo immediately following a traumatic event, and instructed to maintain a daily treatment course for 10 days. When assessed 1 month later, fewer individuals in the propranolol treated group developed PTSD compared with those who received placebo. Furthermore, when tested 3 months later, the propranolol treated group showed fewer signs of hyperarousal in response to trauma reminder cues than those in the placebo group (Pitman, 2002).

Vaiva and colleagues conducted a similar preliminary study in 2003. They gave physically injured victims of motor-vehicle accidents 30 mg of propranolol or placebo 3 times a day for 7 days following the injury. However, when assessed for PTSD two months later, there was no significant difference in rates of PTSD between propranolol and placebo groups. However, the propranolol group had lower levels of PTSD than the

placebo group (Vaiva et al., 2003). While there were many limitations in these pilot studies, the overall findings were considered promising and gave credence to the idea that propranolol may be an effective prophylactic agent for PTSD. Currently, there are a few large-scale randomized controlled trials underway to further determine propranolol's efficacy in preventing PTSD (Pitman & Delahanty, 2005; Stein, Dimsdale, & Hoyt, 2007)

So far, two recent clinical studies have failed to find evidence supporting the use of propranolol to treat PTSD. In a fairly large double-blind, randomized controlled study, Stein and colleagues administered up to 40 mg of propranolol or placebo 3 times a day for 14 days to patients admitted to a surgical trauma center within 48 hours of their traumatic injury (Stein, Dismdale, & Hoyt, 2007). When they assessed for PTSD 1, 4 and 8 months later, they found no significant difference in rates of PTSD between groups that received propranolol and placebo. McGhee and colleagues recently published a retrospective study that examined the prevalence of PTSD in burn patients who were given propranolol to prevent muscle catabolism, compared to burn victims who did not receive propranolol (McGhee et al., 2009). These data, obtained from a military burn center, revealed that the prevalence of PTSD in patients that got propranolol was not statistically different from patients that did not receive propranolol. From these data, the authors concluded that propranolol is not an effective prophylactic for PTSD.

Taken together, these preliminary clinical trials are largely inconclusive regarding the effectiveness of propranolol in preventing PTSD in humans. While the rationale for this approach is based on sound preclinical research, an alternative literature suggests a reexamination of this approach is warranted. Despite ample preclinical evidence showing

that intra-amygdala application of propranolol impairs long-term memory of inhibitory avoidance learning (Gallagher et al., 1977; Lennartz et al., 1996; but see Izquierdo et al., 1992), there is also evidence showing that NE in the amygdala is not important for the consolidation of Pavlovian fear conditioning. Debiec and Ledoux (2004) infused propranolol into the amygdala immediately after cued fear conditioning, and failed to find a retention deficit, even though they used a dose that has been consistently shown to disrupt inhibitory avoidance retention. There is also evidence showing that *systemically* administered propranolol has no effect on the consolidation of Pavlovian fear conditioning. Lee et al. (2001) gave rats systemic injections of epinephrine, amphetamine, and two β-adrenergic receptor antagonists, sotalol and propranolol, immediately following Pavlovian fear conditioning of a simple cue. None of these manipulations affected conditioned fear retention. Similarly, Debiec and Ledoux (2004) failed to find an effect of systemic propranolol on memory retention when given immediately after cued fear conditioning.

Almost all the preclinical evidence supporting a role of NE in fear memory consolidation comes from studies using the inhibitory avoidance paradigm, a task that is dependent on the hippocampus as well as the amgydala (Roozendaal et al., 1999; Izquierdo et al., 1997), whereas, studies showing no effect of NE in fear memory formation used Pavlovian fear conditioning to a simple cue, an amygdala-dependent but hippocampal-independent task (Bast et al., 2003; Selden et al., 1991; Kim & Fanselow, 1992). Grillon and colleagues (2004) argued that inhibitory avoidance learning is more like contextual fear conditioning (which is both amygdala- and hippocampal-dependent) than cued fear conditioning, and hence, hypothesized that context fear would be

susceptible to disruption by propranolol. In support of this hypothesis, they demonstrated in humans that pre-training oral propranolol administration disrupted long-term retention of context fear but had no effect on retention of cued fear (Grillon et al., 2004). These findings, together with evidence obtained from rodents (Debiec & LeDoux, 2004; Lee et al., 2001), suggests that hippocampal-dependent fear memories are susceptible to β-adrenergic influence, whereas hippocampal-independent forms of fear memories are not.

The overall goal of the present study was to examine the efficacy of systemically administered propranolol in disrupting hippocampal-dependent versus hippocampal-independent forms of fear memory. Our laboratory has developed an odor-guided Pavlovian fear conditioning task that is amygdala-dependent (Walker, Paschall, & Davis, 2005), rapidly acquired, and well remembered over time (Glover, Paschall, & Davis unpublished results; Paschall & Davis, 2002). The current study used this paradigm to compare the effects of systemically administered propranolol on the consolidation of three different kinds of Pavlovian fear conditioning tasks, which are either hippocampal-independent (i.e., delay fear conditioning) or amenable to hippocampal influence (i.e., context and trace fear conditioning). It was hypothesized that systemic propranolol will disrupt the consolidation of context and trace fear conditioning, but not delay fear conditioning.

There is a small literature suggesting an important role of dosage and route of administration in the differential effects of propranolol on fear memory formation. Schneider and colleagues (2000) found that systemically administered propranolol dose- and time-dependently *enhanced* inhibitory avoidance retention – a paradoxical observation, considering that local administration of propranolol into the amygdala has

consistently been shown to impair inhibitory avoidance retention. Interestingly, this enhancement was observed with a dose of 10 mg/kg but not 4 mg/kg, and when propranolol was given immediately, but not 2 hrs after training. To explain the differing effects of local versus systemic propranolol on inhibitory avoidance retention, they offered that system-wide blockade of β-adrenergic receptors might oppose the action of local β-adrenergic blockade. This argument was based on an in vitro electrophysiology study which found that in the presence of the β-adrenergic agonist, isoproterenol, tetanic stimulation of the medial amygdala resulted in enhanced short-term potentiation (STP) whereas, stimulation of the lateral amygdala resulted in a suppression of STP (Watanabe et al., 1996). Hence, it is possible that NE modulation of fear memory depends on a global balance between excitatory and inhibitory processes, which are differentially affected by locally versus systemically administered propranolol.

Simson and colleagues (2001) examined the effect of various doses (4, 7, 10 mg/kg) of systemically administered propranolol on spontaneous activity of central amygdala (CeA) neurons. They found that a high dose (10 mg/kg) of propranolol increased CeA spontaneous activity, whereas low (4 mg/kg) and intermediate (7 mg/kg) doses decreased spontaneous activity. They proposed that systemic propranolol dose-dependently affects two opposing (excitatory and inhibitory) modulatory circuits, which tonically influence amygdala spontaneous activity. Based on their findings, they postulated that high doses of propranolol increases spontaneous activity via selective blockade of inhibitory circuits, and low doses of propranolol decrease spontaneous activity via a blockade of excitatory circuits. This interpretation might explain the findings of Schneider et al. (2000) that a high dose (10 mg/kg) of systemically

administered propranolol produced an enhancement in long-term retention of inhibitory avoidance learning, possibly via disinhibition of CeA activity. Importantly, these findings suggests that low doses of propranolol might be more efficacious at disrupting fear memory, possibly through a preferential blockade of excitatory amygdala activity. The current study explores this possibility by examining the effects of various doses of systemically administered propranolol on fear memory retention. Furthermore, given the observation that the greatest inhibition of spontaneous activity occurred 15 min after systemic propranolol administration (Simson et al., 2001), we examined whether or not time of administration is a factor in the efficacy of systemic propranolol in disrupting fear memory retention.

The current project is centered upon the idea that disrupting fear memory in the immediate aftermath of a traumatic experience might be a useful approach for treating certain PTSD symptoms. However, this approach will not help people whose debilitating fear memories were formed well in the past. However, a phenomenon called reconsolidation blockade, whereby stable fear memories may be disrupted if perturbed immediately after retrieval, may prove useful for eliminating long-term fear memories (Nader, 2000). A burgeoning literature suggests that propranolol may be particularly promising as a prophylactic for PTSD via reconsolidation blockade (Debiec & Ledoux, 2004, 2006; Kindt et al., 2009; Przybyslawski et al., 1999; Soeter & Kindt, 2010). Importantly, unlike its effects on consolidation, propranolol has been shown to disrupt the reconsolidation of hippocampal-independent cued fear memory in rodents (Debiec & Ledoux, 2004 & 2006) as well as skin conductive conditioning in humans (Kindt et al., 2009; Soeter & Kindt, 2010). Hence, in addition to its effects on consolidation, the

current project examines the effects of systemic propranolol on reconsolidation of Pavlovian fear conditioning.

Methods

Subjects

Male Sprague-Dawley rats (N=178) (Charles River, Raleigh, NC), weighing between 300 and 350 grams at the time of testing, were group housed four to a cage, and maintained on a 12:12 hour light / dark cycle with food and water available ad libitum. All behavioral procedures took place during animals' light cycle.

Drug

Propranolol, purchased from Tocris (Ellisville, MO), was dissolved in physiological saline. Both drug and saline were injected intraperitoneally (i.p.) and delivered in a volume of 0.1ml/100g body weight.

Apparatus

Rats were trained and tested in two identical 8 x 15 x 15 cm Plexiglas and wire mesh cages as previously described by Cassella and Davis (1986). Background noise (60 dB wideband) and startle stimuli (50 ms white-noise bursts; rise decay, 5 ms) were delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound-level measurements were made with a Brüel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (type 4176) located 7 cm from the center of the speaker, which approximates the distance of the rat's ear from the speaker during testing. Startle response amplitudes were quantified using an Endevco (San Juan Capistrano, CA) 2217E accelerometer. Cage movement produced by the rat's startle response resulted in

displacement of the accelerometer, the output of which was integrated, producing a voltage output proportional to the velocity of cage movement. This signal was amplified by an Endevco model 104 amplifier and digitized on a scale of 0–2510 units by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 (Apple Computers, Cupertino, CA) computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 300 ms after onset of the startle-eliciting noise burst.

Olfactory Apparatus

The olfactory fear conditionined apparatus has been described in detail elsewhere (Paschall & Davis, 2002). In brief, a continuous flow of air was delivered from a compressed-air cylinder at a rate of 1.0 L/min through a small port (1.3-mm lumen diameter) positioned just above a 12.5 mm diameter opening in the top of each cage. For delivery of the olfactory stimulus, a computer-controlled solenoid (Model H15-03; Coulbourn Instruments, Allentown, PA) was opened for 4s, thereby diverting clean air from the compressed-air cylinder into and through a sealed 135-cm^3 glass jar containing 20 ml of 5% (vol/vol) amyl acetate (i.e., the odorant) in propylene glycol solution. The inlet and outlet ports of the glass jar were positioned above the solution such that clean air from the tank mixed with the amyl acetate-containing vapor. The output was then mixed in a 3:5 ratio with clean air before flowing into the cage.

The chamber was actively exhausted into the building's ventilation system at a rate of 0.0114m^3/s. Thus, a volume of air equal to the chamber's total volume was vented every 25 s. Previous results with fear-conditioned rats indicate that with theses procedures startle amplitude returns to baseline levels within 30 s of solenoid closure

(Paschall & Davis, 2002). Cages were cleaned daily with warm tap water and 95% alcohol, and were air dried overnight.

The unconditioned stimulus was a 0.5 s 0.4 mA scrambled shock delivered through the four floor bars as described by Walker and Davis (1997). The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads, Newton, CT).

Behavioral Procedures

Fear conditioning

Acclimation session. On each of two consecutive days, rats were placed in the startle chamber and after a 5 min-acclimation period, received 30 presentations of startle stimuli (95-dB noise burst) separated by a 30-s ntertribal interval. Rats were removed from the chamber immediately after the last startle stimulus presentation. Their mean startle amplitudes were calculated, and used as their pre-training startle baseline. Rats were then divided into treatment groups in which the mean-pretraining baselines were equivalent across groups.

Fear conditioning. The next day, rats were returned to the same startle chambers in which they were matched. After 5-min of acclimation, rats received a single odor-shock pairing (4-s odor that co-terminates with a 0.5-s, 0.4 mA footshock) (Experiments 3 & 6) or 2 odor-shock pairings with a 2-min ntertribal interval (Experiments 1, 2, 4, & 5). For trace fear conditioning, the odor and footshock were separated by a 15-s trace interval (Experiments 4, 5, & 6). Immediately thereafter, rats were removed from the chambers and given an i.p. injection of either propranolol (1, 3, or 10 mg/kg) or saline (Experiments 1, 3, 4, & 6), or returned to their home cage (Experiments 2 & 5).

Reactivation session. Twenty-four hrs after training, rats in Experiments 2 and 5 were returned to the conditioning chamber and after a 5-min acclimation period were presented with a single 4-s odor CS. Immediately thereafter, rats were removed from the chambers and either given either an i.p. injections of propranolol (1, 5, or 10 mg/kg) or saline.

Fear-potentiated startle test. Seven days after training or reactivation, rats were returned to the startle cages, and after 5 min, were presented with 30 startle stimuli (leaders). Thirty seconds after the final leader stimulus, rats received 30 startle-eliciting noise bursts presented alone (noise-alone trial) and 10 noise bursts presented 3.2-s after onset of the 4-s odor (odor-noise trials). The two trial types were presented in a balanced mixed order, with 30-s ntertribal interval.

Statistical Analyses

The mean startle amplitude on noise-alone and on odor-noise test trials was determined and a percent change score was calculated for each rat. Difference scores were calculated as: (odor-noise minus noise alone trials). Percent fear-potentiated startle was calculated as: [(odor-noise minus noise-alone trials) / (noise-alone trials)] × 100. Changes in baseline startle from the pre-conditioning acclimation session to the post-conditioning test session, shown elsewhere to reflect context conditioning, were similarly calculated (i.e., mean startle amplitude of the 30 noise alone trials which occurred at the beginning of the test session divided by the mean startle amplitude of the 30 noise alone trials of the second acclimation session X 100). Depending on the experiment, all data were analyzed by analysis of variance (ANOVA) or repeated measures followed by individual mean comparison using Tukey's post hoc tests. A significance level of $p < 0.05$ was used for all results.

Results

Experiment 1

Propranolol (1, 3, or 10 mg/kg) had no effect on the consolidation of delay fear conditioning

This experiment examined the effect of immediate post-training systemic propranolol on the consolidation of delay fear conditioning to a discrete cue. Figure 1 shows the mean startle amplitude for noise alone (black), and odor-noise (white) trials, and the difference between the two (stripe) of rats that were administered (i.p.) propranolol [1 mg/kg (n = 5), 3 mg/kg (n=5) or 10 mg/kg (n=5)] or saline (n=5) immediately after training. An ANOVA was carried out with Trial Type (odor-noise vs. noise alone) as a within-subjects factor, and Dose as a between-subjects factor. There was a significant trial effect $F(1,3) = 57.91$, $p < .05$, indicating successful one trial fear conditioning, but no significant dose effect $F(1,3) = .28$, $p > .05$ and no trial × dose interaction $F(1,3) = 1.12$, $p > .05$. Thus, neither dose of propranolol impaired consolidation of delay fear conditioning.

Experiment 2

Low dose (1 mg/kg) of propranolol disrupted reconsolidation of delay fear conditioning, but intermediate (5 mg/kg) and high (10 mg/kg) doses did not

This experiment examined the effect of 1, 5, and 10mg/kg of systemically administered propranolol on the reconsolidation of delay fear conditioning. For the first study, rats were returned to the conditioning chamber 24 hrs after training and, after a 5-min acclimation period, were presented with 10 noise alone trials followed by 1 test trial of either an odor-tone (reactivation) or an 11th noise alone trial (no reactivation).

Immediately thereafter, rats were administered (i.p.) saline (n=10) or propranolol (10 mg/kg, n=10). Data from the reactivation session are represented in Figure 2a, which shows the mean startle amplitude for the 10 noise alone trials (dark gray), the test trial (light gray), and the difference between the two (gray stripes). It is apparent that rats startled much more in the presence of the odor relative to their mean startle during noise alone trials. On the other hand, rats did not show an appreciable difference in startle between the first 10 noise-alone trials and the 11th noise alone trial. Thus, it can be concluded that the reactivation trial (odor-noise) caused memory retrieval.

Rats were tested for fear memory retention 7 days after the reactivation session. Figure 2b shows the mean startle amplitude for noise alone trials (black), odor-noise trials (white), and the difference between the two (black and white stripes) for rats given saline (n=10) or propranolol (10 mg/kg, n=10) immediately after an odor-noise test trial (reactivation, n=5) or noise alone test trial (no reactivation, n=5). A two-factor ANOVA was carried out with Treatment (saline vs. propranolol) as one factor, and Retrieval (reactivation vs. no reactivation) as another factor. There was no main effect of Treatment $F(1,20) = .12$, $p < .05$, and no main effect of Retrieval $F(1,20) = .03$, $p > .05$, and no Treatment × Retrieval interaction $F(1,20) = 4.21$, $p > .05$. Thus, at this dose propranolol did not block reconsolidation.

Next, we examined the effect of a low (1 mg/kg) and intermediate (5 mg/kg) dose of propranolol on the reconsolidation of delay fear conditioning. A separate group of animals were fear conditioning, returned to the chamber 24 hrs later, and presented with a reactivation trial (10 noise alones followed by a single odor-noise trial). Immediately thereafter, rats were administered (i.p.) saline (n=23) or propranolol, 1 mg/kg (n=24) or 5

mg/kg, n=21), then tested 7 days later. Data from the reactivation session are represented in Figure 3a, which shows the mean startle amplitude for the 10 noise alone trials (dark gray), the odor-noise test trial (light gray), and the difference between the two (white). Rats startled more in the presence of the odor relative to their mean startle during noise alone trials indicating one again that the odor-noise trial caused memory retrieval.

Figure 3b shows the mean percent potentiation of startle for rats given saline or propranolol. A one-way ANOVA comparing three treatment groups (saline, propranolol 1 mg/kg, and propranolol 5 mg/kg) revealed a significant difference among groups, $F(2,65) = 4.10$, $p < .05$. A Tukey's posthoc revealed a significant difference between saline and propranolol (1 mg/kg), $p < .05$. There was no significant difference between saline and propranolol (5 mg/kg). Together, these findings show that a low dose (1 mg/kg) of systemic propranolol disrupts the reconsolidation of delay fear conditioning. However, an intermediate (5 mg/kg) or high (10 mg/kg) dose of propranolol had no effect on delay fear memory reconsolidation.

Experiment 3

Low dose (1 mg/kg) of propranolol disrupted consolidation of delay fear conditioning when administered 15 min before but not immediately after training

This experiment is based on a study by Simson et al. (2001), which showed that a low dose (4 mg/kg) of systemic propranolol resulted in decreased spontaneous activity in the CeA. Interestingly, the greatest inhibition of spontaneous activity occurred 15 min after systemic propranolol administration (Simson et al., 2001). The current experiment examines the idea that administering a low dose of propranolol 15 min before training would result in diminished amygdala activity at the time of learning. It is predicted that

this protocol would result in a memory deficit, either via a disruption of acquisition or consolidation of fear conditioning.

Rats were given a low dose (1 mg/kg) of propranolol or saline (n=14) either 15 min before (n=8) or immediately before (n=8) a single odor-shock pairing. Figure 4a shows the mean startle amplitude for noise alone (black), odor-noise (white), and the difference (black and white stripes) for each group. Figure 4b shows the same data expressed as mean percent scores. The saline groups were combined in both graphs, because there was no significant difference between rats that got saline immediately before or 15 min before training. A one-way ANOVA comparing the four treatment groups (saline immediate, saline 15 min, propranolol immediate, propranolol 15 min), revealed an overall significant difference among groups, $F(2,20) = 4.62$, $p < .05$ (difference score), $F(2,20) = 4.45$, $p < .05$ (percent score). A Tukey's posthoc test for difference scores revealed that the saline group was significantly different from the group that got propranolol 15 min before training, $p < .05$. For percent scores, the posthoc test showed a significant difference between the group that got propranolol immediately before training and the group that got propranolol 15 min before training, $p < .05$.

To determine if these findings reflect an effect on acquisition or consolidation, rats were tested for short-term memory immediately after training. Thirty seconds after the odor-shock pairing, some rats were presented with 10 noise alone trials with 30-s ntertribal intervals (leaders), followed by 5 odor-noise trials and 10 noise alone trials in a balanced mixed order, with 30-s ntertribal interval. Figure 5 shows the results of the short-term memory test. Figure 5a shows mean startle amplitudes for the first 10 noise alone trials (Leaders), the odor-noise trials, and the noise alone trials, which were

intermixed with the odor-noise trials, for rats given i.p. administration of either saline (n=6, black) or propranolol (1 mg/kg, n=6) (white) 15 min before training. Saline treated rats and propranolol treated rats startled similarly across trial conditions. Both groups startled more in the presence of the odor relative to leaders and noise alone trials. Figure 5b shows the mean percent potentiation of startle from noise alone trials to odor-noise trials. Saline treated rats showed 20% more fear-potentiated startle than the propranolol treated rats. However, this difference was not statistically significant. An independent-samples t-test was conducted to compare the percent scores of saline (M = 62.18, SD = .47) and propranolol (M = 40.71, SD = .44) groups. There was no significant difference among groups, $t(10) = .85$, $p > .05$. Based on these findings, it is concluded that there is no impairment in short-term memory. This suggests that the long-term memory deficit observed when propranolol (1 mg/g) was given 15 min before training reflects an impairment of memory consolidation, rather than acquisition.

Experiment 4

High dose (10 mg/kg) of propranolol had no effect on the consolidation of delay or trace fear conditioning

The goal of this study was to test the hypothesis that propranolol would disrupt hippocampal-dependent fear memories but not hippocampal-independent fear memories. In a typical Pavlovian fear conditioning task, the CS and US overlap in time, and this protocol is usually deemed *delay fear conditioning*. It has been established that the hippocampus is not necessary for delay fear conditioning when a discrete, simple cue is used as the CS (REF). In trace fear conditioning, the CS and US are separated by a time interval (typically on the order of seconds). There is a considerable literature showing

hippocampal as well as amygdala involvement in trace fear conditioning (REF). Because of this, we tested the effect of systemically administered propranolol (10 mg/kg) on the consolidation of delay and trace fear conditioning. Rats were fear conditioned with 2 odor-shock pairings. For delay conditioning, the 4-s odor co-terminated with a 0.5-s, 0.4 mA footshock), and for trace conditioning, the odor and footshock were separated by a 15 s interval. Immediately after the final odor-shock presentation, rats were immediately given i.p. injections of either saline(delay; n=5, trace; n=6) or propranolol (10 mg/kg; delay; n=6, trace; n=6). Rats were tested 7 days later for their startle response during a 4-s odor presentation (odor-noise 0s), or 15-s following the cessation of a 4-s odor cue (odor-noise 15s). Percent scores were calculated relative to noise alone trials that were inter-mixed and balanced between odor-noise trials with 30-s ntertribal intervals.

Figure 6 shows that mean percent scores ($\pm$SEM) for all groups. Data are shown for startle test trials that either overlapped in time with the 4-s odor alone trials (odor-noise 0s), or were presented 15-s (odor-noise 15s) or 30s after odor alone trials. Figure 6a shows the percent potentiation of startle for rats trained in delay fear conditioning and administered either saline (black) or propranolol (10 mg/kg) (white) immediately after training. Rats in both treatment groups showed appreciable fear-potentiated startle across all test conditions. However, both the saline and propranolol groups startled most when tested in the presence of the odor cue (odor-noise 0s). Interestingly, the propranolol treated rats startled appreciably *more* than the saline treated rats at the 0-s test interval. An ANOVA was carried out with Test Interval (0s, 15s) as a within-subjects factor, and Treatment (saline vs. propranolol) as a between-subjects factor. There was a significant

main effect of Test Interval $F(1,9) = 12.26$, $p < .05$, but no significant treatment effect $F(1,9) = 2.4$, $p > .05$, and no Test Interval × Treatment interaction $F(1,9) = 0.82$, $p > .05$.

Figure 6b shows percent scores for rats trained in trace fear conditioning and administered saline (dark gray) or propranolol (light gray) immediately after training. Again, rats in both treatment groups showed fear-potentiated startle across all test conditions. Similar to rats trained in delay conditioning, both the saline and propranolol groups startled most when tested in the presence of the odor cue (odor-noise 0s). In addition, the propranolol treated rats startled *more* than the saline treated rats at the 0s test interval. An ANOVA revealed a significant Test Interval effect $F(1,10) = 12.21$, $p < .05$, but no significant treatment effect $F(1,10) = 0.31$, $p > .05$, and no Test Interval × Treatment interaction $F(1,10) = 1.26$, $p > .05$. Propranolol did not disrupt the consolidation of delay or trace conditioning. Together, these results suggest that a high dose (10 mg/kg) of propranolol does not disrupt the consolidation of delay or trace fear conditioning. On the other hand, 10 mg/kg of propranolol appears to *enhance* fear memory retention of both delay and trace fear conditioning, although this apparent enhancement did not reach statistical significance.

Experiment 5

High dose (10 mg/kg) of propranolol disrupted the reconsolidation of trace but not delay fear conditioning

Here, we tested the effect of systemically administered propranolol (10 mg/kg) on the reconsolidation of delay and trace fear conditioning. Rats were trained and tested similar to the previous study, with the exception that drug treatment was administered 24 hrs after training and immediately following a single odor-noise recall trial. Figure 7a

shows the percent potentiation of startle for rats trained in delay fear conditioning and administered either saline (black) or propranolol (10 mg/kg) (white) immediately after a reactivation (odor alone) trial. Rats in both treatment groups showed good fear-potentiated startle only when tested in the presence of the odor cue (odor-noise 0s). Propranolol treated rats startled more than the saline treated rats during this test condition. Neither treatment group showed appreciable fear memory when startle was probed 15-s after the odor cue presentation. An ANOVA was carried out with Test Interval (0s, 15s) as a within-subjects factor, and Treatment (saline vs. propranolol) as a between-subjects factor. There was a significant main effect of Test Interval $F(1,9) = 10.59$, $p < .05$, but no significant treatment effect $F(1,9) = 1.18$, $p > .05$, and no Test Interval × Treatment interaction $F(1,9) = 0.13$, $p > .05$. Thus, 10 mg/kg propranolol had no effect on reconsolidation of delay fear conditioning.

Figure 7b shows percent scores for rats trained in trace fear conditioning and administered saline (dark gray) or propranolol (light gray) immediately after reactivation. The saline treatment group showed appreciable fear-potentiated startle when tested in the presence of the odor cue (odor-noise 0s), but not during the 15-s test interval (odor-noise 15s). The propranolol treated rats did not show appreciable fear-potentiated startle at any of the test intervals. An ANOVA revealed a significant Test Interval effect $F(1,10) = 8.41$, $p < .05$, but no significant treatment effect $F(1,10) = 1.51$, $p > .05$, and no Test Interval × Treatment interaction $F(1,10) = 2.21$, $p > .05$. Although these results suggest that a high dose (10 mg/kg) of propranolol impairs the reconsolidation of trace fear conditioning, but not delay fear conditioning, they did not reach significance with this

number of subjects. Furthermore, similar to what was observed in consolidation, propranolol appears to *enhance* memory retention of delay fear conditioning.

Experiment 6

Low (1 mg/kg) and high (10 mg/kg) doses of propranolol disrupts consolidation of context fear conditioning. Low (1mg/kg), but not high (10 mg/kg) dose of propranolol disrupts the consolidation of trace fear conditioning.

This study further examines the hypothesis that propranolol will disrupt hippocampal-dependent fear memories but not hippocampal-independent fear memories. Our laboratory previously demonstrated that single-trial odor mediated fear conditioning produces measurable context conditioning, which can be quantified as changes in baseline startle from the pre-conditioning acclimation session to the post-conditioning test session (i.e., mean startle amplitude of the 30 noise alone trials which occurred at the beginning of the test session divided by the mean startle amplitude of the 30 noise alone trials of the pre-training baseline startle test X 100). Using this method, we examine here the effect of immediate post-training systemic administration of propranolol (1 & 10 mg/kg) versus saline on the consolidation of trace and context fear conditioning.

Figure 8 shows the mean startle amplitudes for rats that were administered (i.p.) saline (n=6, black), 1 mg/kg (n=7, white), or 10 mg/kg (n=4, light gray) of propranolol immediately after single-trial trace fear conditioning (4-s odor and 0.5-s, 0.4 mA footshock separated in time by a 15-s interval). Figure 8a shows their mean startle amplitudes during the 30 noise alone trials of the pre-training baseline startle test (Pre-train BL), the 30 noise alone trials which occurred at the beginning of the test session (Post-train BL), the 10 odor-noise test trials (odor-noise 0s), and the 30 noise-alone test

trials that were intermixed with odor-noise test trials (noise-alone 30s). The saline treated rats showed a considerable increase in startle responding from pre-train to post-train baseline startle testing, reflecting context fear conditioning. This apparent increase was not observed in the low dose propranolol treatment group. Both groups given saline and a high dose (10 mg/kg) of propronolol, startled appreciably more in the presence of the odor CS relative to noise-alone trials. However, the low dose propranolol (1 mg/kg) treatment group startled similarly from odor-noise to noise-alone trials. These observations suggest that propranolol (both low and high doses) disrupted context fear conditioning, and that a low dose, but not a high dose of propranolol disrupted consolidation of trace fear conditioning.

Figure 8b shows the mean startle amplitudes for noise alone trials (black), odor noise trials (white), and the difference score (black and white stripes) for each treatment group. A one-way ANOVA comparing the difference scores among treatment groups approached, but did not reach a statistical difference, $F(2,13) = 2.02$, $p > .05$. Figure 8c shows the mean startle amplitude for pre-train baseline startle (dark gray), post-train baseline startle test (white), and the difference between the two (medium gray). A one-way ANOVA comparing the difference scores showed a significant difference among groups, $F(2,14) = 3.98$, $p < .05$. A Tukey's posthoc test showed a signifant difference between the saline group and the high dose (10 mg/kg) propranolol group.

Overall, these results show that single-trial trace fear conditioning produces considerable context fear as indicated by increased startle during the post-train baseline test compared to pre-train baseline test. Importantly, this was only observed in the saline group, but not in either of the propranolol treatment groups, suggesting that propranolol

disrupts the consolidation of context fear conditioning. Also, it appears from these data that 1 mg/kg but not 10 mg/kg propranolol disrupts consolidation of trace fear conditioning, although this did not reach statistical significance.

Discussion

Propranolol has received widespread attention as a possible pharmacological tool to prevent or treat unwanted fear memories in PTSD. However, in spite of a wealth of preclinical evidence showing that propranolol disrupts the consolidation of inhibitory avoidance training, it has yet to be demonstrated that propranolol disrupts the consolidation of Pavlovian fear conditioning. Yet, propranolol has proven to be effective in disrupting reconsolidation of Pavlovian fear memory (Debiec & Ledoux, 2004, 2006; Kindt et al., 2009; Soeter & Kindt, 2010). It is possible that NE modulates the consolidation of certain hippocampal-dependent fear memories, but not hippocampal-independent cued fear memories. Furthermore, cued fear memory retrieval might be more susceptible to noradrenergic influence than cued fear memory encoding.

Our laboratory has developed an odor-guided Pavlovian fear conditioning task that is amenable to hippocampal influence when the odor and shock are separated in time (trace fear conditioning), or when a single odor-shock pairing is presented in a distinct context (context fear conditioning). Using this model, the present study examines the effect of systemic propranolol on the consolidation and reconsolidation of delay, trace, and context fear conditioning. Furthermore, taking into consideration studies implicating dosage as a critical factor in propranolol's amnesic efficacy, this study tested a range of propranolol doses. Based on previous findings, we hypothesize that propranolol would disrupt the consolidation of context and trace fear conditioning, but not delay fear

conditioning, that propranolol would disrupt the reconsolidation of delay, trace, and context fear, and that low doses of propranolol would be more efficacious in disrupting consolidation and reconsolidation of fear memory than high doses. On the whole, the results of the present study support our hypotheses.

The present results demonstrate that when propranolol is administered systemically immediately after delay fear conditioning, it does not impair fear memory retention, regardless of the dosage (tested with 1, 3, and 10 mg/kg) (Figure 1). These data are consistent with the findings of Debiec & LeDoux (2004) as well as Lee et al. (2001) who independently demonstrated that systemically administered propranolol (10 mg/kg and 2 mg/kg respectively) failed to disrupt the consolidation of delay fear conditioning to an auditory cue, using freezing behavior as a measure of retention. The present findings add to this small literature, using the olfactory modality and the fear-potentiated startle model.

Simson and colleagues (2001) showed that a low dose (4 mg/kg) of propranolol produced decreased spontaneous activity in the CeA, and the greatest inhibition occurred 15-min after systemic propranolol administration. Interestingly, we observed an impairment in fear memory retention when 1 mg/kg of propranolol was administered 15-min, but not immediately before delay fear conditioning (Figure 4). This deficit could reflect a disruption of either the acquisition or the consolidation of fear memory. The present results indicate that short-term memory was spared in rats that were administered 1 mg/kg of propranolol 15-min before training (Figure 5), despite showing a marked impairment in long-term retention (Figure 4). However, the expression of short-term fear memory was not robust in either group, and the propranolol treatment group expressed

20% less fear-potentiated startle than the saline group, although this difference was statistically nonsignificant. Nevertheless, an interpretation of these results is limited considering previous findings showing an impairing effect of propranolol on the acquisition (given immediately before training) and expression (given immediately before testing) of olfactory fear conditioning (Kroon & Carobrez, 2009), and on the expression of fear-potentiated startle to a light-shock association (Walker & Davis, 2002). Furthermore, there is a growing literature showing a possible involvement of the CeA in synaptic plasticity (Bahar et al., 2003; Goosens & Maren, 2003), suggesting that decreased spontaneous activity in the CeA at the time of training would likely result in an acquisition deficit (see Paré, Quirk & LeDoux, 2004). Before making a firm conclusion about the present findings, further research is needed to disentangle propranolol's effect on acquisition versus consolidation.

Despite growing evidence that neither a low dose (2 mg/kg – Lee et al., 2001, 1mg/kg – present findings) nor a high dose (10 mg/kg – Debiec & Ledoux, 2004, present findings) of propranolol disrupted the consolidation of cued fear conditioning, it has been demonstrated that propranolol (10 mg/kg) disrupted the reconsolidation of Pavlovian fear conditioning to an auditory cue, as measured by freezing (Debiec & LeDoux, 2004, 2006). To test the generality of those findings on the olfactory-mediated fear-potentiated model, and to explore the role of dose on this effect, the present study examined the effect of immediate post-recall i.p. administration of propranolol (1, 5, and 10 mg/kg) on long-term retention of delay fear conditioning. In contrast to the findings of Debiec and LeDoux (2004), we failed to find a reconsolidation blockade by propranolol when administered at a dose of 10 mg/kg (Figure 2), or 5 mg/kg (Figure 5). On the other hand,

the current findings showed a marked impairment of reconsolidation when a low dose (1 mg/kg) of propranolol was administered (Figure 3). This study provides the first evidence that propranolol's effect on fear memory reconsolidation might be dose-dependent.

It is not clear why propranolol differentially affects consolidation and reconsolidation of cued fear memory. If one assumes that reactivation of fear memory recapitulates neuronal events seen after acquisition, then it would be predicted that propranolol would not affect the reconsolidation of cued fear conditioning, similar to its lack of effect on consolidation. Findings from several experiments using a variety of species and learning models show that consolidation and reconsolidation share several molecular and circuitry requirements (i.e., Bozen et al., 2003; Child et al., 2003; Debiec et al., 2002; Kida et al., 2002; Koh & Bernstein, 2003; Nader et al., 2000; Sangha et al., 2003). On the other hand, a formidable literature suggests that reconsolidation represents at least a partial but not a total recapitulation of consolidation, and that the two processes might be mediated by distinct molecular mechanisms and/or distinct brain regions (i.e., Bahar et al., 2004; Hernandez et al., 2002; Kelly et al., 2003; Lee et al., 2004; Nyberg et al., 1996; Salinska et al., 2004; Taubenfeld et al., 2001; Tronel & Sara, 2002). The present finding that propranolol disrupts the reconsolidation but not the consolidation of cued fear memory is consistent with the idea that these two processes involve distinct mechanisms. Our findings, together with the findings of Debiec and LeDoux (2004), raises the question of whether or not the retrieval of cued fear memory is subject to hippocampal involvement, lending cued fear memory to the modulatory influence of NE. More experiments that explicitly test the role of the hippocampus in reonsolidation of conditioning are needed to address this question.

This project explored the central hypothesis that hippocampal-dependent fear memories are susceptible to post-training noradrenergic influence, whereas hippocampal-independent fear memories are not. The present study tested the effects of systemically administered propranolol on the consolidation and reconsolidation of trace (hippocampal-dependent) versus delay (hippocampal-independent) fear memory. The results show that a high dose (10 mg/kg) of propranolol appears to impair the reconsolidation (Figure 7b) but not the consolidation (Figure 6b) of trace fear conditioning, although this apparent deficit did not reach statistical significance. On the other hand, 10 mg/kg of propranolol did not impair the consolidation (Figure 6a) or the reconsolidation (Figure 7a) of delay fear conditioning, replicating our previous findings.

Interestingly, when propranolol was administered post-training, it appeared to enhance the retention of both delay and trace fear conditioning compared to saline treated rats, although this difference was not statistically significant (Figure 6). This pattern of responding was also observed when propranolol was administered post-recall, but only in the delay fear conditioned rats (Figure 7). Nevertheless, further research is needed before it can be definitively concluded that propranolol is enhancing fear memory retention. If these findings were to be replicated, they would be consistent with the findings of Schneider et al. (2000) that systemic propranolol enhanced retention of inhibitory avoidance learning. Schneider et al. (2000) found that the enhancing effects of propranolol were dose-dependent, whereby 10 mg/kg of propranolol enhanced retention, but 4 mg/kg of propranolol did not. Similarly, the present study demonstrated that low doses of propranolol (1 and 3 mg/kg) had no effect on the retention of delay fear

conditioning, and a high dose (10 mg/kg) of propranolol either had no effect on retention (Figure 1) or it possibly enhanced retention (Figure 6a).

The possibility that 10 mg/kg of propranolol disrupted the reconsolidation but not the consolidation of trace fear conditioning supports the idea that consolidation and reconsolidation are differentially susceptible to noradrenergic influence. On the other hand, these findings do not support the idea that propranolol would disrupt the consolidation of trace fear conditioning because it is a hippocampal-dependent form of fear memory. To further examine the hypothesis that propranolol will disrupt hippocampal-dependent fear memories but not hippocampal-independent fear memories, we took advantage of a previously described observation that context fear conditioning can be quantified as changes in baseline startle from the pre-conditioning acclimation session to the post-conditioning test session (McNish et al., 1997; McNish et al., 2000). Using this method, we examined the effect of low (1 mg/kg) and high (10 mg/kg) doses of propranolol on the consolidation of single-trial trace fear conditioning and context fear conditioning.

We have previously observed that single-trial olfactory fear conditioning produces appreciable context fear (Glover & Davis, unpublished findings). The present results confirm these observations. The saline treated rats showed a considerable increase in startle responding from pre-train to post-train baseline startle testing, reflecting context fear memory (Figure 8a & 8c). It is not surprising that trace fear conditioning produces significant context fear, given previous findings that longer CS-US trace intervals produces stronger conditioning to the surrounding context, whereas shorter trace intervals tend to produce stronger CS conditioning (Marlin, 1981).

Interestingly, the propranolol treated group showed considerably less context fear, and 10 mg/kg of propranolol produced the greatest impairment. These findings are consistent with those of Grillon et al. (2003), who demonstrated in humans that propranolol disrupted context fear memory but had no effect on cued fear memory. Thus, the present results provide support for the idea that context fear conditioning parallels inhibitory avoidance learning more closely than cued fear conditioning. In addition, the present findings indicate that 1 mg/kg but not 10 mg/kg of propranolol impairs the consolidation of trace fear conditioning, although this apparent deficit did not reach statistical significance. Together, these results support the hypothesis that propranolol disrupts the consolidation of context and trace fear conditioning, two hippocampal-dependent forms of Pavlovian fear conditioning, but not delay fear conditioning, a hippocampal-independent Pavlovian fear conditioning task. Nevertheless, there are some inconsistencies in our findings that warrant further attention.

In Experiment 4, we found that 10 mg/kg of propranolol did not disrupt the consolidation of trace fear conditioning, but appeared to cause an enhancement in memory retention (Figure 6b). However, this possible enhancing effect of propranolol was not replicated in Experiment 6 (Figure 8b). The failure to replicate could be due to the fact that the training protocols were not identical between these two experiments (2x vs. 1x odor-shock presentation). Presumably, it is more likely that memory enhancement would be observed after single-trial fear conditioning compared to two-trial fear conditioning. Yet, our results do not indicate that propranolol enhanced consolidation of single-trial trace fear conditioning (Figure 8b). The fact that we previously demonstrated that 10 mg/kg of propranolol did not impair or enhance the consolidation of delay fear

memory (Figure 1), it is concluded that the apparent enhancing effect of propranolol on retention of delay and trace fear memory, observed in Figure 7 and 8, is not a real phenomenon and may be an artifact of the experimental procedure. Further research is needed to clarify these issues.

Another problematic result is the finding that a high dose of propranolol (10 mg/kg) more effectively disrupted the consolidation of context fear conditioning than a low dose (1 mg/kg) of propranolol (Figure 8c). This result does not support our hypothesis that low doses of propranolol will be more efficacious in disrupting the consolidation of fear memory than high doses. Furthermore, the present finding that 10 mg/kg of propranolol impaired context fear conditioning is contrary to the observation that 10 mg/kg of propranolol enhanced retention of inhibitory avoidance learning (Schneider et al., 2000). Nevertheless, the hypothesis was largely based on a single study that examined the effect of systemic propranolol on spontaneous activity of one part of the amgydala, the CeA (Simson et al., 2001). While it is tempting to link those findings to the behavioral effects of systemically administered propranolol, it is difficult to make an interpretation based on these findings alone. More research is needed to uncover dose-dependent effects of systemically administered propranolol on excitatory and inhibitory processes in the amygdala and its afferents.

Taken together, the present findings show that context fear conditioning and trace fear conditioning are both susceptible to post-training noradrenergic influence, similar to inhibitory avoidance learning, whereas cued fear conditioning is not. In light of the tremendous interest in propranolol as a possible prophylactic in PTSD, it is perhaps surprising that more attention has not been given to the findings that cued fear memories

are impervious to post-training propranolol administration. While the inhibitory avoidance model has done much to galvanize interest in the role of the adrenergic system in the pathophysiology of PTSD, this model by itself does not fully translate to the traumatic experience. In the clinical presentation of PTSD, stimuli associated with the trauma can trigger vivid recollections of the traumatic event, and evoke intense fear responses well after the threat has passed. These fear responses closely resemble those observed in inhibitory avoidance training (i.e., avoidance of cues associated with the traumatic event) as well as Pavlovian fear conditioning (i.e., increased autonomic and reflexive responding to specific trauma-related cues). Propranolol treatment for PTSD might effectively counter generalized fear to the environment where trauma occurred, and decrease avoidance behavior, but a growing preclinical literature predicts that propranolol treatment would leave conditioned fear responses to certain trauma-related cues intact. Additional research is needed to further understand the therapeutic implications for these observations.

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Figure Captions

Figure 1. **Propranolol (1, 3, or 10 mg/kg) had no effect on the consolidation of delay fear memory.** Mean startle amplitude for noise alone (black), and odor-noise (white) trials, and the difference between the two (stripe) of rats that were administered (i.p.) propranolol [1 mg/kg (n = 5), 3 mg/kg (n=5) or 10 mg/kg (n=5)] or saline (n=5) immediately after training. There was no significant difference between groups.

Figure 2. **High dose of propranolol (10 mg/kg) had no effect on the reconsolidation of delay fear memory.** (A) Mean startle amplitude for the 10 noise alone trials (dark gray), the test trial (light gray), and the difference between the two (gray stripes). The reactivation trial (odor-noise) caused memory retrieval. (B) Mean startle amplitude for noise alone trials (black), odor-noise trials (white), and the difference between the two (black and white stripes) for rats given saline (n=10) or propranolol (10 mg/kg, n=10) immediately after an odor-noise test trial (reactivation, n=5) or noise alone test trial (no reactivation, n=5). There was no significance difference between groups.

Figure 3. **Low dose (1 mg/kg) of propranolol disrupted the reconsolidation of delay fear memory, but intermediate (5 mg/kg) and high (10 mg/kg) doses did not. (A)** Data from the reactivation session showing the mean startle amplitude for the 10 noise alone trials (dark gray), the odor-noise test trial (light gray), and the difference between the two (white). **(B)** Mean percent potentiation of startle for rats given saline or propranolol (1 or 5 mg/kg). There was significant difference among saline and propranolol (1 mg/kg), * $p < .05$, but not among saline and propranolol (5 mg/kg).

Figure 4. **Low dose (1 mg/kg) of propranolol disrupted the consolidation of delay fear memory when administered 15-min before but not immediately before training. (A)** Mean startle amplitude for noise alone (black), odor-noise (white), and the difference (black and white stripes) for rats given propramolol or saline, immediately or 15-min before acquisition. Saline group was significantly different from the group that got propranolol 15-min before training, * $p < .05$. **(B)** Same data expressed as mean percent scores. There was a significant difference between propranolol given immediately before training and propranolol given 15-min before training, * $p < .05$.

Figure 5. **Propranolol given 15-min before training did not impair acquisition. (A)** Mean startle amplitudes for the first 10 noise alone trials (Leaders), the odor-noise trials, and the noise alone trials, which were intermixed with the odor-noise trials, for rats given i.p. administration of either saline (n=6, black) or propranolol (1 mg/kg, n=6) (white) 15 min before training. Saline and propranolol rats startled similarly across trial conditions. **(B)** Mean percent potentiation of startle from noise alone trials to odor-noise trials. Saline treated rats showed 20% more fear-potentiated startle than the propranolol treated rats. However, this difference was not statistically significant.

Figure 6. **High dose (10 mg/kg) of propranolol had no effect on the consolidation of delay or trace fear memory. (A)** Mean percent potentiation of startle for rats trained in delay fear conditioning and administered either saline (black) or propranolol (10 mg/kg) (white) immediately after training. Both the saline and propranolol groups displayed good

delay fear conditioning. **(B)** Mean percent scores for rats trained in trace fear conditioning and administered saline (dark gray) or propranolol (light gray) immediately after training. Rats in both treatment groups showed fear-potentiated startle across all test conditions.

Figure 7. **High dose (10 mg/kg) of propranolol disrupted the reconsolidation of trace but not delay fear memory. (A)** Mean percent potentiation of startle for rats trained in delay fear conditioning and administered either saline (black) or propranolol (10 mg/kg) (white) immediately after a reactivation (odor alone) trial. Rats in both treatment groups showed good fear-potentiated startle only when tested in the presence of the odor cue (odor-noise 0s). **(B)** Mean percent scores for rats trained in trace fear conditioning and administered saline (dark gray) or propranolol (light gray) immediately after reactivation. Propranolol groups startled less than the saline groups in the presence of the odor CS, although this was not significant.

Figure 8. **Low (1 mg/kg) and high (10 mg/kg) doses of propranolol disrupted the consolidation of context fear memory. Low (1mg/kg), but not high (10 mg/kg) dose of propranolol disrupted the consolidation of trace fear memory. (A)** Mean startle amplitudes during pre-training baseline startle test, post-training baseline startle test, odor-noise test trials , and noise-alone test trials. **(B)** Mean startle amplitudes for noise alone trials (black), odor noise trials (white), and the difference score (black and white stripes) for each treatment group. Rats showed less fear potentiated startle when given low dose but not a high dose of propranolol. **(C)** Mean startle amplitude for pre-train

baseline startle (dark gray), post-train baseline startle test (white), and the difference between the two (medium gray). High dose propranolol produced significantly less fear to the context than saline treatment, $p < .05$.

Figure 1

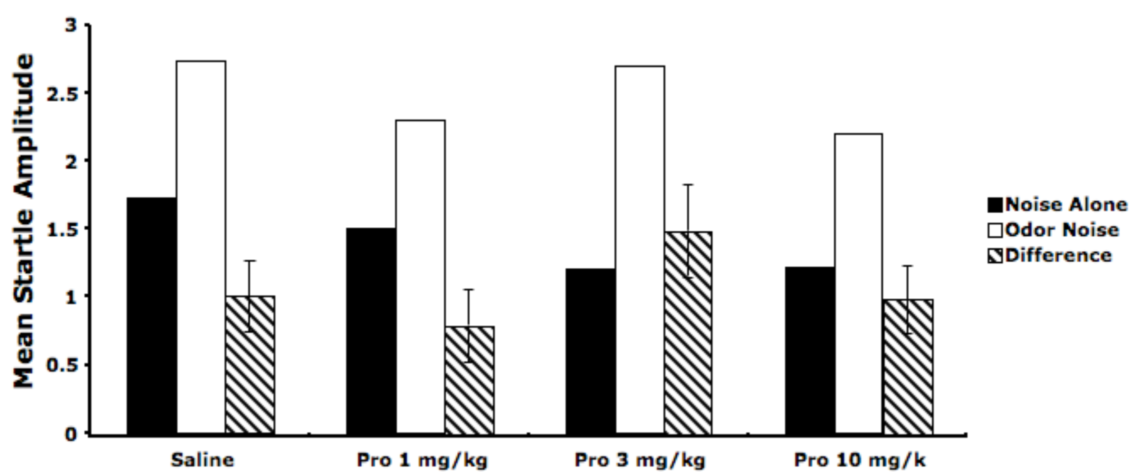
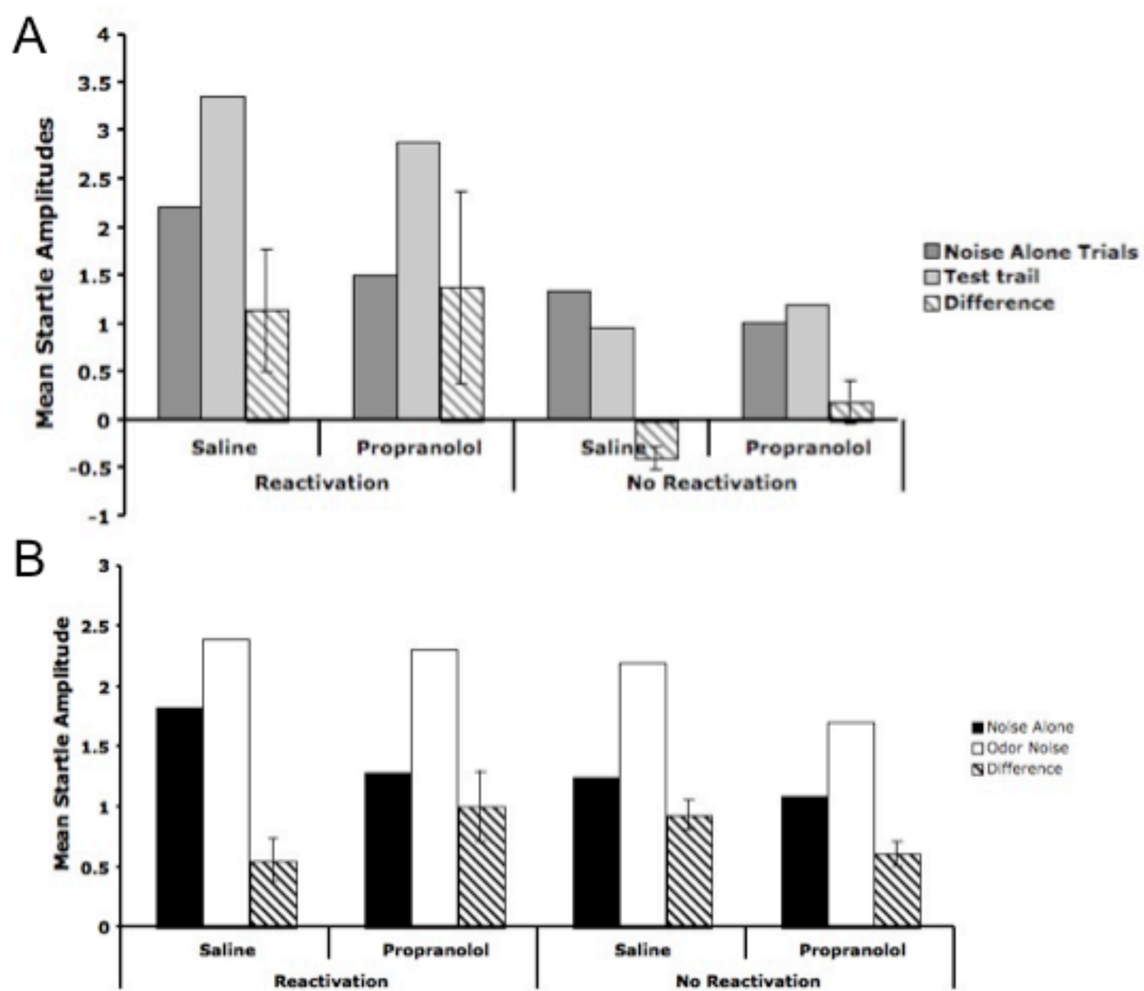


Figure 2



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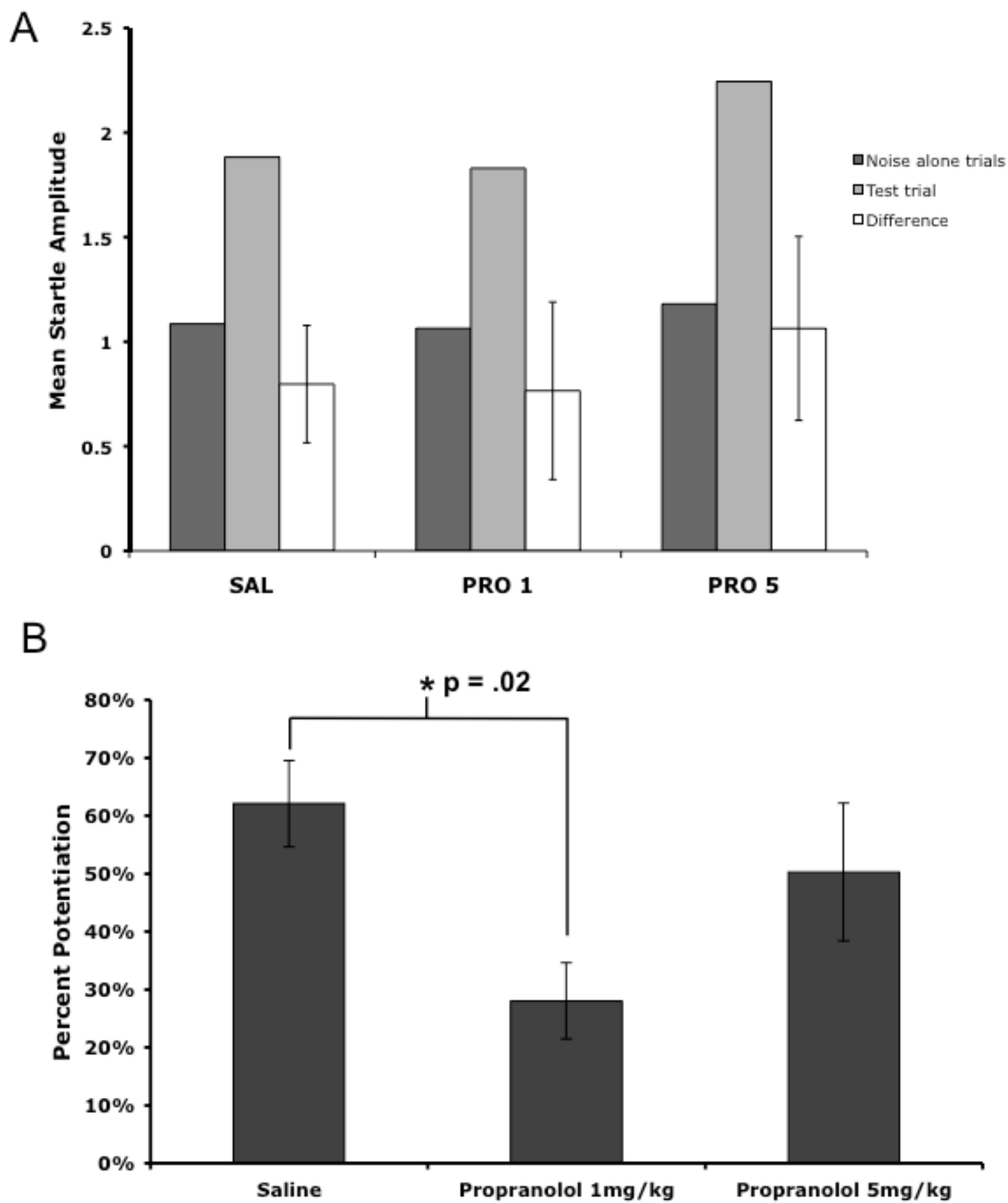

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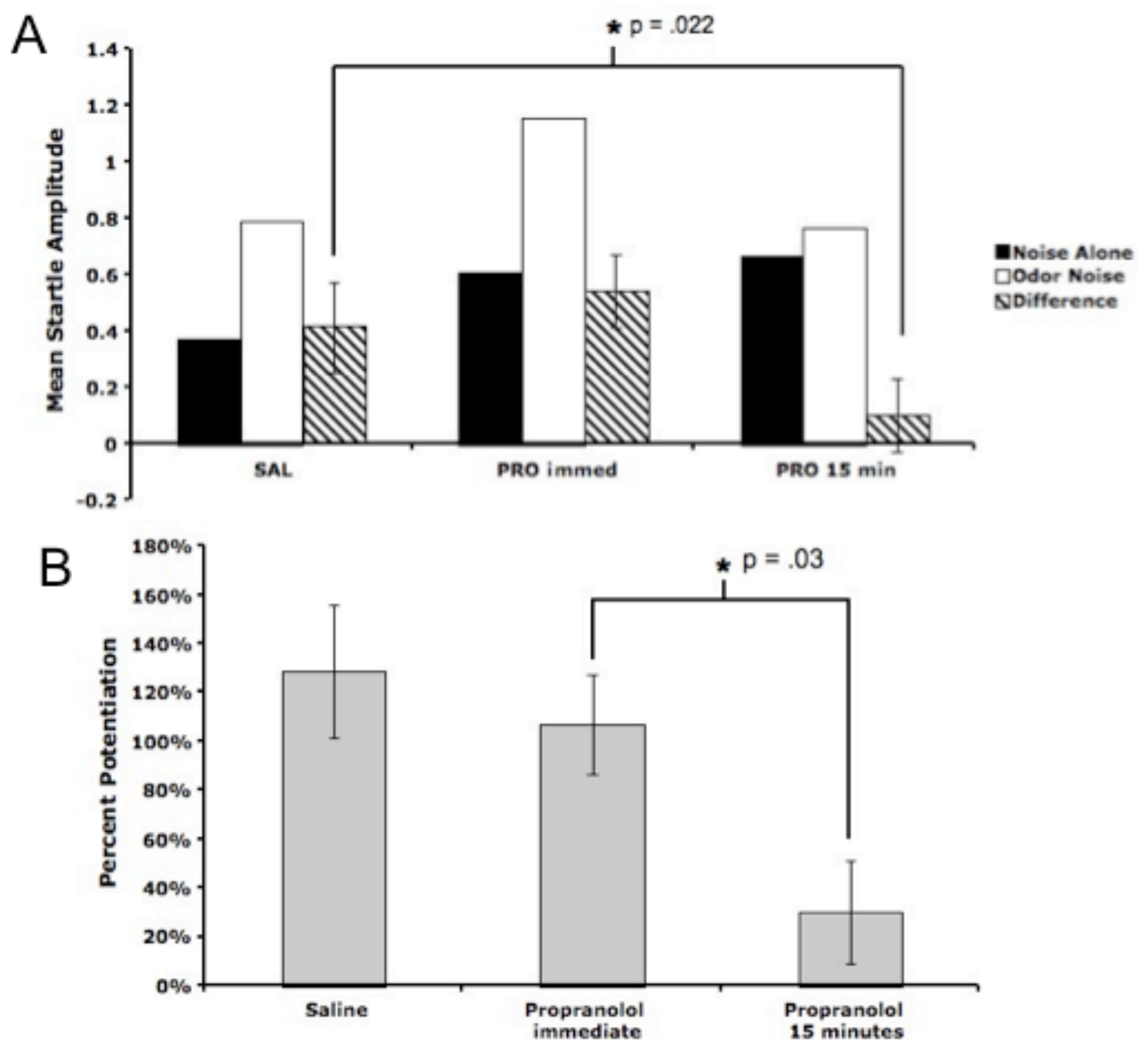


Figure 5

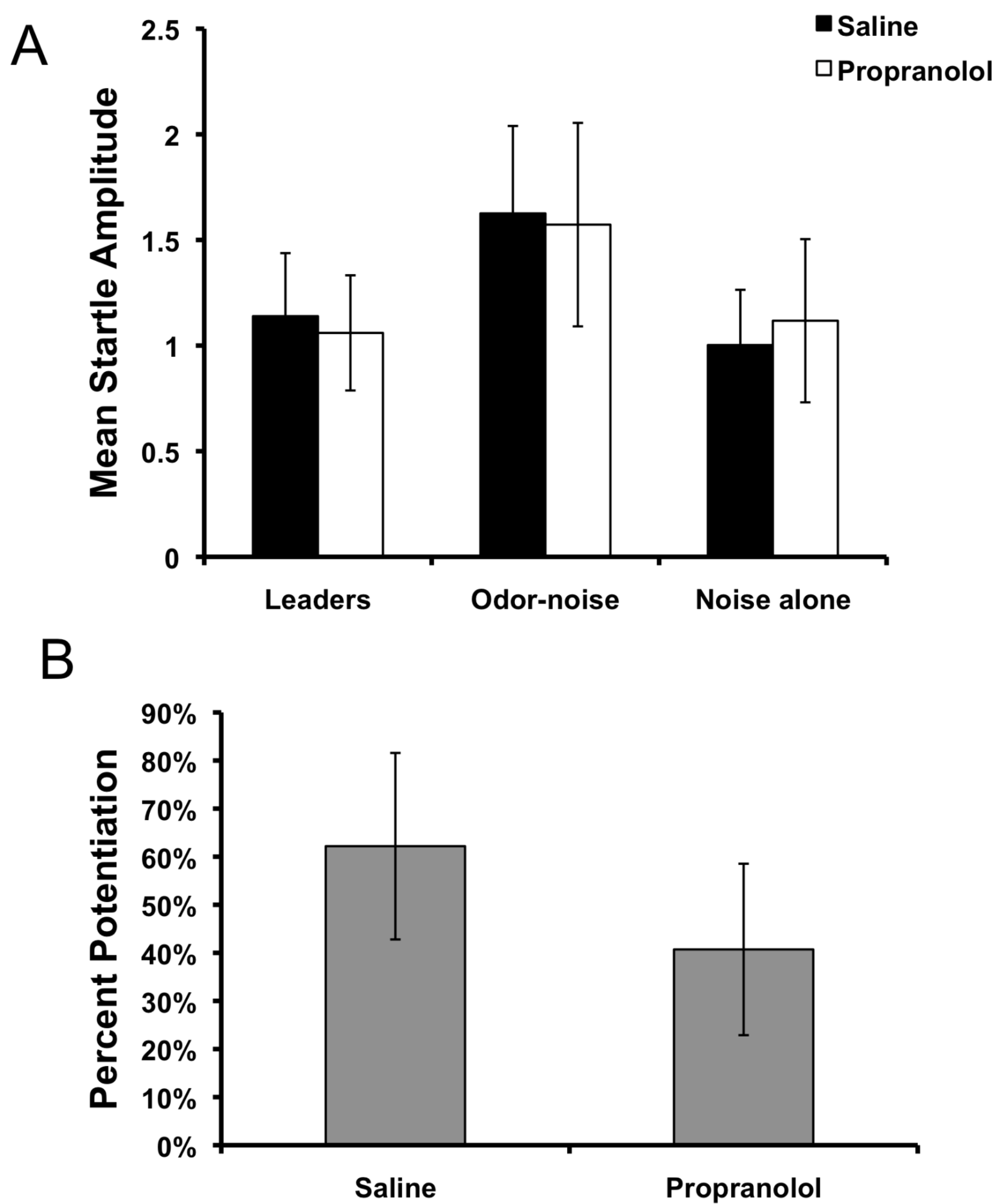
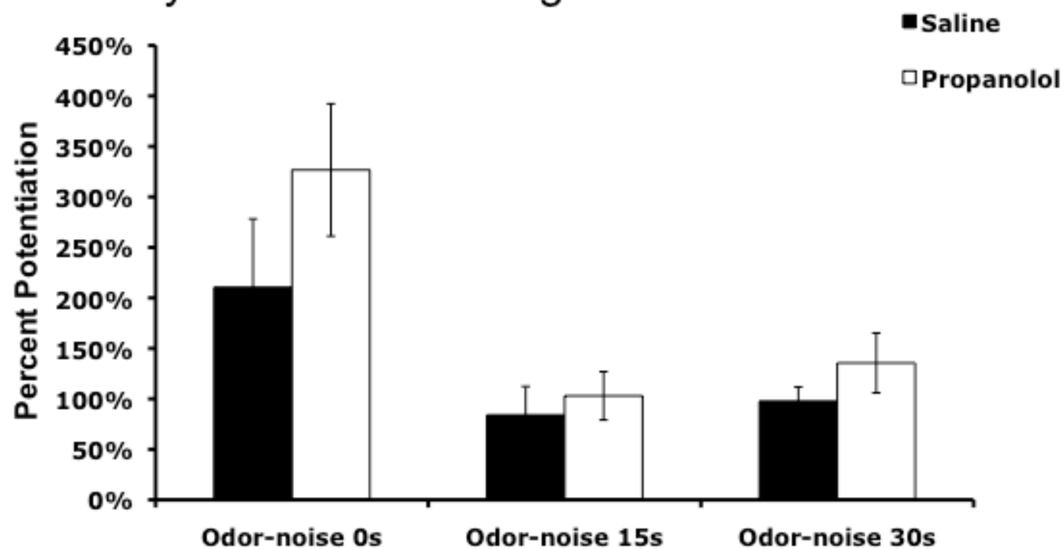


Figure 6



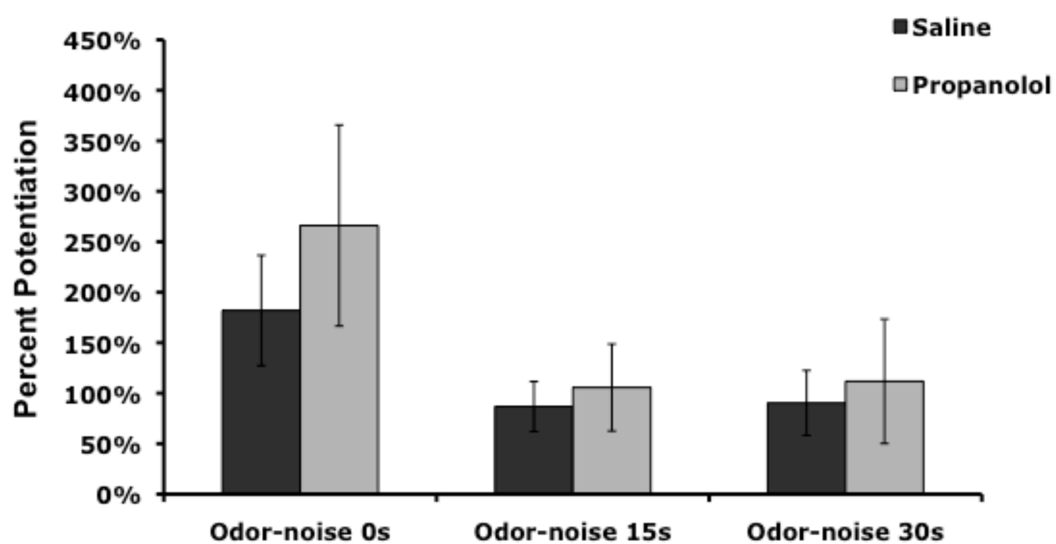
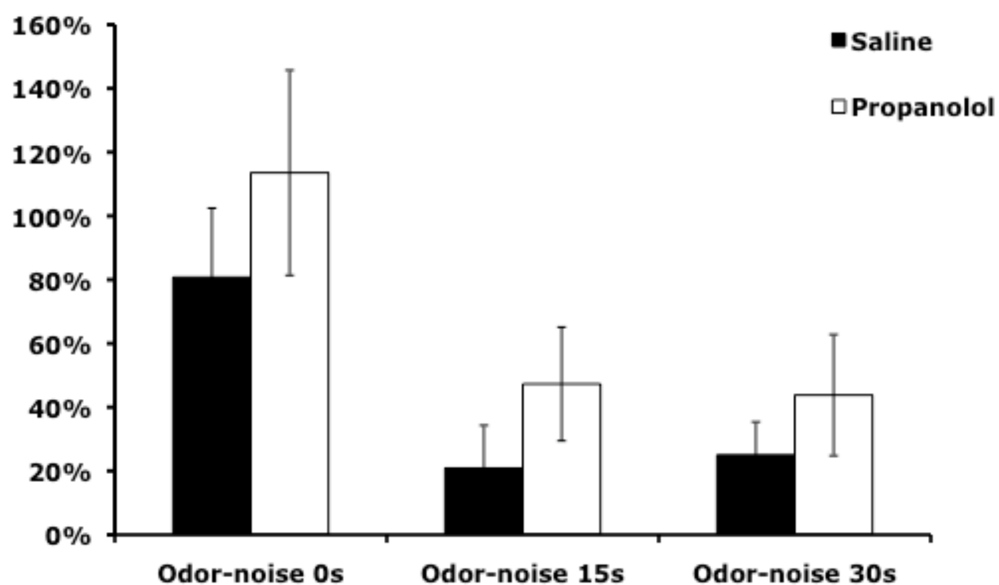


Figure 7



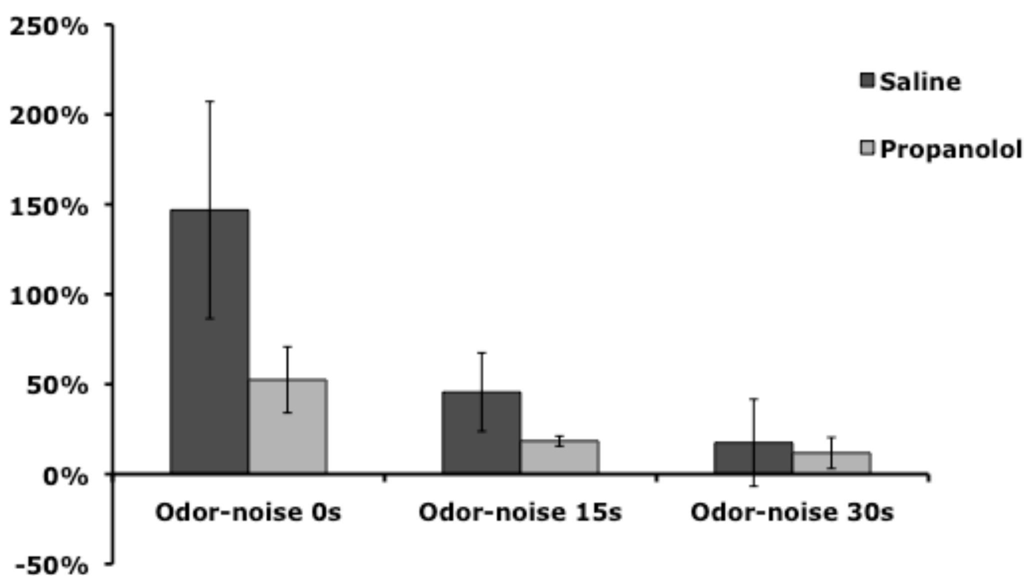
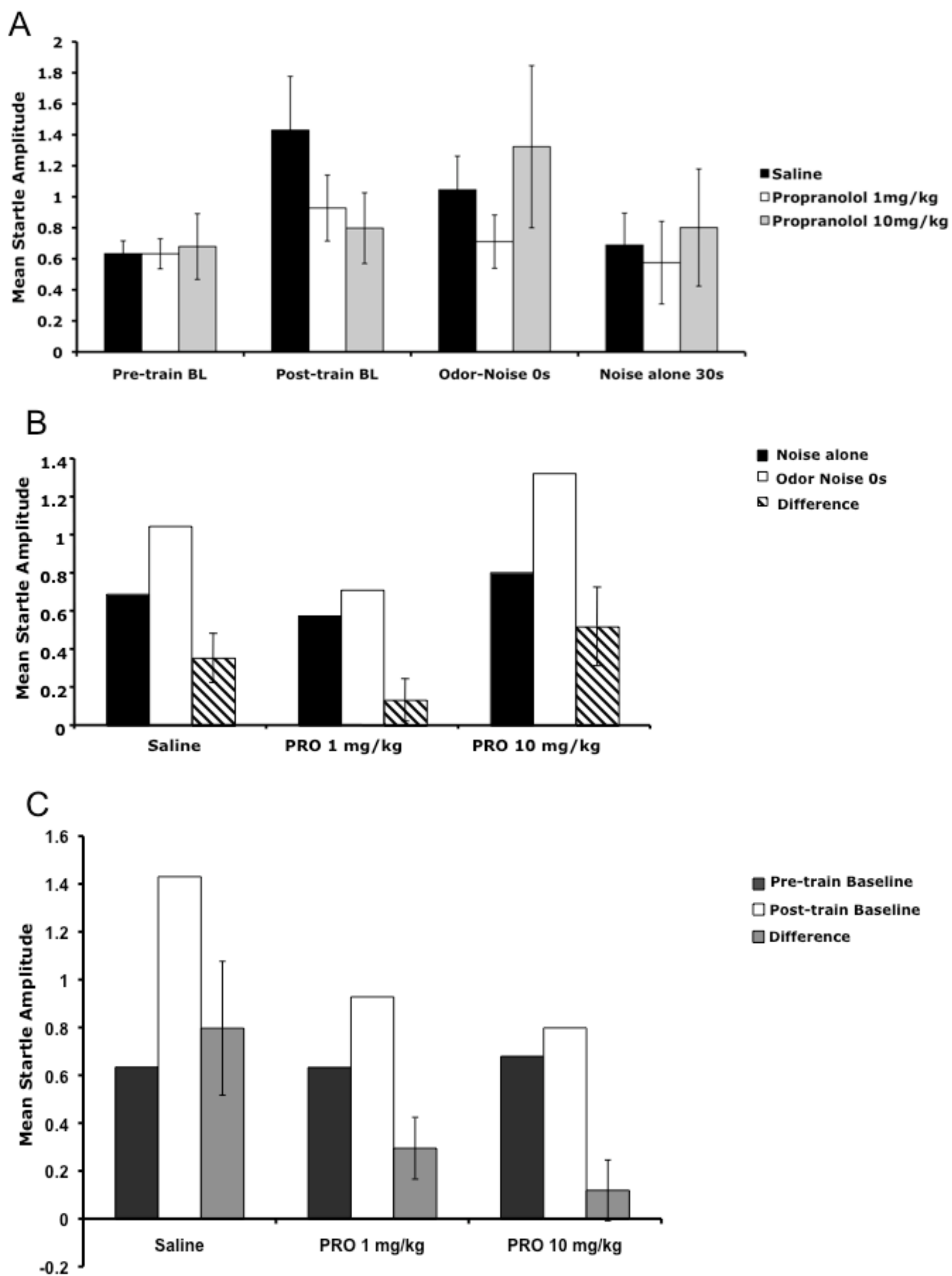


Figure 8



CHAPTER 5

Differing effects of systemic rapamycin on consolidation and reconsolidation of context versus cued fear memories

Abstract

The mammalian target of rapamycin (mTOR) kinase, a regulator of protein translation, has been implicated in synaptic plasticity and learning and memory. Because rapamycin, an mTOR kinase inhibitor, is centrally active following systemic administration and is FDA-approved for use in humans, it has recently attracted interest as a possible prophylactic for PTSD-associated fear memories. Our laboratory has developed an odor-mediated fear-potentiated startle paradigm that is amygdala-dependent and produces robust, long-lasting fear memories after a single training trial. Using this model, systemically administered rapamycin disrupted consolidation and reconsolidation of context fear, but not cued fear in rats. We also observed elevated mTOR signaling activity in the amgydala after a single odor-shock pairing, which was prevented by post-training systemic rapamycin injections. These findings suggest that relative to cued fear memories, context fear memories are more sensitive to the physiological effects of systemic rapamyin on amygdala mTOR signaling. We conclude that while rapamcyin may prove useful in retarding the development of some PTSD-associated memories, a possible lower threshold for disrupting contextual fear memories relative to cued fear memories may have important implications for its clinical usefulness.

Introduction

One of the cardinal symptoms of posttraumatic stress disorder (PTSD) is the presence of intrusive and persistent memories of the traumatic event. Disrupting fear memory formation in the aftermath of a traumatic experience may be a useful approach for mitigating these symptoms. This idea derives from consolidation theory, which holds that newly acquired memories are labile and take time to stabilize into enduring traces (McGaugh, 2000; Dudai, 1996). If this consolidation process is somehow perturbed before the memory is fully stabilized, then, theoretically, the memory trace should be compromised. Indeed, there is ample preclinical and clinical evidence showing that various manipulations (i.e., electroconvulsive shock treatment, certain pharmacological agents), when administered within a short time after a learning experience, can induce retrograde amnesia of that experience (McGaugh, 2000). It is also well established that stable memory traces can become vulnerable to disruption while in an active state of retrieval, a process known as reconsolidation blockade (Misanin, 1968; Nader, 2000). Hence, there is much interest in the clinical promise of drugs that interfere with the consolidation and reconsolidation of fear memories.

The mammalian target of rapamycin (mTOR) kinase modulates the phosphorylation state of the 70-kDA ribosomal S6 kinase (p70s6K) and eukaryotic initiation of factor 4E-binding proteins (4EBPs) proteins, which regulate protein translation (Raught et al., 2001). The mTOR signaling pathway has been implicated in synaptic plasticity (Casadio et al., 1999; Cammalleri et al., 2003; Tang et al., 2002) and learning and memory (Bekinschtein et al., 2007; Blundell et al., 2008; Parsons et al., 2006; Tischmeyer et al., 2003). Rapamycin, a potent inhibitor of mTOR signaling, has

been shown to disrupt both the consolidation and reconsolidation of tone-shock and context-shock fear memories (assessed with freezing) in rats when administered locally into the amygdala immediately after training (Parsons et al., 2006). Importantly, rapamycin has recently been shown to disrupt the consolidation and reconsolidation of context-shock memories (also assessed with freezing) when given systemically either before or immediately after training (Blundell et al., 2008). That systemic administration of rapamycin disrupts fear memories in rodents makes it a promising tool in the pharmacotherapeautic treatment of PTSD. Furthermore, rapamycin is FDA-approved for use in humans, and is already being widely prescribed for various conditions including organ transplantation, cardiovascular disease, and cancer (Plas & Thomas, 2009).

To evaluate the generality of systemic rapamycin effects on fear memory consolidation and reconsolidation, and hence the potential therapeutic efficacy of this drug, we examine here the effects of post-training and post-recall systemic rapamycin injections on single-trial odor-shock and context-shock fear memories in rats. To assess fear memory of both a discrete odor cue and a context, we use an olfactory-mediated Pavlovian fear conditioning paradigm called fear-potentiated startle (FPS), whereby animals show an increased noise-elicited startle response in the presence of an odor conditioned stimulus (CS) that had been previously paired with a footshock unconditioned stimulus (US) (Paschall & Davis, 2002), and to the context where odor-shock conditioning occurred (McNish et al., 1997; McNish et al., 2000).

Elevated phosphorylated p70s6K has been observed in the amygdala after tone-shock and context-shock conditioning, and these learning-related changes were reversed by rapamycin when infused locally into the amygdala (Parsons et al., 2006). To

determine if systemic rapamycin targets this region, we first tested the effect of single-trial olfactory fear conditioning on the relative expression of phosphorylated p70s6K in the amygdala using Western Blot analysis. Then we examined the effect of immediate post-training systemic rapamycin on these expression levels. Together, these experiments utilized an odor-mediated fear-potentiated startle paradigm to further explore the efficacy of a previously described amnesic dose of rapamycin (40 mg/kg) in disrupting the long-term fear memory of both a discrete cue and a distinct context.

Methods

Subjects

Male Sprague-Dawley rats (N=126) (Charles River, Raleigh, NC), weighing between 350 and 400 grams at the time of testing, were group housed four to a cage, and maintained on a 12:12 hour light / dark cycle with food and water available ad libitum. All behavioral procedures took place during animals' light cycle.

Drug

Rapamycin, purchased from LC Laboratories (Woburn, MA), was dissolved in a vehicle made of 5% ethanol, 4% PEG400, 4% Tween 80, and sterile water. Both drug and vehicle were injected intraperitoneally and delivered in a volume of 0.8ml/100g body weight. Rapamycin was administered at a dose of 40 mg/kg, which was chosen based on a previous study by Blundell et al. (2008).

Apparatus

Rats were trained and tested in two identical 8 x 15 x 15 cm Plexiglas and wire mesh cages as previously described by Cassella and Davis (1986). Background noise (60 dB wideband) and startle stimuli (50 ms white-noise bursts; rise decay, 5 ms) were

delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound-level measurements were made with a Brüel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (type 4176) located 7 cm from the center of the speaker, which approximates the distance of the rat's ear from the speaker during testing. Startle response amplitudes were quantified using an Endevco (San Juan Capistrano, CA) 2217E accelerometer. Cage movement produced by the rat's startle response resulted in displacement of the accelerometer, the output of which was integrated, producing a voltage output proportional to the velocity of cage movement. This signal was amplified by an Endevco model 104 amplifier and digitized on a scale of 0–2510 units by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 (Apple Computers, Cupertino, CA) computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 300 ms after onset of the startle-eliciting noise burst.

Olfactory Apparatus

The olfactory fear conditionined apparatus has been described in detail elsewhere (Paschall & Davis, 2002). In brief, a continuous flow of air was delivered from a compressed-air cylinder at a rate of 1.0 L/min through a small port (1.3-mm lumen diameter) positioned just above a 12.5 mm diameter opening in the top of each cage. For delivery of the olfactory stimulus, a computer-controlled solenoid (Model H15-03; Coulbourn Instruments, Allentown, PA) was opened for 4s, thereby diverting clean air from the compressed-air cylinder into and through a sealed 135-cm^3 glass jar containing 20 ml of 5% (vol/vol) amyl acetate (i.e., the odorant) in propylene glycol solution. The

inlet and outlet ports of the glass jar were positioned above the solution such that clean air from the tank mixed with the amyl acetate-containing vapor. The output was then mixed in a 3:5 ratio with clean air before flowing into the cage.

The chamber was actively exhausted into the building's ventilation system at a rate of $0.0114 m^3/s$. Thus, a volume of air equal to the chamber's total volume was vented every 25 s. Previous results with fear-conditioned rats indicate that with theses procedures startle amplitude returns to baseline levels within 30 s of solenoid closure (Paschall & Davis, 2002). Cages were cleaned daily with warm tap water and 95% alcohol, and were air dried overnight.

The unconditioned stimulus was a 0.5 s 0.4 mA scrambled shock delivered through the four floor bars as described by Walker and Davis (1997). The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads, Newton, CT).

Behavioral Procedures

Acclimation session. On each of two consecutive days, rats were placed in the startle chamber and after a 5 min acclimation period, received 30 presentations of startle stimuli (95 dB noise burst) separated by a 30-s intertrial interval. Rats were removed from the chamber immediately after the last startle stimulus presentation. Their mean startle amplitudes were calculated, and marked as their pre-training startle baseline. Rats were then divided into treatment groups that has equivalent mean startle amplitudes across the 30 stimuli (pre-training startle baseline).

Fear conditioning. The next day, rats were returned to the same startle chambers in which they were matched. After 5-min of acclimation, rats received a single odor-shock pairing

(4-s odor that co-terminates with a 0.5-s, 0.4-mA footshock). Immediately thereafter, rats were removed from the chambers and given an i.p. injection of either rapamycin (40 mg/kg) or vehicle (Experiment 1), or returned to their home cage (Experiment 2).

Reactivation session. Twenty-four hrs after training, rats in Experiment 2 were returned to the conditioning chamber and after a 5-min acclimation period were presented with a single 4-s odor CS. Immediately thereafter, rats were removed from the chambers and either given an i.p. injections of rapamycin (40 mg/kg) or vehicle.

Fear-potentiated startle test. Seven days after training or reactivation, rats were returned to the startle cages, and after 5 min, were presented with 30 startle stimuli (leaders). Thirty seconds after the final leader stimulus, rats received 30 startle-eliciting noise bursts presented alone (noise-alone trial) and 10 noise bursts presented 3.2-s after onset of the 4-s odor (odor-noise trials). The two trial types were presented in a manner such that after each odor-noise trial, three noise-alone trials were presented at a 30-s ITI, so that noise-alone trials occurred 30, 60, and 90-s after each odor. For some rats, the training and test contexts were identical. For others, the test context was altered (i.e., sandpaper inserts over the shock bars, Velcro on the sides, and 2 metal-link chains suspended from the top). These alterations have been previously shown to reliably produce discriminable context conditioning (McNish et al., 1997).

Western Blots

Rats received an acclimation session for two consecutive days. Twenty-four hrs later, rats were returned to the chambers and after 5-min of acclimation, received a single odor-shock presentation that was either Paired (4-s odor that co-terminates with a 0.5-s, 0.4-mA footshock) or Unpaired (odor and footshock separated by a 2-mininterval).

Immediately thereafter, rats were removed from the chambers, returned to their homecages and sacrificed via decapitation either 30 or 60 min later (Paired 30 min, Paired 1 hr, Unpaired 30 min, Unpaired 1 hr). Separate control groups were placed in the conditioning chamber for 7 min, without odor or shock exposure, returned to their homecage and sacrificed 1 hr later (Context 1 hr). Other untrained control groups were never exposed to the conditioning chamber and were sacrificed from their homecage (Homecage controls).

A separate group of rats were trained with a single odor-shock pairing (4-s odor that co-terminates with a 0.5-s, 0.4-mA footshock), and immediately thereafter removed from the chamber and given i.p. injections of either rapamycin (40 mg/kg) or vehicle, then returned to their homecages. They were sacrificed via decapitation 30 min later.

Brains were removed rapidly without perfusions, placed into ice-cold PBS for 5-min, then blocked rapidly over ice into 2-mm thick coronal sections. The BLA and dorsal hippocampus were removed bilaterally using a brain punch tool, homogenized in buffer (5 mM HEPES, .32 M sucrose, and Complete Mini EDTA-free protease inhibitor -- Roche Diagnostics), and stored at -80°C. Protein samples were quantified using a standard BCA assay (Pierce, Rockford, IL). Equal quantities (5-10 μg) of protein per animal were loaded onto polyacrilamide-SDS mini-gels, separated electrophoretically, blotted onto nitrocellulose membranes (BioRad, Hercules, CA), blocked, and incubated overnight at 4°C in primary for phospho-p70s6K (Thr 412) (1:1000; Upstate Biotechnology, Lake Placid, NY). After primary antibody exposure, the membranes were incubated in a secondary antibody (1:5000; Upstate Biotechnology) for 90 min. Bound antibody were detected by SuperSignal West Chemiluminescence (Pierce, Rockford, IL)

in an Alpha Innotech Fluorchem imaging system (Alpha Innotech, San Leandro, CA). Antibody detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH: mouse monoclonal, 1:10,000, Research Diagnostics) was used to control for variations in protein loading. Thus, the relative values are expressed as the protein of interest divided by the loading control.

Statistical Analyses

The mean startle amplitude on noise-alone and on odor-noise test trials was determined and a percent change score was calculated for each rat. Difference scores were calculated as: (odor-noise minus noise alone trials). Percent fear-potentiated startle was calculated as: [(odor-noise minus noise-alone trials) / (noise-alone trials)] × 100. Changes in baseline startle from the pre-conditioning acclimation session to the post-conditioning test session, shown elsewhere to reflect context conditioning, were similarly calculated (i.e., mean startle amplitude of the 30 noise alone trials which occurred at the beginning of the test session divided by the mean startle amplitude of the 30 noise alone trials of the second acclimation session X 100). For Western blotting analysis, a percentage of control score was derived for each rat by dividing each animal's relative optical density score by the home cage control group mean. Depending on the experiment, all data were analyzed by analysis of variance (ANOVA) or repeated measures followed by individual mean comparison using Tukey's post hoc tests. A significance level of $p < 0.05$ was taken for all results.

Results

Systemic rapamycin disrupts the consolidation of context but not cued fear memory

We examined the effects of systemically administered rapamycin on the consolidation of Pavlovian fear conditioning to a discrete odor cue and to a distinct context. Figure 1 shows the startle response of rats that were given an i.p. injection of either rapamycin (40 mg/kg) or vehicle immediately after training, and tested in either the same (n=18: rapamycin, n=10; vehicle; n=8) context in which they were trained or tested in a different (n=32: rapamycin, n=17; vehicle, n=16) context. Systemic administration of rapamycin, given immediately after training, produced a significant deficit in long-term memory (assessed 7 days after training) of context fear, but not cued fear. Figure 1a shows the mean startle amplitude for pre-training noise alone (black), and post-training noise alone (white) trials, and the difference between the two (black and white stripes). An ANOVA was performed with Context Fear as a repeated measures factor (pre-training noise alone vs. post-training noise alone) and Treatment as a between-subject factor (vehicle vs. rapamycin). For animals trained and tested in the same context, rapamycin prevented the increase in startle amplitude that occurred on noise alone trials from the pre- to post-conditioning test sessions. This was confirmed by a significant Context Fear x Treatment interaction, $F(1,16) = 4.57$, $p < .05$. Animals that were tested in a different context did not express context fear memory, as indicated by the lack of a significant main effect of Context Fear, $F(1,31) = .00$, $p > .05$. In this case, there was not a significant Context Fear x Treatment interaction, $F(1,31) = .06$, $p > .05$.

Figure 1b shows the mean startle amplitude for post-training noise alone (dark gray), post-training odor-noise (light gray), and the difference between the two (gray and black stripes). An ANOVA was performed with Cued Fear as a repeated measures factor (post-training noise alone vs. post-training odor-noise) and Treatment as a between-

subject factor (vehicle vs. rapamycin). For animals trained and tested in the same context, rapamycin did not prevent the increase in startle amplitude that occurred on odor noise trials, as indicated by a significant main effect of Cued fear, but not a significant Cued Fear x Treatment interaction $F(1,16) = 11.89$, $p < .05$. Animals that were tested in a different context similarly expressed cued fear memory, as indicated by a significant main effect of Cued Fear $F(1,31) = 31.91$, $p < .05$. However, there was not a significant Cued Fear x Treatment interaction.

Systemic rapamycin disrupts the reconsolidation of context but not cued fear memory

We next tested the effects of systemic rapamycin on the reconsolidation of context and cued fear memory. Figure 2 shows the expression of context (1a) and cued (1b) fear memory for rats that were given an i.p. injection of either rapamycin (40 mg/kg) or vehicle immediately after memory reactivation, and trained and tested in the same (n=11: rapamycin, n=5; vehicle, n=6) versus different (n=10: rapamycin, n=5; vehicle, n=5) context. Systemic rapamycin, given immediately after reactivation, resulted in a deficit in the long-term memory of context fear, but not cued fear. Figure 2a shows the mean startle amplitude for pre-training noise alone (black), and post-training noise alone (white) trials, and the difference between the two (black and white stripes). An ANOVA was performed with Context Fear as a repeated measures factor (pre-training noise alone vs. post-training noise alone) and Treatment as a between-subject factor (vehicle vs. rapamycin). For animals trained and tested in the same context, rapamycin prevented the increase in startle amplitude that occurred on noise alone trials from the pre- to post-conditioning test sessions. This was confirmed by a significant Context Fear x Treatment

interaction $F(1,9) = 6.27$, $p < .05$. Animals that were tested in a different context did not express context fear memory, as indicated by the lack of a significant main effect of Context Fear, $F(1,8) = 2.00$, $p > .05$. There was not a significant Context Fear x Treatment interaction. $F(1,8) = 1.15$, $p > .05$.

Figure 2b shows the mean startle amplitude for post-training noise alone (dark gray), post-training odor-noise (light gray), and the difference between the two (gray and black stripes). An ANOVA was performed with Cued Fear as a repeated measures factor (post-training noise alone vs. post-training odor-noise) and Treatment as a between-subject factor (vehicle vs. rapamycin). For animals trained and tested in the same context, rapamycin did not prevent the increase in startle amplitude that occurred on odor noise trials, as indicated by a significant main effect of Cued fear, $F(1,9) = 6.98$, $p < .05$ but not a significant Cued Fear x Treatment interaction $F(1,9) = .08$, $p > .05$. Animals that were tested in a different context similarly expressed cued fear memory, as indicated by a significant main effect of Cued Fear $F(1,8) = 17.53$, $p < .05$. However, there was not a significant Cued Fear x Treatment interaction, $F(1,8) = .05$, $p > .05$.

Single trial olfactory fear conditioning activates phosphorylation of p70s6K in the amgydala

The role of mTOR signaling in synaptic plasticity and learning and memory is due in part to its role in modulating the phosphorylation state of downstream effector p70s6K, which ultimately regulates protein translation (Raught et al., 2001). To determine whether olfactory fear conditioning would activate this signaling cascade in the rat amygdala the relative expression of phosphorylated p70s6K in amygdala neurons was measured using Western blot analysis. Olfactory-mediated fear-potentiated startle is

amygdala-dependent (Walker, Paschall, & Davis, 2005) and is capable of producing robust fear memory after only a single training episode (Paschall & Davis, 2002). Rats were presented with a single, discrete, odor cue and footshock that were either Paired (4-s odor co-terminating with a footshock) or Unpaired (odor and footshock separated by a 2 min interval), and were sacrificed 30-min or 60-min later [Paired 30 min (n=5), Paired 1-hr (n=6), Unpaired 30-min (n=7), Unpaired 1-hr (n=6)]. Another group was placed in the conditioning chamber for 7-min, without odor or shock exposure, and sacrificed 1-hr later (non exposured 1 hr, n=6).

Figure 3a shows quantitative levels of phosphorylated p70s6K protein expressed relative to homecage and GAPDH loading control and shows a clear increase in phosphorylated p70s6K relative to homecage controls, in the paired group at the 30-min post-training time point (Paired-30). A moderate enhancement was also seen at the 1-hr time point (Paired 1-hr). Similarly a modest increase was observed in the unpaired groups at both time points (Unpaired 30-min, Unpaired 1-hr). Non exposured control groups (Control 1 hr) showed a very similar expression profile as homecage controls. An overall one-way ANOVA comparing all groups approached but did not reach statistical significance, $F(5,29) = 2.08$, $p < .09$. A Tukey's posthoc analysis revealed that homecage and Paired 30 groups approached a significant difference, $p < .08$.

Systemically administered rapamycin decreases learning related p70s6K phosphorylation in the amygdala

We next set out to determine whether the amnesic effects of systemically administered rapamycin were associated with diminished phospho-p70s6K signaling in the amygdala by measuring the effect of immediate posttraining i.p. rapamycin (40

mg/kg, n=7) versus vehicle (n=7) injections on the relative increase in phosphosphorylated p70s6K previously seen 30-min after training. Figure 3b shows a significant increase in phosphorylated p70s6K relative to homecage controls (n=6) in the vehicle group, and diminished expression levels in the rapamycin group. An overall one-way ANOVA comparing homecage, vehicle, and rapamycin groups, revealed a significant difference among groups, $F(3,22) = 5.04$, $p < .05$. A Tukey's posthoc revealed a significant difference between homecage and vehicle, $p < .05$, and between the vehicle and rapamycin, $p < .05$. These results support our previous finding that single-trial olfactory fear conditioning produces enhanced expression of phospho-p70s6K in the amygdala 30 min after training. We further show that systemic rapamycin prevented this enhanced expression in the amgydala.

Discussion

We have developed an odor-mediated Pavlovian fear conditioning task that is amygdala-dependent (Walker, Paschall, & Davis, 2005), rapidly acquired after a single odor-shock pairing, and well remembered over time (Glover, Paschall, & Davis unpublished results; Paschall & Davis, 2002). This is a powerful tool to study fear memory consolidation using Pavlovian fear conditioning because it allows one to precisely pinpoint the critical associative event (the single odor-shock pairing) that triggers learning-related neural changes, and administer the putative consolidation-disrupting treatment immediately thereafter. The current study explored the efficacy of systemically administered rapamycin in disrupting the consolidation and reconsolidation of Pavlovian fear conditioning to a discrete cue and to a context. We chose to explore the signaling pathway controlled by mTOR, which regulates protein translation, because it

has been recently implicated in amygdala-dependent learning in a rapamycin sensitive manner (Parson et al., 2006). Importantly, rapamycin has been shown to disrupt fear memory consolidation and reconsolidation when given the systemic route (Blundell et al., 2008), making it a promising tool in the pharmacotherapeautic treatment of PTSD.

Consistent with the findings of Blundell et al. (2008), who used freezing as a measure of fear, we show here that systemic rapamycin disrupted both the consolidation and reconsolidation of context fear memory as measured by fear-potentiated startle. However, systemic rapamycin had no effect on the consolidation or the reconsolidation of cued fear memory. These findings are inconsistent with those of Parsons et al. (2006) who demonstrated that rapamycin disrupted both the consolidation and reconsolidation of cued (tone-shock) as well as context fear memories (assessed with freezing) in rats when administered locally into the amygdala immediately after training (Parsons et al., 2006). This discrepancy may reflect the different modalities used in these two studies (i.e., tone vs. odor) or, alternatively, a greater sensitivity of discrete cue fear conditioning to intra-amygdala vs. systemic rapamycin administration.

To determine whether systemic rapamycin impacts amygdala mTOR signaling, we first asked whether olfactory fear conditioning is associated with changes in activation of phospho-p70s6 kinase signaling in the amygdala. Using Western blot analysis, single trial olfactory fear conditioning resulted in enhanced expression of phosphorylated p70s6K in amygdala neurons (Figure 3a & b). Moreover, a previously described amnesic dose of rapamycin (40 mg/kg) reduced p70s6K signaling when measured 30-min after training (Figure 3b). Overall, our findings suggest for the first time that mTOR signaling in the amygdala may be physiologically relevant in olfactory fear memory consolidation.

Furthermore, a systemic dose of rapamycin (40 mg/kg) – which robustly disrupts context fear memory – significantly reduced amygdala mTOR signaling, despite having no effect on the behavioral expression of cued fear.

Most studies examining mTOR signaling in synaptic plasticity and learning and memory have targeted hippocampal neurons (Cammalleri et al., 2003; Tang et al., 2002), or exclusively used hippocampal-dependent learning tasks (Bekinschtein et al., 2007; Blundell et al., 2008; Sui et al., 2008). Much less is known, however, about role of mTOR signaling in other brain regions (Sui et al., 2008), and in hippocampal-independent fear learning (although see Parsons et al., 2006). The current study further implicates mTOR signaling in amygdala-dependent Pavlovian fear conditioning, using the olfactory modality and the fear-potentiated startle model.

Research on the neurobiology of fear memories is paving the way for pharmacological intervention as a preventive strategy for new trauma victims (consolidation blockade) or a treatment strategy for existing PTSD patients (e.g., reconsolidation blockage). Because drugs must be administered to humans safely and ethically, it is critical that basic researchers find drugs that are efficacious when administered the systemic route. Nevertheless, there are very few pre-clinical studies that examined the effect of systemic drug treatment on fear memory consolidation and reconsolidation. Of the handful of published studies that took this approach, many have reported a failure to demonstrate an amnesic effect of post-training systemic drug administration on fear memory to a discrete cue. Most notable is the apparent resistance of cued fear conditioning to post-training systemic administration of adrenergic antagonists. For example, Lee et al. (2001) gave rats systemic injections of epinephrine,

amphetamine, and two β-adrenergic receptor antagonists, sotalol and propranolol, immediately following cued fear conditioning. None of these manipulations affected conditioned fear retention as measured by freezing behavior. Similarly, Debiec and Ledoux (2004) failed to find an effect of systemic propranolol on the consolidation of cued fear conditioning when given immediately after training, although they did find a disruption of reconsolidation when given immediately after recall. In spite of a wealth of evidence showing that systemic administration of adrenergic drugs and hormones can modulate the consolidation of inhibitory avoidance training (McGaugh, 2002), it has yet to be demonstrated that systemic administration of adrenergic antagonists effects the consolidation of Pavlovian fear conditioning to a discrete cue. Nevertheless, propranolol has received widespread attention as a possible prophylactic agent to prevent unwanted memories PTSD (Strawn & Geracioti, 2008).

There are other examples in the literature showing that systemic post-training drug treatments fail to disrupt the consolidation of cued fear memory. When Thomson and Sutherland (2005) fear conditioned animals to a discrete auditory stimulus and to a distinctive context, they found that post-training systemic administration of lipopolysaccharide, a proinflammatory cytokine, disrupted memory for the context but not the discrete auditory stimulus as assessed by freezing behavior 48 hrs after training. Interestingly, Grillon and colleagues (2004) observed a similar pattern of results in humans.

Our finding that systemic rapamycin disrupts the consolidation and reconsolidation of context fear but not cued fear memory is consistent with such findings, which altogether show that systemically-administered agents disrupts the consolidation of

hippocampal-dependent (i.e., context fear or inhibitory avoidance), but not hippocampal-independent (i.e., cued fear) forms of fear memories. It is not clear why hippocampal-dependent vs. hippocampal independent forms of fear memories show differential vulnerability to systemic drug treatment. Early fear memory researchers observed a similar pattern when using electroconvulsive shock (ECS) as an amnesic treatment. Immediate post-training delivery of ECS disrupted retention when the latency to enter a shock compartment was measured (inhibitory avoidance). However, under the same conditions, animals showed signs of retention when classically conditioned behavioral (Bueno et al., 1993) and autonomic responses, such as heart-rate suppression, urination, and defecation were measured (Chorover & Schiller, 1966; Mendoza & Adams, 1968; Hine & Paolino, 1970; Springer, 1975; Yaginuma & Iwahara, 1971). Consistent with those results, we have found that post-training ECS blocks context but not cued olfactory-mediated fear potentiated startle (Glover, Paschall, Davis, in preparation). It has been suggested that the differential vulnerabilities of conditioned responses to system wide amnesic treatments is a function of higher threshold to disruption for lower order fear memories compared to higher-order fear memories (Springer, 1975). Further research is needed to address these issues – particularly studies that utilize systemic drug treatments and a range of behavioral techniques to assess memory.

Overall the present study reports differential vulnerabilities of cued and context fear memories to systemic rapamycin treatment. Although systemic rapamycin is efficacious in disrupting mTOR signaling activity in the amgydala when administered immediately after training, it has no effect on the behavioral expression of cued fear memory. It is not clear why the consolidation and reconsolidation of cued fear is

impervious to systemic rapamycin treatment. It is possible that certain forms of fear memories might have a higher threshold for disruption than other forms. Further research could address this issue by doing a dose-response measure of systemic rapamycin on cued fear memory consolidation. It is possible that a higher dose, or perhaps a lower dose, of rapamycin might be more efficacious in disrupting cued fear memory. However, our finding that 40 mg/kg of rapamycin prevented the increase in amygdala mTOR signaling seen after cued fear conditioning, suggests that dosage, by itself, is not the issue. There is also the possibility that timing may be a critical factor. Cued fear memory's sensitivity to systemic rapamycin might be restricted to a certain temporal window. We injected rapamycin within 30 s after the odor-shock presentation or reactivation trial, and while this regimen was sufficient to disrupt amygdala mTOR signaling as well as long-term memory of context fear, it is possible that the critical learning-related changes that support long-term cued fear memory had already occurred. More research is needed to address these issues. Based on our findings, we conclude that although systemically administered mTOR inhibitors may prove useful in retarding the development of some PTSD-associated memories, their possible selectivity for disrupting hippocampal-dependent versus hippocampal-independent fear memories might limit their clinical effectiveness.

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Figure Captions

***Figure 1.* Systemic rapamycin disrupts the consolidation of context but not cued fear memory.** Mean startle amplitudes of rats given an i.p. injection of either rapamycin (40 mg/kg) or vehicle immediately after training, and tested in the same context in which they were trained or tested in a different context. **(A)** *Context Fear*, mean startle amplitude for pre-training noise alone (black), and post-training noise alone (white) trials, and the difference (± SEM) between the two (black and white stripes). Rapamycin treated rats showed significantly less fear-potentiated startle to the conditioning context than vehicle treated rats (* $p < .05$). None of the rats that were tested in a different context expressed context fear memory. **(B)** *Cued fear*, mean startle amplitude for post-training noise alone (dark gray), post-training odor-noise (light gray), and the difference (± SEM) between the two (gray and black stripes). There was no significant difference between rapamycin and vehicle treated rats in their expression of cued fear memory whether tested in the same or a different context from training.

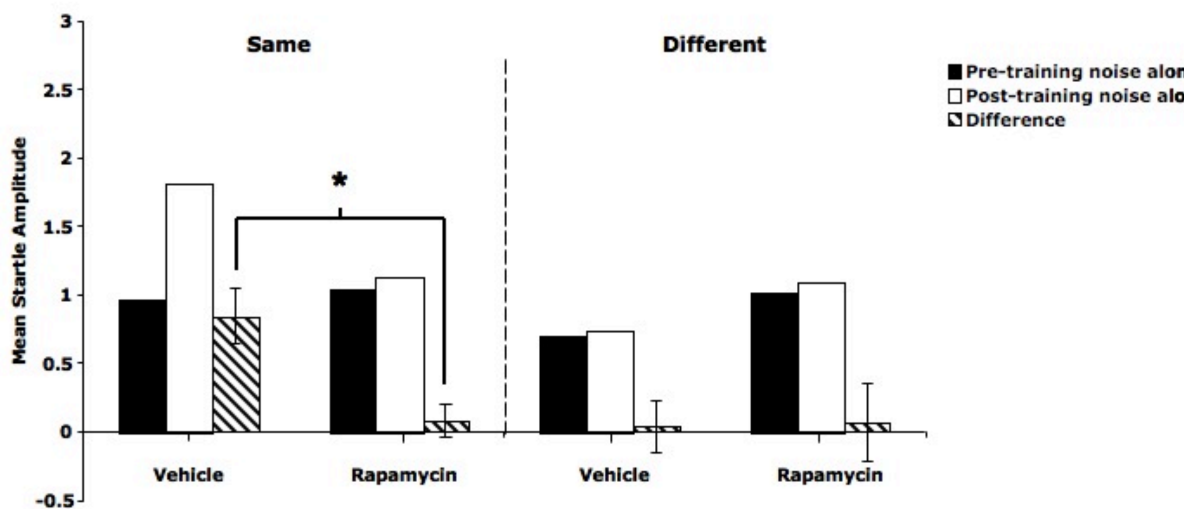
***Figure 2.* Systemic rapamycin disrupts the reconsolidation of context but not cued fear memory.** Mean startle amplitudes of rats given an i.p. injection of either rapamycin (40 mg/kg) or vehicle immediately after memory reactivation, and tested in the same context in which they were trained or tested in a different context. **(A)** *Context Fear*, mean startle amplitude for pre-training noise alone (black), and post-training noise alone (white) trials, and the difference (± SEM) between the two (black and white stripes). Rapamycin treated rats showed significantly less fear-potentiated startle to the conditioning context than vehicle treated rats (* $p < .05$). None of the rats that were tested

in a different context expressed context fear memory. **(B)** *Cued fear*, mean startle amplitude for post-training noise alone (dark gray), post-training odor-noise (light gray), and the difference (± SEM) between the two (gray and black stripes). There was no significant difference between rapamycin and vehicle treated rats in their expression of cued fear memory whether tested in the same or a different context from training.

Figure 3. **Single trial olfactory fear conditioning activates phosphorylation of p70s6K in the amgydala, which was prevented by post-training systemic rapamycin administration.** Bars represent mean optical density values (±SEM) expressed as a percentage of home cage (*HC*) control (black) and GAPDH protein loading control. Representative blots of phosphorylated p70s6K are shown below their corresponding group. **(A)** Rats were presented with an odor cue and footshock that were either *Paired* (white) or *Unpaired* (gray), and were sacrificed 30 min or 60 min later. Another group, *Context*, was placed in the conditioning chamber for 7 min, without odor or shock exposure, and sacrificed 1 hr later (striped). Olfactory fear conditioning is associated with an increase in phosphorylated p70s6K relative to HC, in the paired group at the 30 min post-training time point, as well as moderate enhancement at the 1 hr time point, and in the unpaired groups at both 30 min and 1 hr time points. These observed changes approached but did not reach statistical significance, $p = .08$. **(B)** Immediately after single-trial odor-shock training, rats were given i.p. rapamycin (*RAP*, striped) (40 mg/kg) or vehicle (*VEH*, dotted) and sacrificed 30 min later. There was significant increase in phosphorylated p70s6K relative to *HC* in the *VEH* group (* $p < .05$ compared to HC), and

significantly less expression levels in the *RAP* group (# $p < .05$ compared to VEH and HC).

Figure 1



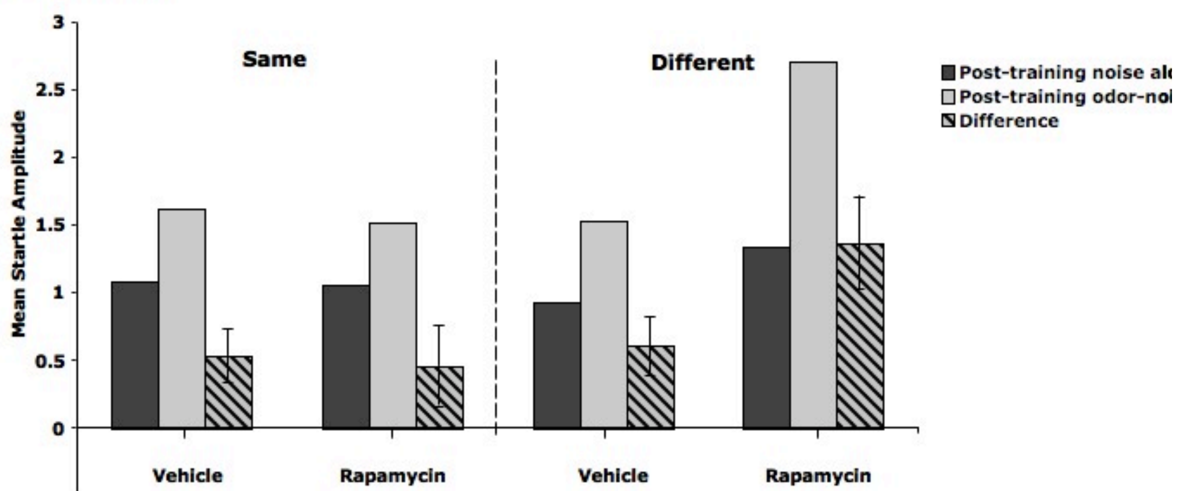
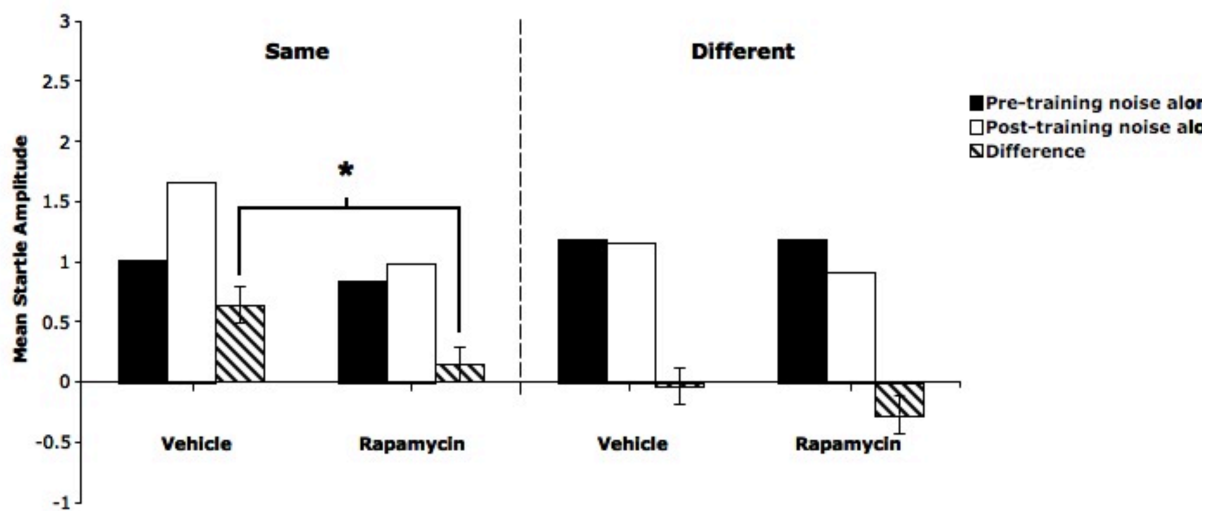


Figure 2



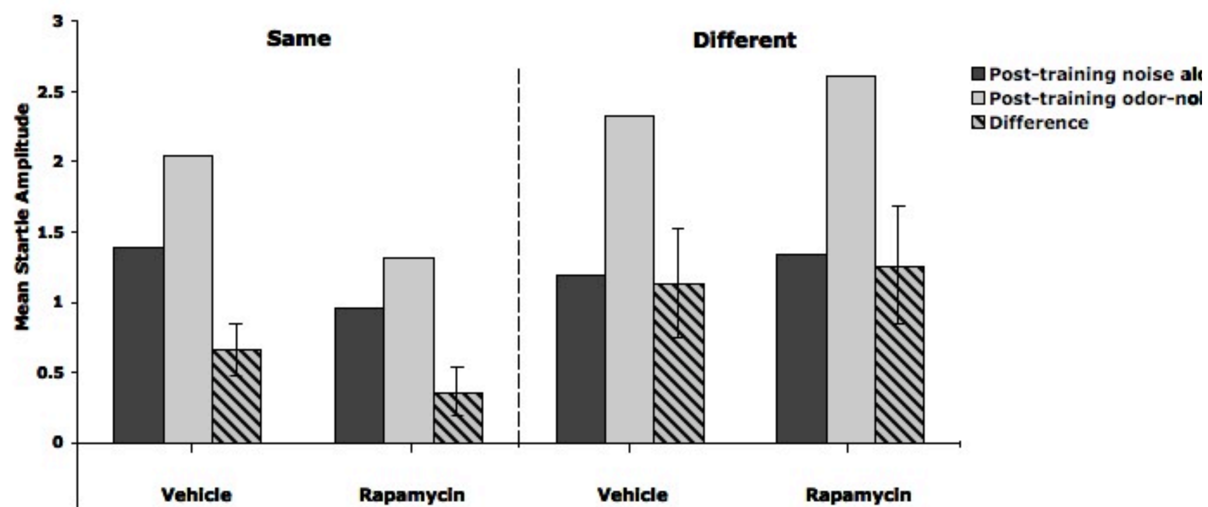
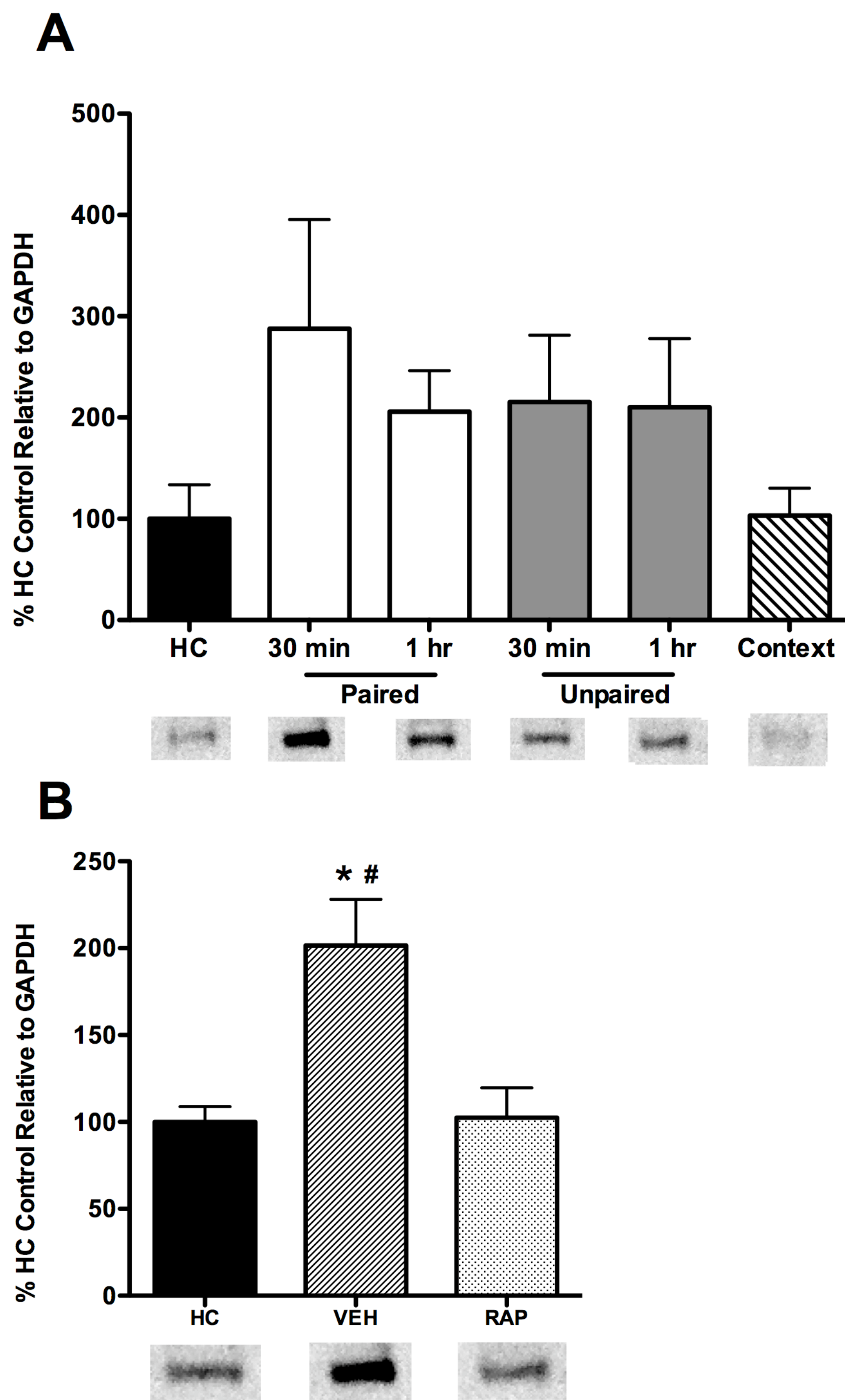


Figure 3



CHAPTER 6

General Discussion

This dissertation project used a rat model of olfactory-mediated fear-potentiated startle to examine the clinical efficacy of two possible pharmacological interventions as well as a novel behavioral intervention that could be implemented in the immediate aftermath of a traumatic event to prevent PTSD or used to reduce extant PTSD symptoms. Overall findings supported our central hypothesis that hippocampal-dependent fear memories are more vulnerable to disruption than hippocampal-independent fear memories. Furthermore, results suggest that treatments given soon after a traumatic experience may have limited effectiveness. However, treatments given soon after the retrieval of traumatic memories hold tremendous clinical promise.

The major findings of this project are that cued Pavlovian fear memories are extremely persistent in the face of various post-training manipulations shown elsewhere to disrupt other forms of fear memories. However, when the conditioning procedure is modified to engage the hippocampus, then Pavlovian fear conditioned memories become susceptible to disruption. In Chapter 2, it was demonstrated that post-training ECS administration disrupted long-term retention of hippocampal-dependent trace fear conditioning, but left hippocampal-independent delay fear conditioning intact. The idea that post-training manipulations differentially effects delay versus trace fear memories was further supported in Chapter 4, where it was shown that systemic propranolol impaired consolidation of trace fear but not delay fear memory. Further support for our hypothesis was provided by findings in Chapters 4 and 5 that propranolol and rapamycin disrupted the consolidation of context fear but not cued fear.

It is not known why hippocamapal-dependent and hippocampal-independent fear memories are differentially vulnerable to disruption. Perhaps the answer lies in where

fear memories are stored. It has been argued that the amygdala stores cued fear memories, but modulates the consolidation of hippocampal- based memories (Fanselow & LeDoux, 1999; Huff & Rudy, 2004). If the amygdala does not modulate itself, then amygdala-dependent cued fear memories would not fall under the modulatory influence of amnesic treatments. Hence, this model predicts that cued fear memories would be resistant to post-training manipulation. Others have argued that the amygdala is not a site of fear memory storage, but only serves to modulate aversive memories stored in other brain regions (Cahill & McGaugh, 1998; Vazdarjanova & McGaugh, 1998). This model predicts that all aversive memories are subject to the modulatory influence of the amygdala and is capable of being disrupted by post-training treatments. Evidence supporting both views has been extensively described (Cahill & Weinberger, 1999; Fanselow & LeDoux, 1999; Maren & Fanselow, 1996; Vazdarjanova, 2000; Vazdarjanova & McGaugh, 1998). What follows is a brief description of relevant findings.

The storage debate rests on two assumptions about the effects of amygdala lesions on the acquisition and expression of fear memories. It is hypothesized that if the BLA is the site of fear memory encoding and storage, then pretraining BLA lesions should block the acquisition of fear memories, and posttraining BLA lesions should cause fear memory retention deficits. Evidence that is consistent and inconsistent with this hypothesis has been demonstrated for Pavlovian fear and instrumental avoidance measures, respectively.

Several studies show that lesions of the BLA given prior to training prevent the acquisition and expression of Pavlovian fear conditioning (e.g., Campeau & Davis, 1995; Hitchcock & Davis, 1987; Maren, 1998, 1999; Maren, Aharonov, & Fanselow, 1996;

Sananes & Davis, 1992). Furthermore, it has been demonstrated that posttraining BLA lesions made 1, 14, or 28 days after training (Maren, Aharonov, & Fanselow, 1996) and 6 or 30 days after training (Lee, Walker, & Davis, 1996) completely blocked the expression of fear conditioning. There is general agreement that lesions of the BLA block the acquisition of instrumental avoidance training (e.g., Cahill & McGaugh, 1990). In contrast, the postulation that the amygdala in not a site of storage for fear memories is based on the general findings that the retention of inhibitory avoidance learning could be assessed in the absence of amygdala influence (e.g, Liang et al., 1982). Some evidence in this regard, shows that when the BLA is lesioned soon (1 or 4 days) but not one month after training, animals demonstrate intact avoidance retention (Parent & McGaugh, 1994; Izquierdo et al., 1997; Parent, Avila, & McGaugh, 1995). These findings implicate a temporary role of the amygdala in inhibitory avoidance learning and, based on the assumption that a brain region that stores a memory must also play a role in the retrieval and expression of that memory retention, they suggest that the amygdala is not the site of memory storage. Overall, by implicating the amygdala as a site of storage for Pavlovian fear conditioning but not for inhibitory avoidance learning, these findings provide the most powerful evidence that these two paradigms engage fundamentally distinct neural processes.

Nonetheless, the interpretation of a storage role of the amygdala is complicated by the fact that the amygdala's circuitry is tightly coupled to the performance of conditioned and unconditioned responses (Davis, 2000). Thus, it can be argued that any lesion of the amygdala can be interpreted as a deficit in unconditioned fear versus a deficit in conditioned fear. However, there is considerable evidence showing that while lesioning

the CE completely abolishes several types of conditioning and unconditioned responses, restricting lesions to the BLA impair conditioned fear but not unconditioned fear (for review, see Davis, 2000). Thus, although the amygdala may play a role in the acquisition, the consolidation, and the expression of conditioned fear, these roles can be dissociated by the clear organization system of amygdalar nuclei.

Importantly, the divergent findings regarding the role of the amygdala in fear memory storage all point to the fundamental notion that Pavlovian fear conditioning and inhibitory avoidance learning engage distinct neural processes which may have different vulnerabilities for post-training disruption. The current project extends the findings of others that cued fear memories are resistant to post-training manipulations. However, when Pavlovian fear conditioning engages hippocampal neural processes, its threshold for disruption is similar to that of inhibitory avoidance. These findings support the idea that certain fear memories are stored in the amgydala and undergo a consolidation process that is different from that of fear memories stored elsewhere.

If proven true, this idea may also explain differential vulnerabilities of consolidated versus reconsolidated fear memories to amnesic treatments. In Chapter 4, it was shown that retention of cued fear conditioning could be disrupted when propranolol is administered immediately after retrieval, but not when given immediately after training. These findings, along with those of Debiec and Ledoux (2004), raise the question of whether the retrieval of cued fear memories engages the hippocampus. Emerging evidence suggests that reconsolidation and consolidation are two fundamentally distinct processes that might be mediated by distinct brain regions (i.e., Bahar et al., 2004; Hernandez et al., 2002; Kelly et al., 2003; Lee et al., 2004; Nyberg et

al., 1996; Salinska et al., 2004; Taubenfeld et al., 2001; Tronel & Sara, 2002). If the retrieval of cued fear memories engage neural system other than the amgydala, then it could be predicted from the above-mentioned amygdala storage hypothesis that cued fear memory reconsolidation would be vulnerable to disruption. Further research is needed to elucidate amygdala and hippocampal involvement in fear memory storage and retrieval. Understanding how these two neural systems interact may be key to finding effective treatments for PTSD.

Much progress has been made in understanding the biological basis of persistent fear memories in PTSD. Nevertheless, many questions remain regarding the functional role of various neural systems in mediating lasting memories after a traumatic experience. The current project underscores the importance of using diverse animal models in order to better represent the varied and complex nature of a traumatic episode. A synthesis of findings from these various approaches offer the best hope for addressing memory-related symptoms in PTSD.

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