Rapid Reacquisition of Contextual Fear Conditioning: The Behavior, Interaction with Alcohol, and Neurobiology

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List of Abbreviations

CS, conditioned stimulus
US, unconditioned stimulus
UR, unconditioned response
CR, conditioned response or conditioned responding
D1R, dopamine 1 receptor
PTSD, post-traumatic stress disorder
AUD, alcohol use disorder
RMANOVA, repeated measures analysis of variance
IHC, immunohistochemistry
IEG, immediate early gene
PFA, paraformaldehyde
NaN_3, sodium azide
PBS, phosphate buffered saline
NGS, normal goat serum
BSA, bovine serum albumin
DAB, diaminobenzidine stain
H4K8ac, histone 4 lysine 8 acetylation
NMDA, N-methyl-D-aspartate
GABA, gamma-aminobutyric acid
CRF, corticotropin-releasing factor
BNST, bed nucleus of the stria terminalis
dBNST, dorsal bed nucleus of the stria terminalis
adBNST, anterior dorsal bed nucleus of the stria terminalis
avBNST, anterior ventral bed nucleus of the stria terminalis
CeA, central amygdala
LA, lateral amygdala
BLA, basolateral amygdala
DG, dentate gyrus of the hippocampus
CA1-3 , CA subfields of the hippocampus
mPFC, medial prefrontal cortex
PL, prelimbic cortex
IL, Infralimbic cortex

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation

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Abstract

The research of Pavlovian conditioning has elucidated much about the basic characteristics and neurobiology of animal memory. A simple associative memory can be formed and extinguished, but conditioned responding often returns following a variety of situations. These post-extinction re-emergences of conditioned behavior have been a point of interest for studying relapse in human disorders. The restoration in conditioned responding despite extinction can mimic the relapse of drug taking in addiction or the return of fear and anxiety behavior in post-traumatic stress disorder. Accordingly, most phenomena of returned conditioned behavior following extinction have been well characterized, but one outcome is less understood. Specifically, post-extinction reconditioning can inconsistently cause both rapid and slow reacquisition of conditioned fear behavior. There is a limited body of work that explains these opposing findings. Thus, the main aim of this dissertation is to further characterize the outcomes and neurobiology of reconditioning in a series of contextual fear conditioning experiments. Chapter 1 comprises a review of the behavioral features, theories, and neurobiology of each post-extinction re-emergence of conditioned responding. Additionally, Chapter 1 places emphasis on reconditioning and rapid reacquisition of fear as a useful tool for studying post-extinction memory modulation, differences between acquisition and reacquisition, and rodent behavior that mimics post-traumatic stress disorder symptoms. Chapters 2 through 4 provide the behavioral findings of my research on rapid reacquisition. Chapter 2 further characterizes the parameters of contextual fear reconditioning that cause rapid reacquisition of fear in two rodent species. In Chapter 3, I explore the

impact of ethanol administration and withdrawal on rapid reacquisition of fear that has important implications for understanding the interaction of alcohol use and fear memory. Chapter 4 probes important brain regions in the fear circuit for their role in rapid reacquisition of fear to see if rapid reacquisition has a distinct neural pattern relative to initial acquisition of fear. Last, Chapter 5 contextualizes my results within the broader findings of the learning and memory field and discusses several important limitations and future directions for this work.

Chapter 1: General Introduction

I. Post-Extinction Re-emergence of Conditioned Responding

Pavlovian conditioning is a paradigm that has been critical to the current understanding of animal and human memory. Since being famously characterized in 1927 by Ivan Pavlov, the field and use of Pavlovian conditioning has grown considerably beyond the ability of a metronome to cause a dog to salivate. Today, Pavlovian, or classical, conditioning is the standard in neuroscience and psychology for studying associative memory.

In general, Pavlovian conditioning consists of pairing a biologically neutral stimulus (e.g., a light or tone that initially does not elicit an overt or standard response), with a biologically relevant stimulus (e.g., food or footshock) that causes the animal to display biologically appropriate responses, called unconditioned responses (UR). Following several pairings of the neutral stimulus, or conditioned stimulus (CS), with the relevant stimulus, or unconditioned stimulus (US), the CS will begin to assume the associative properties of the US. Then CS-alone presentations will cause the animals to recall the US and perform a response in preparation of expected US delivery – the conditioned response (CR), which is generally different than the UR (Holland, 1977). This general setup has led to the creation of paradigms like fear conditioning (Denenberg, 1958; Rescorla & Wagner, 1972) and conditioned place preference (Reicher & Holman, 1977) that have been invaluable to describing the behavioral and neurobiological properties of aversive and appetitive memory and behavior.

With these Pavlovian tasks, researchers have been able to gain great insight into the acquisition and extinction of conditioned behavior and associative memories.

Acquisition, described above, occurs when the animal acquires the CS-US association and CS-alone presentations begin eliciting CRs. In rodents, acquisition of fear CR creates memories that are retained years later (Gale et al., 2004). But CS-alone presentations can also lead to extinction. Extinction occurs when the CS no longer predicts presence of the US and CR subsequently diminishes in response to the CS. The study of acquisition and extinction of Pavlovian conditioning has led to well-defined fear acquisition and extinction circuits (Delamater & Westbrook, 2014; Maren, 2001; Peters, Kalivas, & Quirk, 2009). Using paradigms like fear conditioning, researchers discovered that extinction, while terminating CR and causing no evidence of the original acquisition, does not remove or delete the initial acquisition memory through a variety of post-extinction phenomena.

These post-extinction phenomena include spontaneous recovery, contextual renewal, reinstatement, and reconditioning. This introductory chapter will use research predominately from fear conditioning work to review these post-extinction re-emergences of CR, paying special attention to one outcome of reconditioning – rapid reacquisition. There are several excellent reviews that cover the behavioral traits of each of the above post-extinction outcomes (Bouton, 2002; 2003; Haaker, Golkar, Hermans, & Lonsdorf, 2014; Rescorla, 2004b; Vervliet, Baeyens, Van den Bergh, & Hermans, 2013a; Vervliet, Craske, & Hermans, 2013b). However, this chapter will also review and compare the relevant neurobiology of each to draw meaningful conclusions on the mechanisms used by each. Significantly, this chapter will make a case for a larger focus on rapid reacquisition within the learning and memory field and set the stage for the dissertation research that will be described in

the final section of this chapter.

II. Popular Forms of Post-Extinction Re-emergence of CR and the Neurobiology Underlying Each

a. Spontaneous Recovery

i. Spontaneous Recovery Behavior

Spontaneous recovery is defined as the increasing recovery of CR with the passage of time following extinction. Pavlov first described spontaneous recovery in his seminal 1927 book. This phenomenon has been shown in nearly every conditioning type, including fear conditioning (Leung & Westbrook, 2008; Quirk, 2002). There are also several characteristics that define spontaneous recovery. First, the longer the delay between extinction and retesting, the stronger the appearance of spontaneous recovery is (Quirk, 2002). However, the recovery is never entirely complete. Even if CR appears to be at pre-extinction levels, the recovery is generally quickly lost (Rescorla, 2004b). Additionally, spontaneous recovery decreases with repeated extinction training (Rescorla, 2004b) or by stimuli that act as reminders of extinction (Brooks & Bouton, 1993). Further, it appears to also depend on the spacing of acquisition and extinction (Rescorla, 2004a) and extinction trials (Urcelay, Wheeler, & Miller, 2009). Paradoxically, spontaneous recovery can play a role in enhancing inhibitory learning, as its occurrence during extinction can deepen extinction of CR (Leung & Westbrook, 2008).

There are several theories for the mechanism of spontaneous recovery. The first and most popular theory is that spontaneous recovery occurs due to the

increased ambiguity of the meaning of the CS and temporal relationship of CS to US over time (Bouton, 2002; Rescorla, 2004b). Due to the CS ambiguity following a delay after extinction, the CS once again elicits expression of the initial acquisition memory over retrieval of the inhibitory extinction memory. In this theory, context is viewed as important to spontaneous recovery. Specifically, the temporal context of the inhibitory CS-US association is theorized to change over time and increases CS ambiguity. This theory corresponds with the idea that extinction does not remove acquisition memory, but forms a new inhibitory memory competing for expression. Accordingly, following full spontaneous recovery of CR, there is evidence that the extinction memory still remains due to the rate of re-extinction of the CR (Quirk, 2002).

Alternatively, some have theorized that local performance effects, attentional processes, or stimulus sampling lead to spontaneous recovery. Local performance effects, like fatigue and frustration, can cause an increase in responses and perhaps spontaneous recovery occurs due to factors unrelated to learning. This cause is unlikely to be the entire reason for spontaneous recovery, which is consistently shown in many preparations (Rescorla, 2004b). Some have also proposed that extinction occurs because there is less attending to the CS and spontaneous recovery occurs when the attentional processing of the CS resumes (Robbins, 1990). While others believe that animals use stimulus sampling during acquisition and that the components, not the whole representation, of a CS assume associative strength. Accordingly, during extinction, a random selection of CS elements is believed to be completely extinguished (contradicting the idea that extinction forms a

separate inhibitory memory). But, with the passage of time the components of the CS that were not extinguished cause the recovery of CR (Estes, 1955). For a more thorough review see (Rescorla, 2004b).

ii. Spontaneous Recovery Neurobiology

Research on the neurobiology of spontaneous recovery lags far behind the behavioral understanding of the phenomenon. This research gap is due to the difficulty in interpretation of studies that find differences in spontaneous recovery due to neurobiological manipulation. In many studies, extinction is manipulated and spontaneous recovery is used as a measure of that manipulation, so it is difficult to dissociate what is altering extinction from what is altering spontaneous recovery. However, there are some underlying commonalities of the following studies that connect certain brain regions to spontaneous recovery.

The medial prefrontal cortex (mPFC), a region highly implicated in expression and inhibition of associative learning (Peters et al., 2009), is associated with spontaneous recovery. Spontaneous recovery of conditioned taste aversion caused the mPFC to express conditioning-like levels of c-Fos, an immediate early gene that marks neuronal activity (Chaudhuri, 1997; Knapska & Maren, 2009), relative to extinction (Mickley et al., 2007). This result suggested a recovery of the original memory trace. Also, several studies have shown that lesioning the mPFC can alter spontaneous recovery of Pavlovian and operant conditioning. A lesion of the whole mPFC prior to discriminative contextual fear conditioning impaired spontaneous recovery, but left acquisition and extinction intact (Zelinski, Hong, Tyndall, Halsall, &

McDonald, 2010). Specifically, lesioning the Infralimbic portion of the mPFC (IL) caused enhanced spontaneous recovery of conditioned suppression (Quirk, Russo, Barron, & Lebron, 2000) and appetitive conditioning (Rhodes & Killcross, 2004). Similarly, inactivation of the IL enhanced spontaneous recovery of cocaine self administration (Peters, Vallone, Laurendi, & Kalivas, 2008).

There are two corresponding interpretations of these results. One, as the authors of these studies suggest, this outcome reflects the importance of the IL to extinction. If lesioned, CR returns because the extinction memory cannot be retrieved. Alternatively, this result could show the significance of the IL to spontaneous recovery, such that ablating the IL prevents the IL from disambiguating the associative value of the CS. However, IL lesions have also been found to not effect on spontaneous recovery of fear conditioning (Gewirtz, Falls, & Davis, 1997). Overall, there does appear to be a role of the mPFC in spontaneous recovery, even if extinction-impairing effects largely drive it.

In addition to the mPFC, the amygdala, a region critical for acquisition and expression of conditioning (Klumpers, Morgan, Terburg, Stein, & van Honk, 2014; Maren, 1999; Nader, Majidishad, Amorapanth, & LeDoux, 2001), has shown a role in spontaneous recovery. Like the mPFC, the basolateral amygdala (BLA), but not central amygdala (CeA), showed acquisition-like levels of c-Fos expression in response to spontaneous recovery of conditioned taste aversion (Mickley et al., 2007). This result suggested a subregion-specific involvement of the amygdala in the recovery of CR. Administration of a CB1 (cannabinoid 1 receptor) agonist to the amygdala following fear potentiated startle (Lin, Mao, & Gean, 2006) and inactivation

of the BLA specifically prior to a spontaneous recovery test of cocaine self-administration (Peters et al., 2008) attenuated spontaneous recovery. Taken together, these studies show that the amygdala is involved during spontaneous recovery of a CR.

A few additional regions have also displayed a role in spontaneous recovery. Low frequency stimulation of dorsal anterior cingulate cortex during acquisition prevented spontaneous recovery of eye-blink trace conditioning in rhesus macaques (Klavir, Genud-Gabai, & Paz, 2012); inactivation of the core, but not shell, of the nucleus accumbens dampened spontaneous recovery of cocaine self-administration (Peters et al., 2008); and hippocampal lesions after reactivation of cued fear conditioning (a hippocampus-independent conditioning task) prevented spontaneous recovery relative to animals who did not receive reactivation (Debiec, LeDoux, & Nader, 2002). Refer to Table 1 for a summary of the neurobiology implicated in spontaneous recovery, as well as the other post-extinction forms of CR return.

b. Contextual Renewal

i. Contextual Renewal Behavior

A recurring characteristic of the inhibitory memories formed in extinction is that they are context-dependent, unlike excitatory acquisition memories. This feature was suggested in the contextual control of time on inhibitory CS-US associations in spontaneous recovery, but the context-dependency of extinction is best displayed during contextual renewal. Contextual renewal occurs when the extinguished CS is

Phenomena	Brain Region	Outcome	Citation
Spontaneous Recovery (SR)			
	Amygdala	BLA inactivation attenuated SR of self administration	Peters, Vallone, Laurendi, & Kalivas, 2008
		Acquisition-like c-Fos levels in the BLA, but not CeA following SR of conditioned taste aversion	Mickley et al., 2007
		CB1 agonist during retrieval prevents SR of fear potentiated startle	HLin, Gao, & Gean, 2006
	mPFC	IL inactivation increased SR of self administration	Peters, Vallone, Laurendi, & Kalivas, 2008
		IL inactivation increased SR of conditioned suppression	Quirk, Russo, Baron, & Lebron, 2000
		Acquisition-like c-Fos levels in PL and IL following SR of conditioned taste aversion	Mickley et al., 2007
		Lesion impaired SR, but not acquisition or extinction of discrimination of contextual fear	Zelinski et al., 2010
	Hippocampus	Lesions following reactivation prevented SR of cued fear conditioning	Debiec, LeDoux, & Nader, 2002
	NAc	Core inactivation attenuated SR of self administration	Peters, Vallone, Laurendi, & Kalivas, 2008
	ACC	Stimulation of dACC prevents SR of eyeblink trace conditioning	Klavir, Genud-Gabai, & Paz, 2012
Contextual Renewal			
	Hippocampus	Inactivating the dorsal hippocampus prevents ABC fear renewal, but not ABA renewal	Corcoran & Maren, 2001, 2004
		Pre-training and post-extinction dorsal hippocampus lesion prevent AAB fear renewal	Ji & Maren, 2005

	Hippocampus cont'd	Inactivating the ventral hippocampus (and kappa opioid receptors in VH) prevents AAB fear renewal	Hobin & Maren, 2006; Cole, Richardson, & McNally, 2013
		Post-extinction lesions of the CA1 prevent AAB fear renewal	Ji & Maren, 2008
	Amygdala	Increased c-Fos in BLA following renewal of appetitive instrumental CR and fear CR	Hamlin, Blatchford, & McNally, 2006; Knapska & Maren, 2009
		Increased synaptic plasticity markers in LA following fear conditioning renewal	Lee et al., 2013
		Increased c-Fos in LA and CeA following renewal of appetitive instrumental CR and fear CR	Knapska & Maren, 2009
		Increased Arc in the BLA and LA following fear renewal relative to extinction retention	Orsini & Maren, 2013
		Inactivation of the BLA prevents renewal of Pavlovian alcohol seeking	Chaudhri et al., 2013
	mPFC	Increased c-Fos in the PL following renewal of fear conditioning	Knapska & Maren, 2009
		Decreased Arc in the IL following fear renewal relative to extinction retention	Orsini, Yan, & Maren, 2013
		Pre-conditioning lesions and inactivation of the PR during test blocks ABA fear renewal	Sharpe & Killcross, 2015
		IL lesion prevents renewal of appetitive conditioning	Rhodes & Killcross, 2007
		Inactivation of the PL and IL impair ABA appetitive renewal	Eddy, Todd, Bouton & Green, 2016
		PL inactivation impaired ABA renewal of operant alcohol administration	Willcocks & McNally, 2013

	NAc	Increased c-Fos in shell following renewal of appetitive instrumental CR	Hamlin, Blatchford, & McNally, 2006
Reinstatement			
	Amygdala	Increased c-Fos in medial CeA following reinstatement of fear	Hitora-Imamura et al., 2015
		β-adrenergic receptor agonist facilitates reinstatement of fear	Lin et al., 2011
		CB1R agonist or p300/CBP inhibition during retrieval of conditioned fear inhibits reinstatement	Lin, Moa & Gean, 2006; Maddox, Watts, & Schafe, 2013
		BLA inactivation prevents reinstatement of fear conditioning	Laurent & Westbrook, 2010
		Electrical stimulation following extinction mimics reinstatement of fear	Kellett & Kokkinidis, 2004
	mPFC	Decreased c-Fos in IL following reinstatement of fear	Hitora-Imamura et al., 2015
		IL lesion increased reinstatement of an appetitive task	Rhodes & Killcross, 2004
		D1R antagonist in the IL prevented reinstatement of fear	Hitora-Imamura et al., 2015
	Hippocampus	Lesion impairs reinstatement of fear	Frohardt, Guarraci, & Bouton, 2000; Wilson, Brooks, & Bouton 1995
		Increase of fMRI activity following reinstatement of contextual fear in humans	Lonsdorf, 2014
	BNST	Inactivation and lesion impairs reinstatement of fear	Goode, Kim, & Maren, 2015; Waddell, Morris, & Bouton 2006
		CRF antagonist impairs reinstatement	Waddell, Bouton, & Falls, 2008
Reacquisition			
	Amygdala	Post-conditioning amygdala lesion impairs reacquisition of	Spiegler & Mishkin, 1981; (Not always the

Amygdala cont'd	object-reward associations	case: Maren, Aharonov, & Fanselow, 1996; Kim & Davis, 1993)
	BLA inactivation impairs reacquisition of fear conditioning	Laurent & Westbrook, 2010
	BLA inactivation impairs reacquisition of heroine CPP	Rizos, Ovari, & Leri, 2005
	Inta-BLA antagonism of NMDA and actin rearrangement impairs reacquisition of fear	Laurent & Westbrook, 2009; Motanis & Maroun, 2012
Hippocampus	Post-conditioning dorsal hippocampus lesions impaired reacquisition of matching-task (no EXT)	Sinnamon, Freniere & Kootz, 1978
	Post-conditioning dorsal hippocampus lesions impaired rapid reacquisition of cross maze (no EXT)	Winocur, Moscovitch, Caruana, & Binns, 2005
	Inactivation of dorsal hippocampus, ventral dentate gyrus, and ventral CA1 impaired reacquisition in sub-threshold reconditioning	Fu et al., 2016
	Tetanic stimulation of ventral CA1 impaired reacquisition in sub-threshold reconditioning	Deschaux et al., 2011
	Intra CA1 of p38 MAPK antagonist, protein synthesis inhibitor, and Src tyrosine kinase antagonist inhibits inhibitory avoidance reacquisition	Rossato et al., 2006; Cammarota et al., 2003; Bevilaqua et al., 2005
	Intra CA1 of actin rearrangement inhibitor inhibits contextual fear behavior	Motanis & Maroun, 2012
mPFC	Tetanic stimulation of the mPFC prevents reacquisition in sub-threshold conditioning	Zheng et al., 2013; Deschaux et al., 2011

mPFC cont'd	Post-conditioning lesion of PL impairs reacquisition of eyeblink CR	Oswald, Maddox, & Powell, 2008
	Inactivation of PL impairs reacquisition in sub-threshold conditioning	Fu et al., 2016
	Inactivating PL enhanced reacquisition of operant alcohol CR	Willcocks & McNally, 2013
	Post-conditioning lesion of IL enhanced reacquisition of fear	Morgan, Schulkin, & LeDoux, 2003
	Inactivation of IL prevents reacquisition of heroine CPP	Ovari & Leri, 2008

Table 1. Table of Studies Implicating Brain Regions in Each Post-Extinction Re-emergence of CR. This table summarizes the findings of region specific manipulation on the different types of return of CR. Findings are separated by region and include a brief description of the manipulation, subregion (if relevant), conditioning paradigm. SR = spontaneous recovery; CPP = conditioned place preference; no EXT = no extinction training given before studying CR restoration.

reintroduced outside of the extinction context and CR returns. A typical example is when conditioning of a CS-US association occurs in one context (A) and extinction by CS alone presentations occurs in a separate and distinct context (B). Following extinction of CR, if the extinguished CS is presented again in the original context (A), CR will fully renew. This “ABA” renewal has been seen many times in many rodent preparations of conditioning, including fear conditioning (conditioned suppression: Bouton & Bolles, 1979a; Bouton & King, 1983; Harris, Jones, Bailey, & Westbrook, 2000; Rauhut, Thomas, & Ayres, 2001; Pavlovian fear conditioning: Corcoran & Maren, 2004; Hermann, Stark, Milad, & Merz, 2016; Ji & Maren, 2005; Wang, Jin, & Maren, 2016). ABA renewal is also reliably seen in humans (Alvarez, Johnson, & Grillon, 2007; Effting & Kindt, 2007; Milad, Orr, Pitman, & Rauch, 2005; Neumann, Lipp, & Cory, 2007). Similar types of renewal can also occur during post-extinction CS exposure in a novel context (ABC and AAB renewal: Bouton & Bolles, 1979a; Gunther, Denniston, & Miller, 1998; Harris et al., 2000).

Several interesting characteristics on renewal have been noted. Like spontaneous recovery, spacing acquisition and extinction training and inter-extinction trial time lessens contextual renewal (Huff, Hernandez, Blanding, & LaBar, 2009; Urcelay et al., 2009). However, even after extensive extinction, contextual renewal occurs (Bouton & Swartzentruber, 1989; Rauhut et al., 2001). Also, it is not just spatial locations that can cause contextual renewal, but also interoceptive factors. Both benzodiazepines (Bouton, Kenney, & Rosengard, 1990) and alcohol intoxication (Cunningham, 1979) during extinction can cause state-dependent renewal of CR when drug-free.

There are several theories behind contextual renewal. Two similar theories view context as either a gate or an occasion setter for the expression of the excitatory or inhibitory CS-US memory. In the gating theory, when the inhibitory memory is formed during extinction, the CS becomes ambiguous and the animal requires the presence of the extinction context and the CS to express the inhibitory memory – in other words, the context acts as the gate through which inhibitory memories are expressed (Bouton, 1994). In the occasion setting theory, context acts as a negative occasion setter by signaling that the US does not follow the CS, again context provides the stimuli that disambiguate the appropriate response to the CS (Bouton, 1993; Bouton & Swartzentruber, 1986; Holland, 1992).

Instead, others posit that, rather than gating or setting the occasion, the context is its own CS that is encoded into the associative memory. The context could be part of a compound stimulus or become part of the stimulus configuration during both the formation of the excitatory and inhibitory memory (Pearce & Hall, 1980; Rescorla & Wagner, 1972). Yet, as noted in ABC and AAB renewal, CR can renew in a novel context so the context does not need to be a part of the associative memory for CR to return. For more comprehensive reviews on the behavior of contextual renewal see (Bouton, 2002; 2004; Ji & Maren, 2007; Podlesnik, Kelley, Jimenez-Gomez, & Bouton, 2017; Vervliet, Baeyens, Van den Bergh, & Hermans, 2013a).

ii. Contextual Renewal Neurobiology

For neural mechanism, the dopamine receptor system shows signs of

involvement in contextual renewal. Systemic antagonism of dopamine 1 receptors (D1R) prevented context renewal of alcohol seeking in a Pavlovian discrimination training task (Sciascia, Mendoza, & Chaudhri, 2014) and contextual renewal of cocaine self-administration (Crombag, Grimm, & Shaham, 2002). These findings suggested a role of dopamine in renewal. One could attribute dopamine's involvement to the US being a rewarding substance (Di Chiara & Imperato, 1988), as D1R antagonism also blocked ABA renewal of a instrumental sucrose CR (Hamlin, Blatchford, & McNally, 2006). Yet, the involvement of dopamine in renewal could be due to its role in prediction error signaling (Schultz, 2002); perhaps, post-extinction renewal of CR to the CS enlists prediction error mechanisms, as the most recent CS association was inhibitory.

In terms of brain regions important to contextual renewal, it is unsurprising that the hippocampus, a region critical for spatial processing and memory (Moita, Rosis, Zhou, LeDoux, & Blair, 2004; Sanders, Wiltgen, & Fanselow, 2003), seems to be the major site of focus. Pre-test inactivation (Corcoran & Maren, 2001) or pre-conditioning and post-extinction lesions (Ji & Maren, 2005) of the dorsal hippocampus blocked renewal of fear conditioning CR in a novel context (ABC and AAB renewal, respectively). Yet, neither dorsal hippocampus inactivation (Corcoran & Maren, 2004) nor pre-conditioning lesions of the hippocampus (Frohardt, Guarraci, & Bouton, 2000; A. Wilson, Brooks, & Bouton, 1995) prevented renewal of CR in a context that reliably predicts the CS, like in ABA renewal. These results indicated that the dorsal hippocampus is important for contextual renewal in a novel context that cannot provide additional information of the CS contingency, showing that

contextual occasion setting may be mediated by the hippocampus. Within the dorsal hippocampus, subregions show selective involvement in renewal. Post-fear-extinction lesions of the CA1, but not CA3, impaired AAB renewal of fear, suggesting the CA1 is especially important for the contextual renewal mediated by the dorsal hippocampus (Ji & Maren, 2008). However, both CA1 and CA3 lesions impaired renewal if lesions are made prior to conditioning.

The ventral hippocampus, thought to be important for emotional processing (Fanselow & Dong, 2010), is critical for contextual renewal as well. Inactivating ventral hippocampus during AAB renewal disrupted renewal of fear conditioning (Hobin, Ji, & Maren, 2006). Additionally, infusion of kappa opioid antagonist into the ventral, but not dorsal, hippocampus 24hr prior to renewal test significantly reduced the ABA renewal of fear (Cole, Richardson, & McNally, 2013). This subregion difference shows that while the dorsal hippocampus does not mediate renewal of a context previously associated with the CS, the ventral hippocampus does. This result is likely due to the renewal of an emotional fear response.

The amygdala is also implicated in renewal. Contextual renewal in several appetitive and fear paradigms led to increased c-Fos expression in the BLA (Hamlin et al., 2006, Knapska & Maren, 2009), lateral amygdala (LA), and CeA (Knapska & Maren, 2009); increased Arc in the BLA and LA (Orsini, Yan, & Maren, 2013); and increased synaptic plasticity markers in the LA (Lee et al., 2013). Functionally, if the BLA is inactivated during a renewal test, then renewal of a Pavlovian alcohol-seeking task is impaired (Chaudhri, Woods, Sahuque, Gill, & Janak, 2013). Together, these findings show the importance of the amygdala in contextual

renewal.

In addition to the hippocampus and amygdala, the mPFC plays a part in renewal. Currently, it is theorized that the prelimbic (PL), but not the Infralimbic (IL), subregion of the mPFC is involved in renewal. Contextual renewal of fear conditioning caused increased c-Fos expression in the PL (Knapska & Maren, 2009), but decreased Arc expression in the IL (Orsini et al., 2013). Also, pre-training lesion and inactivation of PL during test sessions blocked ABA fear renewal (Sharpe & Killcross, 2015) and operant alcohol responding renewal (Willcocks & McNally, 2013), while IL lesions enhanced renewal of appetitive conditioning (Rhodes & Killcross, 2007). Conversely, one study found that PL and IL inactivation during test impaired renewal of appetitive conditioning (Eddy, Todd, Bouton, & Green, 2016). However, in this study, Eddy et al. found that PL inactivation exclusively impaired contextual renewal, while IL inactivation impaired both extinction recall in the extinction context and renewal in the conditioning context, which shows a preferential role of the PL in contextual renewal.

The neurobiological research on renewal has also shown how these regions interact to cause contextual renewal. Whole network analyses comparing brain region activity following extinction recall to contextual renewal showed a reversal from negative regional interactions during extinction recall to positive interactions during fear renewal in brain regions important for fear conditioning (IL, amygdala, and hippocampus; Bruchey, Shumake, & Gonzalez-Lima, 2007). Much of the circuit research has focused on hippocampal connectivity. Ventral hippocampus neurons that project to both the PL and BLA (Jin & Maren, 2015) and to either the PL or IL

showed increased c-Fos following fear renewal (Wang et al., 2016). These results show that the hippocampus is critical for communicating contextual information to diverse connections during contextual renewal. Functionally, impairment of the dorsal hippocampus to amygdala connection prevented contextual renewal. Inactivation of the dorsal hippocampus blocked fear renewal and disrupted CS-elicited spike firing in the LA that was specific to the context after extinction (Maren & Hobin, 2007). Further, contralateral inactivation of the BLA and dorsal hippocampus impaired renewal of cocaine self-administration (Fuchs, Eaddy, Su, & Bell, 2007); which shows that the hippocampus-amygdala connection is particularly important for renewal.

In humans, the use of functional magnetic resonance (fMRI) imaging has also shown important neural circuits for renewal. The hippocampus, amygdala, PL, and IL and their connections have all shown increased activation during contextual renewal (Åhs, Kragel, Zielinski, Brady, & LaBar, 2015, Hermann et al., 2016, Lissek, Glaubitz, Uengoer, & Tegenthoff, 2013). In one study, ABA renewal of contextual fear increased brain activity throughout the fear circuit, but ABC renewal specifically showed increased hippocampal connectivity with the amygdala and IL (Hermann et al., 2016). This increased connectivity could indicate, like the animal studies above, that the hippocampus has renewal-type selective involvement when CR renews in a novel context. Overall, the fear circuit does play an important role in how context modulates post-extinction CR. For further research on the neurobiology of contextual renewal, refer to Chen, Wang, Wang, & Li, 2017; Ji & Maren, 2007. Refer to Table 1 for a summary of the neurobiology results of contextual renewal.

c. Reinstatement

i. Reinstatement Behavior

Reinstatement occurs when exposure to the US alone causes reinstated CR to a CS presented later. Like spontaneous recovery, the phenomena was first discovered by Pavlov in 1927, but further popularized by relatively recent works (Delamater, 1997). Like recovery and renewal, reinstatement has been shown in many types of conditioning, including fear conditioning (Rescorla & Heth, 1975), and occurs in human fear conditioning as well (Hermans et al., 2005).

Similar to the phenomena described earlier, reinstatement shows context-dependency. Reinstatement of CR often occurs only if the US was reintroduced in the context in which the extinguished-CS was tested (Bouton & Bolles, 1979c; Bouton & King, 1983). Further, the strength of reinstatement correlates with the strength of contextual conditioning assumed by the context following US exposure (Bouton, 1984; Bouton & King, 1983) and, if the US-exposed test context is separately extinguished, reinstatement is prevented (Baker, Steinwald, & Bouton, 2007; Bouton & Bolles, 1979c). Additionally, extinction of the CS is necessary for reinstatement to a CS presented in the context previously paired with US, showing that extinction and reinstatement are context-dependent processes (Bouton, 1984; Bouton & King, 1986).

Additional CS-context associations may also be involved in reinstatement. Contrary to earlier studies, when the CS is extinguished in a context and the US-alone is delivered in the same extinction context, then reinstatement of CR to the CS

could occur in a distinct context that is not associated with the US (Westbrook, Iordanova, McNally, Richardson, & Harris, 2002). This finding suggests that the extinction context has formed associations with both the CS (during extinction) and US (during reinstatement) and potentially allows a chaining of these associations, so that the CS activates representations of the US and reinstatement of fear to the CS in any context. Additionally, another study found that reinstatement of CR occurred in the extinguished training context when the US was presented in a distinct context following a long, but not short, delay after extinction (McAllister & McAllister, 2006). These findings show the context-dependency of US presentation in reinstatement can generalize to the original training context with the passage of time (which also confounds with spontaneous recovery).

Several theories have been considered to explain reinstatement. One theory postulates that reinstatement occurs via strengthening of the US representation that releases inhibition (Rescorla & Heth, 1975). In extinction, the CS-US association becomes inhibited by the formation of an extinction context-no US association, but upon re-exposure to the US in the extinction context, the US representation and excitatory CS-US association is reinstated (Rescorla, 1979). However, this theory only applies when extinction, reinstatement, and test are in the same context.

The context-dependency of reinstatement to the context in which the US was presented has led to two theories. First, a summation hypothesis conceptualizes the context as an additional stimulus that forms excitatory and inhibitory associations with the US that sum with the CS-US associations to determine the behavioral response to the CS (Bouton & Bolles, 1979a; Bouton & King, 1983). However,

results of reinstatement of partially reinforced stimuli do not support the summation hypothesis (Bouton, 1984; Bouton & King, 1986).

An alternative theory is the retrieval model. Similar to the theory for contextual renewal, this model treats context as an occasion setter for whether CS will elicit expression of the excitatory or inhibitory CS-US association (Bouton, 2004; Bouton, Rosengard, Achenbach, Peck, & Brooks, 1993). This theory can predict most outcomes of post-extinction US exposure, except for the finding of reinstatement of CR to the CS in a novel context (Westbrook et al., 2000), which was explained by the context forming associations with both the CS and US as explained above.

Another hypothesis for reinstatement is the attentional-association theory (Pearce & Hall, 1980; Schmajuk, Larrauri, & LaBar, 2007). In this theory, the CS and context compete for attention when the US is presented. During acquisition an excitatory CS-US association is formed and little attention is given to the context, but during extinction an inhibitory context-US association is formed and attention to context and CS subsequently decreases. However, when the US is presented, the context is attended to and an excitatory context-US association is formed, as well as an attentional shift to the CS during the test. This theory allows reinstatement in: A) the context that US was present by decreasing contextual inhibition and increased attention to the CS for CS-US association reactivation; and B) in a context where the US was not presented by increased attention to the CS and activation of CS-context and US-context associations. In sum, regardless of theory, research shows that reinstatement is a context-dependent form of post-extinction fear restoration. For more in-depth reviews of reinstatement, refer to Bouton, 2002; Haaker et al., 2014

and Vervliet, Craske, & Hermans, 2013b.

ii. Reinstatement Neurobiology

Several studies reveal neural mechanisms and regions involved in reinstatement. Due to the attractiveness of reinstatement as a model of addiction relapse (Shaham, Shalev, Lu, de Wit, & Stewart, 2003), a majority of studies focusing on the neurobiology underlying reinstatement of CR have been in drug self-administration paradigms. Often US-induced reinstatement is not only studied (referred to as drug-primed reinstatement), but also cued- or stress-induced reinstatement, both of which do not encapsulate reinstatement as described above. Due to the complexity of reinstatement definitions and maintaining the scope of this chapter, this section will not focus on reinstatement of drug self-administration CR (for review, refer to Bossert, Marchant, Calu, & Shaham, 2013; Kalivas & McFarland, 2003).

Molecular processes involved in US-induced reinstatement include glutamatergic transmission. Systemic administration of N-methyl-D-aspartate (NMDA) receptor antagonists (but not protein synthesis inhibitors, cannabinoid receptors antagonists, or gamma-aminobutyric acid, GABA, modulators) prevented reinstatement of fear conditioning (Johnson, Baker, & Azorlosa, 2000; Shen, Igarashi, Imamura, Matsuki, & Nomura, 2013). Additionally, systemic d-cycloserine, a partial NMDA agonist, administration following extinction prevented reinstatement, but not renewal (Ledgerwood, Richardson, & Cranney, 2005; Vervliet, 2008; Woods & Bouton, 2006). These studies suggest a selective role of NMDA transmission in

reinstatement of CR.

Brain regions implicated in reinstatement include the amygdala, mPFC, hippocampus, and bed nucleus of the stria terminalis (BNST). In the amygdala, c-Fos expression was increased following reinstatement of fear (Hitora-Imamura et al., 2015) and, in humans, the amygdala was more active after reinstatement of contextual fear conditioning than before (Lonsdorf, Haaker, & Kalisch, 2014). Further, inactivating the BLA prevented reinstatement (Laurent & Westbrook, 2010), while β-adrenergic receptor agonist facilitated reinstatement of fear (Lin, Tseng, Mao, Chen, & Gean, 2011), showing that the amygdala likely plays a direct role in reinstatement.

Additionally, intra-amygdala delivery of a CB1 agonist (Lin et al., 2006) or p300/CBP histone acetyltransferase inhibitor (Maddox, Watts, & Schafe, 2013) during reactivation of acquisition impaired fear reinstatement (while also affecting the excitatory or inhibitory CS-US associations). Also, electrical stimulation of the amygdala following extinction acted to reinstate contextual fear (Kellett & Kokkinidis, 2004). These studies show that, in addition to directly affecting reinstatement, the amygdala is involved in the behavior prior and leading to reinstatement.

Within the mPFC, the IL is critical for reinstatement. The IL had decreased c-Fos expression following reinstatement of fear conditioning (Hitora-Imamura et al., 2015). Lesions of the IL increased reinstatement in an appetite conditioning task (Rhodes & Killcross, 2004), In humans, the ventral mPFC (corollary to the rat IL) was more active prior to reinstatement of contextual fear conditioning than after

reinstatement (Lonsdorf et al., 2014). These findings suggest that the IL is critical to extinction and reversals in extinction, like reinstatement, involve inhibition of the IL. However, intra-IL administration of D1R antagonist prevented reinstatement of fear as well (Hitora-Imamura et al., 2015).

The connection between the mPFC and amygdala also appears to be important to reinstatement. During extinction of fear conditioning, there were depressed evoked field potentials (EFPs) in the mPFC-BLA projection and potentiated EFPs in the reciprocal BLA-mPFC projection, but in reinstatement of fear this pattern was reversed (Vouimba & Maroun, 2011). Additionally, the intra-IL administration of a D1R antagonist that prevented reinstatement of fear also caused a corresponding decrease in medial CeA c-Fos expression (Hitora-Imamura et al., 2015), showing the importance of these connections to mediating fear behavior.

Lesions of the hippocampus (Frohardt et al., 2000) and associated structures (fornix, Wilson et al., 1995) also impaired fear reinstatement, but not renewal or recovery of fear conditioning. In humans, fMRI activity increased in the hippocampus during reinstatement of contextual fear conditioning (Lonsdorf et al., 2014) and in patients with amnesia (damaged hippocampus) showing impaired reinstatement of appetitive conditioning (LaBar & Phelps, 2005). However, the hippocampus is not always necessary for reinstatement, as lesions of hippocampus did not impair reinstatement of appetitive conditioning (Fox & Holland, 1998).

Finally, the BNST is also critical for reinstatement of fear CR. Both lesion (Waddell, Morris, & Bouton, 2006) and inactivation (Goode, Kim, & Maren, 2015) of

the anterior BNST attenuated reinstatement of cued fear conditioning. This BNST-mediated impairment seems to be reinstatement specific, as inactivation of the BNST did not impair renewal of cued fear conditioning (Goode et al., 2015). Additionally, corticotropin-releasing factor (CRF) antagonism in the BNST prevented reinstatement (Waddell, Bouton, & Falls, 2008), suggesting that the BNST's role in reinstatement of fear may be a stress response, as the BNST is critical for sustained fear responses (Davis, Walker, Miles, & Grillon, 2010).

d. Reconditioning and Rapid Reacquisition

i. Rapid Reacquisition Behavior, Alternatives, and Causes

Reconditioning is the post-extinction re-pairing of CS and US. Like many of the phenomena above, reconditioning was first described by Pavlov (1927). He found that a single trial of CS-US pairing after extinction could cause a return of CR. Early on it was found that reconditioning the CS-US association following extinction could lead to a more rapid reacquisition of CR relative to initial acquisition (Konorski & Szwejkowska, 1953; Lauer & Estes, 1955; Smith & Gormezano, 1965). This outcome of reconditioning is called rapid reacquisition and has been shown reliably in many Pavlovian and operant conditioning paradigms (Bouton, Woods, & Pineño, 2004a; McAllister & McAllister, 1994; Napier, Macrae, & Kehoe, 1992; Rescorla, 2001; Todd, Winterbauer, & Bouton, 2012), including fear conditioning in rodents (Leung, Bailey, Laurent, & Westbrook, 2007) and humans (Kindt & Soeter, 2013).

A methodological consideration to note here is that rate of reacquisition can refer to both the within-subjects comparison of initial acquisition to reacquisition

(comparing behavior at two different time points) and the between-subjects comparison of two groups acquiring or reacquiring (comparison of behavior at the same time point). Due to a lack of standardization in the study of reacquisition, both are used interchangeably in many of the papers cited here, but for the discussion of my dissertation studies rate of reacquisition will refer to the latter. Also, for the duration of this dissertation, reconditioning will refer to the procedure that re-pairs CS and US after extinction and reacquisition will refer to the behavioral consequence of the CS-US re-pairing.

Less theory has been applied to reacquisition, but a few models have attempted to explain rapid reacquisition following reconditioning. Many have theorized that rapid reacquisition shows that the original CS-US memory has "savings" that survived extinction and reconditioning leads to a reactivation of the CS-US association (Rescorla, 2002). This theory is supported by evidence that transfer of an instrumental response associated to a reconditioned CS with the same outcome was stronger than a response transferred to a CS that was only conditioned (Rescorla, 2001). Further, just as extinction does not remove the initial excitatory CS-US association, reconditioning does not remove the inhibitory learning from extinction. Following reconditioning, spontaneous recovery was seen in addition to rapid reacquisition (Rescorla, 2001). This result shows further proof that the excitatory and inhibitory CS-US associations, once formed, are not removed, but instead compete for expression.

Also, like the other post-extinction outcomes above, an attentional model, like that described by Pearce & Hall (1980) has been proposed. Specifically, this theory

posits that attention to the CS during extinction is biphasic; initially, the CS is well attended because the predictive value of the CS is high, but after enough CS-alone presentations, the CS's predictive value decreases and so does attention to the CS. In reconditioning, the attention to the CS increases again and, if some of the associative value of the CS survived extinction, then attention and CR would rapidly return. However, this theory assumes that the original CS could lose associative value in extinction, which is unlikely to be true when considering evidence of the previous sections.

Yet the attentional theory of Schmajuk et al. (2007), which includes attention to the context as well as CS and was earlier applied to reinstatement, may apply to reconditioning as well. As before, in extinction, an inhibitory context-US association is formed and over time attention to context and CS declines. But, when the CS and US are re-paired, the CS-US association is reactivated, an excitatory context-US association is formed, and the CS is attended to once again during future tests of the CS. Similarly, US-processing has been implicated as having a strong role in the CS-US association (Rescorla & Heth, 1975) and thus could be important to reconditioning (Weidemann & Kehoe, 2003). In reconditioning, re-exposure to the US would reestablish the US representation and then quickly expose the excitatory CS-US association, especially as it is again directly associated with the CS. Conversely, attention to the context or US alone in these theories of reconditioning seems unnecessary, as the re-pairing of CS and US would make the CS a much more predictive stimuli for attention.

However, there is an alternative outcome of reconditioning which conflicts

with these theories. In some preparations, post-extinction reconditioning leads to an impaired reacquisition that is slower relative to initial conditioning, called slow reacquisition. Slow reacquisition has been shown in conditioned suppression (Bouton, 1986) and taste aversion conditioning in rats (Hart, Bourne, & Schachtman, 1995) and even in fear conditioning in humans (Morís, Barberia, Vadillo, Andrades, & López, 2017). It is interesting that seemingly opposite outcomes can occur following reconditioning, but there are several theories to explain this contradiction.

Bouton and associates have created a body of work describing the conditions of slow versus rapid reacquisition. First, the amount of extinction training can critically influence the outcome of reconditioning. While moderate extinction of CR led to rapid reacquisition, extensive extinction training prior to reconditioning favored slow reacquisition (Bouton, 1986; Leung et al., 2007). This outcome is believed to occur due to the formation of a strong inhibitory CS-US association with over training, as well as establishing the context as a strong negative occasion setter. However, Leung et al., 2007 also showed that this impairment was transient and the rapid reacquisition effect could be seen after a delay in a long-term retention test. This result shows that the original CS-US was not lost or damaged in extinction and with the passage of time the temporal context may favor rapid reacqusition. Yet, some preparations, like the nictitating membrane response, showed rapid reacquisition regardless of extensive extinction (Napier et al., 1992). Additionally, even if the extinction is extremely massive relative to acquisition training (27:1 trial ratio) and rapid reacquisition is prevented, slow reacquisition does not occur (Weidemann & Kehoe, 2003).

Second, slow reacquisition appears to be more likely in Pavlovian paradigms that require few acquisition trials to acquire (i.e., the small amount of conditioning trials in Pavlovian fear conditioning versus the large amount of conditioning trials in eye-blink conditioning/nictitating membrane response). Bouton theorizes massive acquisition of conditioned responding causes not only the formation of excitatory CS-US associations, but also an association of CS-US pairings to further CS-US pairing using sequential learning (Capaldi, 1994). Thus, following extinction, when the CS-US association is re-paired with even a mild reconditioning, a single CS-US may predict further CS-US associations and cause a rapid reacquisition of fear. Accordingly, when the CS-US association was partially reinforced in acquisition, there was higher CR rate in reacquisition following reinforced rather than nonreinforced trials (Bouton, Woods, & Pineño, 2004b; Ricker & Bouton, 1996).

A similar theory also suggests a learning-to-learn effect, such that animals who have already learned a CS-US association may be quicker to reacquire another CS-US association that is not specific to the originally conditioned CS. Ricker & Bouton (1996) showed evidence of learning-to-learn by including learning and learning-naïve controls and showed that learning controls who had previously acquired a CS-US association also acquired a new CS-US association more rapidly. Further, reacquisition of the original CS-US association was slow relative to learning-experienced controls who learned a second CS-US association (Hart et al., 1995). However, the learning-to-learn control confounds with reinstatement, as US alone exposure could be increasing CR to a novel CS that is similar to the originally conditioned CS.

Third, like the three other post-extinction outcomes, context can play a critical role as well. Bouton & Swartzentruber (1989) found that reconditioning in the conditioning context led to rapid reacquisition, but reconditioning in the extinction context led to slow reacquisition. This outcome resembled an ABA renewal and Bouton argued that rapid reacquisition was a form of renewal. As demonstrated in the renewal section above, renewal does not just occur to contexts. The rapid return of CR following reconditioning could be renewal to a state, such as a state of aversive or appetitive behavior or ABA renewal to massive CS-US associations during acquisition. However, there was evidence that rapid reacquisition occurred regardless of context and was only slightly diminished by the extinction context (Willcocks & McNally, 2011). Also, the presence of an extinction-associated cue did not prevent rapid reacquisition (Willcocks & McNally, 2014).

Overall, the slow reacquisition effect is difficult to reconcile with the theory that extinction does not erase the initial excitatory CS-US association. When slow reacquisition occurs, it appears that extinction prevents the ability of the CS-US re-pairing to allow comparable rates of reacquisition with initial acquisition. Yet if slow reacquisition is conceptualized as a form of inhibition, like latent inhibition, that *temporarily* impairs expression of CR (Leung et al, 2007), then slow reacquisition is a less aberrant response in the whole canon.

ii. Neurobiology of Reconditioning and Rapid Reacquisition

Similar to the research on the behavior and theory of reacquisition, the understanding of the neural mechanisms behind reacquisition is limited and mixed. It

is also important to note that the most of studies included here often do not study the neurobiology of *rapid* reacquisition specifically, but instead focus on a more general reacquisition regardless of rate or not following extinction (unless otherwise stated). Despite these inconsistencies, there have been various studies on reacquisition and related findings that implicate certain mechanisms and brain regions. For neural mechanisms, there have been varied results on the role of NMDA neurotransmission in reacquisition. Several studies found that acquisition following some pre-training (similar to reacquisition, except without extinction training) of various paradigms was NMDA-independent, as it was unaffected by systemic NMDA antagonists (inhibitory avoidance: Roesler et al., 1998; water maze: Saucier & Cain, 1995; Bannerman et al., 1995).

The above studies look at "reacquisition" that does not follow extinction and could rely on different mechanisms from the behavior of post-extinction reconditioning. However, in studies of post-extinction reacquisition, d-cycloserine (a partial NMDA agonist) has mixed effects on reacquisition. In some instances, systemic d-cycloserine during extinction training does not impair rapid reacquisition of fear conditioning (similar to renewal; Ledgerwood et al., 2005; Vervliet, 2008), but in others it does impair the rate of reacquisition in ethanol conditioned place preference (Groblewski, Lattal, & Cunningham, 2009). Overall, the role of NMDA in reacquisition is unclear.

In terms of brain regions, the amygdala has received attention as a brain region that may not be necessary for reacquisition of associative memory. When the BLA is lesioned before or after acquisition (Maren, Aharonov, & Fanselow, 1996) or

CeA is lesioned after acquisition (Kim & Davis, 1993), reacquisition (that did not follow extinction) of fear conditioning and fear potentiated startle still occured. However, BLA lesions caused a lasting impairment in expression of conditioning freezing that was still apparent during reacquisition (Maren, Aharonov, & Fanselow, 1996). Also, protein synthesis (a molecular process involved in long-term memory formation; Davis & Squire, 1984) within the BLA is not necessary after post-extinction reconditioning to allow reacquisition of conditioned contextual fear (Motanis & Maroun, 2012).

Inconsistently, post-conditioning lesions of the amygdala also impaired reacquisition of an object-reward association task in monkeys (Spiegler & Mishkin, 1981). This finding was observed in reacquisition following a loss of CR due to lesion, not extinction, however. In post-extinction reconditioning, BLA inactivation led to impaired reacquisition of conditioned fear (Laurent & Westbrook, 2010) and heroin conditioned place preference (Rizos, Ovari, & Leri, 2005). Additionally, NMDA activity (Laurent & Westbrook, 2009b) and actin rearrangement (Motanis & Maroun, 2012) in the BLA were involved in post-extinction reacquisition of contextual fear. Contrasting with the earlier studies, the latter studies suggest that manipulating the BLA can impair reacquisition if the manipulation is more temporally or mechanistically specific. Like NMDA receptor activity, the role of the amygdala in reacquisition is ambiguous.

The hippocampus has a more defined role in reacquisition; in general, lesion or inactivation of the hippocampus impairs reacquisition. In a study that retrained un-extinguished animals following hippocampus lesions, there was an impairment of

reacquisition in a four-choice delayed match-from-sample task by dorsal hippocampus, but not ventral hippocampus lesions (Sinnamon, Freniere, & Kootz, 1978). Similarly, hippocampal lesions showed an impaired rapid reacquisition of memory for spatial location of a food reward in a cross maze (Winocur, Moscovitch, Caruana, & Binns, 2005). Additionally, in studies that closely model reconditioning (sub-threshold conditioning: post-extinction re-pairing CS with US at a weaker intensity than what was used in conditioning), inactivation of the dorsal hippocampus, ventral dentate gyrus, and ventral CA1 area (Fu et al., 2016) and tetanic stimulations of the ventral CA1 (Deschaux, Motanis, Spennato, Moreau, & Garcia, 2011) impaired reacquisition.

The intracellular mechanisms of the CA1 of the hippocampus seem particularly important for reacquisition. Intra-dorsal CA1 administration of p38 mitogen-activated protein kinase antagonist prevented post-extinction reacquisition of inhibitory avoidance CR (Rossato et al., 2006). Similarly, intra-dorsal CA1 infusions of a protein synthesis inhibitor or an mRNA synthesis blocker prior to post-extinction reconditioning impaired reacquisition of inhibitory avoidance (Cammarota, Bevilaqua, Kerr, Medina, & Izquierdo, 2003). However, intra-CA1 protein synthesis inhibitor administration also showed no effect on post-extinction contextual fear reacquisition (Motanis & Maroun, 2012). Finally, the dorsal CA1 required uninhibited Src family tyrosine kinases (Bevilaqua, da Silva, Medina, Izquierdo, & Cammarota, 2005) and actin rearrangement (Motanis & Maroun, 2012) for post-extinction reacquisition of inhibitory avoidance and contextual fear conditioning, respectively. Overall, the hippocampus, particularly the CA1, is critical for reacquisition.

The mPFC is also engaged in reacquisition. Tetanic stimulation of the mPFC prevented reacquisition in the sub-threshold conditioning procedure (Zheng et al., 2013; Deschaux et al., 2011). Interestingly, inactivation of the mPFC also prevented tetanic stimulation of the ventral CA1 from impairing reacquisition (Deschaux et al., 2011), which suggests that the mPFC may be upstream of the hippocampus during the processing reacquisition.

Subregions of the mPFC have been examined as well. The PL region of the mPFC is highly associated with initial acquisition of CR, so it has received the most attention with regards to reacquisition. Although no extinction training was received in this study, post-conditioning lesions of the PL caused reacquisition deficits in eye-blink conditioning (Oswald, Maddox, & Powell, 2008). Similarly, in the sub-threshold conditioning preparation, inactivation of the PL blocked reacquisition expression in a retention test (Fu et al., 2016). It is important to note that these two studies found that PL manipulations did not influence fear expression in general, as CR was able to return with enough retraining (Fu et al., 2016; Oswald et al., 2008). These results show that the PL is important for retrieval of the CS-US association during reacquisition. Yet, others showed that inactivation of the PL enhanced, rather than impaired, reacquisition of operant alcohol conditioning (Willcocks & McNally, 2013).

Unlike the PL, the IL subregion demonstrates an ambiguous role in reacquisition. Inactivation of the IL blocked post-extinction reacquisition of heroin conditioned place preference (Ovari & Leri, 2008), but did not affect post-extinction operant alcohol conditioning reacquisition (Willcocks & McNally, 2013) and even enhanced reacquisition fear conditioning (post-conditioning lesion; Morgan,

Schulkin, & LeDoux, 2003). Although mixed, these data suggest that the mPFC could play a functional part in reacquisition of CR.

III. Similarities and Differences of the Four Post-Extinction Re-emergences of CR

By examining the four forms of post-extinction CR return above there are some critical conclusions that can be drawn. There are many similarities between each, so much so that the names of these outcomes are often used interchangeably for one another in the literature. First and foremost, these phenomena all show that following extinction, the excitatory CS-US association is not removed and shows some savings despite low CR. Re-pairing of CS and US (reconditioning), presenting the US (reinstatement), switching context (contextual renewal), and even the passing of time (spontaneous renewal) can cause the return of CR to the CS after extinction. However, slow reacquisition is somewhat contradictory with the concept of original excitatory CS-US savings.

Two theories also seem to universally explain post-extinction re-emergence of CR. First, in all phenomena, attention to the CS is critical in determining if CR will return. In spontaneous recovery, it is assumed that with the passage of time, attention to the CS that was lost in extinction fluctuates and can recover CR to the CS (Estes, 1955; Robbins, 1990). Similarly, CS presentation in another context, US-alone presentation, and renewed CS-US presentations, can all cause reactivation of the CS-US association and increased attending to the CS to allow CR (Mackintosh, 1975; Pearce & Hall, 1980; Schmajuk et al., 2007).

Second, the context-dependence of inhibitory CS-US associations formed during extinction impacts each phenomenon. In all, testing of an extinguished CS in a context other than the extinction context can cause or influence the return of CR (Bouton, 1993; 1994; 2002; 2004). For spontaneous recovery, it is theorized that the passage of time creates a change in the temporal context. For renewal, exposure to the CS outside of the extinction context renews the CR. For reinstatement, CR is often reinstated only in the context in which the US was presented (by increasing the ambiguity of the extinction context or creating an excitatory context-US association in a novel context). For reacquisition, the rate of reacquisition can be influenced by the context in which reconditioning occurs (rapid if in the conditioning context and slow if in the extinction context).

In concordance with the importance of context to each outcome, all seem to rely on activity in the hippocampus for a return of CR. Despite some contradictory studies, degrading or inactivating the hippocampus prevented spontaneous recovery (Debiec et al., 2002), contextual renewal (Cole et al., 2013; Corcoran & Maren, 2001; 2004; Hamlin et al., 2006; Ji & Maren, 2005; 2008), reinstatement (Frohardt et al., 2000; Lonsdorf et al., 2014; A. Wilson et al., 1995), and reacquisition (Deschaux et al., 2011; Fu et al., 2016; Sinnamon et al., 1978; Winocur et al., 2005).

Additionally, most of these outcomes are also influenced by extinction training intensity. Both spontaneous recovery (Rescorla, 2004b) and reacquisition (Leung et al., 2007) were impaired by extensive extinction training and, if the context in which US was re-exposed was also extinguished, then reinstatement was impaired (Baker et al., 2007; Bouton & Bolles, 1979b). However, extinction intensity did not preclude

contextual renewal (Bouton & Swartzentruber, 1989; Rauhut et al., 2001), showing an important difference between these phenomena in the impact of extinction.

Like the impact of extinction intensity, there are some important differences that these post-extinction CR outcomes display. First, the completeness of CR return can differ. In spontaneous recovery, the recovery of fear was often not complete and quickly diminished without reinforcement (Rescorla, 2004b). Yet, post-extinction renewal of CR to the CS in the acquisition context was comparable to that seen in animals who have not been extinguished (Bouton & Bolles, 1979a) and reacquisition could be even more rapid than initial acquisition (Leung et al., 2007; Weidemann & Kehoe, 2003), suggesting that both show a complete return of the CS-US association.

Additionally, separate extinction “reminder” cues (cues paired with inhibitory learning) differentially affect each post-extinction re-emergence of CR. Spontaneous recovery was blocked by the addition of an extinction-paired CS (Brooks & Bouton, 1993), but the addition of a similar CS had no effect on rapid reacquisition of CR (Willcocks & McNally, 2014).

Similarly, the ambiguity in CS meaning imbued by extinction training is differentially important in each form of return. The increase in ambiguity with the passage of time allows the CS to elicit CR in spontaneous recovery. However, ambiguity impairs rapid reacquisition and does not affect reinstatement. If the CS was partially reinforced during extinction, then rapid reacquisition was reduced (Bouton, Woods, & Pineño, 2004a); whereas partial reinforcement did not affect

reinstatement (Bouton & King, 1986). Overall, the effect of ambiguity on CS representation can differ by CR return type.

Brain regions also show discrepancies in their involvement in each CR return. While the mPFC does seem to be involved in some capacity for all, there are some important subregion differences. For renewal and reacquisition, a consistent result was that injury or inactivation of the PL blocked the return of CR (Eddy et al., 2016; Fu et al., 2016; Oswald et al., 2008; Sharpe & Killcross, 2015; Willcocks & McNally, 2013); but in spontaneous recovery and reinstatement, impairing the IL led to increased CR (Peters et al., 2008; Quirk et al., 2000; Rhodes & Killcross, 2004). This outcome is consistent with evidence showing the PL is critical for the excitatory CS-US association, while the IL is important for the inhibitory CS-US association (Peters et al., 2009; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006).

Importantly, these outcomes of experiments probing the mPFC subregion also speak to the potential mechanism of renewal and reacquisition relative to recovery and reinstatement. Because inhibiting the PL would presumably impact the representation of the excitatory CS-US association, perhaps renewal and reacquisition involve the reactivation of the excitatory association. Conversely, inhibiting the IL would impair the inhibitory CS-US association and, in turn, enhance CR during spontaneous recovery and reinstatement. This neurobiological difference could imply that spontaneous recovery and reinstatement rely more on a failure to retrieve the inhibitory CS-US association than retrieval of the excitatory association. Much more research is necessary to delineate the mechanisms behind each post-extinction form of CR renewal but the idea outlined above provides an interesting

theory.

Likewise, the amygdala appears to be involved in many post-extinction re-emergences of CR, but there is mounting evidence that it is less involved in reacquisition. Damage of the amygdala or inhibition of protein synthesis within the amygdala did not prevent the reacquisition of both fear-potentiated startle and fear conditioning (reacquisition following lesion: Maren et al., 1996; Kim & Davis, 1993; post-extinction reacquisition: Motanis & Maroun, 2012). There are several studies that suggest otherwise, but the above studies show that the amygdala is not always necessary for reacquisition. The findings of a conflicting role of the amygdala in reacquisition are markedly different from those of renewal, reinstatement, and spontaneous recovery, suggesting a unique pattern of neural activity in rapid reacquisition of CR. Additionally, inhibition of the extended amygdala structure, the BNST, impaired reinstatement, but not renewal (Goode et al., 2015), showing another example of amygdala-related structures affecting some, but not all, types of CR return.

IV. Reacquisition versus Acquisition of CR

While the primary focus of this chapter is to cover the different ways CR can re-emerge after extinction, this chapter also places emphasis on reacquisition. Thus, it is important to compare reacquisition to initial acquisition of associative memory. Acquisition and reacquisition are inherently related because both involve the pairing of a CS and US and result in a CR. Yet, there are important differences. First the outcome of reconditioning is not the same as conditioning. As demonstrated above, reconditioning generally causes a more rapid rate of CR in reacquisition than what is

seen in acquisition (Napier et al., 1992). Rapid reacquisition suggests that reconditioning is reactivating and increasing the strength of the excitatory CS-US association relative to conditioning, which has been demonstrated (Rescorla, 2001). Conversely, reconditioning can also lead to a slower reacquisition than initial acquisition (Bouton, 1986), which could provide evidence that extinction does actually diminish some of the original CS-US association, contrary to many other pieces of evidence. Second, while acquisition is context-independent (the CS causes CR in any context), reacquisition outcomes have shown mixed signs of context-dependency. Reconditioning in the conditioning context led to rapid reacquisition, while reconditioning in the extinction context led to slow reacquisition (Bouton & Swartzentruber, 1989). Regardless of the outcome, reacquisition is behaviorally distinct from initial acquisition.

There are also a few neurobiological differences between acquisition and reacquisition. First, reacquisition may not require the same intracellular mechanisms that are critical for initial learning. Glutamatergic transmission through the NMDA receptors is critical for initial acquisition (Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991). However, as described above, NMDA activity can be less critical for reacquisition (Roesler et al., 1998; Saucier & Cain, 1995). Additionally, protein synthesis, which is necessary for initial learning (Davis & Squire, 1984), was not required for reacquisition of CR (Motanis & Maroun, 2012). These works show that reacquisition may become less reliant on mechanisms generally considered to be molecular correlates of learning or may use a distinct set of mechanisms to alter long-term CR.

The last study also reveals that the amygdala can be less critical to reacquisition, as the protein synthesis inhibitor was infused into the BLA. This study and several others described above indicate that the amygdala is less important for reacquisition. Not only does this outcome contrast with other post-extinction forms of CR re-emergence, it also conflicts with the large role of the amygdala in initial acquisition and extinction (Goosens & Maren, 2001; Kim & Davis, 1993; Lee, Choi, Brown, & Kim, 2001; Phillips & LeDoux, 1992; Walker & Davis, 1997). Overall, these differences display important distinctions of reacquisition and provide an important insight into how memory is modulated over time.

V. The Case for Studying Rapid Reacquisition

This chapter demonstrates that reacquisition, in particular rapid reacquisition, deserves further study. First, the differences found when reacquisition is compared to initial acquisition, suggest that the current understanding of the behavior and neurobiology of associative memory is limited. The post-extinction reconditioning of CS-US is more relevant to human associative memories, as strong associative memories and corresponding inhibitory memories are not likely learned in a single, isolated moment. Instead, these memories are more likely reactivated, enhanced, and diminished many times over by multiple exposures to the CS, US, or CS-US pairing throughout one's lifetime. Hence, the rapid reacquisition effect and other forms of memory updating need to be regularly studied along side initial acquisition and extinction studies so the field can better understand how associative memories evolve in humans.

Second, reacquisition is understudied and the interpretations made here

could be largely biased by the few studies that do exist. In particular, the interpretation that reacquisition can be amygdala-independent is potentially misguided, as some reports show a role for the amygdala in reacquisition. Additionally, other regions like the BNST have not received any exploration at all. Thus, our current understanding of the causes and neurobiology of reacquisition is lacking.

Third, the rapid reacquisition of reconditioning is relevant to disorders that include aberrant associative learning components, like addiction and post-traumatic stress disorder (PTSD). The rapid reacquisition of fear is especially relevant to PTSD. PTSD often involves a traumatic experience that results in a persistent memory that is difficult to suppress, resulting in a chronic state of stress and anxiety (DSM-V). One treatment is exposure therapy, in which patients re-experience cues associated with trauma to reduce or extinguish anxiety reactions evoked by those cues. Yet, even after successful treatment, the anxiety reactions evoked by those cues will re-emerge with the passage of time or when mild stressors are encountered (Paunovic & Öst, 2001; Rothbaum & Davis, 2003).

In fear conditioning, although a fear response can be extinguished, fear can be strongly re-established following a very mild conditioning episode, such as in rapid reacquisition. The rapid and massive conditioned response to mild reconditioning relative to conditioning is similar to the hypervigilance and exaggerated response to stressors that subjects with PTSD display (Pitman, 1989; Steinert, Hofmann, Leichsenring, & Kruse, 2015). Additionally, those exposed to trauma are more likely to be exposed to additional trauma and develop PTSD

throughout their lifetimes (Breslau, Chilcoat, Kessler, & Davis, 1999; Kolassa et al., 2010). PTSD that results from multiple trauma exposures is even delineated from PTSD symptomology, as complex PTSD (Herman, 1992). Given that mild stressors cause relapse in PTSD, understanding the neurobiology of how mild reconditioning after extinction re-establishes a robust fear response in rodents is highly significant for translating basic research to the clinical domain and informing future treatment options.

VI. Dissertation Studies

In order to provide a deeper understanding of reacquisition, the following dissertation work will focus on the behavior of, impact of alcohol on, and neurobiology of rapid reacquisition of contextual fear conditioning. My overall hypothesis was that reacquisition would show behavioral and neurobiological differences when compared with initial acquisition and other forms of post-extinction behavior return. The neurobiology of reacquisition was addressed directly with intracranial cannulation (Chapter 4) and indirectly following the administration and withdrawal of first alcohol administration (Chapter 3).

The first set of studies aimed to directly compare the behavior of acquisition and reacquisition and differentiate rapid reacquisition from other post-extinction re-emergences of CR (Chapter 2). In Chapter 2, I hypothesized that the speed of reacquisition relative to initial acquisition would depend on the amount of intervening extinction and that rapid reacquisition would be specific to the CS learned during initial acquisition. First, I compared reacquisition that followed mild reconditioning to initial acquisition that used identical parameters for initial conditioning (mild

conditioning). These studies also compared the impact of moderate versus massive amounts of extinction on the outcome of mild reconditioning, as the extinction amount could alter the rate of reacquisition. This investigation was carried out in two rodent species (mice and rats) to enhance the translatability of the finding. Then, I explored the context-specificity of rapid reacquisition of contextual fear following mild reconditioning to differentiate the phenomenon from reinstatement. I also explored the role of spontaneous recovery and expression of fear behavior during extinction in determining the outcome of reconditioning.

Chapter 3 explored the interaction of ethanol exposure and withdrawal on reacquisition memory, as alcohol modulates acquisition memory. I hypothesized that initial ethanol administration and acute ethanol withdrawal (AEW) would have memory-phase (acquisition versus. reacquisition) or memory-type (excitatory versus inhibitory memory) specific effects due to differences in the neurobiological actions of ethanol administration versus withdrawal. Specifically, I studied the impact of initial acute ethanol administration and AEW on rapid reacquisition of contextual fear relative to initial acquisition. Additionally, I attempted to replicate the previously demonstrated impairment of initial acquisition by AEW and explored the potential influence of AEW during extinction, which has not been studied before.

Chapter 4 investigated the neurobiology of rapid reacquisition and how it differed from initial acquisition. I hypothesized that rapid reacquisition would rely on different or less brain regions of the fear circuit relative to acquisition. First, I examined the expression of well-documented markers of activity in the fear circuit following conditioning and reconditioning. Next, I temporarily inactivated the

amygdala and the BNST with a pharmacological agent immediately prior to mild conditioning and reconditioning to explore the necessity of activity in these regions during acquisition, reacquisition, and the retention of each. Additionally, I attempted to replicate findings of the BNST's importance to the strong acquisition of contextual fear by inactivating the BNST prior to a strong conditioning procedure.

Finally, Chapter 5 summarized the results of each study. Additionally, the chapter related the findings of each experiment to one another and to the broader literature on reacquisition and post-extinction re-emergence of fear that was reviewed above. This chapter also discussed the implications, caveats, and potential future directions of the findings.

Chapter 2: The Behavioral Characterization of Rapid Reacquisition of Contextual Fear Conditioning in C57BL/6J Mice and Long Evans Rats

Parts of the Chapter are adapted from a submitted paper: AR Williams & KM Lattal (2018), "Rapid reacquisition of contextual fear following extinction in mice: Effects of amount of extinction, acute ethanol withdrawal, and ethanol intoxication" for possible publication in Psychopharmacology.

Abstract

Post-extinction restoration of conditioned fear can occur due to a variety of factors, including the re-pairing of CS and US in reconditioning. Reconditioning often results in rapid reacquisition of the conditioned responding, revealing the saving of initial CS-US association that survived extinction. Yet, others find that reconditioning can lead to slow reacquisition when parameters favor expression of the inhibitory memory. This work aimed to further explore the outcome of mild reconditioning of contextual fear in the males of two rodent species, Long Evans rats and C57BL/6J mice. In mice, I found that the rate of reacquisition depends on the amount of extinction training before reconditioning; moderate extinction to behavioral baseline allowed rapid reacquisition of contextual fear, while massive extinction prevented it. Conversely, in rats, I found rapid reacquisition regardless of the magnitude of inhibitory context training. Additionally, rapid reacquisition of contextual fear in rats was not a general enhancement in freezing or a form of reinstatement, nor was it impacted by the rate of extinction of conditioned responding. In all studies, slow reacquisition was not found. Overall, rapid reacquisition of contextual fear usually occurs after mild reconditioning and provides an important paradigm to explore relapse into fear behavior.

Introduction

The study of fear conditioning and extinction has been critical for understanding basic memory processes. For example, research shows that extinction temporarily eliminates conditioned behavior by the formation of an inhibitory memory (Bouton, 2002; 2004; Mowrer, Rescorla, & Klein, 2000; Pavlov, 1927), but not the deletion of the original acquisition memory due to findings of spontaneous recovery, contextual renewal, and reinstatement (reviewed in Chapter 1). A fourth unmasking procedure that has received less attention is reconditioning, which is the post-extinction re-pairing of CS and US (Pavlov, 1927). Although reconditioning is often used as a tool to assess the strength of extinction learning (Bolkan & Lattal, 2014; Hart, Holmes, Harris, & Westbrook, 2014), mechanisms of reconditioning and how it differs from initial conditioning have not been fully characterized in the fear conditioning literature.

There are two contradictory findings following reconditioning after extinction: rapid and slow reacquisition (summarized in Chapter 1). Some argue that rapid reconditioning in fear conditioning or conditioned suppression paradigms are due to other confounding factors (Bouton, 1986; Bouton & Swartzentruber, 1989). Yet, Leung, Bailey, Laurent, and Westbrook (2007) found evidence of both rapid and slow reacquisition of contextual fear conditioning in rats that was dependent upon the amount of intervening extinction training given. They found that reconditioning contextual fear that had been moderately extinguished caused an increase in freezing, but reconditioning fear that was massively extinguished caused a slowed reacquisition relative to a group that received only initial conditioning. Furthermore,

the authors showed that rapid reacquisition by the moderately extinguished group was not due to other processes described by Bouton (1989), such as reinstatement or renewal of fear.

Like extinction, reconditioning does not eliminate the memories that form during the treatment prior; in other words, extinction does not erase initial conditioning and reconditioning does not erase extinction learning (Rescorla, 2001). This result suggests that after the formation of the original excitatory and inhibitory memories, further conditioning and extinction do not remove those memories, but rather modulate and bias the expression of these opposing memories. Further, many associative memories are not formed or modulated in a single cycle of acquisition and extinction, but undergo repeated updating via reconditioning and re-extinguishing. Thus, characterizing post-extinction learning is critical to understanding memory within rodents and humans.

The purpose of the experiments in Chapter 2 was to characterize reconditioning following extinction in two rodent species commonly used in fear conditioning studies (C57BL/6J mice and Long Evans rats). The use of two rodent species also explored the universality and strain-specific effects of reconditioning on rodent behavior. Experiments 1A and 1B characterized reconditioning following a moderate or massive amount of extinction between the initial conditioning and the subsequent reconditioning session in C57BL/6J mice. Similarly, Experiments 2A and 2B investigated the impact of extinction on the rapid reacquisition effect with Long Evans rats. Experiment 2A also explored the possibility that rapid reacquisition of conditioned freezing behavior resulted from a generalized enhancement in freezing

from repeated shock that was not specific to the conditioned context. Finally Experiment 2C investigated the importance of the gradual decay of within-extinction-session conditioned fear to extinction strength and rapid reacquisition. I found that rapid reacquisition of contextual fear 1) occurred persistently in multiple rodent species, 2) had rodent-strain-specific dependency on extinction, and 3) was not a result of nonspecific enhancement in freezing to repeated shock. Thus, the rapid reacquisition provides a unique behavioral tool to study post-extinction enhancements in behavior and the neural mechanisms that allow for updating memory.

Experiment 1A: Rapid Reacquisition of Contextual Fear Following Reconditioning in C57BL/6J Mice (Moderate Extinction).

The purpose of Experiments 1a and 1b was to characterize reconditioning following a moderate or massive amount of extinction between the initial conditioning and the subsequent reconditioning session in mice. The experimental design of both experiments employed a common weak contextual fear conditioning procedure after one of three experimental treatments to contrast initial conditioning and reconditioning simultaneously.

There is evidence for both rapid (Leung et al., 2007; Napier, Macrae, & Kehoe, 1992) and slow (Bouton, 1986; Bouton et al., 2004; Leung et al., 2007) reacquisition following extinction that can depend on the amount of extinction received prior to reconditioning. Much of this work has occurred in rats, which generally show more rapid extinction to a lower asymptote of CR compared to mice,

so it is important to characterize these behavioral effects in mice (Lattal & Maughan, 2012; Tipps, Raybuck, Buck, & Lattal, 2014a).

Methods

Animals and Housing

Twenty-four C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and were housed 4 mice to an individually-ventilated cage. The mice were kept on a 12h light-dark schedule (light started at 0600 and dark started at 1800).

All animals were allowed to acclimate to the vivarium at Oregon Health & Science University (OHSU) for a full 7 days before any handling or behavioral procedures. The vivarium maintained constant (22°C ± 1°C) and humidity (70%) in all animal rooms. All experimental procedures were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985). Food and water were available ad libitum and all behavioral experiments occurred from 0900 to 1500.

Behavior

Apparatus. Fear conditioning occurred in Coulbourn Instruments mouse-conditioning chambers (H10-11M-TC; Allentown, PA) in sound- and light-attenuating chambers with a fan producing 70 dB of background noise. Each chamber was equipped with a circular Plexiglas arena (21.5 cm in diameter and 23 cm in height) placed on a grid floor of stainless steel rods (3.2 mm in diameter, spaced 6.4 mm apart). The grid floor was set to deliver a .35 mA scrambled shock via a 110/120

VAC 50-60 Hz computer-controlled shock generator (Coulbourn H13-15) and an infrared activity monitor (Coulbourn H24-61) fixed to the top of each chamber recorded freezing in Graphic State 3.01 software. The apparatus also contained a house light that was lit as soon as the session commenced and terminated as soon as the session ended. A plastic tray was placed beneath the grid floors to capture any fecal boli or urination. This setting was the context that was conditioned to shock. Before and between each round of behavioral testing, the grid floor, Plexiglas arena, and tray were cleaned with 95 percent ethanol to ensure that the grid floors administered the proper amplitude of shock and to add a distinct smell to the context. Mice returned to the same conditioning chamber on each day to provide consistency in the context.

Habituation. Prior to fear conditioning, all animals were habituated to transport and handling for 3 days. Additionally, mice were acclimated to the antechamber of the procedure room during habituation for 1 hr before and after each day of the behavioral tests.

Freezing Assessment. The level of contextual fear conditioning was assessed by the amount of freezing, the natural conditioned response upon re-exposure to a cue or context associated with shock (Fanselow & Bolles, 1979). Freezing was considered continuous inactivity (with the exception of breathing) for 2 sec. Freezing was measured in real-time by visual time sampling in which the experimenter would assess each animal every 8 s for freezing and hand-score the presence or absence of freezing.

Contextual Fear Conditioning. For all experiments, conditioning was a 12-min session, in which rodents received 4 unsignaled footshocks (2 sec, .35 mA) at 2.5 min, 5 min, 9 min, and 11.5 min into the session. Extinction consisted of a 24-min non-reinforced exposure to the fear-conditioning context. Extinction training was run until animals displayed levels of freezing equal to conditioning naïve mice. Reconditioning was a weak conditioning session that consisted of 3 min session with a single unsignaled footshock (2 sec, .35 mA) delivered 2.5min into the session. Post-reconditioning tests were identical to extinction in that they consisted of 24-min non-reinforced exposure to the fear-conditioning context. All sessions were run at the same time of day separated by 24 hr.

Behavioral Schedule. The mice were subdivided into 3 groups that received different behavioral treatments: Group Recondition (RECOND, n=8), Group Context (CTX, n=8) and Group Condition (COND, n=8). Refer to Figure 1A for a full timeline. Group Recondition (RECOND) received strong initial conditioning followed by moderate extinction and weak reconditioning. Control groups were matched in exposure to the context (Group Context; CTX) or were naïve to the context (Group Condition; COND) before a common weak contextual fear conditioning (as described above for reconditioning) in which all groups received a single footshock in the context. During extinction, CTX provided an indication of when RECOND had reached a behavioral floor, as mice tend to freeze even without exposure to shock (Tipps et al., 2014a). CTX also controlled for an equivalent amount of context exposure, so any effects of context exposure could be distinguished from the effects

of reconditioning. This design allowed for a direct comparison of initial conditioning to reconditioning.

Phase 1: Conditioning (or Control Treatment). On Day 1, RECOND received a 12-min conditioning session, as described above. CTX received a 12-min context exposure with no footshocks. COND did not receive any context exposure or shocks, but was moved into and handled in the procedure room to equate transport and handling among the groups.

Phase 2: Extinction. The following day both RECOND and CTX received extinction training (as described above). COND was handled and returned to the home cage. This treatment continued for five additional days, resulting in a total of six days of extinction (or context exposure in the case of CTX) prior to reconditioning. Six days of extinction was necessary to reach a similar average freezing between RECOND and CTX, which was important before starting the next phase. An additional extinction criterion of freezing below 30 percent was followed for all reconditioning studies to ensure that freezing during tests is due to reconditioning, not initial conditioning (i.e., mice freezing above 30 percent in E6 were removed from analysis). No mice were removed due to this criterion in Experiment 1A.

Phase 3: Reconditioning. On Day 8, all groups (RECOND, CTX, and COND) received reconditioning as described above. For Group RECOND, Day 8 was the second experience of shock in the context and was therefore considered a reconditioning session. For both CTX and COND, this session was the first experience of shock in the context and thus was considered to be an initial

conditioning session. A short session with one shock was chosen to avoid the possibility of all groups reaching a behavioral ceiling (high freezing levels) if given conditioning similar to Day 1 with 4 shocks, as it has been reported that reconditioning can rapidly expose conditioned behavior in one to two trials (McAllister & McAllister, 1988; 1994; 2006; Pavlov, 1927).

Phase 4: Tests. On Day 9 (Test 1), all groups received the same condition as was received on Days 2 through 7: a 24-min context exposure. This day served as a critical day to compare the behavior after reconditioning, conditioning after context exposure, and initial conditioning without context pre-exposure. For Days 10 through 21, all groups were unperturbed in their homecages in the vivarium. On Day 22 (Test 2), all groups received an additional test to show the long-term retention of learned behavior 14 days after reconditioning or conditioning.

Statistics

R-Studio (Boston, MA) and GraphPad Software Prism 6 (La Jolla, CA) were used to run all statistics and create figures, respectively. For this experiment, the extinction sessions were generally analyzed by a two-way, type III-repeated measures analysis of variance (RMANOVA) in which Group was the between subjects measure, Extinction Sessions was the within subjects factor, and average percent freezing for the entire session was the dependent variable. Reconditioning in Phase 3 was analyzed with a two-way RMANOVA comparing the average percent freezing in the 30 sec pre- and post-shock (Time) by Group. Test sessions (Phase 4) were analyzed with a one-way ANOVA (or t-test, if applicable) with percent freezing for the entire session as the dependent measure and Group as the main factor. Any

failure to meet the homogeneity of variances criterion for an ANOVA or t-test (as measured by the Brown-Forsythe Levene's test) was accounted for using a Welch correction. If significance was found for main effects or interactions, Tukey's HSD tests (or Games-Howell for unequal variances amongst groups) were used for simple comparisons between groups and sessions. For all statistical tests, significance was set at $\alpha = 0.05$.

Results

Phase 1: Conditioning

As expected, conditioning (RECOND) caused more freezing during acquisition than context exposure alone (CTX; data not shown). A t-test comparing groups found a significant effect of group ($t_{(14)} = 5.54$, $p < .001$), as RECOND (M = 34.8, SEM = 4.73) froze significantly more than CTX (M = 6.39 , SEM = 2.69) during acquisition.

Phase 2: Extinction

Freezing extinguished over the course of six extinction sessions to similar levels observed in the group that was not fear conditioned (Figure 1B). The two-way RMANOVA revealed a significant main effect of Group ($F_{(1,14)} = 50.29$, $p < .001$), of Extinction session ($F_{(5,70)} = 21.42$, $p < .001$), and a significant Group X Extinction session interaction ($F_{(5,70)} = 16.50$, $p < .001$).

Tukey's post hoc analyses of the Group X Extinction Session interaction demonstrated that group RECOND had significantly greater freezing compared to CTX in E1 ($q_{(14)} = 9.74$, $p < .001$), E2 ($q_{(14)} = 7.59$, $p < .001$), and E3 ($q_{(14)} = 7.08$, $p < .001$). This result showed that three extinction sessions reduced RECOND fear

A.

Group	Acquisition	Extinction	Recondition	Test 1	Test 2
	Day 1	*Day 2-7*	*Day 8*	*Day 9*	*Day 22*
CTX	12 min -	24 min -	3 min +	24 min -	24 min -
RECOND	12 min ++++	24 min -			
COND	Handled				

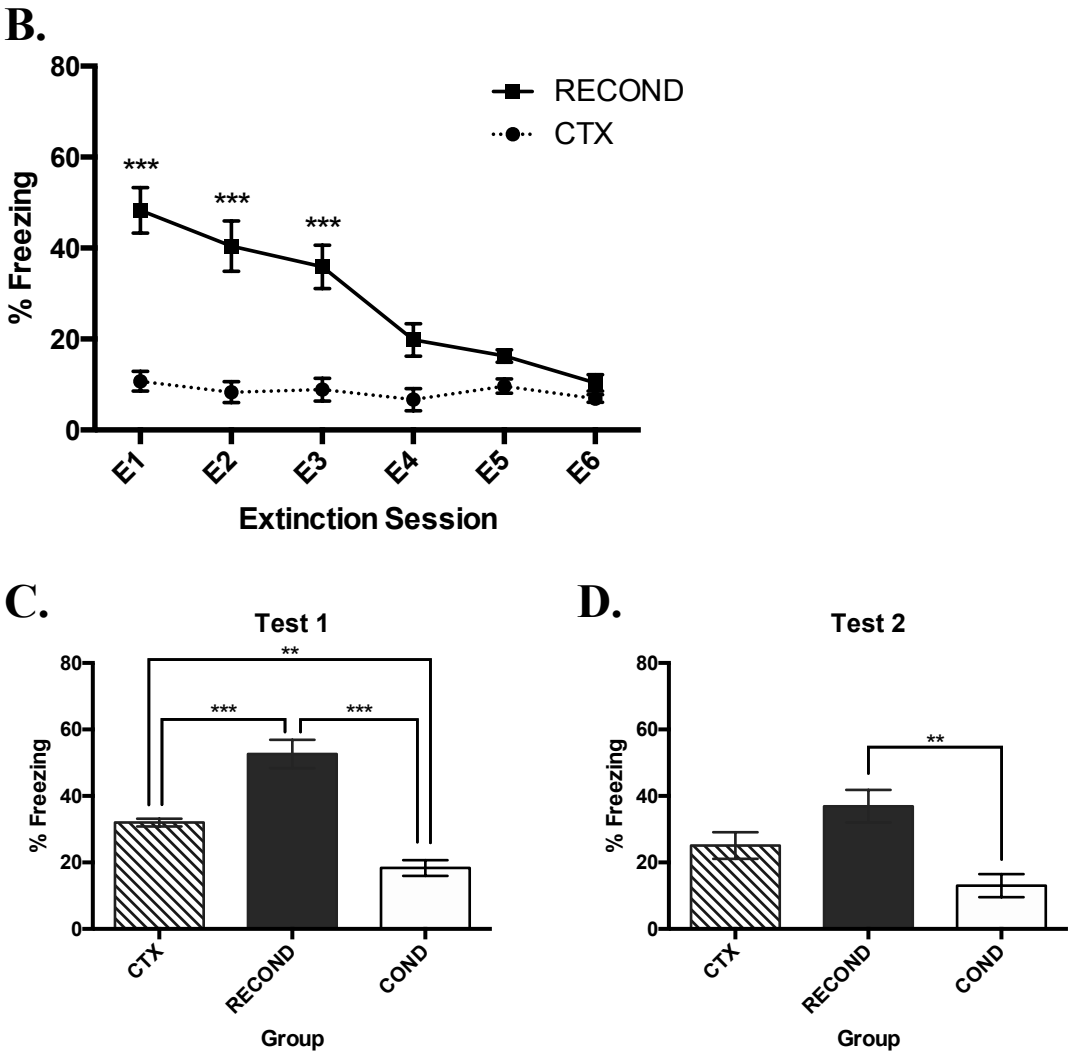


Figure 1. Rapid Reacquisition of Contextual Fear Following Reconditioning in C57BL/6J Mice (Moderate Extinction). (**A**) Overview of the design of Experiment 1A. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .35 mA shock and a minus sign indicates

exposure to the context without shock. **(B)** Mean percent freezing during of each extinction session (E) by mice that receiving fear conditioning (RECOND) or context exposure (CTX) on Day 1 (Day 2- 7). **(C)** Mean freezing from Test 1 (1 day after reconditioning) for Groups CTX, COND (which received no initial conditioning or extinction), and RECOND. **(D**) Mean freezing of Test 2 (administered 14 days after reconditioning) for each group. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$. RECOND, n= 8; CTX, n=8; COND, n=8. Error bars represent standard error of the mean.

behavior to CTX levels and six sessions caused the group to be visually indistinguishable (Figure 1B), which ensured that the next phase of the experiment would begin from a common point of behavior.

A steady decline in freezing over each extinction session by RECOND was also visible when dividing the sessions into 3 min time bins (Figure 2). By E5 and E6, RECOND lowered to visually similar freezing as the conditioning naïve group CTX. The reemergence of some conditioned behavior at the beginning or middle of each extinction session relative to the last 3 min bin of the previous session was likely due to spontaneous recovery after a 24 hr delay (Rescorla, 2004b).

Phase 3: Reconditioning

To assess if reconditioning led to immediate differences in freezing, the average percent freezing for the 30 sec pre- and post-shock (Time) of each Group (RECOND, COND, and CTX) was compared during the brief reconditioning session in a RMANOVA (data not shown). There was a significant effect of Group ($F_{(2, 21)}$ = 7.73, $p < .01$), but not Time ($F_{(1, 21)}$ = .559, p = .463) or a Time X Group interaction ($F_{(2, 21)}$ = .036, p = .964). The main effect of Group was driven by RECOND (M = 17.81, SEM = 5.06) freezing significantly more than COND ($q_{(21)}$ = 3.09, $p < .05$; M = 2.50, SEM = 2.50) and CTX ($q_{(21)}$ = 2.91, $p < .05$; M = 4.06, SEM = 2.86). This result suggested that were group differences due to previous experience with shock in the context that emerged during reconditioning.

Phase 4: Tests

Test 1: Post-Reconditioning Test

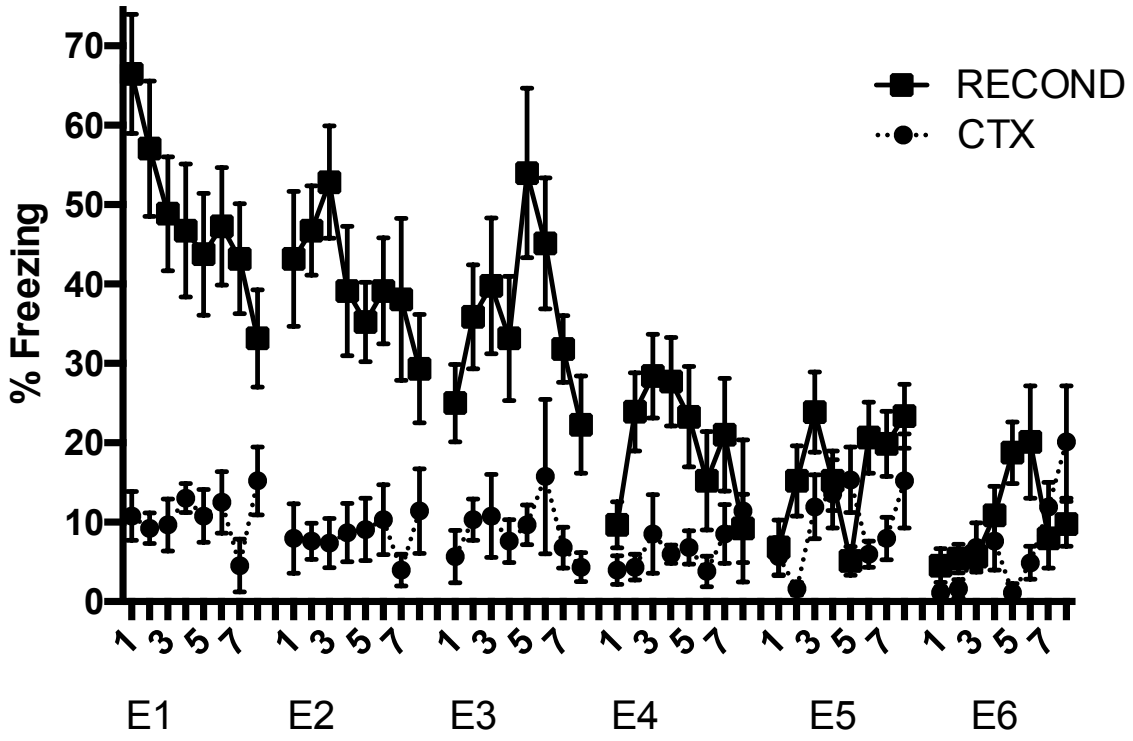


Figure 2. Extinction Sessions in 3 min time bins for Experiment 1A. Mean freezing for each 3 min time bin of each extinction session. RECOND, n=8; CTX, n=8. Error bars represent standard error of the mean.

A Welch corrected one-way ANOVA was used to analyze the data from Test 1 administered 24 hr following reconditioning (Figure 1C). The ANOVA showed a significant main effect of Group ($F_{(2,11.571)}$ =25.71, p < .001). Games-Howell post hoc revealed that the main effect of Group was driven by significantly more freezing by RECOND than both COND (p < .001) and CTX (p < .01), which shows that expression of rapid reacquisition of contextual fear occurs after reconditioning with moderate extinction. I also found that group CTX showed more freezing behavior than COND (p < .001), which suggested that moderate context pre-exposure enhanced acquisition.

Test 2: Long-term Retention Test

Figure 1D shows freezing during Test 2. The one-way ANOVA showed a significant main effect of Group ($F_{(2,21)}$ =8.27, p < .01) that was a consequence of the significantly larger magnitude of freezing displayed by animals in the RECOND group over the COND group ($q_{(21)}$ = 5.75, p < .01). This result showed the persistence of the rapid reacquisition effect over time in a second test.

Discussion

While previous findings of rapid reacquisition have been seen in rats and rabbits in similar learning paradigms (Bouton, Woods, & Pineño, 2004a; Leung et al., 2007; Napier et al., 1992; conditioned suppression and eyeblink conditioning), this experiment demonstrated the first finding of rapid reacquisition of conditioned contextual fear behavior in mice (to the best of my knowledge). Further, I showed that rapid reacquisition persisted to a second test two weeks after reconditioning. Overall, this experiment shows that rapid reacquisition following reconditioning is a

finding that exists across multiple species, now including C57BL/6J mice, and provides a tool to study persistent fear memory.

Experiment 1B: Massive Extinction before Reconditioning Prevents Rapid Reacquisition of Contextual Fear Conditioning

The next experiment explored extensive extinction of freezing behavior beyond the "silent zero" (Pavlov, 1927), during which learning may potentially still occur despite reaching a behavioral floor. This experiment specifically examined how extensive extinction affected reacquisition of contextual fear. I hypothesized that massive extinction would prevent rapid reconditioning and perhaps have an inhibitory effect on reestablishment of contextual fear as was seen in Leung, et al. (2007) in rats.

Methods

Twenty-four naive male C57BL/6J mice were used in this experiment. This experiment was identical to Experiment 1A with the exception of an extended number of days of extinction sessions between Acquisition and Reconditioning (behavioral timeline in Figure 3A). The days of extinction were increased to examine how massive extinction affected reconditioning to the feared context.

For Phase 2, Days 2 through 15 were 24-min context exposures followed by reconditioning (same experimental conditions as Experiment 1A) on Day 16. Hence, there were 14 days of extinction in this experiment as opposed to the 6 days of extinction in Experiment 1A. 24 hr after reconditioning, the animals were given Test

1 (Day 17) and subsequently unhandled for 10 days until Test 2 (Day 27). Both Test 1 and Test 2 were identical to the test sessions in Experiment 1A.

Results

Phase 1: Conditioning

In Experiment 1B, conditioning led to greater within-session acquisition of freezing behavior relative to nonreinforced context exposure. A t-test showed that RECOND (M = 23.27, SEM = 4.06) had significantly larger average percent freezing than CTX (M = 1.51, SEM = 0.51) on Day 1 ($t_{(7.22)}$ =5.31, $p < .01$; data not shown)

Phase 2: Extinction

Figure 3B displays the average amount of freezing for each extinction session, showing extinction far beyond when RECOND froze at levels of conditioning-naïve mice, CTX. A two-way RMANOVA showed a significant effect of Extinction session ($F_{(13,182)}$ =6.48, $p < .001$), of Group ($F_{(1,14)}$ =7.01, $p < .05$), and a significant Group X Extinction session interaction ($F_{(13,182)}$ =8.53, $p < .001$).

The significant interaction seen by the two-way RMANOVA was due to the larger amount of freezing displayed by animals in Group RECOND compared to group CTX in E1 ($q_{(14)}$ = 6.81, $p < .01$) and E2 ($q_{(14)}$ = 5.70, $p < .01$), but not later sessions drove the interaction of Group X Extinction Session found here and provided further evidence of a successful reduction in fear behavior in Group RECOND during massive extinction.

Figure 4 shows each extinction sessions by 3 min time bins. The steady

A.

Group	Acquisition	Extinction	Recondition	Test 1	Test 2
	Day 1	*Day 2-15*	*Day 16*	*Day 17*	*Day 27*
CTX	12 min -	24 min -	3 min +	24 min -	24 min -
RECOND	12 min ++++	24 min -			
COND	Handled				

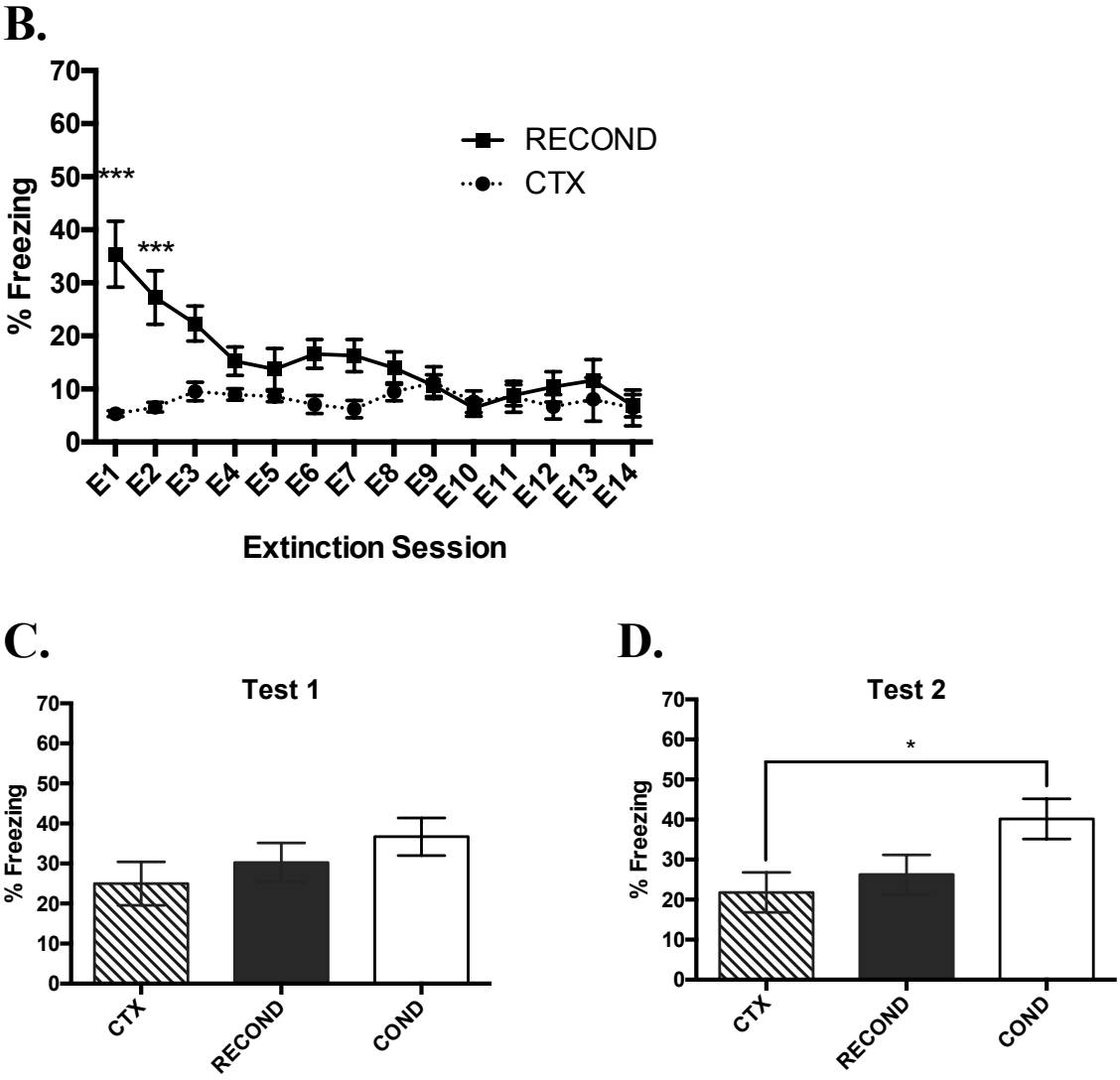


Figure 3. Massive Extinction before Reconditioning Prevents Rapid Reacquisition of Contextual Fear Conditioning (**A**) Overview of the design of Experiment 1B. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .35 mA shock and a minus sign

indicates exposure to the context without shock. (**B**) Mean freezing of each extinction session (E) by mice that receiving fear conditioning (RECOND) or context exposure (CTX) on Day 1 (Day 2- 15). (**C**) Mean freezing of Test 1 (1 day after reconditioning) for each group. (**D**) Mean freezing of Test 2 (administered 11 days after reconditioning) for each group. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$. RECOND, n= 8; CTX, n=8; COND, n=8. Error bars represent standard error of the mean.

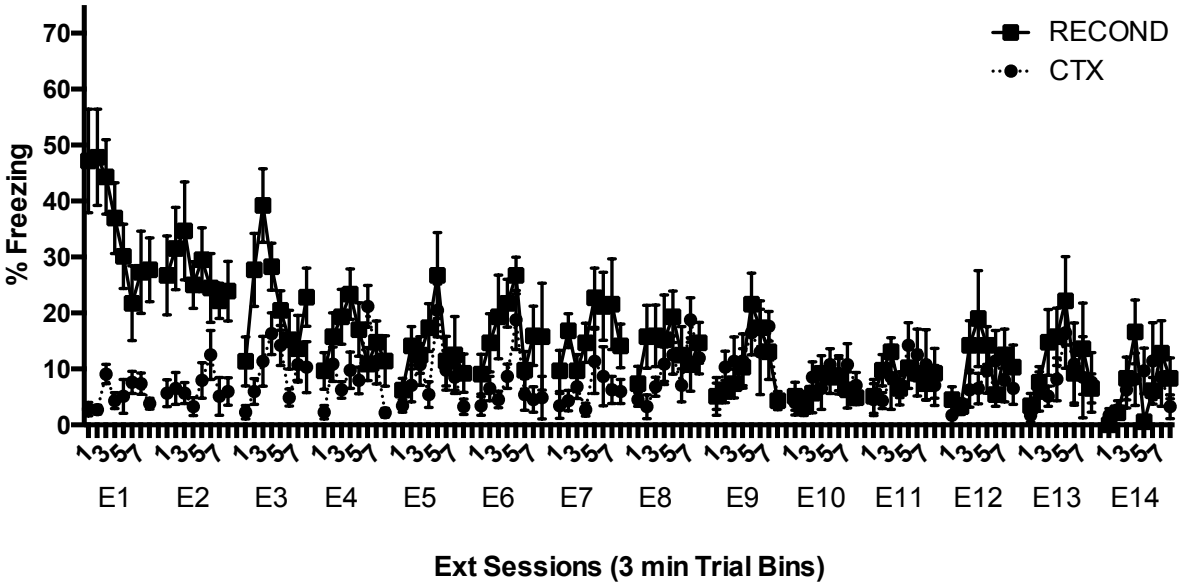


Figure 4. Extinction Sessions in 3 min time bins for Experiment 1B. Mean freezing for each 3 min time bin of each extinction session. RECOND, n=8; CTX, n=8. Error bars represent standard error of the mean.

decline in freezing over each extinction session by RECOND was visible, as well as the convergence of group freezing as RECOND lowered to visually similar freezing as the conditioning naïve group CTX by E5 and E6. The reemergence of some conditioned behavior at the beginning or middle of each early extinction session relative to the last 3 min bin of the previous session was likely due to mild spontaneous recovery after a 24 hr delay (Rescorla, 2006).

Phase 3: Reconditioning

During reconditioning, groups did not differ in their freezing in response to weak conditioning parameters (data not shown). A RMANOVA comparing average percent freezing during 30 sec pre- and post-shock across groups (Time X Group) showed a significant main effect of Time ($F_{(1,21)}$ = 6.27, p < .05; pre-shock: M = 2.08, SEM = 1.44, post-shock: M = 13.54, SEM = 3.89), but not a significant main effect of Group ($F_{(2,21)}$ = 1.55, p = .236; COND: M = 10.94, SEM = 3.93, CTX: M = 9.38, SEM = 5.04, RECOND: M = 3.12, SEM = 2.13) or Time X Group interaction ($F_{(2,21)}$ = .674, p = .520). This finding suggested that reconditioning did not lead to within session differences in reacquisition of freezing behavior, but freezing did increase following shock.

Phase 4: Tests

Test 1: Post-Reconditioning Test

Twenty-four hr following weak reconditioning, all three groups were exposed to the context for 24 min and average freezing behavior was analyzed using a one-way ANOVA for group differences (Figure 3C). The ANOVA did not show a significant Group effect ($F_{(2,21)}$ =1.38, p =.274). Further, no significant difference in

groups was detected when comparing the first 6 min of Test 1 ($F_{(2,21)}$ =2.46, p =.109; COND: M = 41.64, SEM = 5.97, CTX: M = 24.17, SEM = 4.87, RECOND: M = 32.73, SEM = 5.79). This result showed that massive extinction blocked the rapid reacquisition effect seen in Experiment 1A.

Test 2: Long-term Retention Test

The one-way ANOVA comparing the freezing data of all the groups in Test 2, 11 days after reconditioning, revealed a significant main effect of Group (Figure 3D; $F_{(2,21)}$ =3.70, p < .05). Follow up Tukey's HSD revealed that the significant effect of Group was due to the significantly greater magnitude of freezing displayed by Group COND compared to that of Group CTX ($q_{(21)}$ = 3.69, p < .05), revealing a delayed latent inhibition effect of massive context pre-exposure prior to conditioning.

Discussion

This experiment and Experiment 1A demonstrated that rapid reacquisition of contextual fear behavior occurred as a function of how much extinction training is received, such that moderate extinction allowed rapid reacquisition, but massive extinction blocked the enhanced reemergence of fear behavior. Similar findings have been reported (Leung et al., 2007), but this experiment was the first finding of the importance of extinction strength to rapid reacquisition within C57BL6/J mice. However, I did not find evidence of slow reacquisition due to massive extinction, as has been seen in other behavioral models (Bouton, 1986). Given results of rapid and slow reacquisition of conditioned behavior following reconditioning in rats, the following experiments were conducted to replicate Experiment 1A and 1B and extend my findings to Long Evans rats.

Experiment 2A: The Rapid Reacquisition of Contextual Fear is Specific to the Context Reconditioned in Long Evans Rats.

This experiment sought to explore 3 issues: the replication of the rapid reacquisition effect in the Long Evans rat, the context specificity of rapid reacquisition of contextual fear, and the effect of extinction training strength on rapid reacquisition.

I chose to switch to a common rat species used in fear conditioning (Goosens & Maren, 2001; Helmstetter & Bellgowan, 1994; Kim & Fanselow, 1992b) to examine the translatability of my findings to other rodent species, rodent strain differences in fear reconditioning, and differences in the speed of reacquisition seen in rats reported in the literature (i.e., slow or rapid reacquisition; Bouton, 1986, Leung et al., 2007). I also choose to switch to rats because rats quickly extinguish to zero percent conditioned freezing behavior (Laurent & Westbrook, 2009a; Leung et al., 2007; Quirk, 2002), whereas mice tend to show baseline freezing without conditioning (refer to Experiments 1a and 1b) and are difficult to extinguish to zero (Abraham, Cunningham, & Lattal, 2012; Bowers, Xia, Carreiro, & Ressler, 2015; Tipps et al., 2014).

This experiment also examined the context-specificity (or CS-specificity; context is the CS in all experiments) of rapid reacquisition of contextual fear. It is conceivable that the quick reemergence of conditioned freezing following post-extinction context-shock pairing could be due to reinstatement to the US alone (Rescorla & Heth, 1975) or a generalized enhancement of freezing due to repeated shock (e.g., stress enhanced fear learning; Rau & Fanselow, 2009). This experiment

added an additional group that was reconditioned in a distinct context to probe those possibilities.

Methods

Animals and Housing

32 Long-Evans male rats (Charles River Labortories, Wilmington, MA) were purchased at 275-300 g (~9-11 weeks of age) and were housed 2 rats to an individually-ventilated cage. Rats were kept on a reverse 12h light-dark schedule (dark started at 0600 and light started at 1800). The reverse light-dark cycle used for rats differed from the normal light-dark housing conditions of the mice. The switch was made to capture active rodent behavior during their natural waking period, the dark cycle, as rats and mice are nocturnal animals (Rhodes, Garland, & Gammie, 2003). Acclimation and vivarium conditions were identical to Experiment 1.

Behavior

Context A (CTX A). Fear conditioning occurred in operant conditioning chambers (exterior dimensions: 31.8cm L x 25.4 W x 26.7cm H, Med Associates, St. Albans, VT) housed within sound attenuating chambers (Med Associates, St. Albans, VT). The operant chambers were fixed with a grid floor set to deliver a 1 sec, .75 mA scrambled shock and a house light that illuminated to signal the start of the session. Before and after each round of behavior, the grid floors and chamber walls were cleaned with 95% ethanol. Animals were loaded into chambers in red light conditions to maintain the dark circadian cycle.

For Experiment 2A exclusively, the walls of the operant chamber were also fitted with horizontal black and white stripes to provide an additional visual cue.

Context B (CTX B). Fear conditioning for Experiment 2A also occurred in additional operant conditioning chambers (exterior dimensions: 31.8cm L x 25.4cm W x 34.3cm H) housed within sound attenuating chambers (Med Associates, St. Albans, VT). CTX B differed from CTX A in its dimensions, visual cues, cleaning solution, and loading light conditions. These operant chambers were also equipped with inactive levers, cue lights, and food hoppers. Before and after each round of behavior, the grid floors and chamber walls were cleaned with 0.5% bleach. Animals were loaded into chambers in full light conditions to differ from CTX A. The operant chambers were fixed with the same model grid floors as CTX A set to deliver a 1 sec, .75 mA scrambled shock and a house light that illuminated to signal the start of the session.

Behavioral Schedule. In this experiment, 32 naïve male Long Evans rats were run through a modified version of the rapid reacquisition paradigm seen in mice above in Experiment 1A. The length of sessions and number and timing of shocks were the same as each phase in Experiments 1A and 1B. The major differences were the number of Phase 1 conditioning sessions and shock magnitude. Phase 1 increased to 2 days for rats to increase their freezing to levels that could be modulated by various lengths of extinction training. Shock magnitude increased from 2 sec, .35 mA shocks to 1 sec, .75 mA shocks to compensate for rodent size.

There were 4 groups that differed in reconditioning, reconditioning context, and extinction training: reconditioning group with moderate extinction (REC-MOD EXT, n = 8), reconditioning group with massive extinction (REC-MASS EXT, n = 8), reconditioning group with moderate extinction and reconditioning in a different

(switch) context (REC-MOD EXT-SWITCH, n = 8), and conditioning (COND, n = 8). Each group was counterbalanced for contexts with half run in CTX A (n = 4/group) and CTX B (n = 4/group) as their original conditioning context. For the purposes of the simplified behavioral schedule in Figure 5A and the discussion of the methods and results, the original context is CTX A and switch context is CTX B.

Phase 1: Conditioning. Day 1 and 2 (Conditioning) consisted of a conditioning sessions (12 min, 4 footshock) for REC-MASS EXT in CTX A. All other groups were handled and transported to equate treatments. On Day 4 and 5, REC-MOD EXT and REC-MOD EXT-SWTICH received conditioning in CTX A.

Phase 2: Extinction. On Days 3 through 8, REC-MASS EXT received extinction training within the same context as conditioning (CTX A). Concurrently, on Days 6 through 8, REC-MOD EXT and REC-MOD EXT-SWTICH received extinction training within the same context as conditioning (CTX A). This behavioral schedule allowed the massive and moderate extinction groups to receive different amount of extinction training (six vs. three days of extinction), but to both receive their final extinction preceding reconditioning the next day.

Phase 3: Reconditioning. All groups received reconditioning on Day 9. For groups REC-MOD EXT and REC-MASS EXT, reconditioning occurred in the same context as the conditioning context (e.g., if the rat acquired fear conditioning on Day 1 in CTX A, the rat reacquired in CTX A). For REC-MOD EXT-SWTICH, reconditioning occurred in a different context (the “switch” context) from their original conditioning context (e.g., if the rat acquired fear conditioning on Day 1 in CTX A, the rat reacquired in CTX B).

Phase 4: Tests. Day 10 was a post-reconditioning test in the original conditioning context (CTX A; Test 1) and Day 11 was a test in the "switch" context for all groups (CTX B; Test 2). On Day 21, another post-reconditioning test occurred in the original context (CTX A; Test 3) to test for long-term memory.

Results

Phase 1: Conditioning

For all results, the groups are collapsed across contexts as a two-way RMANOVA revealed an insignificant effect of context ($F_{(2, 233)}$ =2.11, p = .12; data not shown; CTX A: M = 21.06, SEM = 2.37, CTX B: M = 18.94, SEM = 1.94) and an insignificant context X session interaction ($F_{(13, 233)}$ =0.75, p = .71).

To assess acquisition differences, a two-way RMANOVA was run showing an insignificant Group effect ($F_{(1,14)}$ =0.15, p = .70) and an insignificant Group X Acquisition session interaction ($F_{(1, 14)}$ = .40, p = .57) between REC-MOD EXT (M = 55.59, SEM = 3.29) and REC-MOD EXT-SWITCH (M = 57.82, SEM = 4.81). There was a significant effect of Acquisition session ($F_{(1, 14)}$ = 35.3, p > .001), suggesting that freezing increased over two days of acquisition (data not shown; session one: M = 46.63, SEM = 2.45, session two: M = 66.78, SEM = 3.83). REC-MASS EXT was not included in this analysis because its acquisition occurred on different days and, thus, would not provide a common test for reliable comparison to the moderate extinction groups. However, a one-way RMANOVA of REC-MASS EXT freezing across acquisition showed a significant effect of Acquisition session ($F_{(1, 7)}$ = 15.03, p > .01; data not shown; session one: M = 35.63, SEM = 4.21, session two: M = 60.20, SEM = 6.97).

A.

Group	Acq		Ext (Acq)		Rec	Test 1	Test 2	Test 3
Exp 2a	*Day 1-2*	*Day 3*	*Day 4-5*	*Day 6-8*	*Day 9*	*Day 10*	*Day 11*	*Day 21*
REC-MASS EXT	CTX A 12 min ++++	CTX A 24 min -	CTX A 24 min -	CTX A 24 min -	CTX A 3 min +	CTX A 24 min -	CTX B 24 min -	CTX A 24 min -
REC-MOD EXT	Handled		CTX A 12 min ++++	CTX A 24 min -	CTX A 3 min +			
REC-MOD EXT-SWITCH	Handled		CTX A 12 min ++++	CTX A 24 min -	CTX B 3 min +			
COND	Handled				CTX A 3 min +			

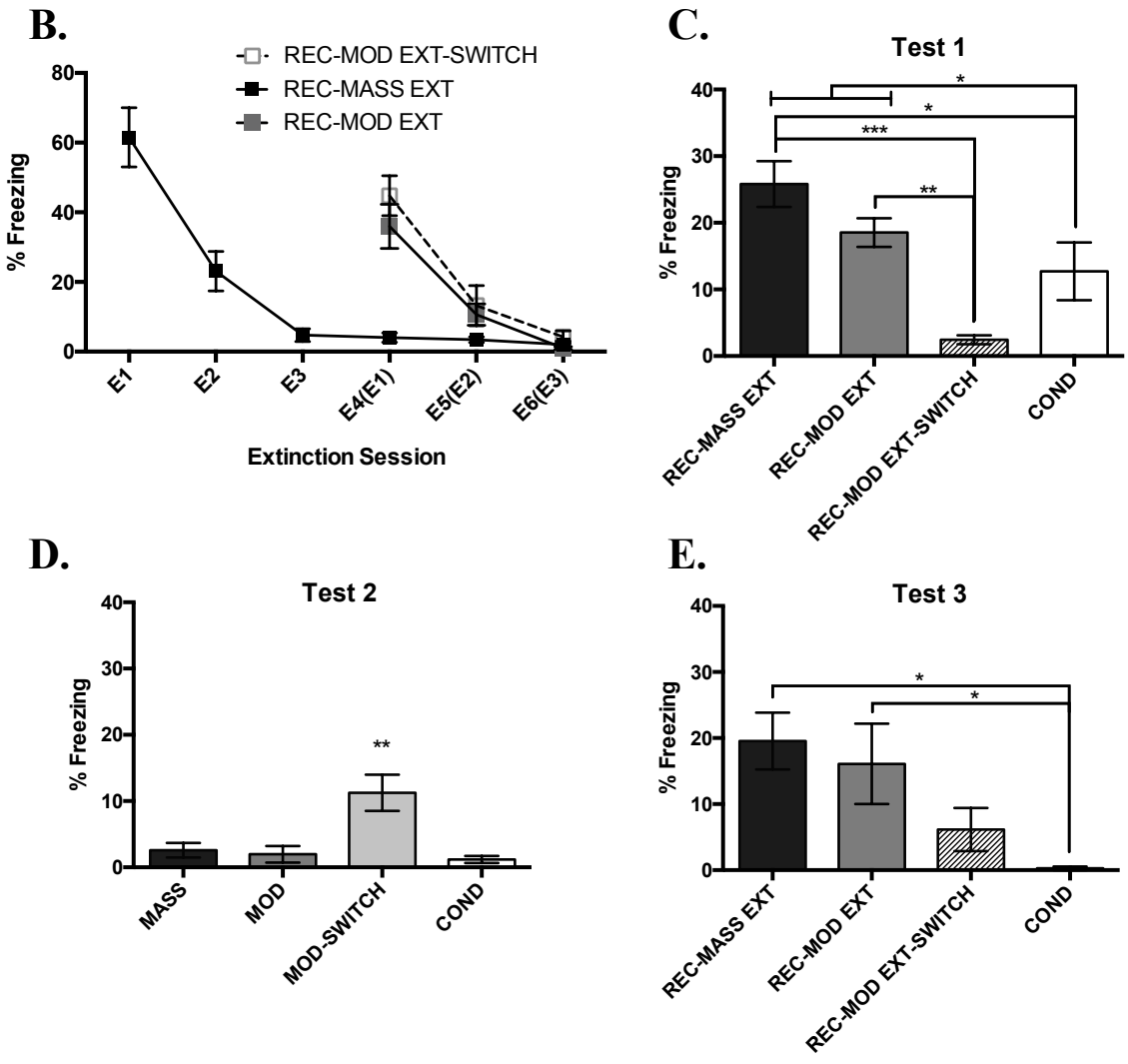


Figure 5. The Rapid Reacquisition of Contextual Fear is Specific to the Context Reconditioned in Long Evans Rats. (A) Overview of design of Experiment 2A.

The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .75 mA shock and a minus sign indicates exposure to the context without shock. CTX A (orginial context) or CTX B (switch context) indicates in which context the session occurred. (**B**) Mean freezing of each extinction session (E1-6 or E1-3) by rats that receiving fear conditioning Days 1 and 2 (REC-MASS EXT) or Days 4 and 5 (REC-MOD EXT). (**C**) Mean freezing of Test 1 (1 day after reconditioning) for each group. (**D**) Mean freezing of Test 2 for each group that occurred in the switch context. (**E**) Mean freezing of Test 3 (administered 12 days after reconditioning) for each group. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$. REC-MOD EXT, n=8 and REC-MASS EXT, n=8; REC-MOD EXT-SWITCH, n=8; COND, n=8. Error bars represent the standard error of the mean.

Phase 2: Extinction

Figure 5B displays that extinction successfully lowered freezing behavior in all REC groups. A two-way RMANOVA comparing REC-MOD EXT and REC-MOD EXT-SWITCH across extinction showed a significant effect of Extinction session ($F_{(2,28)}$ =53.42, p < .001), but an insignificant Group effect ($F_{(1,14)}$ =1.15, p = .31) and an insignificant Group X Extinction session interaction ($F_{(2,28)}$ =0.41, p = .67). Likewise, a one-way RMANOVA comparing REC-MASS EXT across extinction sessions showed a significant effect of Extinction session ($F_{(5,35)}$ =38.12, p < .001). These results suggested that all REC groups successfully extinguished before reconditioning.

Figure 6 shows each extinction sessions by 3 min time bins. The rapid decline in freezing over two extinction sessions by REC groups was visible. Behavior indicative of spontaneous recovery only occurred at the beginning E2 relative to the last 3 min bin of E1 (Rescorla, 2006).

Phase 3: Reconditioning

There was no difference in the average percent freezing between groups during reconditioning (data not shown). A RMANOVA comparing the average freezing 30 sec pre- and post-shock (Time) across all groups found a significant main effect of Time ($F_{(3, 28)}$ =36.05, p < .001; pre-shock: M = .00, SEM = .00; post-shock: M = 33.59, SEM = 5.45), but not Group ($F_{(3, 28)}$ =.487, p = .694; COND: M = 17.19, SEM = 7.11; REC-MASS EXT: M = 12.50, SEM = 6.85; REC-MOD EXT M = 15.62, SEM = 6.80; REC-MOD EXT-SWITCH M = 21.88, SEM = 7.17) or Time X Group interaction ($F_{(3, 28)}$ =.487, p = .694). This finding indicated that freezing during

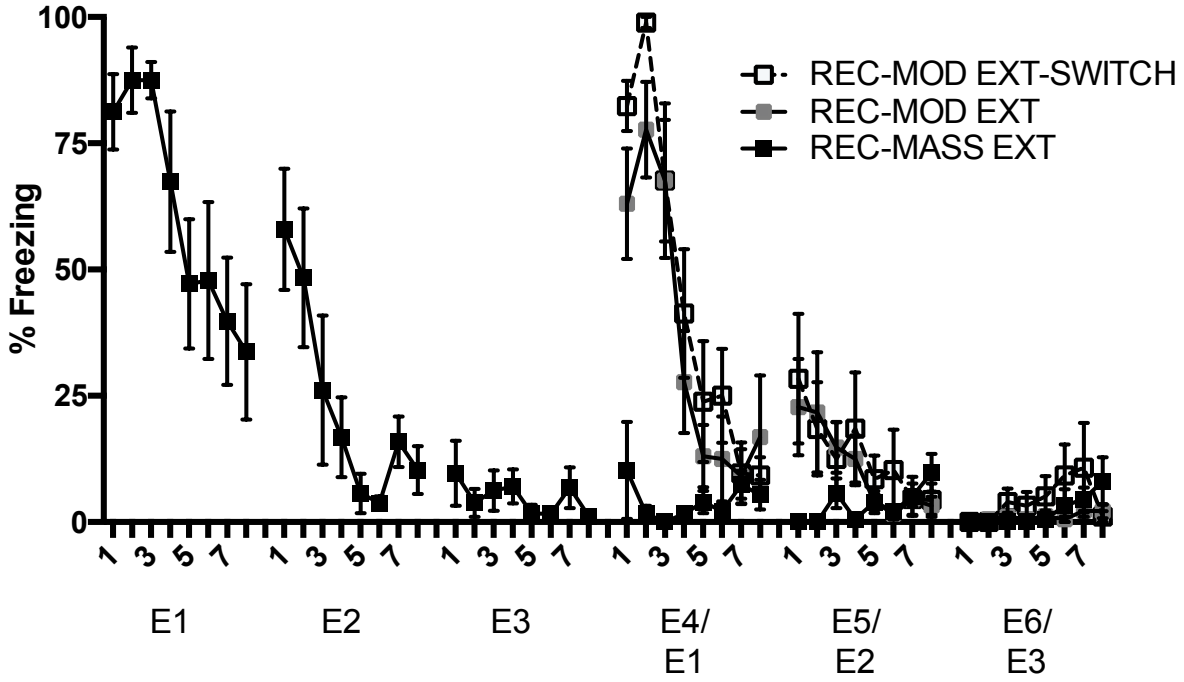


Figure 6. Extinction Sessions in 3 min time bins for Experiment 2A. Mean freezing for each 3 min time bin of each extinction session for; REC-MOD EXT, n=8; REC-MASS EXT, n=8; REC-MOD EXT-SWITCH, n=8. Error bars represent standard error of the mean.

acquisition of weak conditioning did not differ from freezing during reacquisition or a reinstating shock during Phase 3, but shock did increase freezing.

Phase 4: Tests

Test 1: Post-Reconditioning Test

Twenty-four hr following weak reconditioning, all four groups were exposed to the original context (CTX A) for 24 min (Figure 5C). A one-way ANOVA comparing all groups revealed a significant main effect of Group ($F_{(3,28)}$ =10.92, $p < .001$). This effect was driven by REC-MOD EXT and REC-MASS EXT freezing significantly more than REC-MOD EXT-SWITCH ($q_{(28)} = 5.39$, $p < .01$ and $q_{(28)} = 7.82$, $p < .01$, respectively), suggesting that exposing previously conditioned animals to shock in a distinct context did not cause a general enhancement of freezing to an extinguished context. Additionally, the main effect of Group was also due to REC-MASS EXT freezing significantly more to context than COND ($q_{(28)} = 4.39$, $p < .05$), which displayed the rapid reacquisition effect occurred in Long-Evans rats after massive extinction.

Notably during the test, REC-MOD EXT and REC-MASS EXT did not show a significant difference in freezing ($t_{(14)}$= -1.42, $p = .09$; Figure 5C), suggesting that differing levels of extinction did not alter rapid reacquisition as it had in the mice. Due to the lack of a significant difference, REC-MOD EXT and REC-MASS EXT were collapsed into one REC group for all additional analyses. Using a one-way ANOVA for group differences, I found a significant effect of Group (Figure 5C; $F_{(2,29)}$ =13.95, $p < .001$). Post-hoc analysis revealed this effect was driven by significantly more freezing by REC relative to COND ($q_{(29)} = 3.54$, $p < .05$) and REC-MOD EXT-

SWITCH ($q_{(29)}$ = 7.38, p < .01), which again showed the rapid reacquisition of contextual fear occurred in rats and that the effect was specific to the context in which reconditioning occurred.

Test 2: Switch Context Test

The subsequent test in the switch context (CTX B) revealed a significant main effect of Group (Figure 5D; $F_{(2,28)}$ =8.44, p < .001). Follow up Tukey's HSD revealed that the significant effect of Group was due to the significantly greater freezing elicited in the switch context by REC-MOD EXT-SWITCH relative to COND ($q_{(28)}$ = 6.51, p < .01), REC-MOD EXT ($q_{(28)}$ = 5.83, p < .01), and REC-MASS EXT ($q_{(28)}$ = 5.45, p < .01). This result showed that acquisition and rapid reacquisition did not generalize across contextual cues, as COND, REC-MOD EXT, and REC-MASS EXT were not exposed to the switch context, and that RECOND-MOD EXT-SWITCH had formed a distinct contextual fear memory in the switch context.

To compare the level of reinstatement freezing following US exposure in CTX B (switch context) relative to the reacquisition freezing following reconditioning in the CTX A (original context), an additional analysis was run. Although not an ideal comparison because the tests were on two separate days, a one-way ANOVA comparing the freezing of REC-MOD EXT-SWITCH to CTX B on Day 11 to the freezing of REC-MOD EXT and REC-MASS EXT to CTX A on Day 10 found a significant main effect of Group ($F_{(2,21)}$ = 6.66, p < .01), driven by REC-MASS EXT freezing more than REC-MOD EXT-SWITCH ($q_{(21)}$ = 4.75, p < .01). This significant difference showed that a shock in a distinct context from the acquisition context did

not result in a similar magnitude of enhanced freezing as that seen in rapid reacquisition.

Test 3: Long-Term Retention Test

The one-way ANOVA comparing the freezing data of all the groups in Test 3 in CTX A (original context) revealed a significant main effect of Group (Figure 5E; $F_{(2,28)}$ =4.76, $p < .01$). Follow up Tukey's HSD tests revealed that the significant effect of Group was due to the greater magnitude of freezing displayed by REC-MASS EXT and REC-MOD EXT compared to that of COND ($q_{(28)} = 4.74$, $p < .05$ and $q_{(28)} = 3.89$, $p < .05$), showing that similar to mice, rats showed a persistent rapid reacquisition effect that last 12 days beyond reconditioning.

Discussion

This experiment demonstrated several important characteristics of reacquisition. First, it replicated the persistent and rapid reacquisition effect of contextual fear in Long Evans rats, as seen in Experiment 1A and Leung et al. (2007). Importantly, this experiment also revealed that rapid reacquisition of contextual fear was context specific; such that rapid reacquisition only occurred after the re-pairing of the context previously paired with shock in acquisition.

Unlike Experiment 1A and 1B, this experiment did not show that speed of reacquisition occurs like a function of the amount extinction prior to reconditioning. Instead, massive extinction resulted in rapid reacquisition following reconditioning, while moderate extinction did not show rapid reacquisition initially. Although, massive and moderate extinction groups did not differ in the expression of their reacquisition freezing in the initial expression test (Test 1). If compared as one

reconditioning group, they showed rapid reacquisition relative to initial conditioning in Test 1. Additionally, both reconditioning groups (REC- MASS EXT and REC-MOD EXT) showed a persistent rapid reacquisition effect when compared separately with COND in the long-term retention test. This result was surprising as Leung et al. (2007) showed that massive extinction temporarily blocked rapid reacquisition in rats.

Experiment 2B. Rapid Reacquisition Occurs in Rats Following Moderate and Massive Extinction

In the previous experiment, massive extinction prior to reconditioning did not block or slow rapid reacquisition in rats, as seen in mice in Experiment 1B, but instead appeared to enhance expression of reacquisition more so than moderate extinction. Thus, this experiment expanded the difference in extinction training between massive and moderate extinction prior to reconditioning to draw out any differences in reacquisition due to extinction training strength.

Methods

Twenty-four naïve male Long Evans rats were run in a similar experiment as Experiment 2A. This experiment sought to expand upon the effect of extinction training strength on rapid reacquisition and, thus, there were 3 groups: reconditioning with massive extinction (REC-MASS EXT), reconditioning with moderate extinction (REC-MOD EXT), and conditioning (COND).

REC-MASS EXT received conditioning on Days 1 and 2 (Phase 1) followed by twelve days of extinction on days 3 through 14 (Phase 2). REC-MOD EXT

received conditioning on days 11 and 12 (Phase 1) and extinction on days 13 and 14 (Phase 2). Again, this behavioral schedule allowed groups to receive different amounts of extinction (twelve vs. two days), but have a final extinction session on the same day preceding reconditioning.

Day 15 was a reconditioning session (Phase 3) followed by the post-reconditioning test on day 16 (Phase 4). On days 19, 22 and 33 further post-reconditioning tests were run to explore re-extinction and long-term memory. The behavioral schedule is summarized in Figure 7A.

Results

Phase 1: Conditioning

The acquisition of REC-MASS EXT and REC-MOD EXT were assessed in two separate RMANOVAs, as conditioning for each group took place on separate days. The RMANOVA comparing REC-MASS EXT's acquisition of freezing over Day 1 and 2 revealed a main effect of Acquisition session ($F_{(1, 7)}$ =26.7, p < .01; data not shown; session one: M = 41.03, SEM = 5.76, session two: M = 71.07, SEM = 7.56), as did a similar RMANOVA for REC-MOD EXT ($F_{(1, 7)}$ =9.16, p < .05; data not shown; session one: M = 26.95, SEM = 3.55, session two: M = 54.50, SEM = 7.78). This effect showed that the conditioning sessions were successful in increasing conditioned freezing to the context.

Phase 2: Extinction

Figure 7B displays extinction curve for Experiment 2B. A one-way RMANOVA of REC-MASS EXT's freezing over extinction showed a significant effect of Extinction session ($F_{(11, 72)}$ =2.93, p < .01). Conversely, there was not a

A.

	Acq		Ext (Acq)		Rec	Test 1/ Re-Ext 1	Test 2/ Re-Ext 2	Test 3/ Re-Ext 3	Test 4
	Day 1-2	*Day 3-10*	*Day 11-12*	*Day 13-14*	*Day 15*	*Day 16*	*Day 19*	*Day 22*	*Day 33*
REC-MASS EXT	12 min ++++	24 min -	24 min -	24 min -	3 min +	24 min -	24 min -	24 min -	24 min -
REC-MOD EXT	Handled		12 min ++++	24 min -					
COND	Handled								

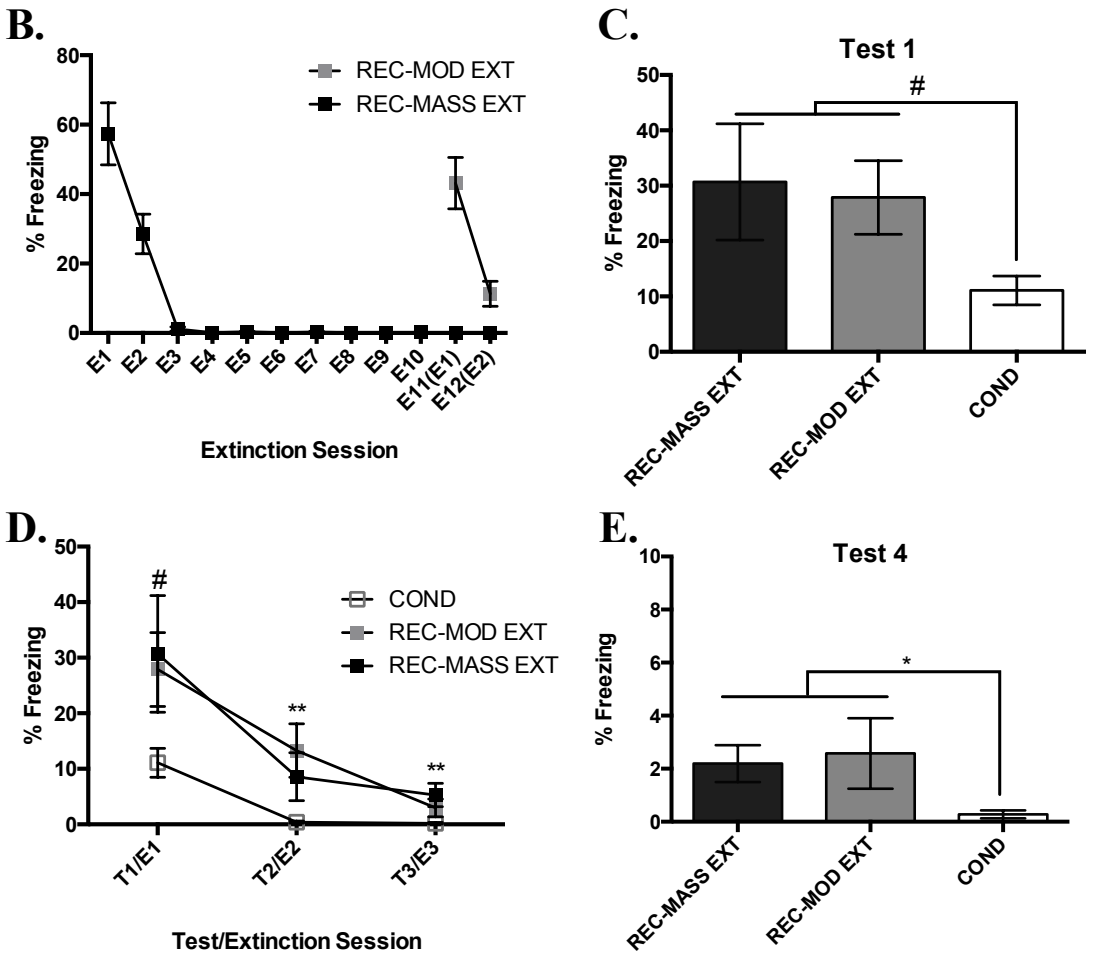


Figure 7. Rapid Reacquisition Occurs in Rats Following Moderate and Massive Extinction. (**A**) Overview of design of Experiment 2B. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .75 mA shock and a minus sign indicates exposure to the context without shock. (**B**) Mean freezing of each extinction session. (**C**) Mean freezing of Test 1 (1

day after reconditioning) for each group. (**D**) Mean freezing across re-extinction (post-reconditioning Test 1, 2, and 3). (**E**) Mean freezing of Test 4 (administered 17 days after reconditioning) for each group. Significance between groups, REC (collapsed REC groups) vs COND, is represented by ** $p < .01$; * $p < .05$; # $p = .051$. REC-MOD EXT, n=8; REC-MASS EXT, n=8; COND, n=8. Error bars represent standard error of the mean.

significant effect of Extinction session on REC-MOD EXT's session average freezing ($F_{(1, 12)}$ =0.24, p = .63). However, if REC-MOD EXT extinction behavior was analyzed by within session 3 min bins (Time), there is a significant effect of extinction over Time ($F_{(15, 105)}$ =14.48, p < .001; Figure 8) and REC-MOD EXT did reach low levels of freezing by the end of E2 (Figure 8; M= .54, SEM= .54), which showed successful extinction in just two extinction sessions. Additionally, Figure 8 shows that behavior indicative of spontaneous recovery only occurred at the beginning E2 relative to the last 3 min bin of E1 (Rescorla, 2006).

Phase 3: Reconditioning

There was no difference in groups' conditioned freezing during reconditioning, but shock increased freezing (data not shown). A RMANOVA comparing average percent time freezing 30 sec pre- and post-shock across all groups (Time X Group) during Phase 3 found a significant main effect of Time ($F_{(1, 21)}$ = 35.67, p < .001; pre-shock: M = 1.04, SEM = 1.04, post-shock: M = 42.71, SEM = 7.13), but did not find a significant main effect of Group ($F_{(2, 21)}$ = .722, p = .497; COND: M = 15.62, SEM = 5.98; REC-MASS EXT: M = 23.44, SEM = 8.68; REC-MOD EXT: M = 26.56, SEM = 9.54) or Time X Group interaction ($F_{(2, 21)}$ = .557, p = .581).

Phase 4: Tests

Test 1: Post-Reconditioning test

Twenty-four hr following weak reconditioning, all three groups were exposed to the context for 24 min. Average freezing behavior was not significantly affected by Group in a one-way ANOVA (Figure 7C; $F_{(2, 21)}$ =2.09, p =.149). However, there was a significant effect of Group ($F_{(2, 21)}$ =3.44, p < .05) when comparing the mean first 6

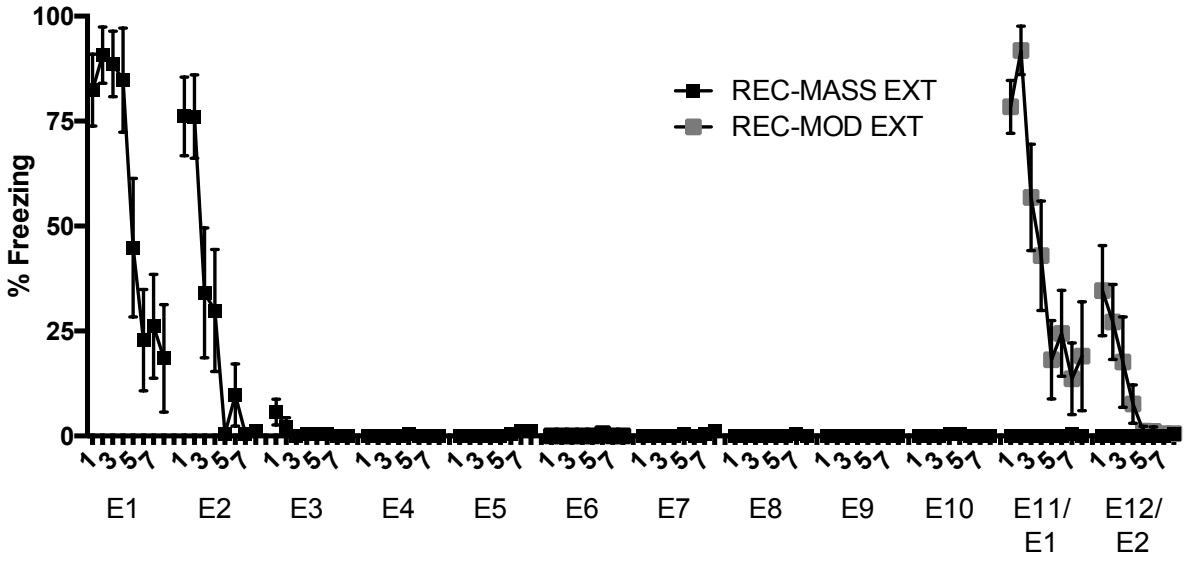


Figure 8. Extinction Sessions in 3 min time bins for Experiment 2B. Mean freezing for each 3 min time bin of each extinction session for; REC-MOD EXT, n=8; REC-MASS EXT, n=8. Error bars represent standard error of the mean.

min of freezing of the test. REC-MASS EXT animals froze significantly more in the first 6 min of the test than COND animals ($q_{(21)}$ = 3.60, p < .05; data not shown; M= 64.72, SEM= 12.32 and M= 30.28, SEM= 6.57, respectively).

It is important to note that REC-MASS EXT and REC-MOD EXT did not differ significantly in their reacquisition ($q_{(21)}$ = 1.01, p = .74). Similar to Experiment 2A, increasing the amount of extinction training, even when increased from 6 days (Experiment 2A) to 12 days (Experiment 2B), did not reduce rapid reacquisition and may, in fact, have an enhancing effect on rapid reacquisition.

Due to the lack of a significant difference between REC groups, REC-MASS EXT and REC-MOD EXT were combined to form a single REC group. I found a trending significance of Group in a t-test; REC froze more than COND when comparing the average freezing for the entire session ($t_{(22)}$= 2.07, p = .051). If the first 6 min of freezing was compared, REC froze significantly more than COND ($t_{(22)}$= 2.6, p < .05; M= 59.86, SEM= 7.46 and M= 30.28, SEM= 6.57, respectively; data not shown). This effect showed that this experiment was somewhat able to replicate the expression of rapid reacquisition once again in rats.

Test 2, 3, and 4: Re-extinction and Long-Term Retention/Spontaneous Recovery Test

Testing continued 3 days after Test 1 (Test 2/Re-Extinction 2) and again another 3 days later (Test 3/Re-Extinction 3). Both Test 2 ($F_{(2, 21)}$ =3.06, p = .068) and 3 ($F_{(2, 21)}$ =2.75, p = .087) had a trending main effect of Group in respective one-way ANOVAs comparing freezing amongst all groups (Figure 7D). However, if REC groups were collapsed into a single REC group, both Test 2 ($t_{(15.09)}$ =-3.31, p < .01;

COND: M = .41, SEM = .17; RECOND: M = 10.94, SEM = 3.17) and 3 ($t_{(15.36)}$ =-2.95, $p < .01$; COND: M = .21, SEM = .14: RECOND: M = 4.13, SEM = 1.32) revealed significantly more freezing by REC than COND. By the last 6 min of Test 3/Re-Extinction 3, groups had insignificant differences in freezing ($F_{(2, 21)}$ =0.660, p = .527) and animals reached low levels of freezing that showed successful re-extinction (data not shown; REC-MASS EXT: M= 2.76, SEM= 2.76, REC-MOD EXT: M= .00, SEM= .00, COND: M= 1.09, SEM= 1.09).

To test for long-term memory and spontaneous recovery of the conditioning and reconditioning, an additional test was run on Day 33 (17 days following reconditioning; Test 4). A one-way ANOVA of the average freezing of all groups did not reveal a significant effect of Group ($F_{(2, 21)}$ =1.99, p = .162). However, when REC groups are collapsed for comparison, I found that the rapid reacquisition effect ($t_{(16.23)}$ =-2.83, $p < .05$) persisted after re-extinction and an extended period of rest, despite low level of freezing (Figure 7E).

Discussion

Once more I found that rapid reacquisition of contextual fear was not impaired by massive extinction, even when massive extinction was increased (Experiment 2A vs 2B). Rather, rapid reacquisition may be enhanced by massive extinction training, as REC-MASS EXT showed a clearer rapid reacquisition effect relative to REC-MOD EXT. As stated above, this outcome was an unexpected result, due to what has been found in mice and rats previously. It is important to note that the rapid reacquisition effect in Experiment 2B was moderate and only detected when reconditioned groups were collapsed or all groups were compared in the first 6 min

of tests. Even so, the results of re-extinction and the long-term retention test suggested that reconditioning did lead to a persistent enhancement in freezing over initial conditioning that endured through repeated exposure to the conditioned context.

Experiment 2C. Gradual Rather than Rapid Suppression of Conditioned Responding During Extinction Does Not Impair Rapid Reacquisition

In Experiments 2A and 2B, I did not see an impairment of rapid reacquisition by massive extinction in rats, despite previous findings showing that massive extinction slowed reacquisition (Bouton, 1986; Leung et al., 2007) and my previous findings preventing rapid reacquisition in mice. I hypothesized that the extinction parameters in Experiments 2A and 2B might not result in extinction strong enough to impair rapid reacquisition due to several possibilities.

First, in Experiments 2A and 2B rats extinguished their freezing behavior quickly and only showed signs of spontaneous recovery 24 hr after the first extinction session, but in no subsequent extinction sessions after (Figure 6 & 8). Some work has shown that preventing conditioned responding during extinction learning can impair extinction learning (Krupa & Thompson, 2003; Mercado, Corcoran, Milad, & Quirk, 2006) and perhaps the lack of freezing seen after two or three extinction session may cause later extinction sessions to have little impact on inhibitory learning. Additionally, a lack of conditioned responding in later extinction sessions could be preventing prediction error mechanisms that allow for extinction (Rescorla, 2000a) or gradual extinction that could cause stronger inhibition of

conditioned responding recovery (Gershman, Jones, Norman, Monfils, & Niv, 2013). A final possibility was that the minimal 24 hr-delay spontaneous recovery at the beginning of each extinction session was impairing extinction memory as the presence of spontaneous recovery had been shown to deepen extinction (Leung & Westbrook, 2008). Thus, Experiment 2C examined if more gradual suppression of conditioned freezing over multiple extinction sessions was necessary to deepen inhibitory learning and impair rapid reacquisition.

Methods

Animals

Sixteen male naïve Long-Evans rats on a Th-Cre +/- background were used in this experiment separated into two groups that differed in extinction session length: reconditioning following 24 min extinction sessions (REC-24 MIN EXT, similar to reconditioning groups above) and reconditioning following 6 min extinction sessions (REC-6 MIN EXT).

Days 1 and 2 consisted of conditioning (Phase 1). For REC-24 MIN EXT, days 3 through 6 consisted of extinction training as described above (24 min of exposure to the context without shock). For REC-6 MIN EXT, Days 3 through 6 also consisted of extinction training, but these sessions were only 6 min of nonreinforced context exposure.

Day 7 was the reconditioning session (Phase 3) followed by the post-reconditioning test on Day 8 for all groups (Phase 4). The behavioral schedule is outlined in Figure 9A.

Results

Phase 1: Conditioning

Conditioning on Day 1 and 2 led to acquisition of the conditioned freezing response over the two sessions in both groups (data not shown). A two-way RMANOVA revealed a significant main effect of Acquisition session ($F_{(1, 14)}$ =17.99, p < .001; session one: M = 33.02, SEM = 4.60; session two: M = 57.39, SEM = 4.31), but did not find a significant main effect of Group ($F_{(1, 14)}$ = .29, p = .865; REC-6 MIN EXT: M = 45.83, SEM = 5.84; REC-24 MIN EXT: M = 44.58, SEM = 5.04) or interaction ($F_{(1, 14)}$ = .014, p = .907), suggesting that the groups showed equally increased freezing behavior during acquisition prior to different extinction training.

Phase 2: Extinction

Despite differences in extinction session timing, extinction caused conditioned freezing to lower in both groups (Figure 9B). A RMANOVA comparing the average percent freezing among groups found a significant main effect of Group ($F_{(1, 14)}$ =17.40, p < .001) and Extinction session ($F_{(3, 42)}$ =67.44, p < .001) and a significant interaction ($F_{(3, 42)}$ =5.66, p < .01).

As expected, the Group effect was driven by significantly more freezing in REC-6 MIN EXT relative to REC-24 MIN EXT across all extinction sessions. REC-6 MIN EXT was given less context exposure in each session (6 min vs 24 min) and thus had more freezing during each subsequent extinction session. The main effect of Extinction session was due to significantly more freezing displayed by both groups in E1 than E2 ($q_{(14)}$ = 6.76, p < .01), E3 ($q_{(14)}$ = 8.90, p < .01), and E4 ($q_{(14)}$ = 10.64, p < .01) as well as in E2 than E4 ($q_{(14)}$ = 3.88, p < .05). Significantly larger freezing

A.

	Acq	Ext	Rec	Test 1
	Day 1-2	*Day 3-10*	*Day 15*	*Day 16*
REC- 6 MIN EXT	12 min ++++	6 min -	3 min +	24 min -
REC-24 MIN EXT	12 min ++++	24 min -		

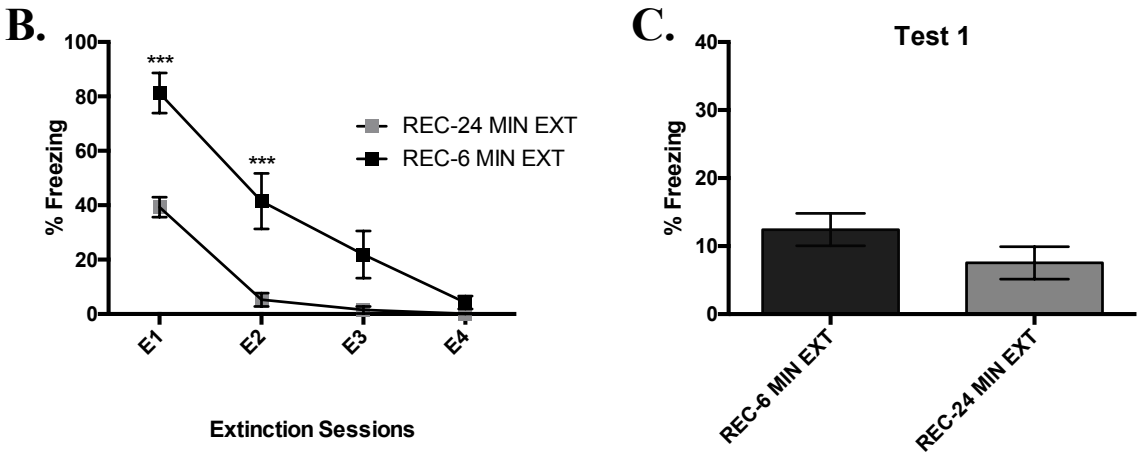


Figure 9. Gradual Rather than Rapid Suppression of Conditioned Responding During Extinction Does Not Impair Rapid Reacquisition. (**A**) Overview of the design of Experiment 2C. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .75 mA shock and a minus sign indicates exposure to the context without shock. (**B**) Mean freezing of each extinction session. (**C**) Mean freezing of Test 1 (1 day after reconditioning) for each group. Significance between groups, is represented by *** $p < .001$. REC-6 MIN EXT, n=8; REC-24 MIN EXT, n=8; COND, n=8. Error bars represent standard error of the mean.

by REC-6 MIN EXT than REC-24 MIN EXT in E1 ($q_{(42)}$ = 12.77, p < .001) and E2 ($q_{(42)}$ = 8.97 p < .001), but not E3 ($q_{(42)}$ = 1.12, p = .213) and E4 ($q_{(42)}$ = .823, p = .999) caused the significant interaction of Group X Extinction session. These results showed that decreasing the time of nonreinforced context exposure led to increased average freezing shown by REC-6 MIN EXT relative to REC-24 MIN EXT over each subsequent extinction session. The REC-6 MIN EXT animals successfully extinguished the conditioned response to near zero percent freezing by E4, while REC-24 MIN EXT displayed near zero average percent freezing by E3.

Figure 10 shows each extinction session by 3 min time bins, in which REC-24 MIN EXT group exhibited a rapid decline in freezing. Behavior indicative of spontaneous recovery only occurred at the beginning of E2 relative to the last 3 min bin of E1 for REC-24 MIN EXT, but REC-6 MIN EXT showed no signs of spontaneous recovery after a 24 hr delay.

Phase 3: Reconditioning

During reconditioning, groups did not show differences in within-reconditioning freezing behavior (data not shown). A RMANOVA comparing the freezing 30 sec pre- and post-shock found a significant effect of Time ($F_{(1, 14)}$ = 7.46, p < .05; pre-shock: M = .00, SEM = .00, post-shock: M = 23.44, SEM = 8.38), but not a significant main effect of Group ($F_{(1, 14)}$ = .299, p = .593; REC-6 MIN EXT: M = 14.06, SEM = 7.56, REC-24 MIN EXT: M = 9.38, SEM = 5.53) or a Time X Group Interaction ($F_{(1, 14)}$ = .299, p = .593). This analysis showed that reacquisition of behavior did not immediately differ following reconditioning, but shock did generally enhance freezing.

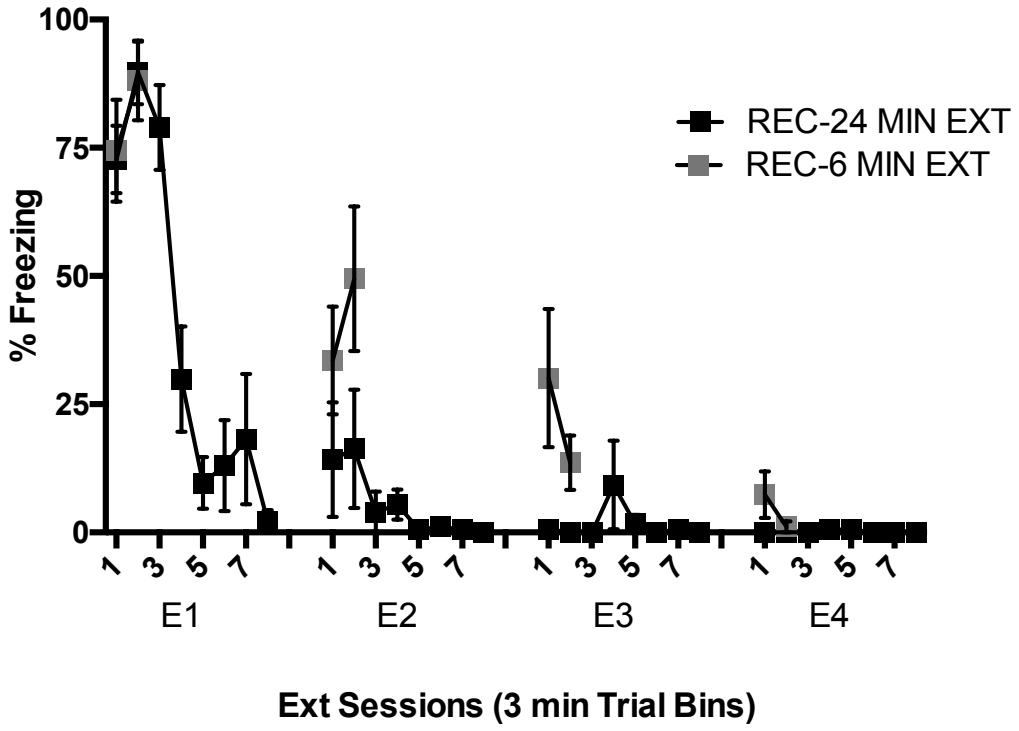


Figure 10. Extinction Sessions in 3 min time bins for Experiment 2C. Mean freezing for each 3 min time bin of each extinction session for; REC-6 MIN EXT, n=8; REC-24 MIN EXT, n=8. Error bars represent standard error of the mean.

Phase 4: Tests

Test 1: Post-Reconditioning Test

Twenty-four hr following weak reconditioning, both groups were exposed to the context and showed no differences in reacquisition behavior (Figure 9C). Freezing behavior was analyzed with a one-way ANOVA that did not find a significant effect of Group ($F_{(1, 14)}$ =, p = .167). This result suggested that varying the length of extinction sessions did not alter reconditioning.

Discussion

In this experiment, I found once again that altering extinction parameters does not alter reacquisition in rats. When rats slowly extinguished their conditioned freezing response over four sessions, they did not show enhanced extinction or impaired reacquisition relative to animals that extinguished quickly in two sessions. This finding indicates that, unlike other previous results (Gershman et al., 2013; Krupa & Thompson, 2003), gradual extinction did not enhance extinction training. Interestingly, the 6 min extinction group did not show spontaneous recovery at all, which could be affecting extinction strength (Leung & Westbrook, 2008) and preventing extinction from impairing reacquisition. It is important to notice that the magnitude of reacquisition was rather small relative to other experiments and perhaps animals were at too low of a behavioral floor (i.e. freezing lowered to the lowest level observed) to observe group differences. Overall, Long Evans rats showed reacquisition of freezing behavior that was independent of extinction learning.

General Discussion

These experiments demonstrated that rapid reacquisition of contextual fear occurs following extinction. Thus, rapid reconditioning appears to involve the unmasking of a context-shock association that is suppressed by extinction. This effect occurred in rats and mice and generally persisted through repeated post-reconditioning tests. Additionally, this work shows that rapid reacquisition of contextual fear is not a result of a generalized enhancement in fear or due to other well-described behavioral phenomena, but is a specific context-associated effect of reconditioning. Interestingly, rats were more impervious to the effects of extinction training on rapid reacquisition, but also displayed less robust rapid reacquisition, than mice. Overall, the rapid reacquisition effect has great potential as a model of persistent memory and associative memory modulation and is a place of future study regarding rodent strain differences during extinction and reacquisition.

Rapid Reacquisition of Contextual Fear Following Reconditioning

In Experiments 1A, 2A and 2B, I found that reconditioning led to the rapid reacquisition of contextual fear in both mice and rats. The finding of rapid reacquisition has been seen in the literature previously (Bouton, Woods, & Pineño, 2004a; Leung et al., 2007; Napier et al., 1992). This work serves to extend these findings in mice, while also characterizing the rapid reacquisition effect in greater depth.

In Experiment 1A, enhanced reacquisition of fear behavior following a mild footshock in mice showed that extinction to a behavioral floor (freezing levels equal to a group that received context exposure in the absence of conditioning) did not

remove the memory of the initial conditioning, but rather that reconditioning can access the original conditioning memory and strengthen its expression. Similar findings have been shown by a variety of post-extinction phenomena (refer to the introduction; Pavlov, 1927; Rescorla, 2004b, Bouton & Bolles, 1979b, Bouton & Bolles, 1980; Rescorla & Heth, 1975). The current findings reinforce reacquisition as yet another way by which to study the way in which fear promoting and inhibitory associative memories interact and compete for expression over manipulation and time.

Importantly, the reconditioned mice in Experiment 1A displayed enhanced fear behavior after a mild footshock compared not only to a group conditioned and exposed to the context for the first time, but also to a group that received initial conditioning with equal context exposure (CTX). This result suggests that the enhanced fear expression of the reconditioned mice was not due to longer context exposure, but was caused by previous experiences of footshock in the context. Similar findings of rapid reacquisition have been found before in contextual fear conditioning in rats (Leung, Bailey, Laurent, & Westbrook, 2007).

In Experiment 2, I used rats to look at the translatability of the rapid reacquisition effect across two rodent strains. Mice have routinely been subjects of fear conditioning studies since the 1950's (Denenberg, 1958; Denenberg, Ross, & Ellsworth, 1959) and reliably show fear conditioning to a variety of conditioned stimuli (Balogh, Radcliffe, Logue, & Wehner, 2002). However, C57BL6/J (B6) mouse freezing behavior is notoriously difficult to extinguish to zero percent freezing, (Lattal & Maughan, 2012; Tipps et al., 2014). The extinction curves seen in Experiment 1

further reinforce the point that mice display baseline freezing behavior. Baseline freezing can complicate studies attempting to examine memory modulation after full extinction by confounding the determination of when mice have fully extinguished unless an additional group of animals is added (CTX group in Experiment 1). Conversely, rats quickly extinguish and display no conditioned freezing behavior (Anglada-Figueroa & Quirk, 2005; Laurent & Westbrook, 2008; Leung et al., 2007; Quirk et al., 2000), which provides a clear indication of full extinction of the conditioned behavior for further testing.

Thus, in Experiment 2, Long Evans (LE) rats were exposed to an updated rapid reacquisition procedure. Like B6 mice, LE rats repeatedly show enhanced freezing behavior following a mild re-pairing of footshock and context relative to rats acquiring for the first time (REC groups relative to COND). Further, LE rats displayed rapid reacquisition under a variety of procedural conditions (Experiment 2A and 2B). Rapid reacquisition occurs in LE rats, similar to B6 mice, despite extinction to a behavioral zero. This finding again suggests that extinction does not remove the original acquisition memory, but merely forms an inhibitory memory that competes for expression in the conditioned context.

However, compared to B6 mice, LE rats displayed less robust rapid reacquisition effects that required collapsing across groups or analyzing later post-reconditioning tests and re-extinction to confirm. Rats and mice have been shown to differ in social and defensive behavior (Blanchard, Griebel, Andrew Henrie, & Caroline Blanchard, 1997; Kummer et al., 2014; Lonstein & Fleming, 2001; Scott, 1966), neurobiology (Routh, Johnston, Harris, & Chitwood, 2009; Whishaw, Metz,

Kolb, & Pellis, 2001), and spatial memory tasks (Akers, Arruda-Carvalho, Josselyn, & Frankland, 2012; Einon, Humphreys, Chivers, Field, & Naylor, 1981; Jonasson, 2005; Whishaw & Tomie, 1996). Additionally, there are several findings of rodent-species differences relevant to the current work. Rats developed long-term contextual fear memory later (Akers et al., 2012) and formed more stable spatial representations over time (Hok, Poucet, Duvelle, Save, & Sargolini, 2016) than mice. These findings suggest that rats have more complex and developed brain regions dedicated to spatial memory that can allow for more precise, long-lasting contextual fear learning.

One particularly relevant finding showed that B6 mice and LE rats showed similar spatial learning under certain conditions, but employed different strategies that tended to be more flexible in LE rats relative to B6 mice (Cressant, Besson, Suarez, Cormier, & Granon, 2007). This difference could be critical for the outcomes seen in rapid reacquisition procedure outlined here, which requires rodents to modulate their learned behavior following both extinction and reconditioning. Perhaps greater flexibility in rats' behavioral responding leads to expression of both promoting and inhibiting fear memory during the reacquisition test as rats prepare to respond to another change in context-shock association. In other words, no shock delivery during the reacquisition test, signals a re-extinction of conditioned responding to which rats quickly adapt. Additional proof of rat behavioral flexibility was the extinction conditioned freezing in two to three sessions (Experiment 2). However, others found that behavioral flexibility, while quicker in rats relative to mice, was similar between the two rodent species in an adaptive decision-making

task (Jaramillo & Zador, 2014). While this finding suggests that both species can adapt to repeated changes in contingencies during a memory task, it also reveals that rats are quicker to adapt to these changes, which is inline with my result and theory. This hypothesis requires further study, but could explain differences in the strength of responding between rodent species.

Overall, the repeated findings of rapid reacquisition following reconditioning in both rodent strains suggest that this effect is a reproducible behavioral finding across rodent strains with potentially interesting rodent strain differences in reacquisition response and behavioral adaptability.

Persistence of Rapid Reacquisition Across B6 Mice and LE Rats

The retention tests in Experiment 1A, 2A, and 2B revealed that the rapid reacquisition of reconditioning was persistent up to as much as 17 days later during a second test. This finding suggests that the effect of mild reconditioning relative to mild initial conditioning does not diminish over time and can cause a long lasting enhancement in conditioned freezing despite extinction to zero. The persistency of rapid reacquisition also reveals a similarity to strong acquisition of fear conditioning, which has been shown to be persistent for up to 2 years (Gale et al., 2004), and to other classical (Mueller & Stewart, 2000; Mueller, Perdikaris, & Stewart, 2002; Schreurs, 1993) and operant (Bekinschtein et al., 2007; Diergaarde, Schoffelmeer, & De Vries, 2006; Grimm, Hope, Wise, & Shaham, 2001) conditioning paradigms. This persistency of acquisition also suggests that rapid reacquisition could have a lasting effect on expression of long-term association memory (up to years later). Further

testing at extended time points is needed to confirm the full extent of the persistency of rapid reacquisition.

The research here is significant for understanding how associative memory changes over time and how post-extinction conditioning sessions can strengthen the original associative memory in a persistent manner. Also, in the context of contextual fear conditioning, the persistency of enhanced conditioned fear behavior is relevant to PTSD. Those with PTSD often show a re-emergence of fear behavior despite treatment (Paunovic & Öst, 2001; Rothbaum & Davis, 2003), which can be compared to the persistent reemergence of conditioned fear behavior following reconditioning. Rapid reacquisition could provide a behavioral correlate for which to better understand the behavior and neurobiology of persistent fear memory and the susceptibility of fear behavior reemergence to a mild reminder, such as is seen in PTSD.

A caveat to the persistency of rapid reacquisition effect is that the same cohorts of animals were used to examine the short-term and long-term persistency of rapid reacquisition in all experiments. Repeated behavioral testing can bias results to be consistent across recurring tests (Stafford & Lattal, 2009). Leung et al., however, found that rapid reacquisition was persistent in animals that were tested in a long-term retention test exclusively. Thus, the finding of persistently enhanced freezing to the context is not likely due to repeated testing.

Rodent-Strain-Specific Extinction Dependency of Rapid Reacquisition

Experiment 1A and 1B found that fear behavior following reconditioning operates as a function of the amount of prior extinction received in B6 mice. In

Experiment 1A mice showed rapid reacquisition following reconditioning after moderate extinction. However, in Experiment 1B mice that received massive extinction, or extinction well beyond their behavioral floor (14 extinction sessions), did not show rapid reacquisition following reconditioning. Surprisingly, 14 days of extinction did not produce the slow reacquisition effect that has been found by others (Leung, Bailey, Laurent, & Westbrook, 2007; Bouton & Swartzentruber, 1989). While slow reacquisition was not found after massive extinction, there was no rapid reacquisition of fear behavior. This result showed that a large amount of extinction training impaired the ability of a single reconditioning footshock to strengthen or allow expression of the original conditioning memory, suggesting that additional inhibitory learning occurred beyond a behavioral baseline, similar to the concept of "silent extinction beyond the zero" (Pavlov, 1927).

Contrary to the extinction-dependency of the rapid reacquisition effect in B6 mice, LE rats showed rapid reacquisition regardless of the amount of extinction training received. In Experiment 2B, LE rats exposed to 6 days of extinction (REC-MASS EXT show an equivalent, and possibly even stronger, rapid reacquisition to the context as rats, who were extinguished for only 3 days (REC-MOD EXT). Further, in Experiment 2B, when the extinction training discrepancy between reconditioning groups was dramatically increased, LE rats extinguished for 12 days had similar, if not greater, rapid reacquisition as rats that were extinguished for only 2 days.

This finding contrasted from what was seen in B6 mice and what others have shown in LE and other strains of rats (Bouton, 1986; Bouton, Woods, & Pineño,

2004a; Leung et al., 2007). As mentioned earlier in the discussion, mice and rats show differences in a number of behavioral tasks, especially behavioral flexibility. One important difference between the behavior of B6 mice and LE rats in these experiments was the speed of extinction and amount of spontaneous recovery in each extinction session. In my experiments, LE rats fully extinguished conditioned responding in two to three sessions and showed spontaneous recovery of the acquisition memory in only the first extinction session. Meanwhile, B6 mice required six sessions of extinction to extinguish and showed signs of spontaneous recovery in several of the initial extinction sessions (Figures 2 and 4).

I hypothesized that the rapid extinction behavior of LE rats could be interfering with extinction strength due to its minimal within-extinction-session spontaneous recovery (Leung & Westbrook, 2008) and rapid loss of conditioned responding (Krupa & Thompson, 2003; Mercado et al., 2006; Rescorla, 2000a) that could be preventing prediction-error driven inhibitory learning during extinction (Rescorla, 2000b; Wagner, 1970). However, shortening the extinction session length to allow for gradual extinction of conditioned responding did not cause any differences in extinction strength as measured by its effect on reacquisition (Experiment 2c). Again, LE rats showed reacquisition that was impervious to changes in extinction training. This finding might reflect rats' superior behavioral flexibility (Cressant et al., 2007) and ability to quickly change behavior to new context contingencies despite efforts to over-train extinction.

Experiment 2C contrasted with studies that showed that the speed of extinction altered conditioned behavior in other post-extinction phenomena, such as

spontaneous recovery and reinstatement (Cain, Blouin, & Barad, 2003; Gershman et al., 2013). The result could suggest that rapid reacquisition is its own unique phenomenon that differs in its extinction-dependency from other post-extinction restorations of conditioned responding in LE rats. However, more work is necessary to confirm these results. Conversely, other studies have shown that that gradual extinction and expression of conditioned responding during extinction may not be necessary for learning of extinction (Ouyang & Thomas, 2005; Zimmerman & Maren, 2010). Perhaps this experiment did not use the ideal method to enhance extinction learning in order to impair rapid reacquisition.

Experiment 2C was also unable to confirm if the presence of spontaneous recovery during extinction sessions strengthens extinction (Leung & Westbrook, 2008). Interestingly, the LE rats receiving shorter extinction session lengths displayed no signs of spontaneous recovery. Potentially, the brief extinction session reactivated the original conditioning memory rather than extinguished it (Lee, Milton, & Everitt, 2006). Reactivation would result in no spontaneous recovery of fear due to reactivation rather than extinction. However, this theory is unlikely to be correct as four brief extinction sessions resulted in a full extinction of conditioned freezing. Additionally, while a 24 hr delay between extinction sessions often causes an initial rebound in conditioned behavior that declines with repeated extinction (Quirk, 2002; Rescorla, 2004b), the experimental design was not ideal to study spontaneous recovery due to rather small delays between extinction sessions (Rescorla, 2004b). Additionally, Experiment 2C did not specifically examine the contrast of acquisition and rapid reacquisition, so I cannot conclude that length of extinction sessions is not

a determining factor of speed of reacquisition relative to acquisition within rats. Thus, future studies are necessary to examine the role of within-extinction-session spontaneous recovery in extinction strength and its ability to impair rapid reacquisition in LE rats.

One key difference in the behavioral schedule between the rodent strains was the number of acquisition sessions during conditioning. LE rats underwent two acquisition sessions in order to increase freezing to a level that could be extinguished over multiple extinction sessions; while B6 mice only received one acquisition session in order to acquire substantial fear behavior. The increase in acquisition sessions in LE rats could have caused rapid reacquisition to be the more likely outcome of reconditioning. Ricker and Bouton (1996) showed that conditioning paradigms that require more conditioning session for acquisition favored rapid reacquisition following reconditioning due to sequential learning. Sequential learning is when CS-US pairings start to predict further CS-US pairing due to extensive acquisition training (Capaldi, 1994). Thus, the experimental design for rats could be favoring rapid reacquisition and preventing massive extinction from impairing the effect. Additional experiments will be necessary to examine the possibility of sequential learning in LE rats.

Another methodological consideration between the rodent species was the phase of the circadian cycle reconditioning occurred. Behavioral manipulations occurred during the light cycle for B6 mice and during the dark cycle for LE rats. As both rodent species are nocturnal, the rats were presumably more active and alert than the mice when exposed to conditioning (Rhodes, Garland, & Gammie, 2003).

Additionally, corticosterone, a critical stress response molecule in rodents, shows circadian patterns of expression, with larger corticosterone expression at during the evening (particularly high corticosterone immediately before the dark cycle) than the morning hours during 12 hr light-dark conditions (Kakihana & Moore, 1976; Ulrich-Lai, Arnhold & Engeland, 2006). Also, corticosterone increases following shock (Weinstock et al., 1998). Thus, delivering a conditioning shock at different phases of the circadian rhythm when corticosterone is unequal could bias the animal's initial response to and memory shock. This procedural difference could bias the LE rats to have different behavior and could explain the resistance to massive extinction training. Studies examining rapid reacquisition in mice during the dark phase will be necessary to explore circadian influences on reacquisition.

Additionally, the behavioral preparation here differed from a similarly conducted study that found that massive extinction impaired rapid reacquisition in LE rats (Leung et al., 2007). In the work by Leung et al., both their conditioning and reconditioning sessions were a short 3 min context exposure with one shock (delivered 1 min into the session) and their extinction was twice-daily with an extinction criterion requiring the absence of freezing across the initial minute of exposure. Perhaps, procedural differences, such as weaker initial conditioning and twice-daily extinction sessions, led to changes in the strength of both conditioning and extinction memories that allowed rapid reacquisition to be impaired by massive extinction training. The evidence of slow reacquisition found by the design in Leung et al. (2007) is relevant to the theory that additional conditioning training during initial

conditioning in Experiment 2 might have allowed sequential learning to favor rapid reacquisition in rats, regardless of extinction training differences.

An important consideration of the rodent strain difference in the extinction-reacquisition interaction found here is that perhaps a strain differences are driving the effect rather than species difference is driving this effect. For example, Blanchard et al., 1997 found that C57/BL6N mice displayed similar predator responses as most rats, while other strains of mice differ considerably in their behavior, and that this similarity could be due to the degree of domestication in both of B6 mice and rats. Additionally, strains of both mice (Balogh et al., 2002) and rats (Graham, Yoon, Lee, & Kim, 2009) display differences in fear conditioning from one another. Strain differences in classical conditioning of both species will be an important focus of further studies of the extinction-dependency of reacquisition speed.

It is interesting that none of these experiments reveal evidence of slow reacquisition as has been seen in others (Bouton, 1986; Bouton, Woods, & Pineño, 2004a), especially when extinction training is massive (Leung et al., 2007). In the literature, there is evidence that slow reacquisition can be more likely after fear reconditioning (Bouton, 1986; 2003) and other paradigms where few conditioning trials are necessary to acquire (Ricker & Bouton, 1996). Yet, rapid reacquisition repeatedly occurred in my studies, and even when extinction was extended in rats. One large difference between these studies and the ones cited above is that context was used as the conditioned stimulus rather than a discrete cue. Bouton (2002) discusses how important contextual cues may be to the slow or rapid reacquisition of

CR through a mechanism similar to renewal, particularly when CS-US associations become a part of the contextual cues and predict further CS-US pairings. In this theory, a single shock in the context could cause the animals to expect more shocks and a renewal of fear behavior. These experiments were designed with context as the primary conditioned stimulus and thus it is difficult to examine if additional contextual cues modulate whether reacquisition was slow or rapid in my experiments. However, the initial conditioning parameters were not overly extensive, even in the rats, and thus did not favor the possibility of renewal or sequential learning causing the enhancement of fear behavior.

Moderate Context Pre-exposure Facilitates Conditioning, While Massive Context Pre-Exposure Impairs Conditioning.

In all experiments with mice, an additional context pre-exposure conditioning group was included to equate context exposure and provide baseline freezing behavior in mice. In Experiment 1A, 7 days of exposure of the context (CTX) enhanced fear behavior 1 day after a mild foot shock relative to a group that had not seen the context until conditioning (COND). This result is consistent with the context pre-exposure facilitation effect (Rudy & O'Reilly, 1999), in which context pre-exposure allows the animals to better learn the distinct spatial configuration of the context and pair said context with a single footshock, and perceptual learning of CS alone exposures prior to conditioning (Mackintosh, Kaye, & Bennett, 2018; McLaren, Kaye, & Mackintosh, 1989). However, this effect needs to be confirmed with further groups that control for context specificity of the pre-exposure facilitation. Yet, the finding that the rapid reacquisition effect was larger than the effect of context pre-

exposure further demonstrated that the increased freezing following reconditioning is due to fear conditioning history not exposure to the context. However, the contrast of context pre-exposure and rapid reacquisition was not explored in rats and perhaps context exposure effects could be driving the rodent strain differences reported above.

Conversely, in Experiment 1b, exposure to the context 15 days prior to conditioning inhibited retention of acquisition compared to the group of mice that was naïve to the context prior to conditioning seen in the long-term retention test. This finding mimics findings of latent inhibition, which occurs when CS is overexposed and made familiar before conditioning (Lubow & Moore, 1959) and has also been seen in contextual fear conditioning (Killcross, Kiernan, Dwyer, & Westbrook, 1998; Leung et al., 2007). However, generally latent inhibition is only seen shortly after conditioning and dissipates with time to reveal the effect of conditioning (Killcross, Kiernan, Dwyer, & Westbrook, 1998). These findings will need to be studied in further depth to understand the effects of massive context exposure upon weak conditioning. One interpretation that can be made is that strength of conditioning depends on the amount of prior context exposure; similar to the way that reconditioning depends on the amount of extinction received in mice. Hence, mild pre-exposure to the context enhanced the conditioning of a single footshock, but massive context pre-exposure caused a latent-inhibition-like impairment of conditioning.

Conditioning-Context-Specificity of Rapid Reacquisition

There are many possible alternative causes for rapid reacquisition. Above, the possibility of sequential learning (CS-US pairing predicting more CS-US pairing; Bouton, 2002a; Capaldi, 1994; Ricker & Bouton, 1996) was discussed. Another possibility is that rapid reacquisition is a form of reinstatement. The shock delivered during reconditioning could have acted as a reminder of the original CS-US association and caused reinstatement of the fear behavior as opposed to an actual reconditioning of the CS-US association (Bouton & Bolles, 1979). Likewise, rapid reacquisition could be a result of a generalized enhancement of freezing behavior due to repeated exposure to shock. Rodents may have become sensitized to shock and exposure to further shock in any context could enhance fear learning, like in Stress Enhanced Fear Learning of rats (Rau & Fanselow, 2009).

In order to address some of these possibilities, I ran Experiment 2A in rats. Animals that received their reconditioning shock in a distinct context from the conditioning context (REC-MOD EXT-SWITCH) did not show rapid-reacquisition-like freezing in the original context (Figure 5B) or in the distinct reconditioning context (Figure 5C). In fact, these rats appeared to show no generalization across contexts. REC-MOD EXT-SWITCH showed near zero percent freezing in the original context (CTX A), suggesting that extinction of the original context was still intact and unaltered by the receiving of shock. In the distinct “switch” context (CTX B), REC-MOD EXT-SWITCH showed freezing similar to rats receiving shock for the first time (COND) and their freezing was markedly less than what is seen in rapid reacquisition, suggesting that they formed a separate acquisition memory for a

distinct context. Therefore, rapid reacquisition is CS-specific, or occurs only when the original context is reconditioned.

These studies did not directly address some additional theoretical causes of rapid reacquisition: sequential learning, learning-to-learn, or the ability of rapid reacquisition to reactivate and strengthen the original excitatory memory. Yet, this experiment shows that rapid reacquisition cannot be conflated with reinstatement to the US nor can it be considered a general enhancement in freezing to any context. Rapid reacquisition of contextual fear is likely its own unique phenomena caused by context-specific learning.

In conclusion, I propose that the rapid reacquisition effect is a distinct post-extinction behavioral finding that occurs in a CS-specific and persistent manner in response to reconditioning of contextual fear in two rodent species. The paradigm described herein provides a model for which to study mechanisms of memory modulation, persistent fear memory, and rodent strains differences in extinction and reacquisition.

Chapter 3: Effects of Acute Ethanol Withdrawal and Acute Ethanol Administration on Rapid Reacquisition of Contextual Fear Conditioning

Parts of the Chapter are adapted from a submitted paper: AR Williams & KM Lattal (2018), "Rapid reacquisition of contextual fear following extinction in mice: Effects of amount of extinction, acute ethanol withdrawal, and ethanol intoxication" for possible publication in Psychopharmacology.

Abstract

Many studies show that acute ethanol intoxication and withdrawal impair initial acquisition or extinction of learned behaviors. Yet, the effects of initial alcohol intoxication and withdrawal on post-extinction memory are not currently studied. One post-extinction restoration of conditioned responding with particular relevance to relapse of fear in PTSD, rapid reacquisition, is particularly understudied. This chapter investigated the impact of acute ethanol withdrawal (AEW) and initial ethanol administration on rapid reacquisition of contextual fear. Rapid reconditioning, but not initial conditioning or extinction, was impaired by AEW. In contrast, acute ethanol administration impaired generally impaired the acquisition and reacquisition of freezing, with an especially strong impairment on acquisition over reacquisition. Additionally, acute ethanol intoxication showed a biphasic response of freezing expression. These findings show that acute ethanol withdrawal and administration may differentially affect different phases of conditioning. Results are discussed in terms of current ideas about acquisition, extinction, and reacquisition and ethanol's effects on memory and neurobiology.

Introduction

Alcohol use disorders (AUDs) and post-traumatic stress disorder (PTSD) are highly comorbid conditions (Blanco et al., 2013) and this comorbidity tends to cause more severe symptoms in both disorders (Blanco et al., 2013; Herman, 1992; Saladin, Brady, Dansky, & Kilpatrick, 1995). There are multiple theories for why this comorbidity exists (e.g., stress, self-medication, high-risk population, etc.; Brown, Read, & Kahler, 2003; McCauley, Killeen, Gros, Brady, & Back, 2012; Stewart, 1996), yet the direct links between AUDs and PTSD are still unknown. To begin to elucidate AUD and PTSD comorbidity, it is important to understand how preclinical models of both disorders interact.

In the rodent laboratory, fear conditioning procedures are used to model some of the memory aspects of PTSD, such as the formation of a long-term fear memory and conditioned behavior to fear-related cues (Pitman, 1989; Rothbaum & Davis, 2003). Additionally, with repeated presentations of the previously fearful stimulus, the extinction of conditioned fear behavior models some aspects of exposure therapy. Many studies have found that extinction causes relatively temporary changes in behavior, with the suppressed behavior returning over time (spontaneous recovery; Pavlov, 1927; Rescorla, 2004b), changes in context (renewal; Bouton & Bolles, 1979b), re-exposure to the unconditioned stimulus (US; reinstatement; Bouton & Bolles, 1980; Rescorla & Heth, 1975), or after a weak reconditioning episode. In general, reconditioning involves the re-pairing of CS and US and can result in the rapid reacquisition of fear behavior (Bouton, Woods, & Pineño, 2004a; Leung et al., 2007), suggesting that the CS-US association is

suppressed but not erased by extinction. Rapid reacquisition of fear behavior is of interest because it mimics the susceptibility of those with post-traumatic stress disorder (PTSD) to relapse after a mild stressor and to become hypervigilant, which are symptoms that worsen in those with comorbid AUDs (Saladin et al., 1995). Little is known about the mechanisms and circuitry of reconditioning and how ethanol intoxication or withdrawal may interact with rapid reacquisition of fear.

Ethanol acts on many different cellular and molecular processes and has complicated behavioral effects. Ethanol intoxication can impair fear conditioning (Broadwater & Spear, 2013; Gould, 2003; Melia, Ryabinin, Corodimas, Wilson, & LeDoux, 1996) and extinction (Bisby et al., 2015; Broadwater & Spear, 2013; Holmes et al., 2012; Lattal, 2007). Yet, ethanol intoxication can sometimes promote fear conditioning depending on the dose and timing of ethanol administration (Bruce & Pihl, 1997; Gulick & Gould, 2007), where mild intoxication can enhance fear conditioning, but a larger intoxicating dose can impair development of a similar memory. Further, ethanol has selective effects, depending on whether conditioning involves contextual or discrete cues (Gould, 2003; Kitaichi et al., 1995; Melia et al., 1996), which is thought to be caused by ethanol's particularly strong influence on the hippocampus and hippocampus-dependent tasks, like contextual fear conditioning (Ryabinin, Melia, Cole, Bloom, & Wilson, 1995). Additionally, ethanol can create an internal context that may signal the operation of acquisition or extinction contingencies (Cunningham, 1979; Lattal, 2007).

Although acute alcohol intoxication has been shown to alter fear conditioning and extinction, less is known about the effects of withdrawal from alcohol on these

learning processes. Withdrawal from alcohol is generally assumed to create a negative internal state that is alleviated through the negative reinforcing effects of alcohol (De Witte, Pinto, Ansseau, & Verbanck, 2003; Heilig, Egli, Crabbe, & Becker, 2010; Koob & Le Moal, 2008). Similar to intoxication, withdrawal from ethanol has been found to both enhance and impair fear conditioning and extinction (Bertotto, Bustos, Molina, & Martijena, 2006; Borlikova, Elbers, & Stephens, 2006; Quiñones-Laracuente, Hernández-Rodríguez, Bravo-Rivera, Melendez, & Quirk, 2015; Ripley, O'Shea, & Stephens, 2003). Further, ethanol withdrawal has also been shown to have specific effects on the brain that are distinct from ethanol intoxication, in particular on learning-related brain regions such as the mPFC, amygdala, and hippocampus (reviewed in Vilpoux, Warnault, Pierrefiche, Daoust, & Naassila, 2009; White & Best, 2000).

Acute ethanol withdrawal (AEW), which is the peak of withdrawal following the first exposure to ethanol, results in both physical and psychological alterations (Karadayian & Cutrera, 2013; Karadayian, Busso, Feleder, & Cutrera, 2013), including alterations in brain activity (Kozell, Hitzemann, & Buck, 2005; Vilpoux et al., 2009) and impairments in initial contextual fear conditioning in C57BL/6J mice (Tipps, Raybuck, Buck, & Lattal, 2015). Little is known about effects of AEW on later learning, such as extinction or post-extinction re-emergence of behavior, processes which tend to be altered in patients with PTSD (Rothbaum & Davis, 2003). While AEW does not model AUD, it can provide insight into how the first withdrawal from ethanol impacts mechanisms of fear learning.

Given the lack of research on the interaction of initial withdrawal and extinction and post-extinction conditioned behavior, this Chapter sought to characterize the effects of AEW on extinction (Experiment 3), post-extinction rapid reconditioning of contextual fear (Experiment 4), and initial conditioning (Experiment 5; replication of Tipps et al., 2015), as well as the effect of initial ethanol administration on rapid reacquisition of contextual fear conditioning (Experiment 6). Overall, I found that AEW's effects on contextual fear memory were modest, but learning phase specific, and deserve further systematic examination.

Experiment 3: AEW Does Not Affect Extinction of Contextual Fear

Chronic forms of withdrawal can impair extinction of conditioned fear behavior (Ripley et al., 2003), but the effect of initial withdrawal from ethanol (i.e., AEW) upon inhibitory memory formation remains unknown. AEW is capable of impairing initial contextual fear conditioning (Tipps et al., 2015); thus, this experiment sought to explore the effect of AEW on the formation and expression of contextual fear extinction.

Methods

Animals and Housing

Thirty-two C57BL/6J male mice (n= 8/group) were purchased from Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and allowed to acclimate to the vivarium at Oregon Health & Science University (OHSU) for a full 7 days before any handling or behavioral procedures were begun. All experimental procedures were approved by the OHSU Institutional Animal Use and Care Committee and were

conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985). The housing conditions were 4 mice to a cage. The room temperature was constant at 22°C ± 1°C and the mice were kept on a 12h light-dark schedule. Food and water were available ad libitum and all behavioral experiments occurred from 0900 to 1400, during their light cycle.

Apparatus. The fear conditioning room contained four Coulbourn Instruments mouse-conditioning chambers (H10-11M-TC; Allentown, PA) in sound- and light-attenuating chambers with a fan producing 70 dB of background noise. Each chamber was equipped with a circular Plexiglas arena (21.5 cm in diameter and 23 cm in height) placed on a grid floor of stainless steel rods (3.2 mm in diameter, spaced 6.4 mm apart). The grid floor was set to deliver a .35 mA scrambled shock via a 110/120 VAC 50-60 Hz computer-controlled shock generator (Coulbourn H13-15) and an infrared activity monitor (Coulbourn H24-61) fixed to the top of each chamber recorded freezing in Graphic State 3.01 software. The apparatus also contained a house light that was lit as soon as the session commenced and terminated as soon as the session was over. This setting was the context (CTX) that was conditioned to shock. Before and between each round of behavioral testing, the grid floor, Plexiglas arena, and tray were cleaned with 95 percent ethanol. Mice were always returned to the same conditioning chamber on subsequent days to provide consistency in context.

Freezing Assessment. The level of contextual fear conditioning was assessed by the amount of freezing, the natural conditioned response upon re-

exposure to a cue or context associated with shock (Fanselow & Bolles, 1979). Freezing was considered continuous inactivity (with the exception of breathing) for 2 sec. Freezing was measured in real-time by visual time sampling in which the experimenter would assess each animal every 8 s for freezing and hand-score the presence or absence of freezing.

Acute Ethanol Withdrawal

Acute ethanol withdrawal (AEW) was defined as 6 hr following a 4 g/kg intraperitoneal (IP) injection of 20% v/v ethanol (Tipps et al., 2015). In other words, AEW is the withdrawal from first alcohol administration. This procedure has previously caused signs of alcohol withdrawal in many inbred strains of mice (increased handling-induced convulsions), especially in withdrawal sensitive lines like DBA/2J (Metten & Crabbe, 1994). The strain used in this Chapter, C57BL/6J, shows low withdrawal sensitivity, but in response to AEW, B6 mice have previously shown impaired contextual fear conditioning (Tipps et al., 2015). Animals in the control saline groups (SAL) received a sham injection of saline of a proportionate volume 6 hr prior to behavior. Animals who did not receive complete injections on Day 2 were removed from results and analyses (n = 2). Injections were given in the morning of the light cycle 6 hr prior to behavior.

General Behavioral Procedure

Prior to conditioning, mice were habituated to handling, injections, and transport for 3 days. Generally, contextual fear conditioning consisted of a 12-min context exposure (described in the Apparatus section above) with four 2 sec, .35 mA footshocks at 2.5, 5, 9, and 11.5 min into the session. Extinction consisted of 24 min

of non-shocked context exposure 24 hr after conditioning. Test sessions were identical to extinction sessions (24-min nonreinforced exposure to the context).

Behavioral Schedule

The full behavioral schedule is in Figure 11A. All animals received contextual fear conditioning on Day 1. On the following day, half of the mice received extinction 6 hr following ethanol (EXT-AEW) or saline injection (EXT-SAL). The other half received the same injections, but remained in their homecages during extinction (NoEXT-AEW or NoEXT-SAL). On Day 3, all groups received a test session to assess the fear memory associated with the context.

Statistics

The main dependent variable was the percent time the animal spent freezing. R-Studio (Boston, MA) and GraphPad Software Prism 6 (La Jolla, CA) were used to run all statistics and create figures, respectively. Freezing behavior was compared for using analysis of variance (ANOVA) with main factors of Group (Extinction and No Extinction), Treatment (AEW or Saline), and, if a repeated measures ANOVA (RMANOVA), Time (3 min bins or Extinction sessions). Any failure to meet the homogeneity of variances criterion for an ANOVA (as measured by the Brown-Forsythe Levene's test) was accounted for using a Welch correction. If significance was found for main effects or interactions, Tukey's HSD tests (or Games-Howell for unequal variances amongst groups) were used for simple comparisons between groups and sessions. A priori hypothesis exploring group differences were conducted with simple t-tests. For all statistical tests, significance was set at α 0.05.

Results

Conditioning

Prior to AEW or extinction treatment on Day 2, I determined group assignments by balancing the average percent freezing displayed by each animal during acquisition (data not shown). A two-way ANOVA, comparing percent freezing during acquisition across future Treatment (AEW or Saline) x future Extinction (Ext or NoExt) did not find a significant effect of Treatment ($F_{(1,26)}$ = 0.008, p = 0.931), Extinction ($F_{(1,26)}$ = 0.040, p = 0.843) or Treatment x Extinction ($F_{(1,26)}$ = 0.086, p = 0.772), confirming groups were balanced before manipulation the next day (freezing by group; EXT-AEW: M = 27.24, SEM = 4.77, EXT-SAL: M = 28.75, SEM = 5.58, NoEXT-AEW: M = 29.19, SEM = 2.36, NoEXT-SAL: M = 28.38, SEM = 2.11).

Extinction During AEW

Comparing the effects of AEW directly on extinction, an RMANOVA comparing the between-subjects factor of Treatment (AEW or Saline) x the within-subjects factor of Time bin (eight 3-min time bins in the 24-min session) in the groups that received extinction (No Extinction groups are not included as they were not exposed to extinction) found a significant effect of Time ($F_{(1,13)}$ = 29.60, p < .001), which suggests that extinction successfully lowers the percent time spent freezing within a session (Figure 11B). The ANOVA did not find a significant effect of Treatment upon extinction of freezing ($F_{(1,13)}$ = 0.917, p = 0.356) nor a significant Time X Treatment interaction ($F_{(1,13)}$ = 0.208, p = 0.656), showing that AEW did not alter the expression of freezing during initial retrieval or the decrease in freezing that occurred over the course of extinction.

A.

Group	Acquisition	Extinction	Test
	Day 1	***Day 2***	*Day 3*
EXT-AEW	12 min ++++	**🡻24 min -**	24 min -
EXT-SAL	12 min ++++	**🡻24 min -**	24 min -
NoEXT-AEW	12 min ++++	**🡻Handled**	24 min -
NoEXT-SAL	12 min ++++	**🡻Handled**	24 min -

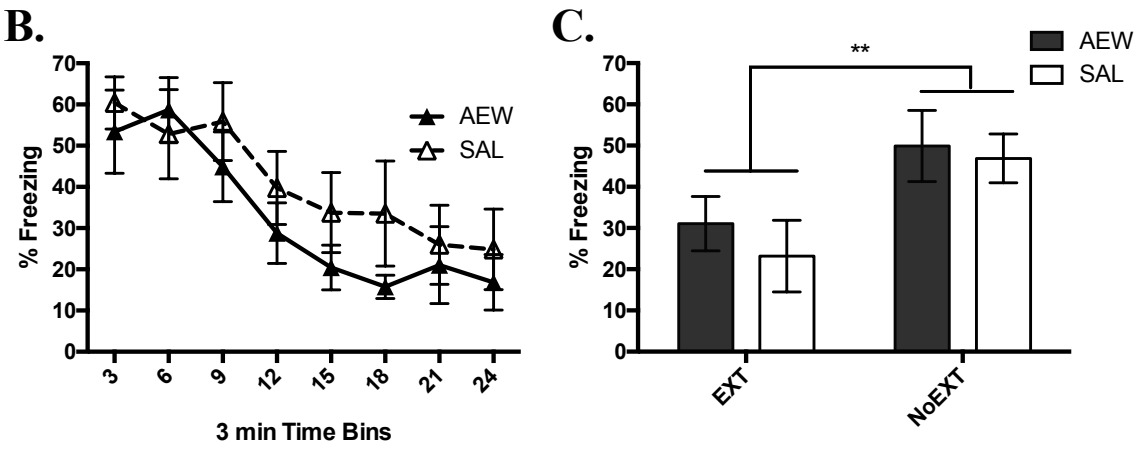


Figure 11. AEW Does Not Affect Extinction of Contextual Fear. (**A**) Overview of the design of Experiment 3. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .35 mA shock and a minus sign indicates exposure to the context without shock. 🡻 indicates the administration of ethanol or saline prior to the session. (**B**) Mean percent freezing during the extinction session (Day 2) in which animals were 6 hr post acute ethanol injection (EXT-AEW) or saline injection (EXT-SAL). (**C**) Mean percent freezing during the test session (Day 3) of all groups; EXT-AEW, n = 8; EXT-SAL, n = 7; NoEXT-AEW, n = 7; NoEXT-SAL, n = 8; **, $p < .01$. Error bars represent standard error of the mean.

Test Following Extinction During AEW

In the test comparing the retention of extinction under AEW (Figure 11C), a two-way ANOVA comparing Extinction training (EXT or NoEXT) and Treatment (AEW or Saline) found a significant main effect of Extinction training ($F_{(1,26)} = 7.97$, $p < .01$) on average percent freezing, but not of Treatment ($F_{(1,26)} = 0.541$, $p = 0.468$) or an Extinction x Treatment interaction ($F_{(1,26)} = 0.109$, $p = 0.744$; Figure 11C), suggesting that acute ethanol withdrawal affected neither the development nor the expression of extinction 24 hr later.

Discussion

Overall, this experiment showed that AEW did not impair extinction of contextual fear conditioning, which differs from AEW's impairment of contextual fear acquisition (Tipps et al., 2015). This discrepancy suggests that AEW may act on specific brain regions relevant to promoting, but not inhibiting conditioned fear memory and behavior. This explanation is plausible as others have shown withdrawal resulted in a distinct pattern of brain activity relative to ethanol intoxication (Kozell et al., 2005; Vilpoux et al., 2009). Given this result, I examined the impact of AEW on post-extinction reconditioning in the next experiment.

Experiment 4: AEW Moderately Impairs Rapid Reacquisition of Contextual Fear

In Chapter 2, I described the behavioral characteristics of rapid reacquisition of contextual fear. I showed that rapid reacquisition is a unique post-extinction outcome that differs behaviorally from initial acquisition. Therefore, Experiment 4

aimed to examine the effect of AEW on reacquisition relative to its effects on initial acquisition. I hypothesized that AEW would impair rapid reacquisition of conditioned contextual fear, as previously AEW impaired acquisition (Tipps et al., 2015) and both are processes of promoting fear memory formation.

Methods

Most methods described in Experiment 3 were replicated in Experiment 4, with the exception of modifications to the behavioral procedure. Forty-eight male C57BL/6J mice were used (6 groups, n=8/group). The behavioral procedure included reconditioning, which was a 3 min context exposure with a single 2 sec, .35 mA footshock delivered at 2.5 min into the session. The reconditioning session is milder than the conditioning session to avoid all animals reaching a high level of freezing in test sessions. Again, test sessions were identical to extinction sessions (24-min nonreinforced exposure to the context).

Behavioral Schedule

Animals underwent the rapid reacquisition procedure (described in Chapter 2) while under AEW to assess the effects of initial withdrawal on reacquisition of conditioned contextual fear behavior (full schedule in Figure 12A).

For Phase 1 on Day 1, mice in group RECOND experienced contextual fear conditioning, while group CTX received nonreinforced exposure to the context for an equal amount of time (12 min). For Phase 2 on Days 2 through 7, RECOND and CTX both had extinction training, as described in Experiment 1A and 3. Five mice were removed from this study for not meeting the extinction criterion established for all reconditioning studies (freezing below 30 percent in the final extinction session).

Mice in group COND were handled and transported an equal amount without exposure to context or shock on Days 1 through 7. For Phase 3, on Day 8, all mice received reconditioning following AEW (RECOND-AEW, CTX-AEW, and COND-AEW) or Saline (RECOND-SAL, CTX-SAL, and COND-SAL) treatment. The following day, a test session occurred for all animals (Phase 4).

Statistics

The statistical analysis of this experiment was similar to Experiment 3, except reconditioning was analyzed with a three-way RMANOVA comparing Group (RECOND, CTX, or COND) X Treatment (AEW or SAL) X Time (Pre-shock or Post-shock) to compare the average freezing 30 sec before and following shock. This analysis was included to examine the direct effect of AEW on shock-induced freezing behavior.

Results

Phase 1: Conditioning

A two-way ANOVA comparing average percent freezing across Group (RECOND and CTX) and future Treatment (AEW or SAL) during conditioning found that there was a significant effect of Group ($F_{(1, 23)}$ = 39.42, p < .001; data not shown; RECOND: M = 24.00, SEM = 3.63, CTX: M = 2.15, SEM = .63), but not Treatment ($F_{(1, 23)}$ = 0.385, p = .541; AEW: M = 9.42, SEM = 3.94, SAL: M = 14.13, SEM = 3.66) nor a Treatment x Group Interaction ($F_{(1, 23)}$ = 0.038, p = .846). The significant Group effect was driven by RECOND responding significantly more to context-shock pairing than CTX to nonreinforced context exposure. The lack of a significant

Treatment effect or interaction showed that treatments were balanced before extinction training.

Phase 2: Extinction

To compare extinction between groups with different conditioning experience, I ran a three-way RMANOVA comparing the between-subjects measures of Group (RECOND and CTX) and Treatment (AEW or Saline) and the within-subjects factor of Session (Extinction Sessions 1 through 6; Figure 12B). Animals had not received different treatment (AEW or saline) during extinction, but future treatment was still included as a factor in the extinction analysis to ensure that there were no major differences in treatment groups prior to reconditioning. The three-way RMANOVA found a significant main effect of Group ($F_{(1, 23)}$ =28.57, $p < .001$), Extinction Session ($F_{(5,115)}$ =9.28, $p < .001$) and a Group X Extinction Session interaction ($F_{(5, 115)}$ = 22.24, $p < .001$), but not significant effect of Treatment ($F_{(1, 23)}$ = .631, $p = .435$) or any other interaction.

The significant Group X Extinction Session effect was again driven by RECOND groups freezing more than CTX groups in E1 ($q_{(25)}$ = 13.03, $p < .001$), E2 ($q_{(25)}$ = 6.61, $p < .001$), and E3 ($q_{(24)}$ = 5.91, $p < .01$) as revealed by post-hoc analyses, but not in later extinction sessions when groups converged. Unsurprisingly, this interaction showed that groups initially differed significantly in their freezing due to their different behavioral treatments; i.e., conditioning led to significantly more freezing than mere context exposure. It also showed that by three extinction sessions groups were statistically indistinguishable. However, after 6 sessions groups were visually similar in freezing (Figure 12B).

A.

Group	Acquisition	Extinction	Recondition	Test
	Day 1	*Day 2-7*	***Day 8***	*Day 9*
CTX-AEW	12 min -	24 min -	**🡻3 min +**	24 min -
CTX-SAL	12 min -	24 min -	**🡻3 min +**	24 min -
RECOND-AEW	12 min ++++	24 min -	**🡻3 min +**	24 min -
RECOND-SAL	12 min ++++	24 min -	**🡻3 min +**	24 min -
COND-AEW	Handled	Handled	**🡻3 min +**	24 min -
COND-SAL	Handled	Handled	**🡻3 min +**	24 min -

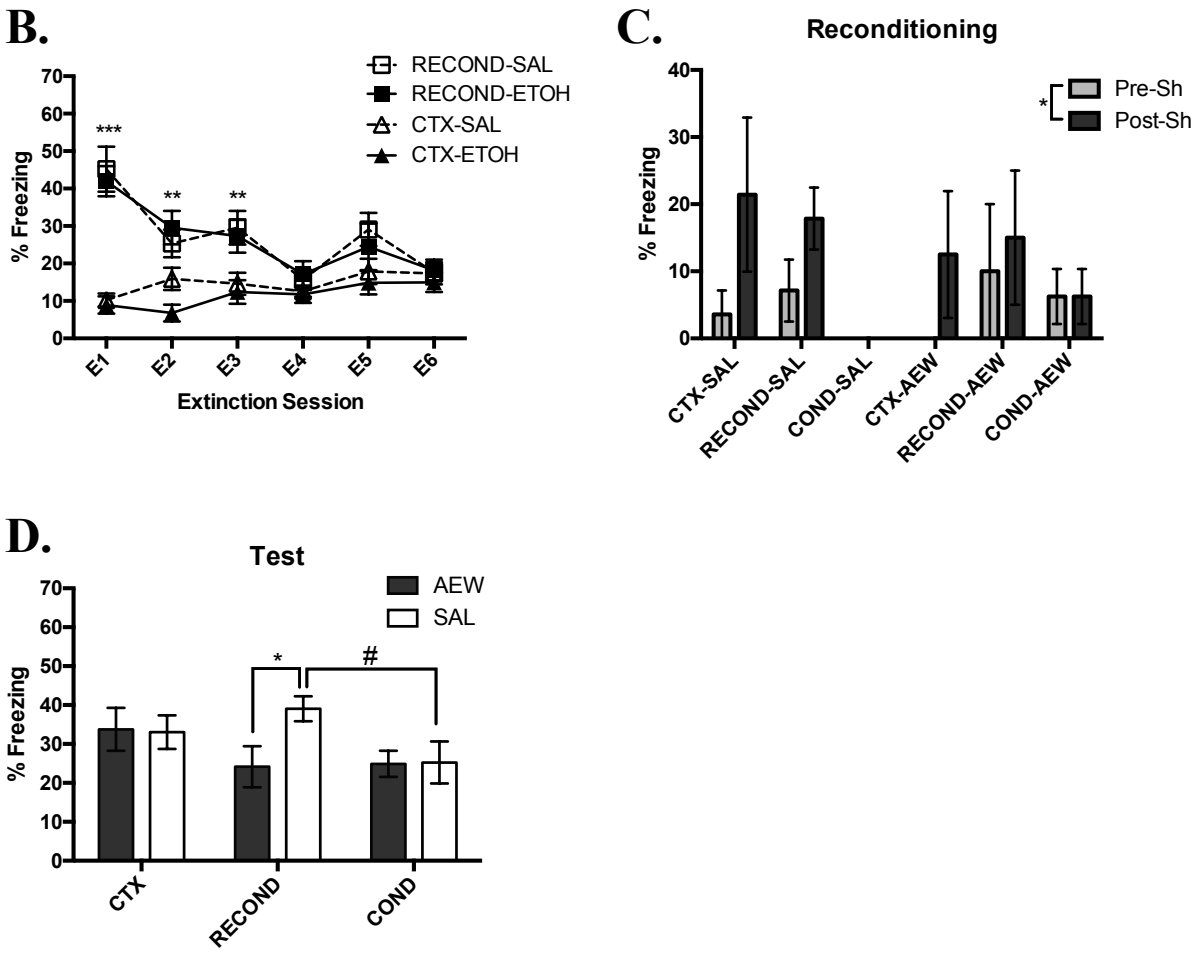


Figure 12. AEW Moderately Impairs Rapid Reacquisition of Contextual Fear.

(**A**) Overview of the design of Experiment 4. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .35 mA shock and a minus sign indicates exposure to the context without shock. 🡻 indicates

the administration of ethanol or saline prior to the session. (**B**) Mean percent freezing during extinction sessions (Days 2-7) of RECOND and CTX groups. (**C**) Mean percent Freezing of all groups during the 30 sec pre and post shock during Phase 3 under AEW or SAL(Day 8). (**D**) Mean percent freezing of all groups during the test session (Day 9) 24 hrs following reconditioning under AEW or SAL. CTX-AEW, n = 8; CTX-SAL, n = 7; RECOND-AEW, n = 5; RECOND-SAL, n = 7; COND-AEW, n = 8; COND-SAL, n = 8; #, $p = .054$; *, $p < .05$ **, $p < .01$; ***, $p < .001$. Error bars represent the standard error of the mean.

Phase 3: Reconditioning During AEW

To assess the effects of AEW directly during reconditioning, a three-way RMANOVA comparing the freezing to context in the 30 sec pre- and post-shock (Time) among Group (RECOND, COND, and CTX) and Treatment (AEW or Saline) was run. There was a significant main effect of Time ($F_{(1, 37)}$ = 4.57, p < .05; Figure 12C). There was also a trending main effect of Group ($F_{(2, 37)}$ = 2.72, p = .078), driven by a trend in higher freezing in RECOND relative to COND ($q_{(40)}$ = 1.89, p = .081). There was no significant main effect of Treatment ($F_{(1, 37)}$ = .00, p = 1.00), suggesting that AEW did not alter the ability of animals to freeze during reconditioning. Additionally, there were not any significant interactions.

Phase 4: Test Following Reconditioning During AEW

Figure 12D shows the mean freezing of all groups in the test assessing the strength of acquisition or reacquisition of contextual fear conditioning under AEW or sham conditions 24 hr before. In a two-way ANOVA comparing Group (RECOND, CTX, and COND) and Treatment (AEW or saline), there were not significant effects of Group ($F_{(2,37)}$ = 2.10, p = .136), Treatment ($F_{(1,37)}$ = 1.14, p = .292), or Group X Treatment interaction ($F_{(2,37)}$ = 1.55, p = .225). Given the results from experiments above and from Tipps et al. (2015), I had three a priori hypothesis that were tested with unpaired t-tests.

In Tipps et al. (2015), AEW impaired contextual fear conditioning and thus I expected that COND-AEW would show significantly impaired fear memory in the test session compared to COND-SAL. However, there was no significant difference between groups ($t_{(14)}$ = .062, p = .952, Figure 12D). This finding indicated that AEW

might not always impair contextual fear conditioning. I also hypothesized that rapid reacquisition following reconditioning would occur in saline groups. An unpaired t-test comparing RECOND-SAL and COND-SAL was not significant, but trending on significance ($t_{(13)}$ = 2.11, p = .054; Figure 12D), which suggested that there was a mild rapid reacquisition of fear behavior in reconditioned animals when compared to conditioned animals.

The final a priori hypothesis was that AEW would impair the rapid reacquisition of fear because previous work suggests that AEW impairs initial conditioning (Tipps et al., 2015). An unpaired t-test comparing RECOND-AEW and RECOND-SAL was significant ($t_{(10)}$ = 2.56, p < .05; Figure 12D) because animals that were reconditioned while experiencing AEW showed significantly less retention of contextual fear than animals reconditioned under normal conditions.

Discussion

This experiment demonstrated that AEW had a relatively mild effect on rapid reacquisition following extinction. Reconditioning under AEW caused a moderate impairment of the reacquisition during the retention test 24 hr later. I also somewhat replicated the rapid reacquisition effect during the test 24 hr after reconditioning in saline animals. Surprisingly, the impairing effect of AEW on initial acquisition (Tipps et al., 2015) was not replicated, which indicates that AEW might not always affect contextual conditioning. However, this experiment did use relatively weak conditioning parameters (3 min context exposure with 1 shock) that might not have allowed levels of freezing that could be impaired by AEW. The next experiment explored the interaction of conditioning strength and AEW.

Experiment 5: AEW Does Not Affect Contextual Fear Conditioning

Due to the lack of acquisition impairment by AEW in the last experiment, the goal of Experiment 5 was to examine if initial acquisition must be strong in order to be affected by AEW as seen previously in Tipps et al. (2015).

Methods

The methods of Experiment 5 are identical to Experiment 3 and 4 with the exception of modifications to the behavioral procedure. Thirty-two male C57BL/6J mice were used (4 groups, n=8/group). Strong conditioning is described above as contextual fear conditioning (12 min session with four footshocks). Weak conditioning, however, consists of a short 3 min context exposure with a single delivery of footshock, identical to the parameters of reconditioning described above.

On Day 1, half of the mice received strong conditioning either under AEW (4 Shock-AEW) or control conditions (4 Shock-SAL). The other half experienced AEW (1 Shock-AEW) or control conditions (1 Shock-SAL) during weak conditioning. The following day all animals were tested for their acquisition memory in a test session (24 min of nonreinforced context exposure). Refer to Figure 13A for the full schedule.

Results

Conditioning During AEW

To assess if AEW impaired freezing response during conditioning, a two-way ANOVA comparing the freezing during acquisition between Group (1 Shock or 4 Shocks) and Treatment (AEW or Saline) was run. I found a significant main effect of group ($F_{(1,28)}$ = 88.42, p < .001; Figure 13B), but not a significant effect of treatment

($F_{(1,28)}$ = .09, p = .77). This analysis demonstrates that 4 context-shock pairings resulted in a stronger within-conditioning-session freezing response than a shorter, 1 shock conditioning session. AEW had no effect on the freezing response to strong or weak conditioning.

Test Following Acquisition During AEW

Freezing behavior in the test of retention of strong or weak conditioning is displayed in Figure 13C. A two-way ANOVA comparing Group (1 Shock or 4 Shocks) and Treatment (AEW or Saline) found a significant main effect of Group ($F_{(1,28)}$ = 17.11, p < .001), but not Treatment ($F_{(1,28)}$ = .091, p = .765) or Treatment X Group interaction ($F_{(1,28)}$ = 1.07, p = .310). This result demonstrated that acquisition expression increased with an increase in shock number, but neither weak nor strong acquisition was affected by AEW during conditioning.

Discussion

This experiment replicated the acquisition findings in Experiment 4, but not those previously found by Tipps et al. (2015). I again found that acquisition, whether strong or weak, was unaffected by AEW. These results suggested that rapid reacquisition was impacted more than acquisition by AEW. In summation with past findings, these results showed that AEW's effects on acquisition could be mixed and perhaps specific to only some conditioning parameters.

A.

Group	Acquisition	Test
	Day 1	*Day 2*
1Shock-AEW	**🡻3 min +**	24 min –
1Shock-SAL	**🡻3 min +**	24 min –
4Shock-AEW	**🡻12 min ++++**	24 min –
4Shock-SAL	**🡻12 min ++++**	24 min –

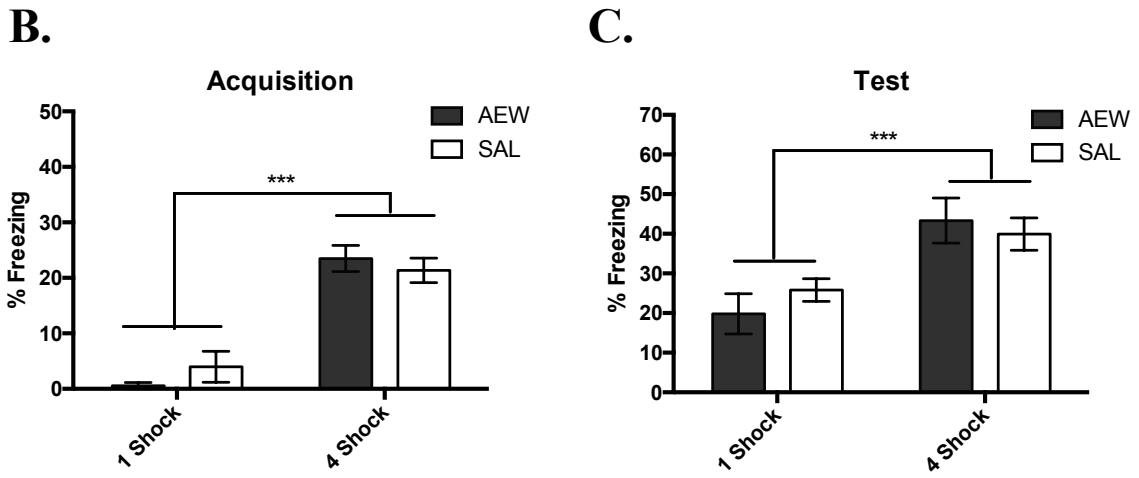


Figure 13. AEW Does Not Affect Contextual Fear Conditioning (A) Overview of the design of Experiment 5. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .35 mA shock and a minus sign indicates exposure to the context without shock. 🡻 indicates the administration of ethanol or saline prior to the session. (**B**) Mean percent freezing during strong (4 Shock) or weak (1 Shock) conditioning (Day 1) in which animals were 6 hr post acute ethanol injection (AEW) or saline injection (SAL). (**C**) Mean percent freezing during the test session (Day 2) 24 hrs following strong conditioning. 1 Shock-AEW, n = 8; 1 Shock-SAL, n = 8; 4 Shock-AEW, n = 8; 4 Shock-SAL, n = 8. ***, $p < .001$. Error bars represent the standard error of the mean.

Experiment 6: Acute Ethanol Administration Generally Impairs Fear Learning

Initial ethanol intoxication and its effects on conditioned fear memory have been more thoroughly explored than AEW and fear memory, but the impact of initial intoxication on rapid reacquisition is unknown. This experiment examined the effect of initial acute ethanol administration on rapid reacquisition of contextual fear. I hypothesized that ethanol administration would likely impair acquisition (as seen in similar behavioral preparations in Gould, 2003) and might impair rapid reacquisition, as both are mechanisms promoting fear memory.

Methods

To assess the effects of acute ethanol administration on rapid reacquisition, Experiment 4 was replicated, but on Day 8 animals either received acute ethanol administration (RECOND-INTX, CTX-INTX, and COND-INTX) or saline (RECOND-SAL, CTX-SAL, and COND-SAL) treatment. Acute ethanol administration in this chapter was defined as 5 min following 1.5 g/kg IP injection of 20% v/v ethanol. This dose has previously been used as an intoxicating dose in C57BL/6J strain (Crabbe, Cameron, Munn, Bunning, & Wahlsten, 2001) and has caused impairments of both contextual fear conditioning and extinction memories (Gould, 2003; Lattal, 2007). Forty-eight male C57BL/6J mice were used (6 groups, n=8/group). Refer to Figure 14A for the full schedule.

Results

Phase 1: Conditioning

Prior to extinction, percent freezing during acquisition was compared across groups and future treatment. A two-way ANOVA comparing Group (RECOND vs

A.

Group	Acquisition	Extinction	Recondition	Test
	Day 1	*Day 2-7*	***Day 8***	*Day 9*
CTX-INTX	12 min -	24 min -	**↓3 min +**	24 min -
CTX-SAL	12 min -	24 min -	**↓3 min +**	24 min -
RECOND-INTX	12 min ++++	24 min -	**↓3 min +**	24 min -
RECOND-SAL	12 min ++++	24 min -	**↓3 min +**	24 min -
COND-INTX	Handled	Handled	**↓3 min +**	24 min -
COND-SAL	Handled	Handled	**↓3 min +**	24 min -

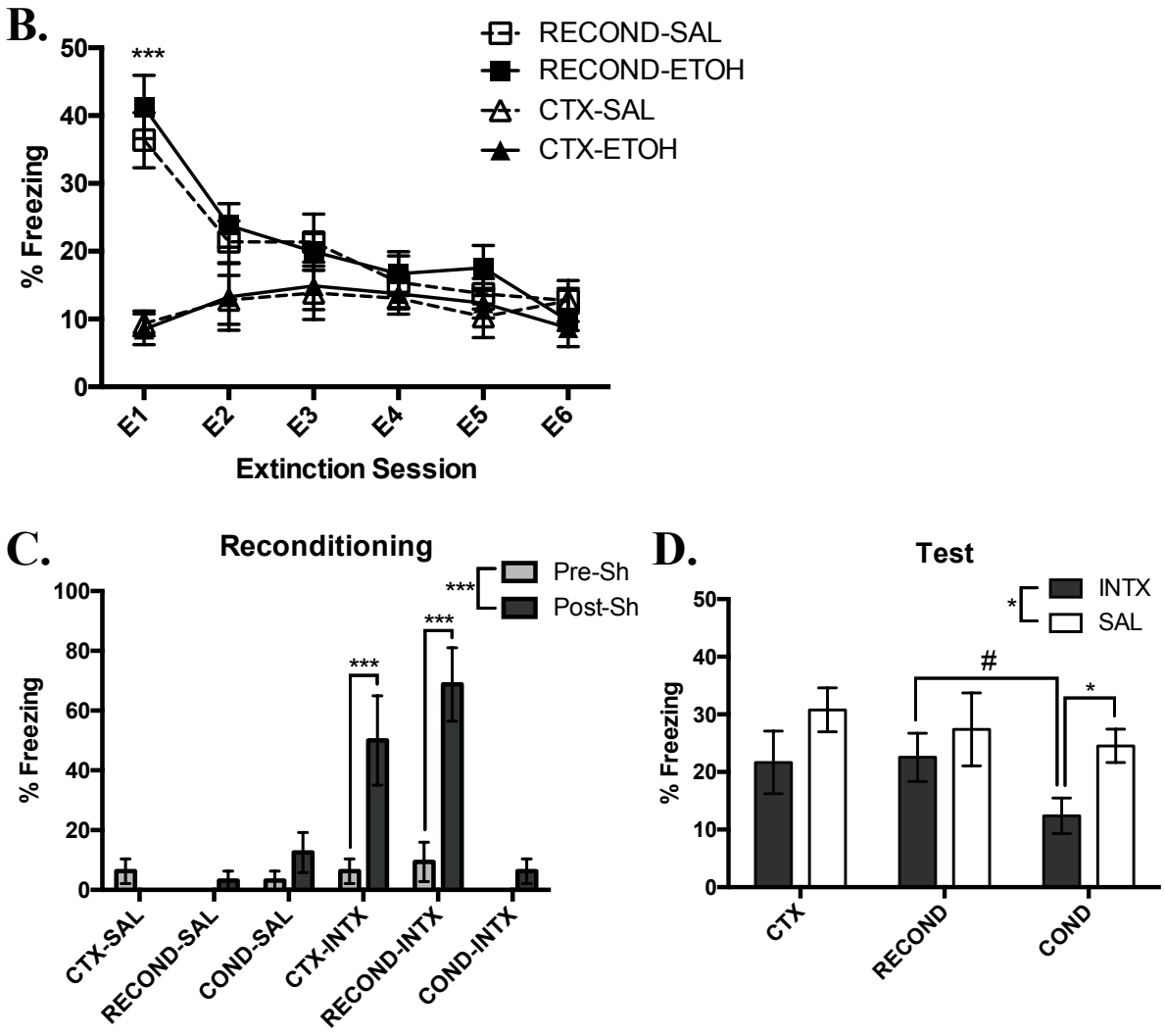


Figure 14. Acute Ethanol Administration Generally Impairs Fear Learning. (**A**) Overview of the design of Experiment 6. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .35 mA

shock and a minus sign indicates exposure to the context without shock. 🡻 indicates the administration of ethanol or saline prior to the session. (**B**) Mean percent freezing during extinction sessions (Days 2-7) of RECOND and CTX groups. (**C**) Mean percent freezing for 30 sec pre and post shock of all groups during Phase 3 (reconditioning) under acute ethanol administration (INTX) or SAL (Day 8). Further significant main effects and interactions are detailed in text. (**D**) Mean percent freezing of all groups during the test session (Day 9) 24 hrs following reconditioning under acute ethanol administration (INTX) or SAL. CTX-AEW, n = 8; CTX-SAL, n = 8; RECOND-AEW, n = 8; RECOND-SAL, n = 8; COND-AEW, n = 8; COND-SAL, n = 8; #, $p = .071$; *, $p < .05$ **, $p < .01$; ***, $p < .001$. Error bars represent the standard error of the mean.

CTX) and Treatment (INTX vs. SAL) found a significant effect of Group ($F_{(1,28)}$ = 53.44, p < .001; data not shown; RECOND: M = 19.81, SEM = 2.11, CTX: M = 2.77, SEM = .83), but did not show significant effects of future Treatment ($F_{(1,28)}$ = .042, p = .834; INTX: M = 11.05, SEM = 3.01, SAL: M = 11.53, SEM = 2.40) nor Group x Treatment interaction ($F_{(1,28)}$ = .398, p = .533). This finding indicated that conditioning caused significantly more freezing than context exposure (RECOND > CTX), but that the groups were balanced across treatments.

Phase 2: Extinction

Extinction of RECOND and CTX was analyzed in a three-way RMANOVA (Figure 14B), which found a significant main effect of Group (RECOND > CTX; $F_{(1,28)}$ =12.57, p < .01), Extinction Session ($F_{(5,140)}$ = 17.09, p < .001) and a Group X Extinction Session interaction ($F_{(5,140)}$ = 24.73, p < .001), but not a significant effect of Treatment ($F_{(1,28)}$ = .060, p = .808).

The significant Group X Extinction Session effect was again driven by RECOND groups freezing more than CTX groups in E1 ($q_{(30)}$ = 12.54 , p < .001) as revealed by post-hoc analyses, but not in later extinction sessions when groups converge. This finding demonstrated that groups had statistically similar freezing by extinction session 2, but extinction was ran for 6 sessions to maintain procedural similarity to previous experiments.

Phase 3: Reconditioning During Acute Ethanol Administration

To assess for the effects of acute ethanol administration directly on reconditioning, a three-way RMANOVA comparing the freezing to context in the 30 sec pre- and post-shock (Time) among Groups (RECOND, COND, and CTX) and

Treatment (INTX or Saline) was run (Figure 14C). There was a significant main effect of Group ($F_{(2, 42)}$ =4.12, p < .05) driven by RECOND freezing significantly more than COND ($q_{(45)}$ = 4.37, p < .01). There was also significant main effect of Time ($F_{(1, 42)}$ =34.10, p < .001). The final main effect was a significant main effect of Treatment ($F_{(1, 42)}$ =19.92, p < .001), in which INTX groups froze more than saline groups, suggesting that acute ethanol administration, unlike AEW in the experiments above, generally enhanced the freezing response of all groups during conditioning. In addition to main effects, there was a significant Group X Treatment interaction ($F_{(2, 42)}$ = 8.40, p < .001), Group X Time interaction ($F_{(2, 42)}$ = 4.21, p < .05), Treatment X Time interaction ($F_{(1, 42)}$ = 27.13, p < .001), and Group X Treatment X Time three-way interaction ($F_{(2, 42)}$ = 8.15, p < .01).

The three-way interaction was driven by RECOND-INTX showing significantly more post-shock freezing than RECOND-SAL ($q_{(42)}$ = 9.74, p < .001) and CTX-INTX showing significantly more post-shock freezing than CTX-SAL ($q_{(42)}$ =6.85, p < .001), whereas COND-INTX did not freeze significantly more post-shock than COND-SAL ($q_{(42)}$ = .00, p = .999). Additionally, the REC and CTX treatment differences were insignificant before shock (all $q_{(42)}$ < 0.5, p > .98). The interaction was also driven by REC-INTX ($q_{(42)}$ =9.92, p < .001) and CTX-INTX ($q_{(42)}$ =5.78, p < .001) freezing more following shock than before shock, whereas others group did not significantly increase their freezing following shock. In the 30 sec post-shock, there was additional significant differences: RECOND-INTX froze more than COND-INTX ($q_{(42)}$ = 8.97, p < .001), COND-SAL ($q_{(42)}$ = 9.57, p < .001) and CTX-SAL ($q_{(42)}$ =

10.04, $p < .001$); and CTX-INTX froze more than COND-INTX ($q_{(42)} = 4.93$, $p < .001$), COND-SAL ($q_{(42)} = 4.81$, $p < .001$), and REC-SAL ($q_{(42)} = 7.38$, $p < .001$).

Importantly, these interaction effects showed that intoxicated mice had much stronger freezing response during a reconditioning shock and a context pre-exposed conditioning shock when compared to saline-treated mice (Figure 14C) and ethanol administration might facilitate short-term rapid reacquisition during reconditioning and acquisition during conditioning of a pre-exposed context.

Phase 4: Test Following Reconditioning During Acute Ethanol Administration

In the test following reconditioning (Figure 14D), a two-way ANOVA comparing Group (RECOND, CTX, and COND) and Treatment (ethanol or saline) found a significant effect of Treatment ($F_{(1,42)} = 5.72$, $p < .05$), but not of Group ($F_{(2,42)} = 1.74$, $p = .187$) or Group X Treatment interaction ($F_{(2,42)} = .339$, $p = .715$). The significant effect of Treatment was driven by the general dampening effect of administration on freezing behavior in the subsequent test day.

Many examples in the literature show that intoxication impairs initial contextual fear conditioning (Gould, 2003; Kitaichi et al., 1995), thus I predicted decreased freezing in COND and CTX groups who were intoxicated during conditioning (Day 8, Reconditioning day for RECOND). An unpaired t-test revealed significantly lower freezing behavior by COND-INTX when compared to COND-SAL ($t_{(14)} = 2.88$, $p < .05$; Figure 14D), but a non-significant difference between CTX-INTX and CTX-SAL ($t_{(14)} = 1.38$, $p = .191$), suggesting that context pre-exposure might prevent impairment of conditioning by acute ethanol administration. I also

hypothesized that if initial conditioning and reconditioning memories could both be affected by AEW under certain conditions, they might be similarly impacted by ethanol administration. Thus, I examined the difference in RECOND-INTX and RECOND-SAL in the test and found there was not a significant difference in their freezing behavior ($t_{(14)}$ = .639, p = .533). I also hypothesized that I would replicate the rapid reacquisition effect in the non-intoxicated animals, but surprisingly when comparing RECOND-SAL and COND-SAL there was not a significant differences in freezing behavior ($t_{(14)}$ = .411, p = .687). There was however a trending difference between RECOND and COND groups within the ethanol administration condition ($t_{(14)}$ = 1.96, p = .071; Figure 14D).

Discussion

This experiment showed that ethanol administration had a direct enhancing effect on fear conditioning acquisition and reacquisition that later caused a general impairment of expression of conditioned freezing the following day, with a particularly strong impairment on initial acquisition relative to reacquisition. The impairment of acquisition expression by intoxication has been demonstrated in the literature previously (Gould, 2003; Gulick & Gould, 2007; Kitaichi et al., 1995), but this experiment was the first time a direct comparison to reacquisition expression showed that ethanol administration's memory-impairing effects were minimal on fear memory reacquisition and, in this case, actually somewhat facilitated rapid reacquisition.

General Discussion

The major conclusion from these experiments is that initial withdrawal from ethanol has modest, but specific, effects on memory. Specifically, I found that AEW moderately impaired the expression of rapid reacquisition of fear following post-extinction reconditioning, but had no effect on extinction or conditioning. Further, there was a general biphasic effect of acute ethanol administration on both acquisition and post-extinction reacquisition, with an especially strong impairment of initial acquisition expression.

AEW Moderately Impairs Rapid Reacquisition, but Not Initial Acquisition or Extinction of Contextual Fear Conditioning

Experiment 4 demonstrated that post-extinction reconditioning caused rapid reacquisition of fear that was reduced by AEW. However, in Experiment 4, I failed to replicate AEW-induced impairment of conditioning, as was seen previously (Tipps et al., 2015). Conditioning had relatively weak parameters (3 min context exposure and a single .35 mA shock) compared to Tipps et al. (2015). Thus, Experiment 5 compared the effects of AEW on strong (4 shock-context pairings) and weak (1 shock-context pairing) contextual fear conditioning and found that AEW impaired neither weak nor strong conditioning. It is important to note that the behavioral procedures of Experiment 5 did not perfectly replicate those used by Tipps et al. (2015). These results could suggest that AEW's effect on fear conditioning is very specific, similar to other findings of ethanol intoxication (Gulick & Gould, 2007; Experiment 6).

One must also consider the saliency of the two conditioning sessions and their vulnerability to impairment by AEW. Despite the identical parameters of conditioning and reconditioning sessions in Experiment 4, initial conditioning was likely a relatively more salient event compared to reconditioning in terms of the expectancy (first CS-US pairing session vs. second; Mackintosh, 1975; Rescorla & Wagner, 1972). Due to the increased saliency, initial conditioning could be more impervious to the memory-impairing effects of AEW. However, it is also theorized that rapid reacquisition occurs from a reactivation and strengthening of the original CS-US association (Rescorla, 2001; 2002), which should make reacquisition more resistant to memory-impairing drugs. Future studies should explore the effects of AEW on saliency and memory updating events.

These findings are not the first instance of different types or reoccurrences of learning relying on different brain regions or mechanisms. It has been shown that both short and long-term memory (Bekinschtein et al., 2007; McGaugh & Dawson, 1971; Yeh, Lin, & Gean, 2004) and conditioning- and retrieval-induced plasticity (Alberini, 2005; Hertzen, 2005), rely on similar neural mechanisms, yet have a distinct pattern of molecular processes. Further, the acquisition of initial extinction requires activity in the BLA, while re-extinction does not (Laurent, Marchand, & Westbrook, 2008). Hence, it is entirely likely that acquisition and reacquisition of contextual fear could recruit a different set of brain regions or mechanisms that are selectively susceptible to the effects of AEW. Accordingly, both protein synthesis and actin rearrangement are important for fear acquisition in the BLA and CA1, but

only actin rearrangement, not protein synthesis, appears to be involved in fear reacquisition (Motanis & Maroun, 2012).

In Experiment 3, AEW had no effect on the development or expression of extinction when tested 24 hr later. These results differ from the effects of acute ethanol administration and chronic ethanol withdrawal, which often impair extinction learning (Bisby et al., 2015; Holmes et al., 2012; Lattal, 2007, Bertotto et al., 2006). This finding suggested that the first withdrawal from ethanol might not impact inhibitory learning. Additionally, AEW might cause a specific pattern of neural activity that spares extinction. For example, AEW induces increased in activity in the prelimbic cortex of the mPFC (Kozell et al., 2005), a region implicated in the formation and expression of fear conditioning memories (Peters, Kalivas, & Quirk, 2009; I. Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006), But, the impact of AEW on IL, which is important for extinction of behavior (Laurent & Westbrook, 2009), is unknown. The molecular signature of AEW in conjunction with my behavior results imply that AEW could have a distinct pattern of neural activity that might preferentially affect circuits associated with fear-promoting learning. However, the conflicting findings of AEW and contextual fear acquisition suggest that AEW's impact on fear-promoting learning is moderate or selective.

Acute Ethanol Administration Generally Impairs Conditioning

In Experiment 6, acute ethanol administration caused a general impairment in conditioned freezing behavior. The dose of ethanol administered here has known effects on body temperature (Mihalek et al., 2001), anxiety-like behavior in the elevated plus maze (Mihalek et al., 2001; Homanics Quinlan & Firestone, 1999), and

locomotion (Homanics Quinlan & Firestone, 1999; Correa et al., 2004) in the C57BL6/J mouse. Additionally, 30 min after ethanol administration at this dose the blood ethanol concentration of B6 male mice is 95 ± 3 mg% (Middaugh et al., 1992), showing ethanol administration at this somewhat low dose affects behavior and physiology. Ethanol-intoxication-induced impairment of acquisition has been shown before (Gould, 2003; Melia et al., 1996; Stragier et al., 2015). Specifically, this dose of ethanol administration has impaired cued and contextual fear conditioning (Gould, 2013) Yet, this impairment in freezing was less severe in mice that received context pre-exposure or in mice that received extinction prior to reconditioning. This suggests that a history of exposure to a context may overcome some of the impairing effects of acute ethanol administration. Additionally, acute ethanol administration impaired initial acquisition over reacquisition of contextual fear.

During acute ethanol administration, there was a general increase in freezing when shock was paired with context, but the increased freezing did not persist into a long-term memory difference during the test. Specifically, acute ethanol administration facilitated reacquisition and context pre-exposed acquisition of freezing during reconditioning, but showed minimal effects on both during the 24 hr retention test. Conversely, animals in AEW did not differ in shock-induced freezing during acquisition and reacquisition (Experiment 4 and 5) or in the freezing response to the context during extinction (Experiment 3). This suggests that ethanol administration, but not withdrawal, may increase conditioned responding to shock. However, work has shown that ethanol withdrawal, not intoxication, causes

increased sensitivity to stressors and tactile sensitivity (Rassnick, Koob, & Geyer, 1992; Smith, Hostetler, Heinricher, & Ryabinin, 2016).

The selective impact of ethanol administration on acquisition and AEW on reacquisition provides additional evidence that acquisition and reacquisition rely on different neurobiology. Additionally, there is a potential circuit-based explanation for ethanol's disparate effects on specific phases of fear memory. The hippocampal place cells, for example, show an initial reduction in neural activity in response to both acute and chronic ethanol intoxication (White & Best, 2000) and intoxication-induced reductions in hippocampal activity have been shown to interfere with contextual fear conditioning (Melia et al., 1996). Conversely, ethanol withdrawal causes increased hippocampal activity (Kozell et al., 2005; Matsumoto et al., 1993). Perhaps, acute ethanol intoxication's reduction in hippocampal activity causes impairments of conditioning and extinction (Lattal, 2007), as both rely upon hippocampal activity (Peters et al., 2009; Rudy & O'Reilly, 1999; Stafford, Raybuck, Ryabinin, & Lattal, 2012), but only mildly affects reconditioning. Whereas, AEW, which has causes increased hippocampal activity (Kozell et al., 2005), impairs only the rapid reacquisition effect. This theory will have to be studied in more depth, but the biphasic response of the hippocampus, and likely other brain regions, to ethanol administration could explain the memory-specific impairments of acute ethanol administration and withdrawal.

However, several caveats to these experiments should be explored. In Experiment 6, I did not find rapid reacquisition following moderate extinction in the saline treated animals. Similar results are seen in other post-extinction CR

outcomes, like spontaneous recovery, which does not always occur following a delay in extinction (Rescorla, 2004b). The lack of a rapid reacquisition effect suggested that the parameters in Experiment 6 were not conducive to rapid reacquisition and could have prevented a rapid reacquisition impairment by ethanol administration. There were key differences in Experiment 6 that could block rapid reacquisition. Foremost, the timing of administration of saline injection immediately (Exp. 5), as opposed to 6 hrs (Exp. 3), prior to reconditioning could have prevented rapid reacquisition of fear behavior via stress, which has been known to impair learning (Kim & Diamond, 2002). Also, the extinction curve of reconditioning animals was much steeper than in previous studies and by the second extinction session the reconditioning animals were statistically equivalent to conditioning-naïve CTX animals. Perhaps, the reconditioning animals were over-extinguished and this blocked rapid reacquisition. Interestingly, the mice exposed to acute ethanol administration during reconditioning did show a trending rapid reacquisition effect. Further studies are needed to confirm which conditions allow for rapid reacquisition.

Additionally, in the acute ethanol withdrawal studies, the strain of mouse used here, C57BL/6J, is a withdrawal resistant line and thus may not have shown as robust behavioral effects of AEW as another strain (Metten & Crabbe, 1994). Ethanol administration similar to the dose used in this study has not been shown the cause conditioned place preference in C57BL/6J (Cunningham et al., 1992) and this strain of mice has shown less discrimination of ethanol at this dose compared to other strains of mice (Shelton & Grant, 2002). Even so, AEW did cause a moderate impairment of rapid reacquisition of fear following reconditioning in B6 mice, which

suggested that memory was impaired by initial withdrawal from alcohol, even in a withdrawal resistant strain. Further, strains of mice that are more withdrawal prone display poor conditioning and spontaneous recovery of contextual fear, such as the DBA/2J strain (Balogh et al., 2002; Lattal & Maughan, 2012; Tipps et al., 2014). Thus, the B6 mouse was not entirely unfit for these studies.

Also, the stimulus properties of ethanol were not explored here. Multiple studies have shown that ethanol administration can operate as an internal stimuli that can also be paired with a learning contingency (Cunningham, 1979b; 1979a; Lattal, 2007). Thus, it is possible that AEW created an internal stimulus that signaled the operation of conditioning, extinction, or reconditioning contingencies. The stimulus properties of AEW should be a point of future study, as perhaps rapid reacquisition might show state-dependent expression. However, if ethanol administration was acting as an internal stimulus in Experiment 6, both acquisition and reacquisition would be impaired in the drug-free test, but instead acquisition is preferentially impaired by acute ethanol administration. This outcome suggests that internal state caused by alcohol may not be conditioned during some phases of contextual learning, such as reacquisition.

These experiments did not also address the potential confounds of locomotion and stress. Alcohol is known to affect locomotion and can cause both increases and decreases in locomotion (Mihalek et al., 2001; Homanics, Quinlan & Firestone, 1999), depending on the dose and strain of rodent. Unfortunately, the only locomotion measured here was the absence of movement. Thus, I do not known how locomotion differed following ethanol administration or withdrawal from ethanol

administration from controls. As the memory of the context is a measure of the absence of locomotion, ethanol's affects on locomotion are potential confounds. Additionally, while the animals here were habituated to injections, they were not habituated to ethanol injections. Alcohol injections in rodents could be both painful and stressful. Thus, the sensation and stress of ethanol administration and withdrawal could also act as an internal stimulus that was conditioned in addition to the context.

Importantly, withdrawal following a single exposure to ethanol does not model AUD, such that chronic ethanol withdrawal causes different effects relative AEW. Accordingly, many brain regions that show activation following repeated or chronic ethanol withdrawal, (Borlikova et al., 2006; Olive et al., 2001), do not show activation following initial withdrawal or AEW (Borlikova et al., 2006; Kozell et al., 2005; Vilpoux et al., 2009). Therefore, we cannot expect that a single experience of ethanol withdrawal and a formation of a relatively mild fear memory will mimic the human pattern of AUD-PTSD comorbidity. Neither can I conclude that the ethanol-induced differences in brain activity discussed here are critical to AUD-PTSD comorbidity.

Similarly, the conclusions drawn about neurobiology of acquisition, extinction, and reacquisition from AEW and ethanol administrations's behavioral effects suffer from indirect study. I did not directly measure the alcohol-induced changes in neurobiology or directly correlate those changes with behavioral impairments. Rather, examples of the neurobiological effects of ethanol administration and withdrawal found in the literature were referenced to make conclusions. However, some neural patterns, such as the biphasic response of the hippocampus to alcohol,

have been repeatedly reported and suggest that ethanol administration's and AEW's neurobiological differences can lead to differences in behavior and memory, as was seen here. Thus, the impact of AEW and administration on memory can inform the initial role of alcohol use on fear memory phases and pinpoint brain regions for further study.

One conclusion that is clear from these experiments and the work of others is that the study of the interaction of alcohol and memory is mixed. For example, alcohol administration does not always impair conditioning, but can actually enhance depending on the dose (Gulick & Gould, 2007). Also, repeated withdrawal has been shown to impair contextual fear conditioning by several authors (Ripley et al., 2003; Stephens, Brown, Duka, & Ripley, 2001), but others have found that contextual fear conditioning is unaffected (Borlikova et al., 2006) or even enhanced (Bertotto et al., 2006) by withdrawal. Even my laboratory's own results are conflicting with regard to the effect of AEW on conditioning (Tipps et al., 2015). Thus, this chapter serves as a call for a systematic assessment of the conditions under which ethanol impairs (or enhances) memory so that the field can better understand the effects of alcohol on memory at large and improve current preclinical understanding of early PTSD-AUD comorbidity progression.

Chapter 4: The Neurobiology Underlying Rapid Reacquisition of Contextual Fear Conditioning

Abstract

The neurobiology of post-extinction reconditioning is mostly understudied and mixed. Further, the understanding of the brain regions and mechanisms involved in the post-extinction rapid reacquisition versus initial acquisition of contextual fear is limited. This chapter sought to explore the fear circuit for its differential involvement in acquisition and rapid reacquisition of contextual fear. I found that acquisition led to greater activation of the fear circuit than rapid reacquisition, as measured by the immunoreactivity of post-mortem tissue for markers of neuronal activity. Specifically, the bed nucleus of the stria terminalis (BNST), hippocampus, and amygdala all showed increased activity markers following acquisition, while only the hippocampus showed increased activity markers following rapid reacquisition. Also, pharmacological inactivation of the BNST impaired rapid reacquisition and strong acquisition, but not weak acquisition of contextual fear. However, temporary inactivation of the amygdala did not impair rapid reacquisition or weak acquisition of contextual fear. Overall, rapid reacquisition shows a unique pattern of neural activity that relies on the fear circuit in a different manner relative to acquisition.

Introduction

Rapid reacquisition of fear conditioning is a form of post-extinction reemergence of conditioned behavior that has received limited behavioral characterization and even less characterization of its underlying neurobiology. Rapid reacquisition is one of two outcomes following reconditioning in which conditioned freezing behavior is more rapidly reacquired relative to animals acquiring fear for the first time. The behavior of rapid reacquisition of contextual fear is more thoroughly covered in Chapters 1 and 2. In brief, rapid reacquisition is a unique post-extinction re-emergence of conditioned fear that can lead to a persistent enhancement in freezing in multiple rodent species. Studying the neurobiology of rapid reacquisition can provide insight into the regions that are necessary during relearning and updating of memory that leads to persistent behavioral changes.

Relatively little work has focused on the neurobiology of rapid rate of reacquisition of contextual fear compared to initial acquisition. However, those studies that exist, in combination with the extensive research on the neurobiology of conditioned fear acquisition and extinction, reveal brain regions that are potential targets for involvement in rapid reacquisition. The brain regions examined in this chapter for their potential role in reconditioning are the amygdala, hippocampus, bed nucleus of the stria terminalis (BNST), and medial prefrontal cortex (mPFC) and their subregions.

The amygdala has long been implicated in emotional processing in human and animal models (Davis, 1994; M. Davis & Whalen, 2001; Phelps, 2006). Also, the amygdala has been shown to be critical for most fear conditioning paradigms,

including cued, contextual, and fear potentiated startle (Goosens & Maren, 2001; Kim & Davis, 1993; Lee et al., 2001; Phillips & LeDoux, 1992; Walker & Davis, 1997). The amygdala is composed of several subdivisions important for fear modulation. Subregions of interest include the central nucleus of the amygdala (CeA), the lateral amygdala (LA), basolateral amygdala (BLA), and a group of GABAergic cells between the BLA and CeA, the intercalated cell masses (ITC). Each one is speculated to have a different role in fear conditioning, such as receiving sensory input associated with fear (LA; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; Maren & Quirk, 2004), expression of fear behavior (CeA; Maren & Quirk, 2004; Maren, Phan, & Liberzon, 2013; Wilensky, Schafe, Kristensen, & LeDoux, 2006a; LeDoux 2000), and acquiring and inhibiting fear behavior (BLA and ITC; Amano, Duvarci, Popa, & Paré, 2011; Helmstetter & Bellgowan, 1994; Maren et al., 2013; J. Muller, Corodimas, Fridel, & LeDoux, 1997).

The amygdala has also been studied for its role in reacquisition of conditioned fear (reviewed in Chapter 1). Several studies indicated that subregions of the amygdala, while necessary for initial fear acquisition, were not necessary for reacquisition of fear-potentiated startle (CeA; Kim & Davis, 1993) or post-extinction reacquisition of fear to a new tone (basal amygdala; Anglada-Figueroa & Quirk, 2005). Additionally, post-training BLA lesions that impaired acquisition expression lessened, but did not fully prevent, reacquisition of non-extinguished cued fear (Maren et al., 1996). Further, protein synthesis, a process generally thought to be important for long-term memory formation (Davis & Squire, 1984), was not needed for contextual fear reacquisition within the BLA (Motanis & Maroun, 2012).

Conversely, others found that NMDA activity (Laurent & Westbrook, 2009b) and actin rearrangement (Motanis & Maroun, 2012) in the BLA were involved in post-extinction reacquisition of contextual fear. Thus, the amygdala requires further investigation of its role in reacquisition. Specifically, the amygdala's role in rapid reacquisition relative to acquisition, which was not examined in these studies, is unknown.

Similar to the amygdala, the mPFC has also been implicated in fear learning (Laurent & Westbrook, 2008; Rozeske, Valerio, Chaudun, & Herry, 2015) and has subdivisions with different roles in expressing contextual fear. The prelimbic cortex (PrL) in the dorsal region of the mPFC has been shown to be important for fear learning expression (Corcoran & Quirk, 2007). Conversely, the infralimbic cortex (IL) in the ventral region of the mPFC is required for inhibiting expression of fear during extinction (Laurent & Westbrook, 2009a; Quirk et al., 2000; Vidal-Gonzalez et al., 2006). Yet, the role of the mPFC in reacquisition of fear is mixed. A procedure that mimicked some aspects of the rapid reacquisition, but used a sub-threshold US to recondition fear, showed that tetanic stimulation of the mPFC (Deschaux et al., 2011; Zheng et al., 2013) or inactivation of the PL (Fu et al., 2016) impaired reacquisition. Yet, inactivating the PL enhanced reacquisition in operant ethanol administration (Willcocks & McNally, 2013) and other preparations similarly showed mixed effects of PL and IL inactivation on reacquisition (reviewed in Chapter 1).

Another region of particular importance to contextual fear conditioning is the hippocampus (Phillips & LeDoux, 1992; Rudy & O'Reilly, 1999). Traditionally, the trisynaptic substructures (dentate gyrus or DG, CA3, CA1, and the subiculum) have

been studied for their separate roles in hippocampus-dependent memory. The DG has indicated a role in pattern separation and retrieval of contextual fear memories (Deng, Aimone, & Gage, 2010; Lee & Kesner, 2002). The CA3 appears to involved in the earliest processing of a contextual representation during acquisition, while the CA1 is important for consolidation and retrieval of the contextual fear memory (Daumas, 2005; Lee & Kesner, 2002).

Generally, the hippocampus is also important for reacquisition (reviewed in Chapter 1). Relevant to the current study, hippocampus lesions prevented rapid reacquisition of a spatial navigation task (Winocur et al., 2005). Additionally, tetanic stimulation of the hippocampus impaired reacquisition in sub-threshold fear reconditioning (Fu et al., 2016). However, the role of the hippocampus in the rate of reacquisition of contextual fear following reconditioning has yet to be examined.

A final region of interest is the anterior BNST (Alheid & Heimer, 1988), a member of the extended amygdala that shares similar connections and neuroanatomical makeup with the CeA (Alheid, DeOlmos, & Beltramino, 1995). The BNST is critical for the conditioning of sustained anxiety to long-duration cues, such as contextual fear conditioning (Davis et al., 2010; Schulz & Canbeyli, 1999; Sullivan et al., 2004; Waddell et al., 2006), but not cued fear conditioning for which the CeA is necessary (Zimmerman & Maren, 2011). The BNST is also important for CRF- and light- potentiated startle (Lee & Davis, 1997; Walker & Davis, 1997), as well as stress-induced reinstatement of drug seeking (Erb & Stewart, 1999) and reinstatement of conditioned cued fear behavior (Goode et al., 2015; Waddell et al., 2006). The BNST has not been investigated for its involvement in rapid reacquisition

of contextual fear or reacquisition in general, but its importance in sustained anxiety and contextual fear acquisition makes it a likely candidate.

This chapter used two markers of neural activity to investigate each of these regions for a role in rapid reacquisition of contextual fear relative to initial acquisition: c-Fos and histone 4 lysine 8 acetylaton (H4K8ac). The functional relevance of two regions that showed significant c-Fos and H4K8ac expression following acquisition and rapid reacquisition of contextual fear was assessed using temporary pharmacological inactivation during reconditioning. These experiments found that rapid reacquisition has a distinct neural pattern that might not rely on the same brain regions as acquisition.

Experiment 7: Rapid Reacquisition Has a Distinct Pattern of Immediate Early Gene and Histone Acetylation Expression in the Brain Relative to Acquisition

To examine the role of multiple regions in rapid reacquisition, brain tissue was collected following reconditioning. This tissue was processed via immunohistochemistry for the presence of two activity markers in the mPFC, amygdala, hippocampus, and BNST. The first activity marker was the immediate early gene (IEG), c-Fos, as it is reliably used as a marker for neural activity in many studies (Guzowski, 2002; Morgan, Cohen, Hempstead, & Curran, 1987; Vann, Brown, Erichsen, & Aggleton, 2000; Weitemier & Ryabinin, 2004) that is only expressed when there is neuronal activity. c-Fos is also necessary for the molecular processes involved learning and memory (Fleischmann et al., 2003) and previously has been used to identify regions important for contextual fear conditioning (Huff et

al., 2006; Knapska et al., 2012). Finally, the timing of c-Fos activation has been thoroughly studied and its relatively quick response time to stimuli allows experimenters to narrow down possible stimuli or experimental manipulations that caused IEG activation (the highest level of c-Fos protein expression is about 1 to 3 hr after the manipulation; Morgan et al., 1987; Weitemeir & Ryabinin, 2004).

The second activity marker was histone acetylation, specifically histone 4 lysine 8 (H4K8ac). Histone acetylation is also involved in gene transcription and expression (McQuown et al., 2011; Peixoto & Abel, 2013; Wang, Xia, Weiss, Refetoff, & Yen, 2010), which is upstream of protein synthesis and, thus, heavily implicated in learning. Further, this specific form of histone acetylation is associated with transcriptional activation (Kouzarides, 2007) and enhanced c-Fos expression and long-term memory (McQuown et al., 2011). This experiment allowed for the direct comparison of impact of acquisition and reacquisition on the brain regions important for fear learning.

Methods

Animals and Housing. 24 Long-Evans male rats (Charles River Laboratories, Wilmington, MA) were purchased at 275-300 g (~9-11 weeks of age) and were housed 2 rats to an individually-ventilated cage. Rats were kept on a reverse 12h light-dark schedule (dark started at 0600 and light started at 1800). All animals were allowed to acclimate to the vivarium at Oregon Health & Science University (OHSU) for a full 7 days before any handling or behavioral procedures were begun. The vivarium maintained constant temperature (22°C ± 1°C) and humidity (70%) in all animal rooms. All experimental procedures were approved by

the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985). Food and water were available ad libitum and all behavioral experiments occurred from 0900 to 1500.

Behavior

Apparatus. Fear conditioning occurred in operant conditioning chambers (exterior dimensions: 31.8cm L x 25.4 W x 26.7cm H, Med Associates, St. Albans, VT) housed within sound attenuating chambers (Med Associates, St. Albans, VT). The operant chambers were fixed with a grid floor set to deliver a 1 sec, .75 mA scrambled shock and a house light that illuminated to signal the start of the session. Before and after each round of behavior, the grid floors and chamber walls were cleaned with 95% ethanol. Animals were loaded into chambers in red light conditions to maintain the dark circadian cycle.

Freezing Assessment. The level of contextual fear conditioning was assessed by the amount of freezing, the natural conditioned response upon re-exposure to a cue or context associated with shock (Fanselow & Bolles, 1979). Freezing was considered continuous inactivity (with the exception of breathing) for 2 sec. Freezing was measured in real-time by visual time sampling in which the experimenter would assess each animal every 8 s for freezing and hand-score the presence or absence of freezing.

Behavioral Schedule. For all experiments, conditioning was a 12-min session, in which rodents received four unsignaled footshocks (1 sec, .75 mA) at 2.5 min, 5 min, 9 min, and 11.5 min into the session. Extinction consisted of a 24-min

non-reinforced exposure to the fear-conditioning context. Reconditioning was a weak conditioning session consisting of 3 min session with a single unsignaled footshock delivered 2.5 min into the session. All sessions were run at the same time of day separated by 24 hr.

There were 4 groups that differed in conditioning history: reconditioning group with moderate extinction (REC- EXT, n = 6), reconditioning group with no extinction (REC-NO EXT, n = 6), conditioning only group (COND, n = 8), and a handled, behavior-naïve group (HAND, n = 6). The full behavioral schedule is detailed in Figure 15A.

Phase 1: Conditioning. Day 1 and 2 consisted of a conditioning sessions for REC-EXT and REC-NO EXT. All other groups were handled and transported to equate treatments.

Phase 2: Exitnction. On Days 3 through 5, REC-EXT received extinction training, while REC-NO EXT was handled and returned to the homecage without extinction training.

Phase 3: Reconditioning. All groups received reconditioning, as described above, on Day 6. For groups REC-EXT and REC-NO EXT, Day 6 was a reconditioning session, but for COND this day was its first conditioning and exposure to the context. HAND was handled and returned to the homecage as in all previous phases. 50-90 min following the end of Phase 3 all animals were scarified and perfused for brain collection.

Brain Removal and Cryoprotection

Animals were sacrificed via isoflurane until all breathing had stopped and then thoracotomy was used as a secondary form of euthanasia. Rats were then perfused with 4% paraformaldehyde (PFA) and phosphate buffered serum (PBS). Brains were removed and placed in 4% PFA in PBS for no more than 24 hr. For cryoprotection, brains were then placed in a solution of 20% sucrose and .1% sodium azide (NaN3) in PBS until the brain was fully saturated with sucrose (generally 24 hrs). The brains were then transferred to 30% sucrose and .1% NaN_3 in PBS for no more than 1 month before sectioning. The brains were sectioned at 35 microns and then placed in well plates of PBS and 0.1% NaN_3 until further processing via immunohistochemistry.

Immunohistochemistry (IHC)

Materials. Rabbit anti c-Fos IgG (F7799, Sigma-Aldrich, Saint Louis, MO) and goat anti-rabbit biotinylated IgG antibodies (BA-1000, Vector Laboratories) were purchased for c-Fos detection. Vectastain ABC Kit (PK-400, Vector Laboratories) and Metal Enhanced Diaminobenzidine (DAB) Kit (PI-34064, Fischer Scientific) were used for immunoreaction detection.

Rabbit anti H4K8 Acetyl-Histone H4 Lys8 (2594S, Cell Signaling, Danvers, MA) and goat anti-rabbit IgG F(ab')2-Alexa Fluor 488 (Thermo Fisher Scientific) antibodies were purchased for H4K8ac histone acetylation detection.

Tissue Selection for Processing. Due to the large amount of tissue collected to include all regions of interest, not all of the tissue could be processed at once and multiple rounds of IHC were run. In order to have a balanced

representation, tissue was processed by region in which all animals were included (e.g., every other slice of mPFC for each animal was processed simultaneously).

DAB IHC Schedule. All procedures used here were adopted from the Ryabinin laboratory (Weitemier & Ryabinin, 2004). All incubations were done at room temperature on a plate rotating at ~40 rpm. Slices in 12 well plates and net wells were first treated with 3 X 5 min washes in PBS to wash away NaN_3. A 15 min incubation in 0.3% hydrogen peroxide in PBS blocked endogenous peroxidase activity and was followed by three additional PBS washes. The slices were then incubated in a blocking solution for 4 hr, which consisted of a 1:10 solution of normal goat serum (NGS, S-1000, Vector Laboratories) into 0.1% triton in PBS (PBS/Triton). NGS was the blocker of choice in these molecular assays due to the animal (goat) in which the secondary antibody was raised. Following blocking, the slices were incubated overnight (~15 hrs) in primary antibody, anti c-Fos rabbit IgG, that was at a concentration of 1:15000 in PBS/Triton/bovine serum albumin (BSA).

The following day commenced with 3 X 5 min PBS washes and subsequently with the secondary antibody incubation. The secondary antibody solution consisted of 1:200 concentration of biotinylated goat anti-rabbit IgG in PBS/Triton. This incubation lasted for 1 hr and was followed with 3 X 5 min PBS washes.

Next, a Vectastain ABC kit was used to bind to secondary-antibody-bound cells via a biotin/avidin interaction. The ABC solution was a 1:200 concentration of both chemicals A (avidin) and B (biotin; the exact composition of which is proprietary information) into PBS/triton. This mixture was made 30 min prior to application and the incubation lasted for 1 hr. This step was followed by PBS washes and

application of a 1X solution of diaminobenzidine (DAB) in peroxidase (H_2O_2) buffer that provided intense coloration of immunopositive cells. Unlike previous steps, this incubation was not done on the rotating plate. The incubation time in DAB was around 1 min, a time chosen from pilot data for the optimal for strong signal and minimal background (data not shown). This incubation time was kept consistent for all plates. Immediately after DAB incubation, the wells were placed into ultraviolet (UV) purified water to stop the reaction and prevent the tissue from saturating.

The slices were then placed onto glass Superfrost slides (Fisher Scientific) via a mounting solution (composed of UV purified water, gelatin, acetic acid, and 95% ethanol) and allowed to dry overnight. After drying, the slides were dehydrated in sequential steps of ethanol at increasing concentrations: 10 min in 70%, 95%, and then 100% ethanol. Next, the slides were dehydrated in the final step by submersion into Citrasolv (Fisher Scientific) and immediately cover slipped with glass covers and Cytoseal 60 mounting media (Fisher Scientific).

Fluorescent IHC Schedule. Fluorescent IHC was similarly run as DAB IHC above with several exceptions. Well plate IHC was employed again and slices were first treated with 3 X 10 min washes in 0.1M Tris washes to wash away NaN_3. Slices were then incubated in 1% sodium borohydride in Tris for 30 min followed by Tris washes (at least 3 washes) until the sodium borohydride reaction was removed. Prior to the primary antibody, blocking was performed by incubating the slices in 5% NGS in a 0.3% Triton-Tris solution for 45 min. Then, slices received an overnight incubation in the primary antibody, 1:1000 rabbit anti H4K8ac, in Triton-Tris and 1% NGS.

The following day, the primary antibody was washed away in 3 X 10 min Tris washes. Another round of blocking (45 min in 5% NGS) occurred prior to secondary antibody incubation. The secondary antibody incubation was 1 hr submersion in 1:1000 goat anti-rabbit Alexa Fluor 488 and 5%NGS in Triton-Tris. During and following the secondary antibody, slices were protected from light at all times. The slices were then washed in Tris and 3 X 5 min in PBS. Following washes, slices were mounted on glass Superfrost slides in the mounting solution used above. Slices were not dehydrated, but instead cover-slipped with Vectashield mounting medium with DAPI (H-1200, Vector Laboratories) shortly after mounting on the slide. DAPI labeled the neurons with a fluorescence of 360 nm.

Tissue Imaging and Cell Counting. The processed tissue was imaged using the Leica DM4000B microscope (Leica Microsystems Inc., Buffalo Grove, IL) on brightfield settings for DAB or with laser light set to excite Alexa Fluor 488 for fluorescent markers. Each image was taken at a consistent intensity, field diaphragm, aperture, and magnification for each image and region (I= 8;A= 25; FD= 32 for DAB and I= 100; FD= 6; Σ=100X for fluorescence). All images used for cell counting were taken with a 10x objective, except for the hippocampus, which required a 5x objective to capture the region. Each image was collected using QImage Micro-Publisher 3.3 RTV camera and software (QImaging, Surrey, BC, Canada) at the same exposure (exposure= 84.1 ms for DAB and exposure = 1.87 for fluorescence).

Each image was analyzed using ImageJ software (NIH, Bethesda, MD). The images were adjusted through a threshold procedure, in which images were

converted to an 8-bit image prior to the application of a threshold mask. The threshold mask was chosen using neighboring brain regions with only nonspecific background staining as control regions. A consistent threshold range (for DAB, 80-120; for fluorescence, 20-50 on a dark background) and circularity (0.7 to 1.00) were used through out the regions. The lower limit of pixels required for a stained cell depended on the region, but was consistent within each region (60 pixels for mPFC regions, 50 pixels for amygdala and BNST, and 15 pixels for the hippocampus because the images were at a lower magnification).

Both whole regions (expect the amygdala) and subregions were analyzed for group differences. The chosen location (relative to bregma) for each subregion was: IL (3.00 mm); PrL (3.35 mm); adBNST – dorsal subregion of anterior BNST (0.00 mm); avBNST – ventral subregion of anterior BNST (0.00 mm); CeA (-2.76 mm); BLA (-2.76 mm); LA (-2.76 mm); CA1 (-3.00 mm); CA2 (-3.00 mm); CA3 (-3.00 mm); DG (-3.00 mm). The Paxinos-Watson rat atlas was used to confirm the bregma location of slices and only slices within .35 mm of the bregma location listed above were considered for assessment of staining. Two to four slices were counted and averaged for each animal to get the average amount of target protein expression captured by IHC. Due to the loss of integrity of the tissue of a few animals, the total numbers of animals per group for each region are shown in Tables 2 and 3. For H4K8ac analyses, the CeA tissue was too degraded to include in analyses.

Statistics

R-Studio (Boston, MA) and GraphPad Software Prism 6 (La Jolla, CA) were used to run all statistics and create figures, respectively. Behavior was analyzed with

either a one-way ANOVA with Group as the main factor or as a repeated measures ANOVA (RMANOVA) with Group as the between-subjects factor and Time as the within-subjects factor (Extinction sessions or 30 sec pre- and post-shock). The dependent measure for behavior was average percent time for an entire session or for a specified time bin. c-Fos or H4K8ac detection was analyzed with a one-way ANOVA with Group as the main factor and average protein counts per animal as the dependent measure. Any failure to meet the homogeneity of variances criterion for an ANOVA or t-test (as measured by the Brown-Forsythe Levene's test) was accounted for using a Welch correction. If significance was found for main effects or interactions, Tukey's HSD tests (or Games-Howell for unequal variances amongst groups) were used for simple comparisons between groups and sessions. For all statistical tests, significance was set at $\alpha = 0.05$.

Results

Behavior

Phase 1: Conditioning

Over two contextual fear conditioning sessions, both REC-EXT and REC-NO EXT acquired conditioned freezing to the context (Figure 15B). A two-way RMANOVA comparing Group X Acquisition Session showed a significant within-subjects effect of Acquisition Session ($F_{(1,10)} = 36.86$, $p < 0.001$; session one: M = 61.24, SEM = 2.44, session two: M = 83.20, SEM = 3.92), but not a main effect of Group ($F_{(1,10)} = 0.32$, $p = 0.583$; REC-EXT: M = 73.83, SEM = 4.76, REC-NO EXT: M = 70.61, SEM = 4.49) or an interaction ($F_{(1,10)} = 0.386$, $p = 0.548$). This result

A.

Group	Acquisition	Extinction	Reconditioning	Euthanasia
	Day 1-2	*Day 3-5*	*Day 6*	*Day 6*
REC-EXT	12 min ++++	24 min –	3 min +	50-90 min later
REC-NO EXT	12 min ++++	Handled		
COND	Handled	Handled		
HAND	Handled	Handled	Handled	

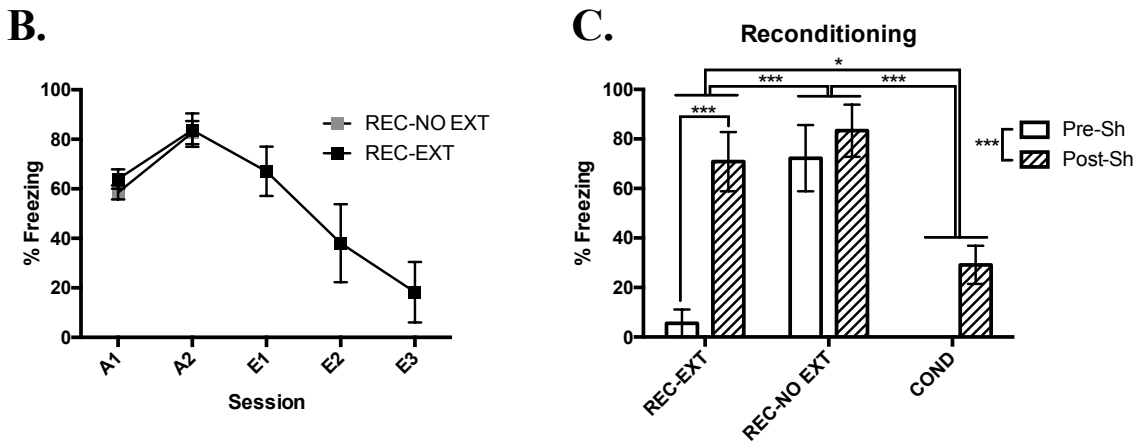


Figure 15. Rapid Reacquisition Behavior Prior to IHC. (**A**) Overview of the design of Experiment 7. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .75 mA shock and a minus sign indicates exposure to the context without shock. (**B**) The acquisition and extinction curve of REC-EXT and REC-NO EXT as shown by the mean percent freezing for each session; A = acquisition session, E = extinction session (**C**) Mean percent freezing in the 30 sec pre and post shock on Day 6 during. Rapid reacquisition of freezing relative to initial acquisition occurred in both REC groups, but only in REC-EXT relative to pre-shock freezing. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$. REC-EXT, n = 6; REC-NO EXT, n = 6; COND, n = 6; HAND, n = 6. Error bars represent standard error of the mean.

showed that conditioning increased freezing behavior over two sessions, but that there were no group differences due to initial acquisition.

Phase 2: Extinction

During three sessions of extinction, animals in REC-EXT decreased their conditioned freezing response (Figure 15B). A one-way RMANOVA revealed a significant within-subjects effect of Extinction Session on session average percent freezing ($F_{(2,10)}$ = 16.69, p < 0.001) that was driven by the significantly lower freezing in E3 compared to E1 ($q_{(15)}$ = 3.7948, p < .05). While E3 session average freezing did not show animals extinguishing to 0% freezing, all animals in REC-EXT were near 0% freezing in the last 3 min of E3 (M = 2.89, SEM = 2.89), which showed animals successfully extinguished before reconditioning.

Phase 3: Reconditioning

During reconditioning, REC-EXT showed rapid reacquisition of conditioned freezing following the reconditioning shock relative to COND and REC-NO EXT (Figure 15C). A RMANOVA comparing percent time spent freezing in the 30 sec before and after shock by each Group (Time X Group) found a significant main effect of Group ($F_{(2,15)}$ = 23.46, p < 0.001), within-subjects effect of Time ($F_{(1,15)}$ = 34.41, p < 0.001), and a trending interaction effect ($F_{(2,15)}$ = 3.55, p = 0.054). The main effect of Time was caused by significantly more freezing shown after shock relative to before shock, suggesting that shock increased freezing in all groups. The main effect of Group was driven by significantly more freezing by REC-NO EXT relative to COND ($q_{(15)}$ = 7.10, p < .001) and REC-EXT ($q_{(15)}$ = 4.44, p < .001). Additionally, REC-EXT showed significantly more freezing than COND ($q_{(15)}$ = 2.65, p < .05),

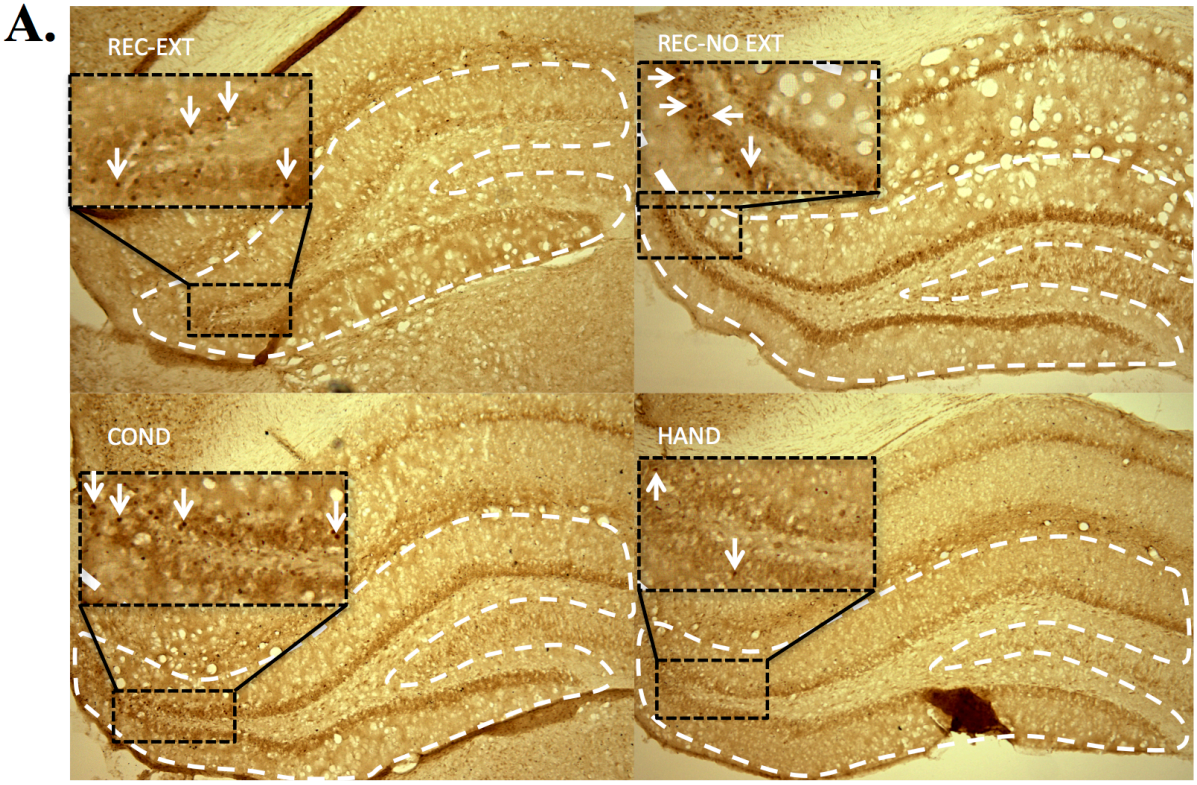
indicating that both reconditioning groups froze more than COND and that a lack of extinction training causes higher freezing in general (REC-NO EXT > REC-EXT). Yet the trending interaction effect showed that only REC-EXT had significantly more freezing post-shock relative to pre-shock ($q_{(15)} = 6.85$, $p < .001$), while COND ($q_{(15)} = 1.07$, $p = .262$) or REC-NO EXT did not ($q_{(15)} = .671$, $p = .957$), showing that only REC-EXT showed a rapid reacquisition effect specific to the reconditioning shock. Animals were euthanized 50-90 min after Phase 3 for IHC.

c-Fos IHC

All regions and subregions were analyzed for group differences in c-Fos positive cells. Representative DAB IHC staining for c-Fos for each group in a significant region (DG) can be seen in Figure 16A. The full summary of results is in Table 2.

Only the hippocampus had a significant difference in c-Fos among groups in whole region analyses (Figure 17A). A one-way ANOVA found a significant main effect of Group ($F_{(3,19)} = 11.1$, $p < .001$) that was driven by significantly more c-Fos positive cells in COND ($q_{(19)} = 7.95$, $p < .001$), REC NO-EXT ($q_{(19)} = 5.40$, $p < .01$), and REC-EXT ($q_{(19)} = 4.84$, $p < .05$) relative to HAND. This finding suggests that the hippocampus is more active during initial acquisition and reacquisition compared to a handling control.

However, when the subregions of the hippocampus were further analyzed, the DG showed a similar, but distinct pattern of expression relative to the overall hippocampus (Figure 17B). In a one-way ANOVA comparing c-Fos-positive cells in the DG among groups there was a significant main effect of Group ($F_{(3,19)} = 16.6$, p


A.
REC-EXT
REC-NO EXT
COND
HAND

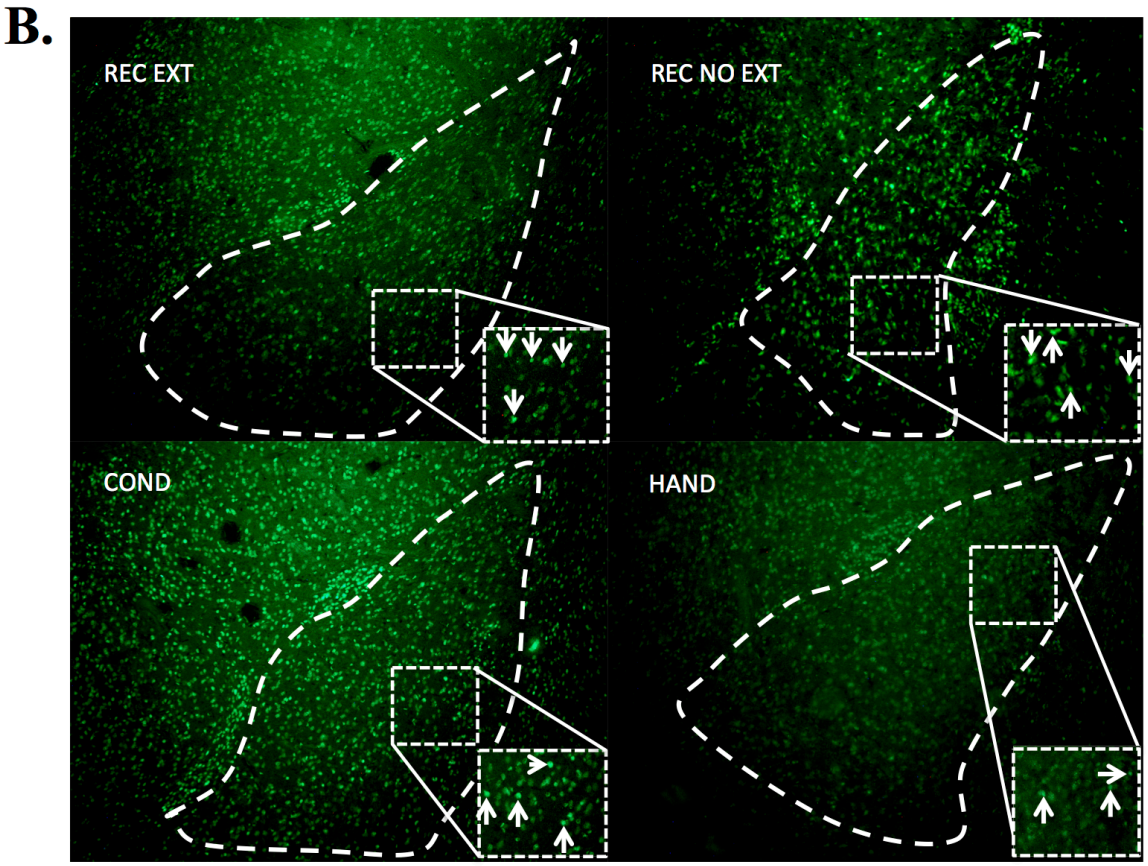

B.
REC EXT
REC NO EXT
COND
HAND

Figure 16. Representative Coronal Slices of c-Fos and H4K8ac Staining. (**A**) The four panels are representative images of average c-Fos staining in DG for each group that resulted from DAB IHC. The large-dashed white line represents what region was counted for the DG. The small-dashed black square shows a magnified region of the slices with white arrows indicating cells showing positive DAB staining (not all stained cells are indicated with an arrow to maintain clarity). (**B**) The four panels are representative images of average H4K8ac staining in LA for each group that resulted from fluorescent IHC. The large-dashed white line represents what region was counted for the LA. The small-dashed white square shows a magnified region of the slices with white arrows indicating cells showing positive fluorescent staining (not all stained cells are indicated with an arrow to maintain clarity).

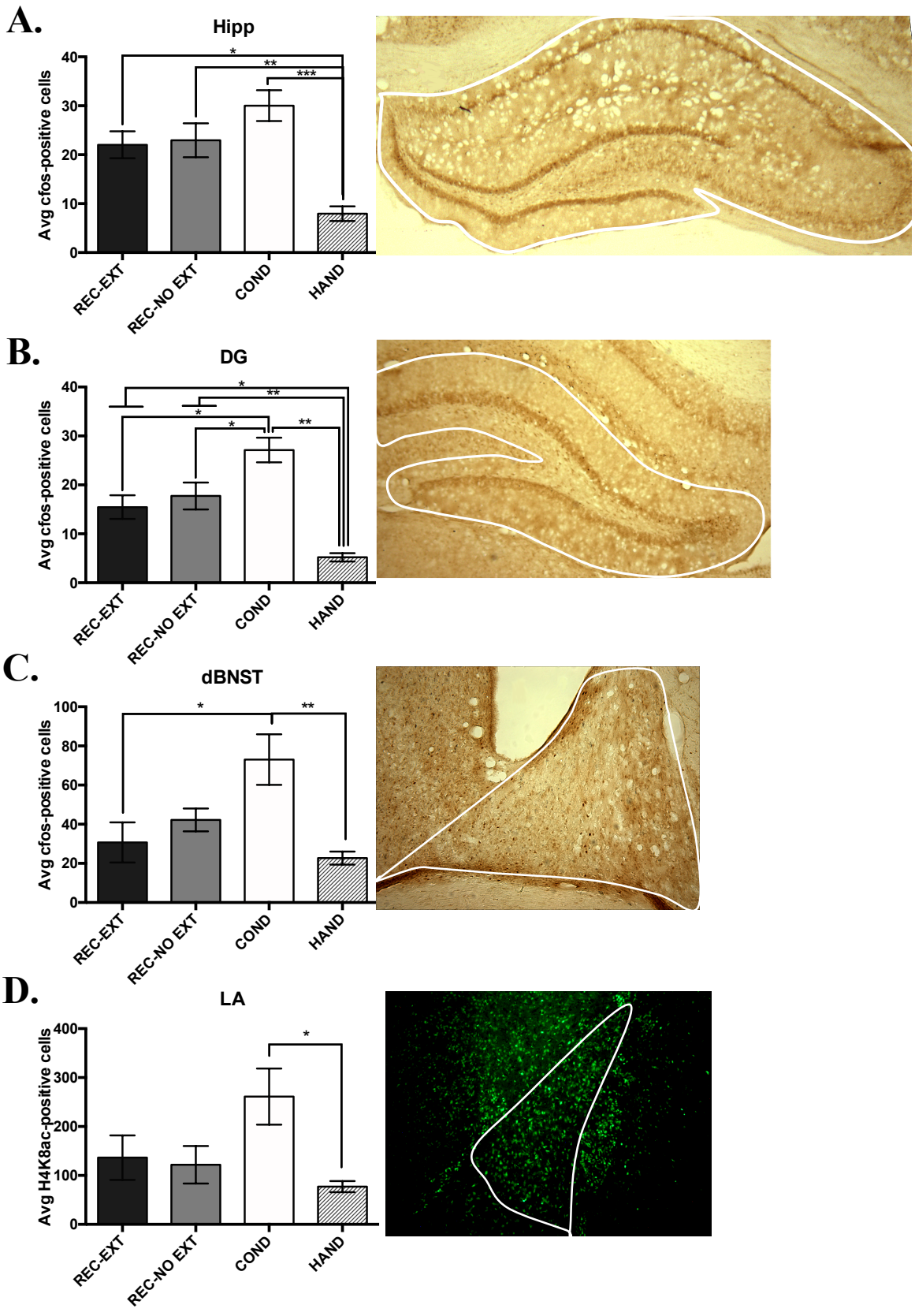
Table 2. Mean ± SEM of c-Fos expression for each sub-region.

	REC-EXT	REC-NO EXT	COND	HAND	ANOVA RESULT
mPFC	294.3 ± 53.3	260.4 ± 26.9	298.6 ± 39.8	206.1 ± 34.5	$F_{(3,20)} = 1.15$, $p = .352$
IL	124.0 ± 20.3	117.3 ± 11.6	131.6 ± 18.8	82.1 ± 13.9	$F_{(3,20)} = 1.75$, $p = .188$
PrL	170.3 ± 33.8	143.1 ± 18.3	167.1 ± 23.7	124.0 ± 20.8	$F_{(3,20)} = .78$, $p = .519$
Amygdala					
LA	14.6 ± 3.8	13.9 ± 2.6	23.4 ± 3.6	17.3 ± 3.0	$F_{(3,20)} = 1.73$, $p = .194$
BLA	32.3 ± 6.5	23.9 ± 5.4	35.8 ± 5.5	41.5 ± 5.9	$F_{(3,20)} = 1.60$, $p = .220$
CeA	6.2 ± 2.9	7.2 ± 1.6	12.5 ± 3.0	5.5 ± 1.0	$F_{(3,20)} = 1.94$, $p = .155$
dHipp	**22.0 ± 2.8* (n=5)**	**23.0 ± 3.5***	**30.0 ± 3.2***	**8.0 ± 1.5**	**$F_{(3,19)} = 11.1$, $p < .001$**
CA1	1.6 ± .23 (n=5)	1.3 ± .42	.71 ± .23	1.1 ± .5	$F_{(3,19)} = .913$, $p = .453$
CA2	.38 ± .19 (n=5)	.52 ± .18	.33 ± .19	.06 ± .06	$F_{(3,19)} = 1.53$, $p = .238$
CA3	4.6 ± 1.4 (n=5)	3.4 ± 1.2	1.8 ± .36	1.6 ± .41	$F_{(3,19)} = 2.22$, $p = .119$
DG	**15.5 ± 2.4*# (n=5)**	**17.7 ± 2.8*#**	**27.1 ± 2.5***	**5.2 ± 0.84**	**$F_{(3,19)} = 16.6$, $p < .001$**
aBNST	84.0 ± 30.1	100.7 ± 18.6	160.1 ± 29.8	76.8 ± 11.4	$F_{(3,20)} = 2.52$, $p = .087$
adBNST	**30.7 ± 10.3#**	**42.22 ± 5.8**	**73.0 ± 12.9***	**22.7 ± 3.3**	**$F_{(3,20)} = 6.16$, $p < .01$**
avBNST	53.3 ± 20.4	58.5 ± 13.2	87.1 ± 19.6	54.1 ± 8.3	$F_{(3,20)} = 0.98$, $p = .418$

Table 2. Mean ± SEM of c-Fos positive cells in every region selected following Day 6 (Reconditioning). n = 6, unless otherwise specified. Bold indicates significance between groups.

* Significantly higher compared to HAND at $p < .05$; ** $p < .01$

Significantly less than COND at $p < .05$; ## $p < .01$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REC-NO EXT
COND
HAND
D.
LA
Avg H4K8ac-positive cells
400
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200
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REC-EXT
REC-NO EXT
COND
HAND

Figure 17. Acquisition leads to Stronger Activity Marker Expression than Rapid Reacquisition. (**A**) The bar graph on the left shows the average c-Fos-positive cells within the entire dorsal hippocampus for each group. The image on the right shows a representative coronal slice of the hippocampus and the solid white line shows the region that was assessed for creating the average c-Fos-positive cell counts for analysis. (**B**) is a similar figure for the DG of the hippocampus and (**C**) is a similar figure for the adBNST. (**D**) The bar graph on the left shows the average H4K8ac-positive cells within the lateral amygdala (LA) for each group. The panel on the right shows a representative coronal slice of the LA and the solid white line shows the region that was assessed for creating the average H4K8ac-positive cells. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$. Group numbers for each region are detailed in Tables 2 and 3. Error bars represent standard error of the mean.

< .001). The main effect of Group was caused by significantly more DG c-Fos expression in REC-EXT ($q_{(19)} = 4.43$, $p < .05$), REC-NO EXT ($q_{(19)} = 5.68$, $p < .01$), and COND ($q_{(19)} = 9.93$, $p < .01$) relative to HAND and significantly more DG c-Fos expression in COND relative to REC-EXT ($q_{(19)} = 5.04$, $p < .05$) and REC-NO EXT ($q_{(19)} = 4.25$, $p < .05$). This effect suggests that both acquisition and reacquisition cause increased DG c-Fos expression, but that initial acquisition causes the strongest activation of DG c-Fos expression.

While the aBNST as a whole did not show significant group differences in c-Fos expression, the adBNST subregion had a significant finding (Figure 17C). A one-way ANOVA comparing adBNST c-Fos expression found a significant main effect of Group ($F_{(3,20)} = 6.16$, $p < .01$) due to significantly more c-Fos positive cells in COND relative to HAND ($q_{(20)} = 5.66$, $p < .01$) and REC-EXT ($q_{(20)} = 4.76$, $p < .05$). Similar to the DG, this result implies that initial acquisition causes a stronger activation of c-Fos in the adBNST relative to reacquisition or simple handling.

H4K8ac IHC

All regions and subregions were analyzed for group differences in H4K8ac positive cells, but only the lateral amygdala subregion showed significant differences. Representative fluorescent IHC staining for H4K8ac for each group in a significant region (LA) can be seen in Figure 16B. A summary of the results of each region is in Table 3.

A one-way ANOVA found a significant main effect of Group on cells positive for H4K8ac within the lateral amygdala ($F_{(3,18)} = 3.64$, $p < .05$; Figure 17D). The significant Group effect was driven by significantly more H4K8ac-positive cells in

Table 3. Mean ± SEM of H4K8ac expression for each sub-region.

	REC-EXT	REC-NO EXT	COND	HAND	ANOVA RESULTS
mPFC	273.6 ± 80.1	510.3 ± 233.8, (n=5)	645.8 ± 253.4	198.5 ± 49.0, (n=4)	$F_{(3,17)} = .788$, $p = .517$
IL	264.5 ± 116.5	157.1 ± 33.8	324.5 ± 122.7	115.2 ± 33.3, (n=4)	$F_{(3,18)} = .958$, $p = .434$
PrL	245.8 ± 117.5	153.3 ± 42.3 (n=5)	285.2 ± 96.3	121.8 ± 34.5, (n=4)	$F_{(3,17)} = .677$, $p = .578$
Amygdala					
LA	**136.1 ± 45.5**	**121.8 ± 38.3,** (n=4)	**261.3 ± 57.3***	**77.2 ± 11.4**	**$F_{(3,18)} = 3.64$, $p < .05$**
BLA	146.8 ± 56.4	63.8 ± 15.4, (n=4)	181.6 ± 46.2	81.42 ± 20.8	$F_{(3,18)} = 1.66$, $p = .212$
dHipp	52.3 ± 20.6 (n=5)	24.0 ± 13.6 (n=5)	66.1 ± 40.0	12.1 ± 4.3	$F_{(3,18)} = 1.06$, $p = .390$
CA1	16.6 ± 9.9 (n=5)	2.7 ± 1.6	23.7 ± 9.7	2.1 ± 0.7	$F_{(3,19)} = 1.74$, $p = .193$
CA2	3.0 ± 1.3 (n=5)	1.8 ± 1.2 (n=5)	4.5 ± 2.6	.8 ± .3	$F_{(3,18)} = .99$, $p = .419$
CA3	2.3 ± .2 (n=5)	1.7 ± .7 (n=5)	2.9 ± 1.1	1.2 ± .5	$F_{(3,18)} = 1.23$, $p = .328$
DG	30.4 ± 10.6 (n=5)	15.0 ± 8.8	40.4 ± 26.9	8.1 ± 3.4	$F_{(3,19)} = .90$, $p = .459$
aBNST	530.0 ± 116.3	405.6 ± 39.1	610.8 ± 162.4	479.4 ± 45.5	$F_{(3,20)} = .685$, $p = .572$
adBNST	227.8 ± 52.5	185.4 ± 19.8	283.4 ± 84.3	191.3 ± 22.2	$F_{(3,20)} = .756$, $p = .532$
avBNST	302.2 ± 69.0	220.2 ± 26.0	327.3 ± 83.2	288.1 ± 30.7	$F_{(3,20)} = .631$, $p = .603$

Table 3. Mean ± SEM of H4K8ac positive cells in every region selected following Day 6 (Reconditioning). n = 6, unless otherwise specified. Bold indicates group differences.

* Significantly higher compared to HAND at $p < .05$; ** $p < .01$

COND relative to HAND controls (q (18) = 4.48, p < .05), which suggested that conditioning significantly activated histone acetylation within the lateral portion of the amygdala.

Discussion

This experiment revealed that rapid reacquisition of conditioned freezing occurred immediately after a reconditioning shock. However, rapid reacquisition caused less activation of brain regions involved in fear learning than acquisition. Specifically, I found initial acquisition led to a significant increase in H4K8ac in the lateral amygdala, while reconditioning did not. Similarly, in the adBNST, acquisition caused an increase in c-Fos expression relative to no-learning controls and reconditioning. Conversely, in the hippocampus and its subregion, the DG, I found that both acquisition and rapid reacquisition led to enhanced c-Fos expression. However, within in the DG, acquisition led to more c-Fos activity than reacquisition. These findings suggest that brain regions heavily implicated in fear learning may become less necessary when fear is relearned. Overall, these results provided target regions associated with acquisition and reacquisition to further examine for their involvement in rapid reacquisition.

Experiment 8: Amygdala activity may not be required for Rapid Reacquisition of Contextual Fear Conditioning

As mentioned in the introduction, the amygdala is well-known for its involvement in fear learning (Amano, Unal, & Paré, 2010; Helmstetter & Bellgowan, 1994; Maren et al., 2013; Maren & Quirk, 2004; Muller et al., 1997; Wilensky, Schafe, Kristensen, & LeDoux, 2006b). Thus, the amygdala is a primary suspect for brain regions involved in rapid reacquisition and the BLA has been previously implicated in reacquisition of contextual fear (Laurent & Westbrook, 2009b; Motanis

& Maroun, 2012). However, the H4K8ac IHC results in Experiment 7 suggested that activity in the lateral amygdala was primary involved in acquisition, but not reacquisition. Experiment 8 was conducted to examine the role of amygdala in rapid reacquisition of contextual fear, which could be an amygdala-independent memory process.

Methods

Animal type and housing conditions were identical to Experiment 7. This experiment was run in two separate cohorts that were analyzed together. Statistical analyses were similar to Experiment 7, but Treatment was now included as a factor in ANOVAs. Additionally, adjusted effect size was calculated with omega-squared in R and the power to detect effects was calculated with G*Power (Faul, Erdfelder, Lang, & Buchner, 2007) for the freezing results in the Phase 4 test. Cohort was also included in all initial analysis to rule out cohort influencing significant outcomes, but was removed from all primary analyses as it was not a significant main effect when comparing its influence on percent freezing on all sessions (pre-reconditioning: $F_{(1,20)} = 2.02$, $p = .170$; post-reconditioning: $F_{(1,8)} = .48$, $p = .508$).

Intracranial Cannula Placement Surgeries. 44 Long Evans male rats (Charles River Labortories, Wilmington, MA) were purchased at 275-300 g (~9-11 weeks of age) and underwent intracranial surgery for placement of guide cannulas (C315GS-5/SPC, 26 gauge, 17 mm, Plastics One, Roanoke, VA) and dummies (C315DCS-5/SPC, 30 gauge, 17.5 mm, Plastics One) above the amygdala. Rats were given an intramuscular injection of ketamine/xylazine (85 mg/kg; 10 mg/kg) for initial anesthesia and then maintained under vaporized isoflurane (1%) for the

remainder of the surgery. Following anesthesia, rats' heads were shaved and then they were placed in a stereotaxic guide (David Kopf Instruments). Guide cannulas were implanted 1 mm dorsal to the amygdala with the following coordinates, relative to bregma: -2.5 mm AP, ±5 mm ML, and -7 mm DV. Guide cannulas were secured with stainless steel screws and acrylic dental cement (Orthojet). The stainless steel dummies were then placed in the guide cannulas to prevent cannula blockage and infection. Prior to lifting anesthesia, rats were given a subcutaneous injection of Carprofen (5mg/kg; 5mg/ml; Putney) and antibiotic ointment (Neosporin) was applied to the sutures. These treatments were repeated for 3 days following surgery and animals were given 7 days to recover prior to behavior.

Behavior. The behavioral schedule was exactly as described in Experiment 7, except different groups were used here and tests occurred in Phase 4 following reconditioning. There were four groups that received one of two behavioral schedules and one of two amygdala treatments. Reconditioning with muscimol + baclofen (REC M+B) received the reconditioning procedure described above with an inactive amygdala during reconditioning, while reconditioning with saline (REC SAL) received the same behavioral schedule with an active amygdala during reconditioning. Conditioning with muscimol + baclofen (COND M+B) received the reconditioning procedure described above with an inactive amygdala during initial conditioning, while conditioning with saline (COND SAL) received the same behavioral schedule with an active amygdala during conditioning.

In brief, in Phase 1 (Days 1 and 2) both REC groups received conditioning as described in Experiment 7, while COND groups were handled and transported an

equivalent amount. For Phase 2 (Days 3-5), REC groups received extinction and COND groups were again handled and transported similarly. In Phase 3 (Day 6), all groups received a microinjection of either muscimol + baclofen or saline prior to receiving the weak conditioning parameters of reconditioning. In Phase 4 (Day 7), 24 hr following reconditioning, all groups were exposed to the context for 24 min (identical to extinction training) for the test of reacquisition of contextual fear.

Drugs. A mixture of GABAa, muscimol (Sigma-Aldrich), and GABAb, Baclofen (Sigma-Aldrich), receptor agonists was prepared to inhibit activity within the amygdala, which has the presence of both types of GABA receptors (Mitrovic, Mitrovic, Riley, Jan, & Basbaum, 1999; Wisden, Laurie, Monyer, & Seeburg, 1992), the major inhibitory neurotransmitter system in the brain. A 0.1mM muscimol + 1.0 mM baclofen mixture (M+B) in sterile saline was used as it has had previous success locally inhibiting neural activity in rodents in similar procedures (Buffalari & See, 2010; Pina, Young, Ryabinin, & Cunningham, 2015; Rogers, Ghee, & See, 2008). Sterile saline was used as the control vehicle for animals not receiving microinjections of M+B.

Microinjections. Microinjectors were made in-house with 32 gauge hallow stainless steel tubing (18 mm, Small Parts Inc.) to reach 1 mm past the end of the guide cannula and into the amygdala. Polyethylene tubing (PE-20, Instech) connected the microinjectors to 10 ul Hamilton glass syringe (Hamilton Company, Reno, NV) on a mircosyringe pump (Fusion 100, Chemyx Inc.). Microinjectors were placed into the amygdala of each animal immediately prior to Phase 3 and .3 µl of M+B or saline was bilaterally infused at an infusion rate of .3 µl/min. Microinjectors

remained in the guide cannula for 1 min following infusion to ensure the entire volume was infused and then rats were placed in the context for conditioning. 4 animals were removed from the main analysis at this step due to broken or clogged guide cannula that prevented bilateral microinjections.

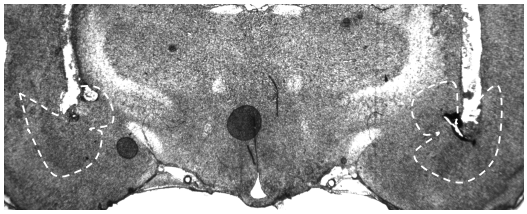
Cannula Placement Confirmation. Several days following Phase 4, rats were euthanized with isoflurane until all breathing had stopped and then brains were removed and placed in 4% PFA in PBS for no more than 24 hr. Brains then underwent cryoprotection and tissue sectioning as described in Experiment 7. Placement of the guide cannula and microinjector location was confirmed on the microscope. Correct amygdala placement was considered to be within the amygdala at -2.40 mm to -3.24 mm around bregma. Only animals with bilateral placement within the amygdala were included in analyses. Figure 18A shows a representative coronal section of guide cannula and microinjector placement in the amygdala.

Results

Cannula Placements

The placement of injectors into the amygdala can be seen in Figure 18B. For this experiment, 5 injectors terminated in the CeA, 8 injectors terminated in the LA, and 9 terminated in the BLA. The placements of the cannulae resulted in the following group numbers: REC M+B, n = 6; REC SAL, n = 4; COND M+B, n= 8; COND SAL, n = 4. Unilateral misses (n = 11) and bilateral misses (n = 7) were removed from primary analyses.

A.



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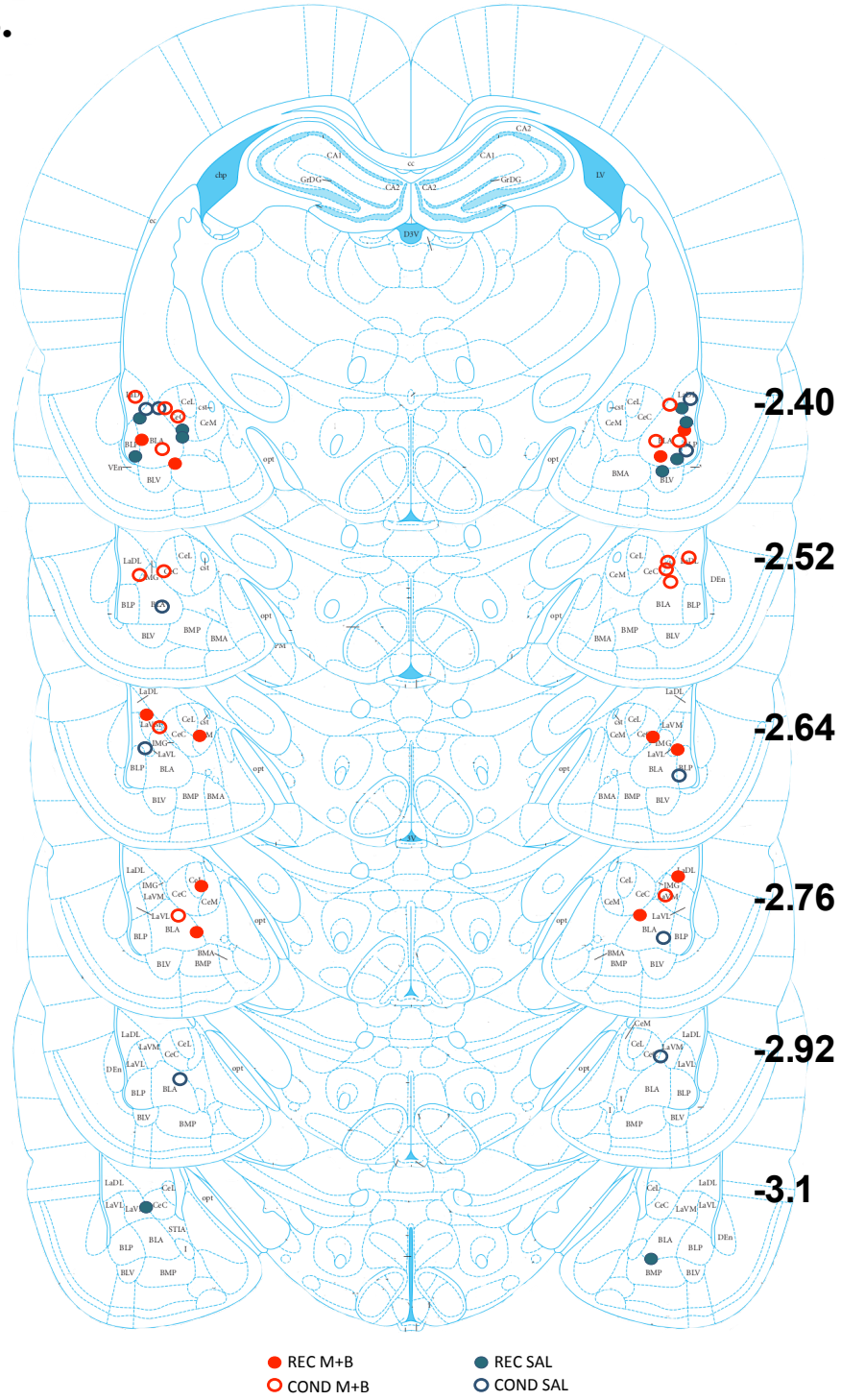

Figure 18. Cannula Placements within the Amygdala. (A) displays a representative coronal slice of the amygdala with cannula tracks ending above the amygdala to allow microinjectors to infuse M+B or SAL into the amygdala. (**B**) is a schematic of all the microinjector placements within the amygdala for Experiment 8 across bregma coordinates -2.40 to -3.1. The filled circles represent REC and empty circles represent COND groups. Red circles represent M+B amygdala treatment and blue circles represent saline treatment.

Phase 1: Conditioning

The full schedule is described in Figure 19A. Conditioning on Days 1 and 2 led to increased conditioned freezing responses (data not shown). A RMANOVA comparing Acquisition Session X Treatment for REC groups found a significant effect of Acquisition Session ($F_{(1,6)}$ = 20.68, p < .01), but not of Treatment ($F_{(1,6)}$ = 0.02, p = .871; M+B: M = 53.35, SEM = 4.30, SAL: M = 56.39, SEM = 3.78), nor an interaction ($F_{(1,6)}$ = 2.37, p = .174). This effect was due to the increase in percent time freezing from acquisition session one (M= 42.99, SEM = 5.05) to two (M = 64.47, SEM = 4.32) that showed that conditioning successfully increased conditioned responding to the context and that groups were balanced by future amygdala treatment.

Phase 2: Extinction

Extinction led to a decrease in percent time spent freezing in both REC groups prior to reconditioning (Figure 19B). A RMANOVA found a significant main effect of Extinction Session ($F_{(2,12)}$ = 21.79, p < .001), but did not find a significant effect of Treatment ($F_{(1,6)}$ = 0.91, p = .377) or interaction ($F_{(2, 12)}$ = 0.496, p = .621). The significant main effect of Extinction session was driven by more freezing in E1 relative to both E2 ($q_{(27)}$ = 4.73, p < .01) and E3 ($q_{(27)}$ = 7.41, p < .01), which showed that extinction successfully lowered freezing in 3 sessions.

Phase 3: Reconditioning

A.

Group	Surgery	Acquisition	Extinction	Reconditioning	Test
	Day - 7	*Day 1-2*	*Day 3-5*	***Day 6***	*Day 7*
REC M+B	Amygdala cannula placement surgery	12 min ++++	24 min –	**3 min +**	24 min –
REC SAL		12 min ++++	24 min –		
COND M+B		Handled	Handled		
COND SAL		Handled	Handled		

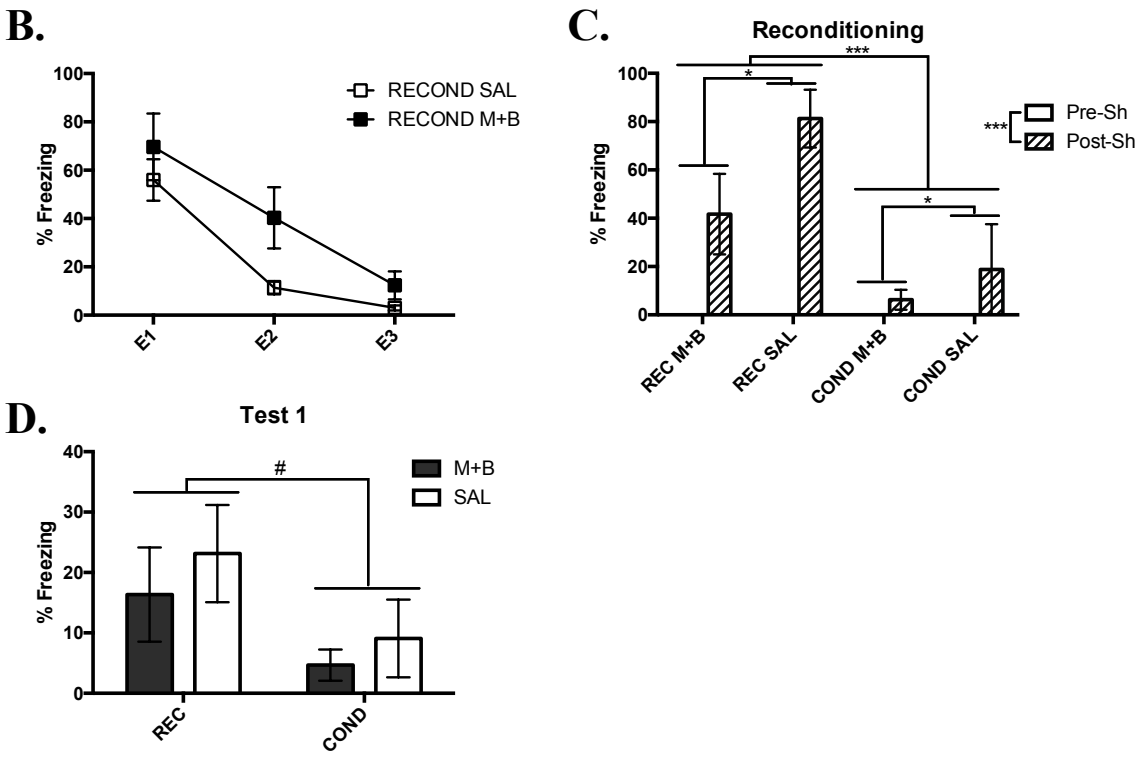

Figure 19. Temporary Inactivation of the Amygdala Does Not Prevent Rapid Reacquisition or Acquisition. (**A**) Overview of design of Experiment 8. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .75 mA shock and a minus sign indicates exposure to the context without shock. (**B**) The extinction curve of REC SAL and REC M+B as shown by the average percent freezing for each session; E = extinction session (**C**) Mean percent freezing in the 30 sec per and post shock on Day 6 during Reconditioning. Rapid reacquisition of freezing relative to initial acquisition occurs in both REC groups, but is dampened by prior amygdala M+B treatment. (**D**) Mean

percent freezing during the Test on Day 7, where expression rapid reacquisition and acquisition were unaffected by amygdala inactivation during Day 6. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$; # p = .052. REC SAL, n = 4; REC M+B, n = 6; COND SAL, n = 4; COND M+B, n = 8. Error bars represent standard error of the mean.

The weak conditioning session on Day 6 led to immediate rapid reacquisition of freezing that was generally impaired by inactivity within the amygdala (Figure 19C). A three-way RMANOVA comparing percent time spent freezing in the 30 sec before and after shock by each Group and Treatment (Time X Group X Treatment) found a significant main effect of Group ($F_{(1,26)}$ = 33.68, p < .001), driven by REC freezing more than COND. There was also a significant effect of Treatment ($F_{(1,26)}$ = 6.29, p < .05) and a significant within-subjects effect of Time ($F_{(1,26)}$ = 74.55, p < .001).

Additionally, there were two significant interactions. There was a significant Group X Time interaction ($F_{(1,26)}$ = 33.68, p < .001), caused by REC freezing more than COND after shock ($q_{(26)}$ = 10.37, p < .001), but not before ($q_{(26)}$ = .00, p = 1.00). There was also a significant Treatment X Time interaction ($F_{(1,26)}$ = 6.29, p < .05) caused by SAL freezing more than M+B after shock ($q_{(26)}$ = 3.03, p < .05) compared to before ($q_{(26)}$ = .00, p = 1.00). However, there was not a significant Group X Treatment interaction ($F_{(1,26)}$ = 3.20, p = .085) or a 3-way interaction ($F_{(1,26)}$ = 3.20, p < .085). These results suggested that rapid reacquisition occured immediately following reconditioning shock and amygdala inactivation generally impaired freezing, but the amygdala-induced impairment of freezing did not completely prevent acquisition or reacquisition from occurring in the M+B treated animals.

Phase 4: Test

In the test following Phase 3, retention of initial acquisition and rapid reacquisition of contextual fear was unimpaired by temporary pharmacological

inactivation of the amygdala during reconditioning (Figure 19D). A two-way ANOVA comparing the effect of Group and Treatment on average percent freezing revealed only a trending effect of Group ($F_{(1, 18)} = 4.34$, $p = .052$), but no effect of Treatment ($F_{(1, 18)} = .82$, $p = .376$) or the interaction ($F_{(1, 18)} = .03$, $p = .850$). Due to relatively small sample sizes in this experiment, the adjusted effect sizes (omega-squared, ω^2) of the previous ANOVA were also calculated. The main effect of Group had a medium effect size ($\omega^2 = .078$), while both Treatment ($\omega^2 = -.004$) and the Group X Treatment interaction ($\omega^2 = -.023$) had small effect sizes (medium $\omega^2 \cong .06$: small $\omega^2 \cong .01$; Kirk, 1996). Additionally, the power to detect a medium sized effect here was quite small (power = .20, where generally power = .80 is desired). This outcome suggested that the effect of Group was somewhat reliable, while Treatment and the interaction were not practically significant or this experiment did not have enough power to see a significant difference in Treatment or interaction with the current sample size.

Additionally, a similar ANOVA comparing the first 6 minutes of the test showed a significant main effect of Group ($F_{(1, 18)} = 4.73$, $p < .05$), but no effect of Treatment ($F_{(1, 18)} = .00$, $p = .992$) or the interaction ($F_{(1, 18)} = .00$, $p = .998$). These results suggested that rapid reacquisition of contextual fear occurred regardless of activity within the amygdala.

Discussion

This experiment indicated that the amygdala might not be involved in the rapid reacquisition of contextual fear. Amygdala inactivation did dampen acquisition and reacquisition of freezing, but did not prevent rapid reacquisition (or acquisition)

during reconditioning or the expression of rapid reacquisition 24 hr later. This result confirmed other findings of the limited involvement of the amygdala in reacquisition (reacquisition without extinction following post-training lesion: Maren et al., 1996; Kim & Davis, 1993; post-extinction reacquisition: Motanis & Maroun, 2012). Yet, the involvement of the amygdala in the *rapid* rate reacquisition of contextual fear relative to acquisition had not been studied in any other work as was done in this experiment.

The lack of acquisition impairment by amygdala inactivation contradicted many studies previously showing the amygdala's importance to the acquisition and expression of contextual fear conditioning (Goosens & Maren, 2001; Lee et al., 2001; Phillips & LeDoux, 1992). Yet, the acquisition parameters here were mild and could be creating a behavioral floor that was difficult to dampen further with amygdala inactivation. These results indicated that the amygdala regions explored might not need to be active for rapid reacquisition of contextual fear to occur.

Experiment 9: The Bed Nucleus of the Stria Terminalis is Involved in Rapid Reacquisition of Contextual Fear Conditioning

The BNST is implicated in anxiety, specifically sustained anxiety responses, such as those necessary during the elevated plus maze (Davis et al., 2010) and contextual fear conditioning (Waddell et al., 2006). The c-Fos IHC results in Experiment 7 also implicate the adBNST as an active region during acquisition, but not rapid reacquisition. To assess if BNST activity was necessary for rapid

reacquisition of contextual fear, I temporarily inactivated the BNST prior to reconditioning in this experiment.

Methods

This experiment was identical to Experiment 8, with the exception of the targeted brain region: the BNST. 62 Long-Evans male rats underwent surgery as described above to place a guide cannula 1 mm above the BNST. The following coordinates were used, 0.0 mm AP, ±4.20 mm ML, and -7.10 mm DV relative to bregma, and the guide cannula was lowered at a 25° angle to prevent injector placement within the lateral ventricles. 2 animals were lost during surgery and 12 animals were removed from the main analysis due to broken or clogged guide cannula that prevented bilateral microinjections.

Correct BNST placement was considered to be within the BNST at 0.12 mm to -0.60 mm around bregma. Microinjector placements within the anterior commissure between the dorsal and ventral BNST were also counted, as seen in the literature (Goode et al., 2015). Figure 20A shows a coronal section of guide cannula and microinjector placement in the BNST. This experiment was run in three separate cohorts that were analyzed together. Cohort was removed from all primary analyses, as it was not a significant main effect when comparing its influence on percent freezing on all sessions (pre-reconditioning: $F_{(2, 17)} = 3.42$, $p = .060$; post-reconditioning: $F_{(2, 33)} = .51$, $p = .606$). While cohort did show a trending effect on freezing pre-reconditioning, cohort was ultimately not included in primary analyses because there was not a significant interaction with future treatment, the other main factor pre-reconditioning, in initial analyses ($F_{(8, 68)} = 2.19$, $p = .130$).

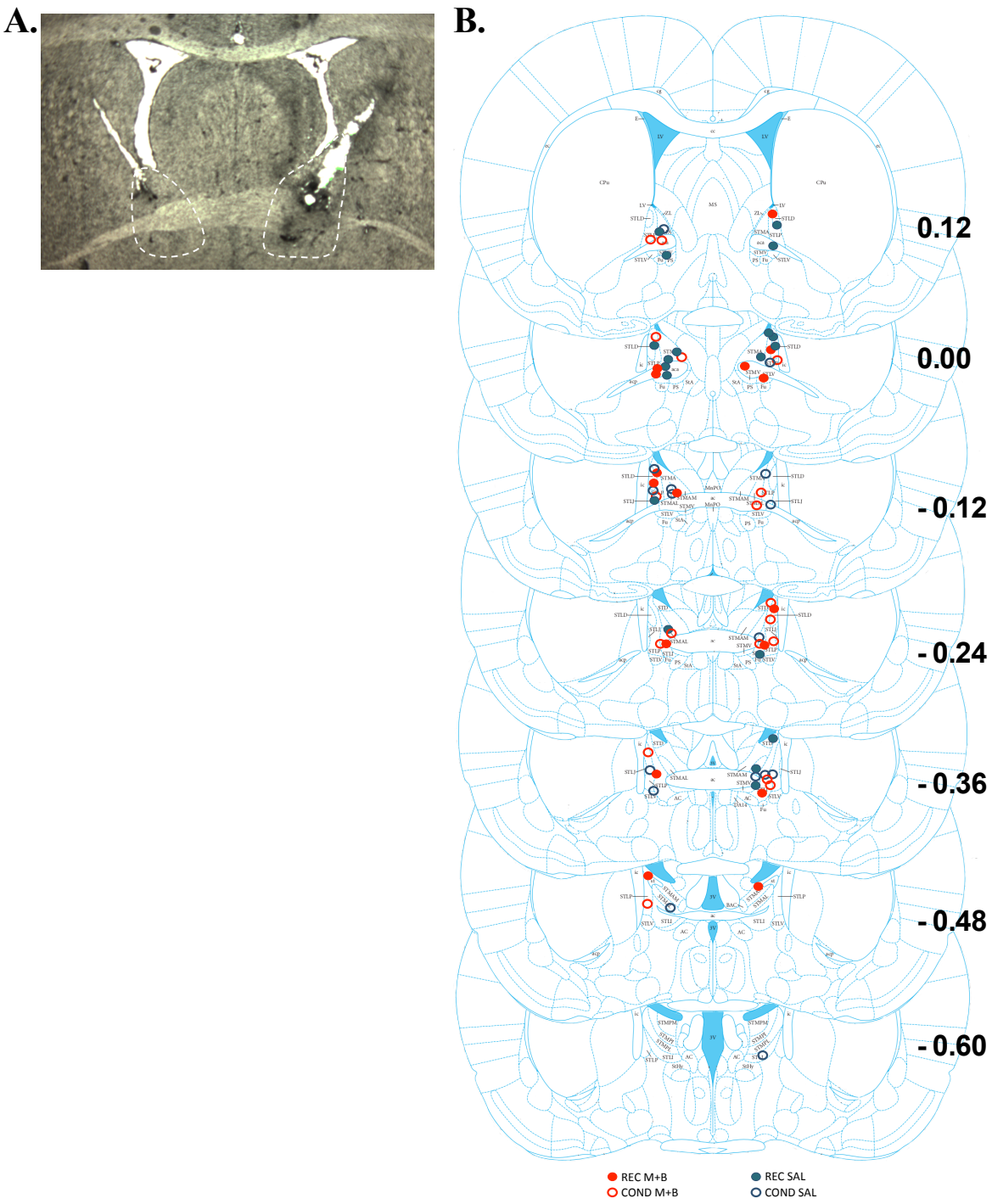

Figure 20. Cannula Placements within the BNST for Experiment 9. (A) displays a representative coronal slice of the BNST with cannula tracks ending above the BNST to allow microinjectors to infuse M+B or SAL. (**B**) Schematic of all the microinjector placements within the BNST for Experiment 9 across bregma

coordinates 0.12 to -0.60. The filled circles represent REC and empty circles represent COND groups. Red circles represent M+B BNST treatment and blue circles represent saline treatment.

Results

Cannula Placements

The placement of injectors into the BNST can be seen in Figure 20B. For this experiment, 23 injectors terminated in the anterior dorsal BNST, 4 injectors terminated in the anterior ventral BNST, 3 terminated in the posterior dorsal BNST, and 6 terminated in the anterior commissure. These placements resulted in the following groups: REC M+B, n = 8; REC SAL, n = 12; COND M+B, n= 7; COND SAL, n = 9. Unilateral misses (n = 9) and bilateral misses (n = 5) were removed from primary analyses.

Phase 1: Conditioning

The full behavioral schedule is in Figure 21A. As in previous experiments, two conditioning sessions led to a significant increase in conditioned freezing behavior in REC groups (data not shown). A RMANOVA comparing the within-subject effect of Acquisition Session and between-subject effect of future BNST Treatment revealed a significant effect of Acquisition Session ($F_{(1, 16)}$ = 27.26, p < .001; session one: M = 46.95, SEM = 2.58, session two: M = 65.97, SEM = 4.04), but no effect of Treatment ($F_{(1, 16)}$ = .01, p = .910; M+B: M = 56.36, SEM = 3.83, SAL: M = 56.52, SEM = 4.00) or an interaction ($F_{(1, 16)}$ = .01, p = .931). These results showed that conditioning led to a successful acquisition of conditioned freezing in groups balanced for future BNST treatment.

Phase 2: Extinction

Extinction training led to a successful reduction in conditioned freezing in both REC groups prior to reconditioning (Figure 21B). A RMANOVA investigating the

A.

Group	Surgery	Acquisition	Extinction	Reconditioning	Test
	Day - 7	*Day 1-2*	*Day 3-5*	***Day 6***	*Day 7*
REC M+B	BNST cannula placement surgery	12 min ++++	24 min –	**🡻 3 min +**	24 min –
REC SAL		12 min ++++	24 min –		
COND M+B		Handled	Handled		
COND SAL		Handled	Handled		

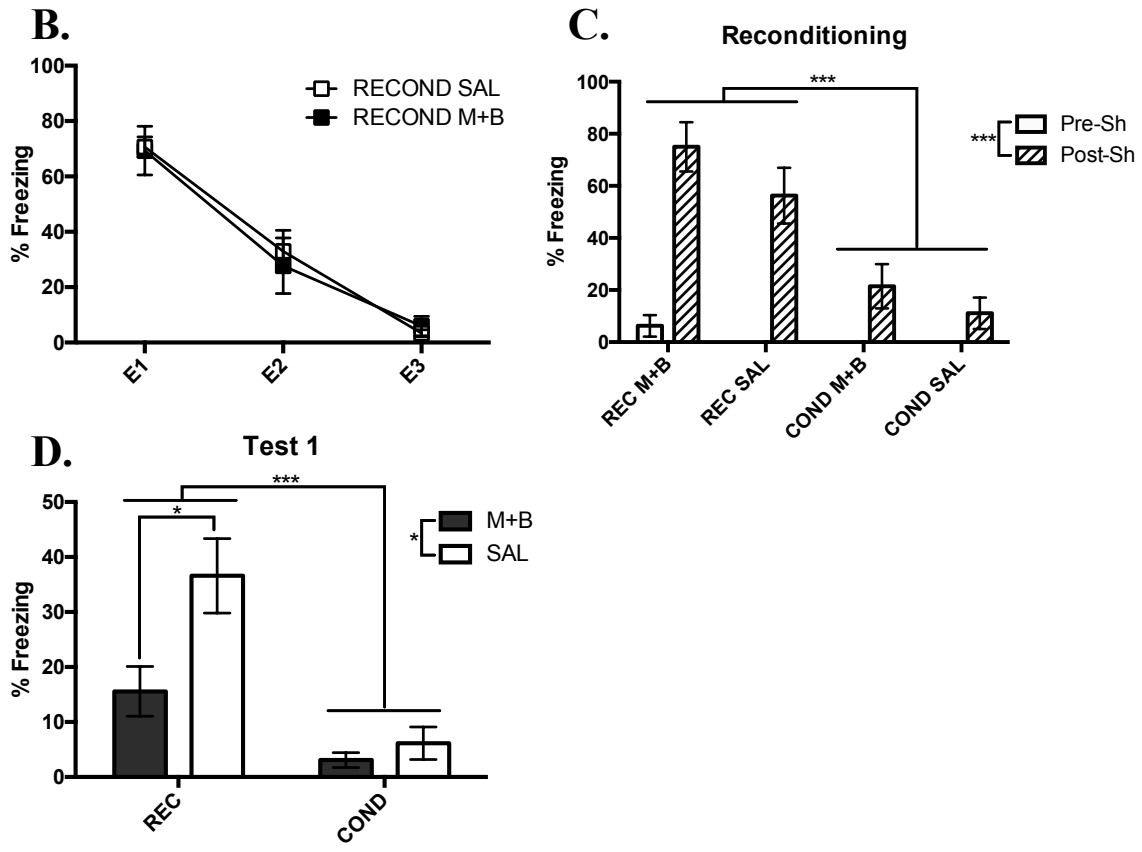

Figure 21. Inactivation of the BNST Prevents Expression of Rapid Reacquisition of Contextual Fear, but Not Mild Acquisition. (**A**) Overall design of Experiment 9. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .75 mA shock and a minus sign indicates exposure to the context without shock. (**B**) Extinction curve of REC SAL and REC M+B as shown by the average percent freezing for each session; E = extinction session (**C**) Mean percent freezing in the 30 sec per and post shock on

Day 6 during. Rapid reacquisition of freezing relative to initial acquisition occurs in both REC groups. (**D**) Mean percent freezing during the Test on Day 7, where expression rapid reacquisition, but not weak acquisition was blocked by BNST inactivation during Day 6. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$. REC SAL, n = 12; REC M+B, n = 8; COND SAL, n = 9; COND M+B, n = 7. Error bars represent standard error of the mean.

effect of repeated Extinction Sessions and future BNST Treatment on freezing behavior revealed a significant within-subject effect of Extinction Session ($F_{(2, 32)}$ = 89.19, p < .001), but no effect of future Treatment ($F_{(1, 16)}$ = .43, p = .522) or an interaction ($F_{(2, 32)}$ = .52, p = .602). The significant effect of Extinction Session was caused by significantly more freezing in E1 relative to E2 ($q_{(32)}$ = 9.29, p < .001) and E3 ($q_{(32)}$ = 15.56, p < .001) and E2 relative to E3 ($q_{(32)}$ = 6.26, p < .001), showing that three Extinction Sessions were necessary to lower freezing behavior prior to reconditioning. This result also suggested that groups had equivalent behavior prior to BNST manipulations.

Phase 3: Reconditioning

A reconditioning shock led to immediate rapid reacquisition of contextual fear that was not altered by BNST inactivation prior to Phase 3 (Figure 21C). A three-way RMANOVA comparing percent time spent freezing in the 30 sec before and after shock by each Group and Treatment (Time X Group X Treatment) found a significant main effect of Group ($F_{(1, 29)}$ = 27.98, p < .001), driven by REC freezing more than COND. There was also a significant within-subjects effect of Time ($F_{(1, 29)}$ = 71.77, p < .001) that was due to more freezing after shock relative to before. However, Treatment was not a significant main factor here ($F_{(1, 29)}$ = 2.98, p = .095).

Additionally, there was a significant Group X Time interaction ($F_{(1, 29)}$ = 24.28, p < .001), caused by REC freezing more than COND after shock ($q_{(29)}$ = 16.78, p < .001), but not before ($q_{(29)}$ = .07, p = .983). These results suggested that rapid reacquisition occurred immediately following a reconditioning shock regardless of

BNST treatment, which indicated that activity within the BNST was not involved in the reacquisition of fear during reconditioning.

Phase 4: Test

Temporary inactivation of the BNST with M+B during reconditioning impaired retention of the rapid reacquisition effect 24 hr later in the test (Figure 21D). A two-way ANOVA found a significant main effect of Group ($F_{(1, 32)}$ = 15.15, p < .001) that was due to greater freezing shown by REC compared to COND. The ANOVA also revealed a significant main effect of Treatment ($F_{(1, 32)}$ = 5.08, p < .05), which was caused by M+B group freezing less relative to SAL treatment. There was not a significant interaction of Group X Treatment ($F_{(1, 32)}$ = 2.82, p = .102), however, a t-test comparing the impact of the two treatments on REC groups revealed a significant impairment of M+B on retention of reacquisition freezing relative to SAL ($t_{(21)}$ = 2.82, p < .05). Interestingly, a t-test comparing COND groups across treatments did not find a significant effect of Treatment ($t_{(16)}$ = .60, p = .555). These results suggest that activity within the BNST during a reacquisition shock is involved in retention of the reacquisition memory 24 hr later.

Discussion

The results here suggested that the BNST was necessary for retention of rapid reacquisition of contextual fear. This conclusion corresponds with the current theory of the BNST's involvement in sustained fear or anxiety-like responses (Davis et al., 2010; Walker, Toufexis, & Davis, 2003), in which animals display sustained anxiety behavior to a context rather than a phasic fear to a cue (like a discrete light or tone conditioned stimulus). Both acquisition and reacquisition of contextual fear

conditioning likely rely on the BNST, as contextual fear leads to sustained fear responses in the presence of the context. Interestingly, I found that BNST inactivation did not impair initial acquisition of contextual fear, which conflicted with previous findings (Waddell et al., 2006). However, as in the last experiment, the conditioning parameters here were mild and might not lead to acquisition of enough freezing behavior to be impaired with pharmacological inactivation.

Experiment 10: The Bed Nucleus of the Stria Terminalis is Involved in Strong Acquisition of Contextual Fear Conditioning

In the previous experiment, BNST inactivation with M+B did not impair initial acquisition of contextual fear, as has been previously found (Sullivan et al., 2004; Waddell et al., 2006; Zimmerman & Maren, 2011). Yet, the parameters of initial acquisition in all previous experiments intentionally resulted in low levels of conditioned behavior in conditioning control groups. Thus, in this experiment, I assessed the role of the BNST in *strong* acquisition of contextual fear by temporarily inactivating the BNST prior to stronger conditioning parameters.

Methods

The methods for this experiment are identical to Experiment 9 except for the behavioral schedule and timing of microinjection into the BNST to assess the role of the BNST initial strong conditioning. There were 2 groups who differed in the BNST treatment: Conditioning with an inactive BNST, COND M+B, and conditioning with an active BNST, COND SAL. 40 Long-Evans male rats underwent surgery as described above to place a guide cannula 1 mm above the BNST. Figure 20A shows

a representative coronal section of guide cannula and microinjector placement in the BNST. The experiment was run in two separate cohorts that were analyzed together. Cohort was removed from primary analyses as it did not have a significant main effect in freezing across all sessions ($F_{(1, 24)}$ = .58, p = .454). Two animals were died during surgery and 6 animals were removed from the main analysis due to broken or clogged guide cannula that prevented bilateral microinjections.

Behavior and Microinjection

In brief, 7 days following surgeries, animals received a microinjection of either M+B or saline into the BNST immediately prior to initial conditioning on Day 1. Initial conditioning was performed like in previous experiment, 12 min context exposure with 4 shocks, but only one day of conditioning occurred instead of two. On Day 2, animals were exposed to the context for 24 min without shock to test their memory of the context and as an initial extinction session (Test1/Extinction Session 1 or T1/E1). Days 3 through 4 were repeated tests, which act as extinction sessions (T2/E2 and T3/E3). The following day, reconditioning (3 min context exposure with 1 shock) occurred (Day 5). Day 6 was a post-reconditioning test, which was identical to treatments on Days 2-4. All sessions were separated by 24 hrs.

Results

Cannula Placements

The placement of injectors into the BNST can be seen in Figure 22. For this experiment, 23 injectors terminated in the anterior dorsal BNST, 2 terminated in the posterior dorsal BNST, and 1 terminated in the anterior commissure. The cannulae

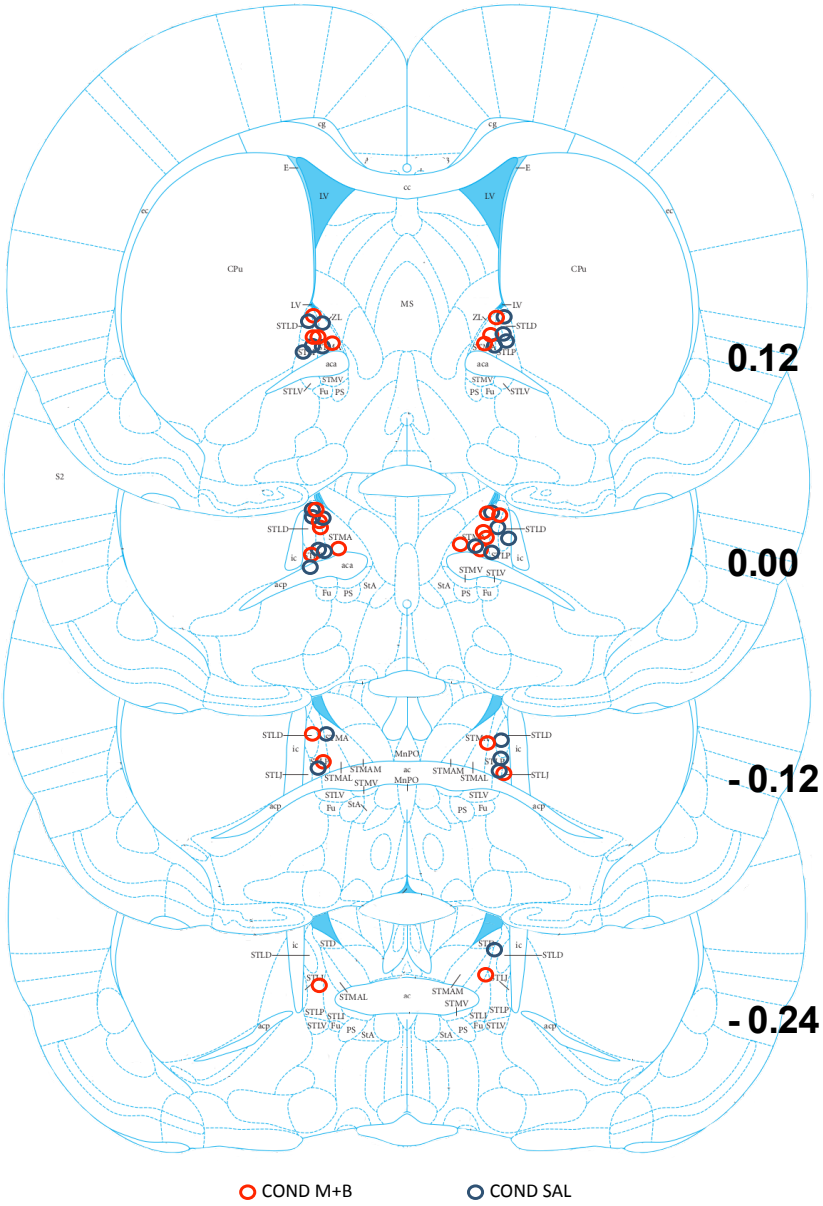

Figure 22. Cannula Placements within the BNST for Experiment 10. This figure is a schematic of all the microinjector placements within the BNST for Experiment 10 across bregma coordinates 0.12 to -0.24. Red circles represent M+B BNST treatment and blue circles represent saline treatment.

placements resulted in the following group numbers: M+B, n= 14; SAL, n = 12. Unilateral misses (n = 3) and bilateral misses (n = 3) were removed from primary analyses.

Conditioning

The full behavioral schedule can be seen in Figure 23A. Inactivation of the BNST caused an enhancement of freezing to shock during initial strong acquisition (Figure 23B). A t-test comparing the percent freezing across Treatment found significantly greater freezing in M+B compared to SAL ($t_{(24)} = 2.52$, $p < .05$). This result suggested that pre-treating the BNST with M+B might enhance conditioned responding to shock.

Test 1

In the retention test, acquisition with BNST M+B pre-treatment caused a marked reduction in the ability to express the acquisition memory 24 hr later (Figure 23C). A t-test comparing the percent freezing in the test across Treatment revealed significantly less freezing by M+B compared to SAL ($t_{(24)} = 2.97$, $p < .01$), which indicated that activity in the BNST was involved in initial acquisition of a strong acquisition memory.

Extinction

If the first test and the two tests following are considered extinction sessions (i.e., for this experiment, Tests 1, 2, and 3 are synonymous with Extinction Sessions 1, 2, and 3; T1-T3 = E1-E3), then extinction training resulted in a decreased in conditioned responding in both treatments (Figure 23D). A RMANOVA showed a

A.

Group	Surgery	Acquisition	Extinction	Reconditioning	Post-Reconditioning Test
	Day - 7	***Day 1***	*Day 2-4*	*Day 5*	*Day 6*
COND M+B	BNST cannula placement surgery	**🡻12 min ++++**	24 min –	3 min +	24 min –
COND SAL					

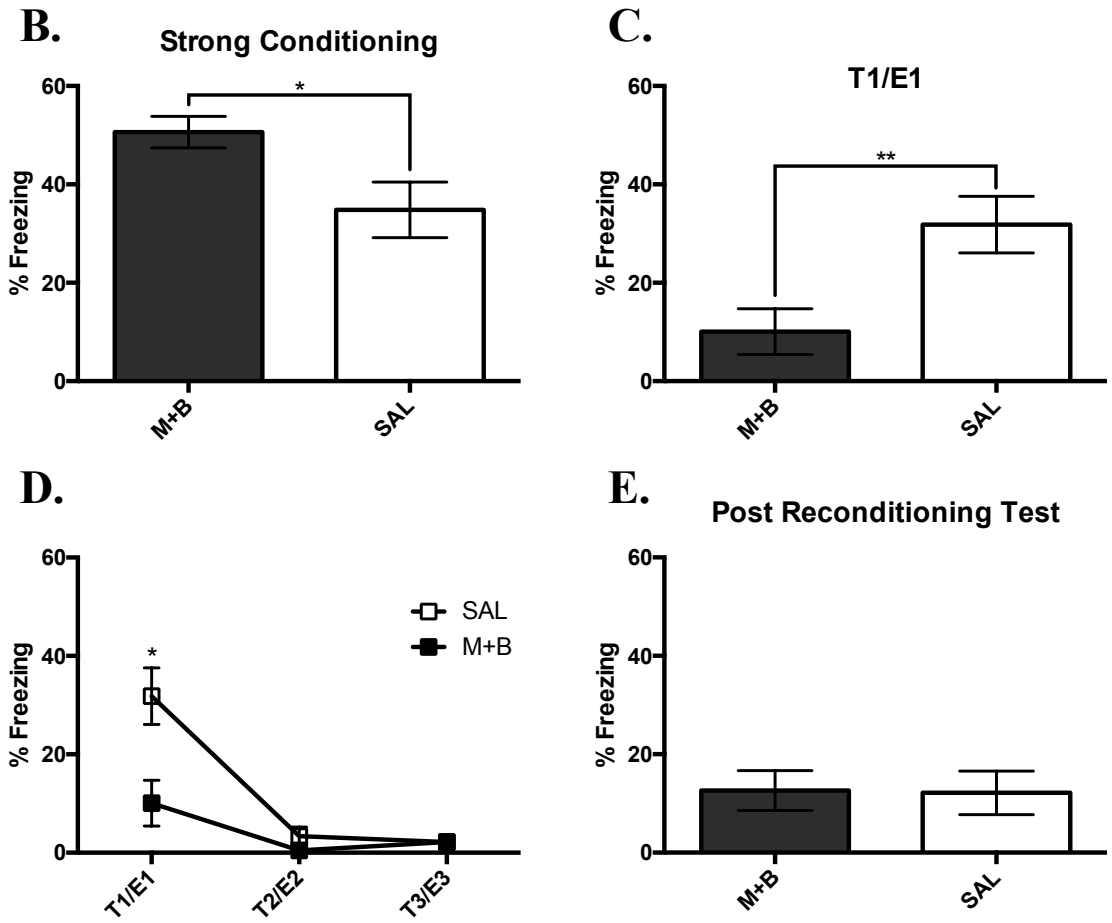

Figure 23. Inactivation of the BNST Prevents Strong Acquisition. (**A**) Overall design of Experiment 10. The times listed represent the total time of exposure to the context for a given session. A plus sign indicates a single .75 mA shock and a minus sign indicates exposure to the context without shock. (**B**) Mean percent freezing during acquisition of strong conditioning with an active or inactive BNST. An inactive

BNST enhanced freezing. (**C**) Mean percent freezing during the expression of acquisition in the test on Day 2, where retention of acquisition was impaired by an inactive BNST during conditioning (**D**) Extinction curve on Days 2 through 4 as shown by a line graph of both groups' decreasing average percent time freezing. (**E**) Mean percent freezing during the test of reacquisition on Day 6 that was unaffected by initial acquisition differences. Significance between groups is represented by *** $p < .001$; ** $p < .01$; * $p < .05$. COND SAL, n = 12; COND M+B, n = 14. Error bars represent standard error of the mean.

significant main effect of Treatment ($F_{(1, 18)}$ = 4.73, p < .05), Extinction Session ($F_{(2, 36)}$ = 22.21, p < .001), and Treatment X Extinction Session interaction ($F_{(2, 36)}$ = 4.22, p < .05). The significant main effect of Treatment was caused by SAL freezing more than M+B (Figure 23D). More freezing in E1 relative to E2 ($q_{(36)}$ = 16.29, p < .001) and E3 ($q_{(32)}$ = 16.73, p < .001) caused the significant within-subjects effect of Extinction Session. The interaction was due to SAL freezing more than M+B in E1 ($q_{(32)}$ = 15.97, p < .001), but not in E2 ($q_{(32)}$ = .09, p = .985) or E3 ($q_{(32)}$ = .00, p = 1.0). This interaction showed that inactivation the BNST during acquisition caused a significant impairment in freezing that dissipated after one extinction session (i.e., after Test 1).

Reconditioning and Post-Reconditioning Test

Reconditioning did not lead to differences in reacquisition of freezing due to previous treatment (data not shown). A two-way RMANOVA comparing percent time spent freezing in the 30 sec before and after shock by each past Treatment during conditioning (Time X Treatment) found a significant within-subjects effect of Time ($F_{(1, 18)}$ = 32.31, p < .001), driven by significantly more freezing after (M = 40.38, SEM = 6.06) shock compared to before (M = .00, SEM = .00). However, there was no effect of past Treatment ($F_{(1, 18)}$ = 1.19, p = .290; M+B: M = 15.18, SEM = 4.70, SAL: M = 26.04, SEM = 6.97) or an interaction effect ($F_{(1, 18)}$ = 1.19, p = .290) on freezing, suggesting that post-shock reacquisition was independent of past BNST inactivation and differences in conditioning.

Additionally, a t-test comparing the expression of reacquisition in a test 24 hr later did not show an effect of previous Treatment of reacquisition freezing ($t_{(24)}$ =

.07, p = .941), which again suggested that all animals were able to reacquire equally despite prior impairment of initial acquisition due to an inactive BNST (Figure 23E).

Discussion

This experiment found inactivation of the BNST immediately prior to strong acquisition initially enhanced freezing responses, but then impaired expression of acquisition in a retention test. These results confirm that the BNST is important for the acquisition of conditioned contextual fear. Also, the BNST showed a biphasic response to M+B inactivation that initially enhanced freezing behavior, but eventually led to an impairment in contextual fear memory. However, this impairment in initial acquisition did not last into a drug-free reacquisition, as both groups reacquired equally regardless of initial acquisition differences. This reacquisition effect differed from earlier parameters as only one instead of two conditioning sessions occurred before reconditioning and reacquisition was not directly compared to initial acquisition.

General Discussion

The major finding from these experiments was that rapid reacquisition likely had a similar, but distinct involvement of brain regions compared to initial acquisition. In brain regions that showed significant group differences in the immunohistochemistry experiment, initial acquisition either caused the only significant increase in or the strongest activation of markers of neural activity relative to rapid reacquisition. Additionally, I found that activity within the BNST was

necessary for acquisition and rapid reacquisition, while activity in the amygdala might not be necessary for rapid reacquisition of contextual fear. Taken together, these results suggest that rapid reacquisition of contextual fear may rely on less brain regions or a similar, but distinct set of brain regions than those involved in acquisition.

The Amygdala May Be Important for the Acquisition, but Not Rapid Reacquisition, of Contextual Fear.

With regards to the amygdala, the findings here suggested that the amygdala was a region important for the acquisition, but less so for reacquisition of contextual fear conditioning. The IHC results for histone acetylation found that the lateral subregion of the amygdala had greater H4K8ac following weak initial acquisition than rapid reacquisition following extinction, reacquisition without extinction, and no-learning controls. This finding suggested that the lateral amygdala was more important for acquisition than reacquisition and might not be involved in reacquisition at all due to the reacquisition groups showing equivalent H4K8ac in the LA to no-learning controls.

An increase of H4K8ac following only acquisition suggested that this region was important for the molecular processes during and leading to the acquisition of a contextual fear memory. The lateral amygdala is a region known for being the entryway for sensory information that will be part of an associative memory (Hiroi & White, 1991; LeDoux et al., 1990; Maren & Quirk, 2004; Nader et al., 2001) and is necessary for cued and contextual fear conditioning (Goosens & Maren, 2001). Particularly relevant to this finding, two studies found that epigenetics processes,

including histone acetylation, within the LA were critical for synaptic plasticity during and consolidation of a fear memory (Maddox et al., 2013; Monsey, Ota, Akingbade, Hong, & Schafe, 2011). Given these findings and the LA's role as the sensory gateway for associative memories, the LA was likely to increase H4K8ac expression immediately following exposure of context and shock pairing.

In addition to the H4K8ac LA results, another piece of evidence that suggested the amygdala is not as involved in reacquisition was the amygdala inactivation study. This study found that inactivation of the amygdala during reconditioning did initially impair conditioned freezing following shock, but did not cause an impairment in retention of the rapid reacquisition effect in the following day during a drug-free context test. This outcome suggested that the amygdala was important for freezing and conditioned fear responses, such that when the amygdala was inactivated, freezing to a naïve or previously conditioned context was dampened. However, activity in the amygdala might not be necessary for rapid reacquisition retention, as rapid reacquisition retention was seen in both groups that had an active or inactive amygdala during reconditioning.

Contrary to many examples in the literature, I did not find an acquisition impairment following amygdala inactivation. Rats were able to express memory of acquisition that was independent of the amygdala's activity status during acquisition. It is highly unlikely that the amygdala is not involved in acquisition of contextual fear following one shock and context pairing, as the amygdala has been shown to be involved in contextual fear conditioning acquisition and expression many times over (Goosens & Maren, 2001; Helmstetter & Bellgowan, 1994; Laurent & Westbrook,

2009b; Lee et al., 2001; Muller et al., 1997; Wilensky, Schafe, Kristensen, & LeDoux, 2006b). Thus, I do not believe that the results of Experiment 8 suggest that the contextual fear conditioning is amygdala-independent. However, the parameters of the conditioning session used here were mild (3 min of context exposure with one shock). These weak parameters led to a small amount of conditioned freezing, which could cause a behavioral floor that was difficult to impair with amygdala inactivation, similar to what was seen with BNST inactivation.

Despite not finding evidence of the amygdala's involvement in acquisition in these studies, the proposed theory that the amygdala may be important for initial learning, but not re-learning is not entirely novel. First, the CeA is necessary for acquisition and expression of fear-potentiated startle, but not reacquisition (Kim & Davis, 1993). Second, the BLA is important for initial extinction, but not re-extinction (Laurent et al., 2008; Laurent & Westbrook, 2010). However, regarding reacquisition of fear, the BLA has shown mixed results. One study showed that reacquisition of fear is a BLA-independent process (post-training lesion of BLA does not completely block reacquisition; Maren et al., 1996). Yet, others have shown that the BLA is involved in the post-extinction reinstatement and reacquisition of cued fear (Laurent & Westbrook, 2010) and the reacquisition of contextual fear (Laurent & Westbrook, 2009b). Additionally, molecular process, like actin rearrangement (but not protein synthesis; Motanis & Maroun, 2012), and NMDA neurotransmission (Laurent & Westbrook, 2009b) within the BLA have displayed roles in reacquisition of learning. These molecular findings suggest that even though reacquisition can occur without activity in the amygdala, it does not necessitate that reacquisition is completely

independent of the amygdala. Cumulatively, the literature suggests that the amygdala is somewhat involved in reacquisition.

Yet, the involvement of the BLA or amygdala as a whole to *rapid* reacquisition of contextual fear relative to acquisition had not been previously examined until this work. Overall, I found that the amygdala did not need to be active for rapid reacquisition of contextual fear to occur and rapid reacquisition did not lead to increased amygdala histone acetylation expression seen in acquisition. These results represent the first finding of the minimal role of the amygdala in rapid reacquisition of contextual fear and lend further evidence to the theory that the amygdala's role in re-learning associative memories is likely specific or minimal.

However, the outcome the amygdala inactivation results needs to be interpreted with caution. The power analysis of the ability to detect a medium effect size with the sample size in Experiment 8 revealed that the study was underpowered for detecting medium-sized (and thus also small-sized) effects. Nonetheless, amygdala inactivation did generally impair freezing during conditioning and reconditioning, indicating that the amygdala was inactivated by M+B and prevented expression of freezing responses, as was seen previously (Kim, Rison, & Fanselow, 1993; Wilensky, Schafe, Kristensen, & LeDoux, 2006). Also, I was able to detect a moderate rapid reacquisition effect (regardless of treatment) in Experiment 8, suggesting that there was enough power to see reliable differences. Thus, the results suggest that the amygdala may not be critically involved in rapid reacquisition, but further study is needed to confirm this finding.

Hippocampal Activity is Associated with Both the Acquisition and Rapid Reacquisition of Contextual Fear Conditioning.

The hippocampus is a region critical for spatial cognition and spatial learning (Jung, Wiener, & McNaughton, 1994; Kim & Fanselow, 1992a; Moser, Moser, Forrest, Andersen, & Morris, 1995; Muller, Stead, & Pach, 1996). The results here further implicate the hippocampus as an important structure for spatial learning and suggest that the hippocampus may also be important for relearning spatial fear. Both acquisition and rapid reacquisition of contextual fear caused increased c-Fos expression in the whole dorsal hippocampus.

Many studies have shown the importance of the dorsal hippocampus in contextual fear conditioning (Corcoran, Desmond, Frey, & Maren, 2005; Lee & Kesner, 2004; Phillips & LeDoux, 1992). Following acquisition contextual fear, markers like c-Fos show increased expression in the hippocampus (Huff et al., 2006) and hippocampal place cells show strong remapping to a context paired with shock in conditioning (Moita et al., 2004). Moreover, many pharmacological inactivation and lesion studies have shown the necessity of the hippocampus in contextual, but not cued, fear conditioning (Corcoran et al., 2005; Phillips & LeDoux, 1992). My c-Fos results, in combination with many in the field before it, confirm the role of the hippocampus in contextual fear.

However, these results also present the first finding of the hippocampal neural activity associated with rapid reacquisition of contextual fear. Following both rapid reacquisition with and without prior extinction, there was an increase in c-Fos expression in the dorsal hippocampus. It is important to note that the hippocampus

was the only region that showed increased activity markers following reacquisition. Due to the extensive proof of the dorsal hippocampus's role in spatial learning, it is likely that the hippocampus is important during learning events that update the associative strength of a context. Accordingly, molecular processes of retrieval within the hippocampus are necessary to strengthen an initial contextual fear memory (Lee, 2008; 2010) and may explain the reacquisition-induced increase in c-Fos. Additionally, many theorize that the hippocampus is a site important for indexing episodic memories and thus may be critical for relearning contextual fear in addition to initial learning (Teyler & Rudy, 2007).

Yet, my results merely correlated hippocampal activity with, but did not show the functional importance of the hippocampus to, reacquisition of contextual fear. However, studies show that the hippocampus is necessary for rapid reacquisition for the memory of the spatial location of a food reward in a cross maze (Winocur et al., 2005) and molecular processes within the hippocampus are important for reacquisition of fear (Bevilaqua et al., 2005; Motanis & Maroun, 2012). Hence, it was entirely possible that the dorsal hippocampus played an important role in rapid reacquisition of fear, even if it was not functionally shown here.

I also found that the DG of the dorsal hippocampus showed an increase in c-Fos positive cells following the acquisition and rapid reacquisition of contextual fear conditioning. The DG was also the site of most c-Fos positive cells among the hippocampus subregions, suggesting the importance of the DG to contextual fear. Unlike the hippocampus as a whole, the DG had significantly more c-Fos in

expression following acquisition compared to both reacquisition groups, suggesting that the DG in particular was critical for contextual fear conditioning.

Previous research found that the DG is critical for pattern separation (Treves & Rolls, 1994) and is the best-known site of adult neurogenesis in rats (Altman & Das, 1965). There has also been evidence that the DG is necessary for the retrieval of contextual fear memories 1 day after contextual fear conditioning (Lee & Kesner, 2002; 2004). Further, the DG tags and recruits adult-generated neurons to update and strengthen remote spatial memories (Trouche, Bontempi, Roullet, & Rampon, 2009). Accordingly, the increase in DG staining following acquisition could be due to neurogenesis or pattern separation following formation of a new spatial fear memory. Whereas, the increase in DG c-Fos following reacquisition could either represent retrieval, potential modification/enhancement, and pattern separation of the original contextual memory. The relatively lower c-Fos expression in the reacquisition groups could be due to habituation of c-Fos following multiple exposures to shock and context (Melia et al., 1994, Watanabe, Stone, & McEwen, 1994), but also could suggest that DG activation was larger in response to initial acquisition over reacquisition and perhaps more critical for initial acquisition.

It is also possible that the c-Fos activation seen in the DG and hippocampus as a whole is due to spatial recognition and processing rather than specific to spatial learning. The hippocampus has shown expression of IEG activity markers in tasks that are known to be hippocampal-independent (Guzowski, 2002; Kubik, Miyashita, & Guzowski, 2007), but spatial processing and spatial learning are intrinsically related and difficult to dissociate here. The inclusion of a handling control group

hopefully mitigated some of the c-Fos expression due to non-learning-specific hippocampal processes, but the handling animals were not exposed to the conditioning context and could not account for activation of place cells specific to the context. Unfortunately, the work here did not include an examination of the hippocampus' functional necessity to rapid reacquisition relative to acquisition. However, the c-Fos outcomes indicate that the hippocampus and DG are associated with acquisition and reacquisition and that the DG is particularly activated by acquisition over reacquisition of contextual fear.

The BNST is involved in Both the Acquisition and Reacquisition of Contextual Fear Conditioning.

The results for the BNST show that it is a region that is critical for both the acquisition and rapid reacquisition of contextual fear. First, the IHC showed that initial acquisition led to increased c-Fos positive cells in the adBNST. The BNST has been implicated in fear and anxiety-like responding (Davis et al., 2010; Sink, Chung, Ressler, Davis, & Walker, 2013) and contextual fear conditioning (Sullivan et al., 2004; Waddell et al., 2006; Zimmerman & Maren, 2011). Specifically, the BNST is theorized to be important for sustained, not phasic, fear responses that occur in response to long duration cues or unpredictable threat (Davis et al., 2010; Fendt, Endres, & Apfelbach, 2003; Walker et al., 2003), such as that seen during contextual fear conditioning. During contextual fear conditioning, shock is not discretely signaled causing animals to show sustained defensive behavior during continuous exposure with the best predictor of shock, the context. Thus, enhanced markers of activity in the BNST following contextual fear acquisition was expected, as the BNST

as a whole had previously shown increased markers of metabolic activity following the expression of contextual fear (González-Pardo, Conejo, Lana, & Arias, 2012). Additionally, the adBNST is particularly important for anxiogenic behavior and stress responses (Herman, Cullinan, & Watson, 1994; Sung-Yon Kim et al., 2013).

Direct manipulation of the BNST prior to acquisition also revealed its importance in initial contextual fear learning. Pharmacologically inhibiting activity within the BNST prior to strong conditioning caused impaired contextual fear acquisition retention. As mentioned previously, this outcome was not the first finding of the BNST's role in contextual fear conditioning, but I did however find that inactivating the BNST only impaired initial acquisition of strong, not mild conditioning. This result is interesting as weak conditioning led to a significant increase in c-Fos expression in the adBNST. One might expect that weak initial acquisition would also be impaired by BNST inactivation. The lack of weak acquisition impairment could be due to the low amount behavior elicited by weak conditioning that could be a behavioral floor. Additionally, the BNST is an heterogeneous brain region, comprised of at least 18 subregions (Ju & Swanson, 1989; Ju, Swanson, & Simerly, 1989), some of which have been proposed to have opposing roles in anxiety. The oval nucleus within the adBNST is thought to be anixogenic, but the remainder of adBNST has shown anxiolytic properties (Sung-Yon Kim et al., 2013). Further, my pharmacological studies targeted all of the BNST, dorsal and ventral, and the significant c-Fos results were found only in the dorsal. Thus, inactivating the whole BNST could cause opposing processes that resulted in no effect on milder contextual fear manipulations.

Interestingly, during strong conditioning, inactivation of the BNST led to increased freezing behavior that later led to weakened expression of acquisition. This finding showed that BNST inactivation has a biphasic response during acquisition and expression. This example was not the first time inactivation of the BNST with GABA agonists caused an increase fear response. Meloni, Jackson, Gerety, Cohen, & Carlezon (2006) found that delivery of muscimol to the BNST led to increased acoustic startle in the presence of a light previously paired with shock (fear potentiated startle) relative to saline. This result, in conjunction with my findings, suggests that the BNST is also involved in inhibiting fear potentiated startle responses, as well as conditioned freezing following contextual fear acquisition and reacquisition. One could theorize that the BNST inhibits responses to unconditioned stimuli. However, this theory is unlikely to be correct as inactivating the BNST only enhanced startle during fear-potentiated, but not to acoustic startle alone, and did not have an effect on other types of fear conditioning (like cued fear conditioning; Sullivan et al., 2004). Additionally, the Meloni et al. result could be an aberrant outcome as others found that fear potentiated startle was unaffected by BNST inactivation via AMPA antagonistm (Walker & Davis, 1997) and lesions (Lee & Davis, 1997). Conversely, this opposing outcome of BNST inactivation on contextual fear conditioning (enhanced freezing during acquisition and subsequently impaired retention of acquired freezing) could again show the differential role of BNST subnuclei in anxiety-like behavior or simply represent a biphasic response to BNST GABA agonism.

Unlike the amygdala, the BNST appears to be important for both the acquisition and *reacquisition* of contextual fear conditioning. Specifically, inactivating the BNST with GABA agonists prior to reconditioning prevented retention of rapid reacquisition of fear. The BNST has previously been shown to be important for the reinstatement of fear conditioning (Goode et al., 2015; Waddell et al., 2006) and stress-induced reinstatement of drug-seeking behavior (Erb & Stewart, 1999). The BNST result here is the first finding of the BNST's involvement in rapid reacquisition of contextual fear. Interestingly, there was not a statistically significant increase in c-Fos expression in the adBNST following reacquisition, conflicting with the BNST inactivation results. This lack of increase again could be due to the diverse nature of the BNST. Or this result could suggest that the BNST was active during reconditioning, but might be important to consolidation of reacquisition. The IHC likely captured c-Fos that represented activity during reconditioning, as brains were preserved roughly over an hour after. Also, in Experiment 9, inactivation of the BNST did not prevent rapid reacquisition during reconditioning, but did impair expression of rapid reacquisition the following day. Thus, perhaps, the BNST is critical for the consolidation and retention of the rapid reacquisition effect rather than for the mechanisms of reacquiring during reconditioning itself.

These results confirm the role of the BNST in contextual fear conditioning and lend additional support to the theory that the BNST is important for sustained anxiety or defensive responses to unpredictable threat even after the immediate threat has passed. In addition to studies that showd the BNST's importance for sustained fear responses in initial contextual fear acquisition (Sullivan et al., 2004; Waddell et al.,

2006) and fear conditioning of similar long-duration cues (Walker & Davis, 1997; Walker, Miles, & Davis, 2009), this chapter presented the importance of the BNST to rapid reacquisition, which involves a large, rapidly acquired sustained fear response that is a disproportionate (relative to conditioned animals) to the stimuli provided (a single context-shock association). Additionally, my results showed the impairment of contextual fear acquisition and reacquisition by BNST inactivation occurred later when the threat is long removed. In conjunction with other work above, these studies further implicated the BNST as the site of sustained fear responses to long-duration or unpredictable cues.

While BNST inactivation during reconditioning did cause a decrease in freezing during rapid reacquisition expression, there was not a significant Group X Treatment interaction to show this impairment was specific to reacquisition. An additional t-test between the treatments was required to reveal that rapid reacquisition was impaired, but not initial weak acquisition. This result found that inactivating the BNST had a moderate effect on rapid reacquisition. However, this finding also indicates that BNST activity is necessary, but not sufficient, for rapid reacquisition and that other regions, such as the hippocampus, are likely involved. Additionally, the heterogeneous nature of the BNST makes it difficult to interpret which subregions of the BNST, or even within the adBNST, are truly important to rapid reacquisition, as it is likely they are playing opposing roles (Sung-Yon Kim et al., 2013). More advanced viral techniques will be needed to elucidate subregion involvement in contextual fear learning.

Additional caveats of the results herein should also be noted. With regard to histology, the markers of neural activity selected here may have limited my findings. While reliably used as a marker of neuronal activity in many studies, c-Fos does not designate a region's functional requirement for contextual fear conditioning (Guzowski, 2002; Kubik et al., 2007). Evidence of c-Fos not corresponding to functional activity was found in the BNST. Following reacquisition, c-Fos was not upregulated, suggesting that the BNST was not recruited by reacquisition. However, the pharmacological inactivation studies show the opposite – that the BNST was necessary for rapid reacquisition of fear conditioning. This finding indicates that c-Fos expression is not a one-to-one correlation with the functional relevance of a brain region during learning. Additionally, the difference in locomotion between the animals during the period where c-Fos was examined could also alter c-Fos expression in brain regions important for motor control during a fear response.

Additionally, my results could be limited by the habituation of c-Fos, which has been found after repeated restraint stress (Melia et al., 1994), as well as in other repeated paradigms. Habituation is proposed to occur through either habituation of cellular expression of c-fos when a cell is reactivated by the same stimulus or by habituation of the population of cells activated by repeated stimulus presentation, such that the population of cells activated the second time is smaller (Girotti et al., 2006). Thus, either type of habituation could potentially occur during repeated context or shock exposure. This pitfall of c-Fos could also explain the findings in adBNST, as rapid reacquisition involved repeated exposure to the context and shock and could have habituated c-Fos expression. Importantly, c-Fos habituation could be

an alternative explanation for the findings of lowered c-Fos activity after reacquisition relative to acquisition in all the brain regions examined here. Decreased c-Fos has been seen throughout the fear circuit following pre-exposure to context, tone, or shock in cued fear conditioning (Radulovic, Kammermeier & Spiess 1998). This work found that simultaneous enhanced c-Fos expression caused by the a tone and shock during pairing was critical for strong acquisition of cued fear conditioning. Habituation of c-Fos would negate the conclusion that the fear circuit is less involved in reacquisition. However, c-Fos did increase in the hippocampus following reacquisition. This outcome suggests that c-Fos did not habituate the contextual fear learning in all regions, particularly in a region critical for contextual fear learning. Additionally, the other marker of neural activity H4K8ac has not demonstrated habituation to repeated experiences and I demonstrated selective enhancement of H4K8ac only following acquisition within the lateral amygdala. Yet, an IEG whose expression does not habituate to repeated treatment, like ΔFosB (Kelz et al., 1999), or use of double labeling might have been more appropriate for examining reacquisition.

Also, H4K8ac is not just associated with learning and can occur for many different processes that involve transcriptional activation. For instance, H4K8ac is upregulated in inflammation (Ashbrook et al., 2015) and antibody (Li, Zan, Xu, & Casali, 2013) responses. This alternative could be represented in the H4K8ac results, where there was very large magnitude of staining within the mPFC, amygdala, and dBNST. The massive H4K8ac staining of these regions could have created a ceiling effect of expression that prevented discernment of reliable group

differences. Additionally, histone acetylation is upstream of processes like protein synthesis, which has been shown to be unimportant to reacquisition in the amygdala (Motanis & Maroun, 2012). This study implies that histone acetylation may not have been an ideal marker for studying reacquisition. However, I was able to obtain significant differences in H4K8ac within the LA after acquisition, so these issues did not wholly prevent interpretation of the results. Additionally, like c-Fos, H4K8ac revealed regions associated contextual fear learning, but did not provide evidence of the regions' functional involvement in acquisition or reacquisition, as H4K8ac expression did not increase in the BNST or hippocampus following contextual fear learning.

With regards to the pharmacological inactivation studies, a caveat include the region inactivated by muscimol + baclofen. The spread of the microinjection likely did not inhibit the entire amygdala and entire BNST, but rather inhibited large sections of the brain regions. Thus, neuronal signaling within these regions was likely impaired, but the entire region was likely not silenced. Thus, these studies could be biased by the section of these brain regions that was inactivated by microinjection of muscimol + baclofen.

Despite these caveats, the results showed that the rapid reacquisition of fear had a distinct pattern of neural activity compared to initial acquisition. First, brain regions showed larger activation to acquisition than reacquisition. I also found evidence that the BNST and hippocampus, but not the amygdala, were likely involved in rapid reacquisition. Inactivation of the BNST during reconditioning led to

impaired rapid reacquisition and rapid reacquisition led to increased c-Fos expression in the hippocampus.

Together, these data suggest that rapid reacquisition does not necessarily recruit and involve the same brain regions as acquisition. Further, the results suggest that updating contextual fear memories with reconditioning is a unique neurological process that leads to rapid reacquisition. The study of the neurobiology of rapid reacquisition and other types of memory updating are necessary to better understand how memory evolves over time and exposure to changes in association.

Chapter 5: Overall Discussion

I. Summary of Results

Overall, these results showed that rapid reacquisition occurred after mild post-extinction contextual fear reconditioning and was likely not due to another form of CR restoration. Rapid reacquisition also displayed differential vulnerability to alcohol administration and a distinct pattern of brain activity relative to initial acquisition, which indicated that reacquisition did not wholly rely on the same mechanisms as initial acquisition. The rapid reacquisition effect presented a persistent enhancement in fear behavior following a small reminder that could be used to study memory modulation and persistent fear in humans.

Chapter 1 reviewed the behavioral features, learning concepts, and neural mechanism of the four post-extinction forms of conditioned response restoration, with particular attention on rapid reacquisition. These outcomes have increased the field's understanding of relapse of conditioned behavior despite extinction treatment. Thus, a comparison and contrast of each outcomes' defining features is critical to informing the research and treatment of human disorders like addiction and PTSD. I also present rapid reacquisition following reconditioning as a unique post-extinction restoration of fear conditioned responding that differs in several important ways from the others and from initial acquisition. Yet, rapid reacquisition has received less research on its underlying causes and neurobiology and thus deserves further exploration. The ability of a single CS-US re-pairing to elicit rapid reacquisition of fear that is considerably larger than acquisition provides an important behavioral correlate for studying PTSD symptomology and neurobiology in animal models.

In Chapter 2, I described the behavioral characteristics of rapid reacquisition in a contextual fear conditioning procedure with two rodent species. I found that rapid reacquisition of fear occurred after mild reconditioning following extinction to a behavioral baseline (moderate extinction). Moreover, rapid reacquisition was a persistent effect that could last for two weeks following reconditioning. I also found that there were important rodent strain differences in the rapid reacquisition effect; rapid reacquisition occurred following moderate, but not massive, extinction in mice, but rapid reacquisition occurred following both massive and moderate extinction in rats. Finally, this chapter showed that rapid reacquisition of contextual fear was context-specific and not due to a general enhancement in freezing following repeated shock or reinstatement. Additionally, the rate of extinction or presence spontaneous recovery during extinction did not seem to affect reacquisition. Slow reacquisition of contextual fear was not seen in any of these studies.

In Chapter 3, I found that acute ethanol withdrawal has mild effects on memory that are learning-phase specific. AEW did not impair the acquisition or expression of excitatory (conditioning) or inhibitory (extinction) context-US associative memory, but did moderately impair the rapid reacquisition of contextual fear. Additionally, acute alcohol administration displayed a biphasic pattern on contextual fear memory; during mild reconditioning under intoxication there was an enhanced general expression of freezing to shock and of rapid reacquisition, whereas 24 hr later, in a drug free test of contextual fear, there was a general dampening effect on contextual fear retention, specifically impacting the retention of initial acquisition. This chapter showed that ethanol administration and withdrawal

might have different learning-phase-specific effects on memory and the implications of the results for learning-related neurobiology.

In Chapter 4, I demonstrated that initial acquisition and rapid reacquisition had similar, but distinct patterns of neural activity. First, there was greater expression of markers of neural activity throughout the fear circuit following acquisition compared to reacquisition. Initial acquisition led to enhanced c-Fos expression in the dorsal BNST and hippocampus and increased H4K8 acetylation in the lateral amygdala, while rapid reacquisition only enhanced c-Fos expression in the hippocampus. Additionally, the enhanced c-Fos expression of acquisition was larger than that of rapid reacquisition in the dentate gyrus, which contained the bulk of the hippocampus c-Fos expression. Second, BNST, but not amygdala, activity was necessary during mild reconditioning for rapid reacquisition to occur. Additionally, I recapitulated the importance of BNST activity during strong condition for initial acquisition of contextual fear. Overall, I found evidence that rapid reacquisition differs behaviorally and neurobiologically with other post-extinction CR outcomes and with initial acquisition. These results are discussed in the context of the current learning and memory literature below.

II. Rapid Reacquisition is a Unique Behavioral Post-Extinction Form of Fear Return

a. Behavior

The results of this dissertation confirmed previous findings of rapid reacquisition and added more depth to the field's understanding of its characteristics

and how it compares to other post-extinction phenomena. As shown in Chapter 1, rapid reacquisition occurs following reconditioning in many paradigms (Bouton, Woods, & Pineño, 2004a; Leung et al., 2007; Napier et al., 1992) and is theorized to show that the original CS-US association has savings that were not lost in extinction and can be enhanced by a mild CS-US pairing (Rescorla, 2001; 2002). My work added to this theory by replicating findings of rapid reacquisition of contextual fear following mild reconditioning in multiple studies (Chapter 2, Experiment 1A, 2A, & 2B; Chapter 3 Experiment 2; Chapter 4, all experiments). Whereas, rapid reacquisition of contextual fear has been demonstrated before (Leung et al., 2007), these results showed the consistency of the finding in different laboratories and in different rodent species. These results included the first findings of the rapid reacquisition effect in mice. Thus, the rapid reacquisition effect has now been noted in rabbits, rats, mice, and humans. In sum, these findings suggest that rapid reacquisition is highly translatable aspect of animal memory and not a species-specific response to conditioning.

Alternatively, there are several other post-extinction outcomes that restore CR and could be behind the findings here (fully described in Chapter 1). In brief, CR can return after extinction due to the passage of time (spontaneous recovery), in a distinct context from the extinction context (contextual renewal), from exposure to the US alone (reinstatement), and from CS-US re-pairing (reconditioning). Additionally, reconditioning can result in both rapid reacquisition (as shown above) and slow reacquisition.

Slow reacquisition of contextual fear was not found in any instances of mild reconditioning. Massive extinction (Chapter 2, Experiment 1B, 2A, & 2B), acute ethanol withdrawal (Chapter 3, Experiment 4), ethanol administration (Chapter 3, Experiment 6), and BNST inactivation (Chapter 4, Experiment 9) caused reacquisition of contextual fear that was equivalent, but not impaired or slower, relative to initial acquisition. Slow reacquisition of contextual conditioning has been demonstrated in rats before (Leung et al., 2007), so it is possible to achieve in this paradigm, unlike what has been shown in eye-blink conditioning (Weidemann & Kehoe, 2003). But the lack of slow reacquisition in each study here shows that specific conditions are necessary to achieve slow reacquisition. Rapid reacquisition and equivalent reacquisition of fear appear to be far more likely outcomes.

I also showed that rapid reacquisition of contextual fear was a persistent effect that survived repeated testing and long-term tests. Rapid reacquisition lasted up to two weeks later in both mice and rats (Chapter 2, Experiments 1A, 2A, & 2B). Leung et al. (2007) displayed similar long-term retention of rapid reacquisition of freezing, but the replication here again suggested a consistent characteristic of rapid reacquisition. It also presents an important difference between rapid reacquisition and other post-extinction phenomena. As mentioned in Chapter 1, rapid reacquisition differs in the “completeness” of the return of post-extinction responding relative to pre-extinction responding. While the return of CR in spontaneous recovery is generally less than pre-extinction responding and quickly dissipates (Rescorla, 2004b), the return of CR in rapid reacquisition can be greater than what it shown in initial acquisition (Bouton, Woods, & Pineño, 2004a; Napier et al., 1992) and this

effect does not dissipate over time, showing that reacquisition is complete and long lasting.

Additionally, the long-term retention tests indicated that rapid reacquisition did not occur due to the passage of time, like it does with spontaneous recovery. It is possible that the small delay between extinction and initial reacquisition testing could have allowed some of the CR to spontaneously recover. This possibility is an important consideration for these studies because the comparison group for reacquisition only received conditioning, but no extinction, prior to the context test. Thus, the comparison group would not display spontaneous recovery. However, following a delay after initial testing, during which all animals would have extinguished some of the CR in a nonreinforced context exposure, the enhanced reacquisition of CR relative to acquisition was still apparent in the long-term retention tests. While spontaneous recovery could play a role in the rapid reacquisition of contextual fear (Rescorla, 2001), it did not seem like the sole cause of rapid reacquisition effect. Additionally, Leung et al. (2007) showed that spontaneous recovery could not explain the increased behavior following reconditioning in a retention test, so the rapid reacquisition found here was likely not due to spontaneous recovery.

This work differentiated rapid reacquisition from reinstatement. Reinstatement occurs following extinction when the US is presented alone and then CR to the CS returns, generally within the context the US was presented (Bouton & Bolles, 1979c). Additionally, when the US is delivered in the same context as the extinction context, reinstatement can occur in a distinct context that is not associated with the US

(Westbrook et al., 2002). Thus, rapid reacquisition of fear following reconditioning that involves US exposure could simply be reinstatement. Specifically, my findings of rapid reacquisition to the same context that was extinguished could be reinstatement as well. However, I showed that if reconditioning occurred in a distinct context (Context B) from the acquisition/extinction context (Context A), rapid reacquisition did not occur due to reinstatement in the distinct context (B), nor did fear return in the original conditioning context (A) that was extinguished prior to reconditioning (Chapter 2, Experiment 2A). These results suggested two conclusions. One, that rapid reacquisition was not due to US-induced reinstatement. Two, rapid reacquisition was not a result of a general enhancement of freezing to any context, as reconditioning in the distinct context did not cause rapid reacquisition there, nor did reconditioning in the conditioning context cause enhanced freezing in a novel context.

Contextual renewal has been shown to influence the outcomes of reconditioning (Bouton & Swartzentruber, 1989), so it could play a role in the findings here. Yet, in contextual fear conditioning, the context is the CS, so it is difficult to study the role of additional contextual cues that could cause renewal of CR in my paradigm. Thus, most results here did not rule out context renewal to additional contextual components as a possible confound in rapid reacquisition, but one finding indicated that it was not the primary cause of rapid reacquisition. When re-exposure to shock occurred in a distinct context (B) from the acquisition/extinction context (A), the animals showed a return of conditioned fear in the distinct context (B), but not the original context that was extinguished (A; Chapter 2, Experiment 2A).

This finding closely resembled contextual renewal, however if the magnitude of the fear return in the distinct context (B) was compared with animals that reacquired in the original context (A), the magnitude was less and similar to animals that were acquiring a single context-shock association for the first time. This finding suggested that post-extinction pairing of distinct context and shock could lead renewal-like phenomena, but that the relatively large return of conditioned fear in rapid reacquisition was an outcome specific to the re-pairing the original context with shock. Further, I believe renewal to be an unlikely explanation as all aspects of extinction sessions were identical to the parameters used in the test for reacquisition (both context and session length were identical) and thus, in the framework of renewal, the test should have favored expression of the extinction over the reconditioning memory.

In addition to the behavioral results, the interaction of acute ethanol withdrawal and rapid reacquisition showed a similarity with another post-extinction return of fear. The explicit interaction of ethanol intoxication and withdrawal on post-extinction CR phenomena (where ethanol or a drug of abuse is not the US) is mostly unstudied. This work presents the first findings of how initial withdrawal and administration impact reacquisition of contextual fear. I demonstrated that rapid reacquisition was impaired by acute ethanol withdrawal (Chapter 3, Experiment 4), whereas administration had more minimally impairing effects on rapid reacquisition (Chapter 3, Experiment 6). Previously, rapid reacquisition of cued fear conditioning was enhanced by chronic ethanol withdrawal prior to any learning (Bertotto et al., 2006). This finding seems contradictory to my results, but A) this study used chronic,

not acute, withdrawal and B) the results could be interpreted as a failure of extinction because chronic withdrawal prior to conditioning caused conditioned freezing behavior that was resistant to extinction training. Additionally, this paper showed that reinstatement of cued fear was also enhanced by chronic ethanol withdrawal, which could suggest that both share a similar mechanism that is altered by chronic withdrawal. Unfortunately, there is not enough work on alcohol's effects on post-extinction memory to draw more conclusions on their similarities and differences, but the results here begin to elucidate how reacquisition is affected by ethanol.

b. Neurobiology

In terms of neurobiology, rapid reacquisition and reacquisition mostly rely on similar brain regions as the other post-extinction forms of fear return (for review, refer to Chapter 1), but there are some important differences. These brain regions include the hippocampus, amygdala, BNST, and mPFC.

Consistently, the hippocampus and its subregions show a role in spontaneous recovery (Debiec et al., 2002), contextual renewal (Corcoran & Maren, 2004; Ji & Maren, 2005), reinstatement (Frohardt et al., 2000; A. Wilson et al., 1995), and reacquisition (Cammarota et al., 2003; Fu et al., 2016). I added to this canon here and also added more detail about the hippocampus' involvement in rapid reacquisition. Rapid reacquisition led to an increase in c-Fos in the dorsal hippocampus following reacquisition of contextual fear relative to handled, learning-naive controls (Chapter 4, Experiment 7). This c-Fos expression was driven largely by the dorsal DG subregion of the hippocampus. The DG is known for its role in pattern separation (Trouche et al., 2009) and the ventral DG has previously been

implicated in a sub-threshold conditioning procedure that shares some similarities with mild reconditioning (Fu et al., 2016). These studies suggest a potential role for the hippocampus and DG in rapid reacquisition.

Interestingly, a corresponding increase in histone acetylation was not seen here. With regards to learning, histone acetylation is seen as one of the upstream processes that allow gene transcription and protein synthesis, which are necessary for long-term memory formation (Davis & Squire, 1984; Federman, Fustiñana, & Romano, 2009). Thus the finding of increased hippocampal activity via c-Fos without increased histone acetylation could suggest that the hippocampus recruited histone-acetylation-independent processes during reacquisition.

Additionally, the findings here corroborate the theory that reacquisition does not always involve the amygdala, unlike the other post-extinction outcomes. Not only did rapid reacquisition not lead to increased c-Fos or histone acetylation within the amygdala (Chapter 4, Experiment 7), temporary inactivation of the amygdala prior to reconditioning did not impair retention of rapid reacquisition the following day (Chapter 4, Experiment 8). Others have similarly found that the amygdala was less necessary for fear potentiated startle or fear conditioning reacquisition (Kim & Davis, 1993; Maren et al., 1996; Motanis & Maroun, 2012). However, one particularly relevant study showed that BLA activity during reconditioning was necessary for reacquisition of conditioned fear (Laurent & Westbrook, 2010). Compared with the role of the amygdala in spontaneous recovery (Mickley et al., 2007; Peters et al., 2008), contextual renewal (Chaudhri et al., 2013; Knapska & Maren, 2009), and

reinstatement (Hitora-Imamura et al., 2015; Laurent & Westbrook, 2010), the lack of clear amygdala involvement in rapid reacquisition here was a notable difference.

The BNST had only previously shown involvement in reinstatement of fear conditioning (Goode et al., 2015), but now I also demonstrated the BNST's necessity for rapid reacquisition of contextual fear conditioning. Temporarily inactivating the BNST prior to reconditioning prevented retention of the rapid reacquisition effect 24 hr later (Chapter 4, Experiment 9). The BNST is relatively understudied in post-extinction CR compared to other brain regions, but it has shown to be important for several types of fear conditioning, specifically those that involve sustained fear responses (Walker et al., 2009) or conditioning stimuli with less clear predictive value (Alvarez, Chen, Bodurka, Kaplan, & Grillon, 2011; Goode & Maren, 2017; Hammack, Todd, Kocho-Schellenberg, & Bouton, 2015).

The results further added to the BNST's role in sustained fear responses to ambiguous CSs (context) and showed an important similarity between the neurobiology of rapid reacquisition and reinstatement. Not only were both reacquisition (Chapter 4, Experiment 9) and reinstatement impaired by BNST inactivation (Goode et al., 2015), both were enhanced by chronic ethanol withdrawal (Bertotto et al., 2006), which causes many long term changes to neurobiology (De Witte et al., 2003; Holmes et al., 2012). Accordingly, first withdrawal (AEW) affected rapid reacquisition in the findings here (Chapter 3, Experiment 4). Both reacquisition and reinstatement involve exposure to the US, which likely leads to similar neural mechanisms for the return of conditioned fear responding. However, reinstatement appears to be more amygdala-dependent than reacquisition (Hitora-Imamura et al.,

2015; Kellett & Kokkinidis, 2004; Laurent & Westbrook, 2010; Lin et al., 2011; Maddox et al., 2013). Interestingly, the BNST compensated for BLA in the expression conditioned fear that resulted from overtraining of contextual fear conditioning when the BLA was lesioned (Poulos, Ponnusamy, Dong, & Fanselow, 2010). Thus, these results and my own imply that rapid reacquisition could bypass the amygdala and instead recruit the BNST to allow the retention and expression of rapid reacquisition.

Also of note, there was no detectible difference in activity in the mPFC following reacquisition. Rapid reacquisition led to c-Fos and histone acetylation levels that were identical to handled controls (Chapter 4, Experiment 7). Previous work has demonstrated a role of the mPFC within spontaneous recovery (Mickley et al., 2007; Rhodes & Killcross, 2004), contextual renewal (Eddy et al., 2016; Rhodes & Killcross, 2007), reinstatement (Hitora-Imamura et al., 2015; Rhodes & Killcross, 2004), and reacquisition. In the post-extinction sub-threshold conditioning procedure tetanic stimulation of the mPFC blocked reacquisition (Deschaux et al., 2011; Zheng et al., 2013). The latter studies were not explicitly investigating rapid reacquisition of fear following an identical CS-US re-pairing, which could explain the difference. Additionally, the mPFC's functional necessity to rapid reacquisition was not examined, so it is possible that the mPFC could still play a role. These results suggest that rapid reacquisition of contextual fear is a distinct post-extinction fear restoration that differs both behaviorally and neurobiologically from other outcomes.

The neurobiological findings also have implications for the fear circuit during reacquisition. Refer to the circuit diagram in Figure 24 for the connectivity between

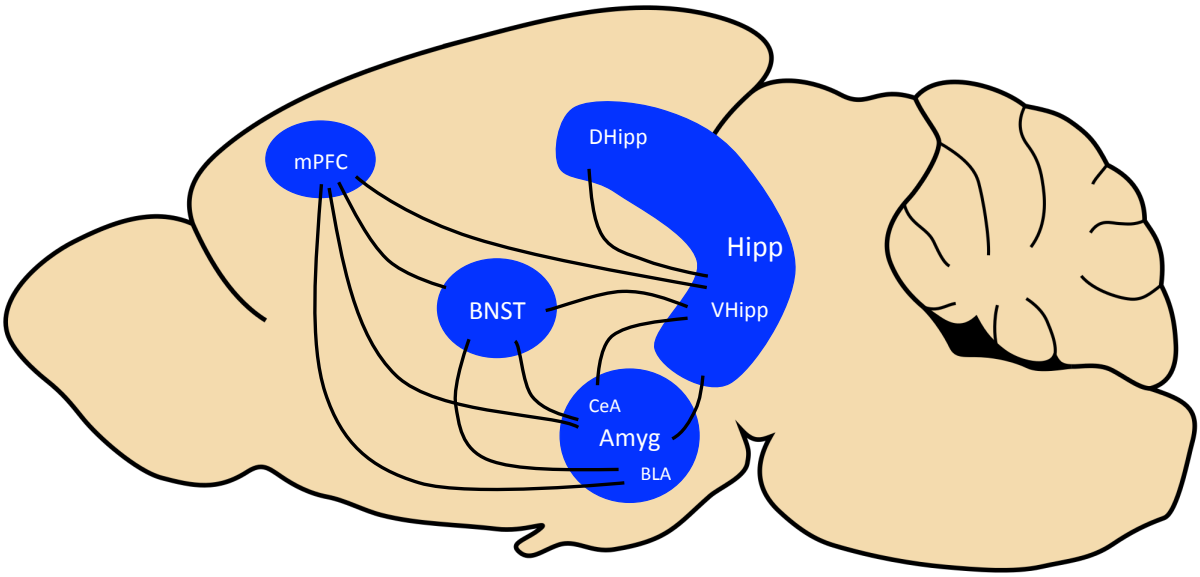

Figure 24. Circuit Diagram of Brain Regions Potentially Involved in Rapid Reacquisition. The figure shows a sagittal section of the rat brain with the target brain regions and their connections with one another. As described in text, the BNST afferent and efferent connections may be more critical to rapid reacquisition than amygdala connections. mPFC = medial prefrontal cortex; BNST = bed nucleus of the stria terminalis; Amyg = amygdala, CeA = central amygdala; BLA = basolateral amygdala; Hipp = hippocampus; Dhipp = dorsal hippocampus; Vhipp = ventral hippocampus.

the brain regions of interest to this research. By inactivating the dBNST we prevent GABAergic input from the CeA and glutamatergic input from the BLA, ventral hippocampus, and mPFC from acting at the BNST (Stamatakis et al., 2014; Tannenholz, Jimenez & Kheirbek, 2014). Further inhibition of the BNST inhibits the projections to the ventral tegmental area, the lateral hypothalamus, and paraventricular nucleus and likely prevents important stress and threat monitoring responses (Stamatakis et al., 2014). Inactivation of the BNST also inhibits the BNST reciprocal connections with the amygdala and hippocampus (Stamatakis et al., 2014; Tannenholz, Jimenez & Kheirbek, 2014).

Inactivating the amygdala prevents successful excitatory input from the ventral hippocampus, mPFC, and thalamus (Stamatakis et al., 2014; Muller et al., 2012; Marek et al., 2013). Also, the amygdala projections to the BNST (from the CeA and BLA), mPFC (Marek et al., 2013), and hippocampus would be silenced as well as the CeA output to similar regions as the BNST (Alheid, DeOlmos, & Beltramino, 1995).

I find that the BNST, but not amygdala, may be important for rapid reacquisition of fear. Thus, upon reacquisition perhaps the ventral hippocampus and mPFC inputs to the BNST and the BNST outputs to the ventral tegmental area, the lateral hypothalamus, and paraventricular nucleus are most critical for rapid reacquisition. Whereas, the amygdala afferent and efferent connectivity may be less critical. This hypothesis is entirely possible as the output structures of the BNST and CeA are similar and perhaps the BNST acts as the necessary conduit of information to these output structures during reacquisition of contextual fear. Accordingly,

studies have suggested that the BNST could be important for fear responses that bypass the amygdala (Poulos, Ponnusamy, Dong, & Fanselow, 2010).

One may note that I examined the dorsal, but not ventral, hippocampus, which shares less connectivity with the limbic and frontal centers discussed here (Tannenholz, Jimenez & Kheirbek, 2014; Xu et al., 2016). Yet, the dorsal hippocampus is critical for spatial cognition and is interconnected with the ventral hippocampus (Tannenholz, Jimenez & Kheirbek, 2014). Thus the dorsal hippocampus has indirect connections with the amygdala, BNST, and mPFC and well as some direct minor connections (Jin & Maren, 2015).

III. Rapid Reacquisition is Not a Recapitulation of Initial Acquisition

a. Behavior

There are several ways in which reacquisition differed from initial acquisition. As mentioned in Chapter 1, rapid reacquisition generally differs in its rate, context-dependency, and neurobiology from acquisition. The findings here confirmed and added to this list of differences. In Chapter 2, I demonstrated that reacquisition of contextual fear tended to be more rapid than acquisition (Experiment 1A, 2A & 2B). In mice, massive extinction prior to mild reconditioning caused reacquisition that was equivalent to mice acquiring for the first time, while rats showed rapid reacquisition relative to initial acquisition regardless of extinction intensity. Additionally, I showed rapid reacquisition following moderate extinction in the studies of Chapter 3 and 4 as well (with the exception of Chapter 3, Experiment 6). Thus, while reacquisition can

appear similar to acquisition, it tends to be more rapid than acquisition, suggesting that the two are behavioral distinct.

Also acquisition and reacquisition differed in their vulnerability to ethanol administration and withdrawal. Much research has shown the impact of alcohol administration and withdrawal on initial acquisition (refer to the introduction of Chapter 3), but this work was the first exploration of the explicit interaction of ethanol and reacquisition. Like others (Gulick & Gould, 2007), I found that ethanol administration can both enhance and impair acquisition (Chapter 3, Experiment 6). Specifically, alcohol administration had a biphasic effect that enhanced freezing responses during context-shock pairings, but impaired retention of freezing in a drug free test. Similar to acquisition, I found that rapid reacquisition could also be generally enhanced and impaired by ethanol dministration, although the impact of ethanol administration on the retention of reacquisition was markedly less than on acquisition.

Unlike ethanol administration, acute ethanol withdrawal selectively impaired retention of the rapid reacquisition effect. I did not find an effect of AEW on acquisition or extinction of contextual fear conditioning (Chapter 3, Experiment 3 & 5). Contrary to my findings, previous research showed that AEW impaired acquisition of contextual fear (Tipps et al., 2015). These outcomes show that the effects of AEW on acquisition memory are mixed and likely moderate, but they also indicate that reacquisition may be more liable to the effect of first withdrawal from alcohol. However, this result presents a finding that is contradictory with the theory that rapid reacquisition results of a reactivation and strengthening of a CS-US

association (Rescorla, 2001), but could also suggest that reacquisition and acquisition have different neurobiological mechanisms that are differentially affected by the pharmacological activity of AEW. Additionally, it suggests that different phases of learning have differential vulnerability to alcohol administration and withdrawal, which can inform interactions of alcohol and fear memory in humans. Overall, my hypothesis that AEW would have memory-phase (acquisition versus extinction versus reacquisition) specific was confirmed. The differences in the neurobiology of acquisition and reacquisition that could contribute to differing vulnerability to AEW (and to some extent administration) are explored below.

b. Neurobiology

Although the neural mechanisms underlying acquisition and reacquisition of contextual fear overlap, they appear to be distinct. Differences in their reliance on NMDA transmission and the amygdala were discussed in Chapter 1. The results of Chapter 4 revealed additional novel differences in their neurobiology. First, acquisition led to a significantly larger activation of the fear circuit compared to reacquisition. Following mild contextual fear conditioning there was enhanced c-Fos expression in the dorsal BNST and enhanced histone acetylation in the lateral amygdala relative to handled controls. Meanwhile, mild reconditioning did not lead to enhanced expression of either markers in these regions. Mild reconditioning did enhance c-Fos expression in the hippocampus and DG. Yet in the DG, the reacquisition-induced increase in c-Fos expression was significantly lower than acquisition (Chapter 4, Experiment 7). These results showed that the first pairing of context and shock caused larger activation of the fear circuit relative to subsequent

re-pairing and suggested that rapid reacquisition might rely on alternative or less brain regions. This enhanced activation of the fear circuit by acquisition is logical in terms of prediction error and the salience of a first association among conditioned and unconditioned stimuli (Rescorla & Wagner, 1972; Wagner, 1970), which would be much stronger during initial acquisition than reacquisition. This outcome also corresponds with other findings of different or less recruitment of neural regions involved in the re-learning of a memory (Kim & Davis, 1993; Laurent & Westbrook, 2010; Winocur et al., 2005).

Additionally, the results here demonstrated that retention of rapid reacquisition could occur when the amygdala was inactivated during reconditioning. The amygdala is a region known to be critical for the acquisition and expression of fear conditioning (Goosens & Maren, 2001; Kim & Davis, 1993; Lee et al., 2001; Phillips & LeDoux, 1992; Walker & Davis, 1997). Yet, previous finding showed that the amygdala can be less important for reacquisition (Kim & Davis, 1993; Motanis & Maroun, 2012). I also found that inactivation of the amygdala prior to reconditioning did not impair retention of rapid reacquisition (Chapter 4, Experiment 8). Additionally, only acquisition, but not reacquisition of contextual fear led to enhanced histone acetylation in the lateral amygdala. Not only did this latter result suggest a lack of amygdala involvement in reacquisition, it also indicates differences in mechanism. None of the brain regions examined show an increase in histone acetylation following reacquisition, which hints that reacquisition may not need histone acetylation (within the amygdala specifically) to occur. This outcome corresponds

with findings that show protein synthesis within the amygdala is not necessary for reacquisition (Motanis & Maroun, 2012).

It also appears the mPFC may not be involved in reacquisition, as markers of activity did not increase in the mPFC following reacquisition. However, acquisition also did not cause an increase in activity markers in the mPFC. This outcome could be explained by findings that show that the mPFC is not involved in the acquisition of conditioned fear, but rather in determining when to express conditioned fear following conditioning (Corcoran & Quirk, 2007; Morgan & LeDoux, 1995). Thus, acquisition would not lead to increased c-Fos or histone acetylation in mPFC, while expression of fear behavior, which was not examined by IHC, might. I did hypothesize that pervious experience to extinction might enhance activity in the PL and IL during reconditioning, as both inhibitory and excitatory associations would be competing for expression. Yet, rapid reacquisition did not lead to enhanced markers of activity in the mPFC subregions.

In contrast to the amygdala and mPFC, both acquisition and reacquisition of contextual fear recruited the BNST. Rapid reacquisition and strong acquisition are both impaired by inactivating the BNST during reconditioning and strong conditioning, respectively (Chapter 4, Experiment 9 & 10). These results added to the previous findings of the BNSTs involvement in contextual fear acquisition (Sullivan et al., 2004; Waddell et al., 2006; Zimmerman & Maren, 2011) and sustained fear (Walker et al., 2003; 2009). These results also correspond with the theory that the BNST is critical for fear responses to conditioned stimuli that have poor predictability for the shock (Goode & Maren, 2017). In contextual fear

conditioning, animals are exposed to the context for extended times without shock relative to cued conditioning, which results in context being a conditioned stimulus with less predictive value that a discrete cue and causes animals to show sustained defensive behavior while in the context.

Further support of the role of the BNST in conditioned fear to less predictable CSs was also shown in my studies. While strong acquisition of contextual fear was impaired by BNST inactivation, mild acquisition of contextual fear was unaffected (Chapter 4, Experiment 9 & 10). Mild conditioning to a behavioral floor could cause this result, but there are additional interpretations. These results also correspond with the findings that conditioning of contextual fear was only impaired by BNST inactivation when the shock was delivered after a long exposure to the context (10 min), but not when shock was delivered shortly after context exposure begins (1 min; (Hammack et al., 2015). My mild conditioning parameters consisted of shock delivered 2.5 min into exposure to the context and, thus, could have caused a form of BNST-independent contextual fear, as the context had increased predictive value that required less sustained responding.

Additionally, these results suggest that the BNST may be critical for the consolidation and retention of contextual fear learning. Specifically, BNST inactivation did not prevent the initial expression of strong acquisition or rapid reacquisition of freezing during conditioning or reconditioning, but later impaired retention of both. Thus, a mechanism within the BNST that occurs after the pairing of context and shock was likely impaired, such as consolidation. The importance of the BNST to the consolidation of fear memory will need to be explored more.

Interestingly, the c-Fos results suggested that the dorsal BNST would be involved in only initial acquisition, as c-Fos expression was enhanced following conditioning, but not reconditioning. However, the increased dorsal BNST c-Fos could be a general response to footshock, not learning, as footshock has been shown to increase BNST c-Fos (Erb, Lopak, & Smith, 2004). Also, the lack of BNST c-Fos activity during reconditioning would correspond with the theory that short-term expression of rapid reacquisition during reconditioning is unaffected by BNST inactivation (as seen above).

Similar to the BNST, both acquisition and reacquisition demonstrated differences in hippocampal activity. However, as stated above, the DG of the hippocampus, showed larger c-Fos expression following acquisition compared to reacquisition. This result implies that the reacquiring of contextual fear requires less activity in the hippocampus and agrees with the DG proposed role in spatial learning. The DG is implicated in pattern separation (Deng et al., 2010), which would be critical during the first context and shock pairing. Additionally, the DG is involved in retrieval of contextual fear memories (Lee & Kesner, 2004), which would be important during reacquisition when the initial context-shock association is likely reactivated. Perhaps, retrieval requires less DG activity than new pattern separation.

The alcohol results in Chapter 3 also gave indirect insights into potential differences in the neurobiology of acquisition and reacquisition. Acute ethanol withdrawal caused impaired rapid reacquisition of contextual fear, but not extinction or acquisition (Chapter 3, Experiments 3, 4, & 5). Brain regions implicated in AEW include the amygdala, BNST, PL, and CA3 of the hippocampus, as all show AEW-

induced increases in c-Fos activity (Kozell et al., 2005). Thus, the AEW impairment of rapid reacquisition could suggest an involvement of these brain regions in reacquisition. In addition to the BNST inactivation results demonstrated above, the AEW impairment of reacquisition also supports the BNST's importance to rapid reacquisition. AEW can cause increased c-Fos expression in the BNST that could alter natural BNST activity required for rapid reacquisition and can result in impaired rapid reacquisition. However, one would expect that AEW modulation of BNST activity would also affect acquisition and I did not replicate AEW-induced impairment of acquisition. Additionally, the increase in amygdala activity following AEW would also suggest that the amygdala might be involved in the AEW impairment of rapid reacquisition. Contrarily, I showed that amygdala activity might not be functionally necessary for rapid reacquisition, which suggested that amygdala might not be behind the impairment of rapid reacquisition by AEW.

While reacquisition did not increase c-Fos expression in the mPFC or its subregions, the PL subregion, a region involved in expression of fear conditioning memories (Peters et al., 2009; Vidal-Gonzalez et al., 2006), could play a role because it is affected by AEW. The lack of c-Fos expression again could be due to the PL's involvement in expression, not acquisition of conditioning (Corcoran & Quirk, 2007; Morgan & LeDoux, 1995). Additionally, the hippocampus is further implicated in rapid reacquisition. Several reports showed that the hippocampus is activated by various types of ethanol withdrawal, including AEW (Kozell et al., 2005; Matsumoto et al., 1993; Vilpoux et al., 2009). I found that rapid reacquisition also led

to increased c-Fos in the hippocampus. Thus, the over-activation hippocampus could be playing an important role in AEW's inhibition of rapid reacquisition.

In contrast to AEW, acute ethanol administration preferentially impacted initial acquisition over rapid reacquisition (Chapter 3, Experiment 6). Acute ethanol administration increases c-Fos expression in the IL, CeA, BNST and decreases c-Fos in hippocampus (Ryabinin et al., 1995). Acute ethanol administration-induced activation could suggest that these brain regions are more involved in acquisition than with reacquisition. I showed that the amygdala might not be necessary for rapid reacquisition of contextual fear, but the CeA is known to be important for fear expression following initial acquisition (Wilensky, Schafe, Kristensen, & LeDoux, 2006). Thus, the CeA could mediate acute administration's impairment on fear acquisition expression. Additionally, the BNST's importance for acquisition of contextual fear was shown here and in many other reports, so it too could mediate the ethanol-administration-induced acquisition impairment. Intriguingly, both a subregion of the amygdala, CeA, and the BNST are critical members of the extended amygdala circuit that are recruited during the transition to alcohol dependence (Gilpin & Roberto, 2012; Koob, 2008). Thus, the differential impact of AEW and administration on aspects of fear learning requires further exploration with regards to these brain regions on which both AEW and ethanol administration have shown a demonstrated impact.

The hippocampus also likely plays a role in the ethanol-administration-induced impairment of acquisition. Hippocampal place cells show a reduction in neural activity in response to both acute and chronic ethanol intoxication (White &

Best, 2000) and intoxication-induced reductions in hippocampal activity have been shown to interfere with contextual fear conditioning (Melia et al., 1996). Additionally, I showed that the hippocampus had increased c-Fos activity following acquisition and the c-Fos increase in the DG was larger for acquisition compared to reacquisition. This result could suggest that acute ethanol administration's reduction in c-Fos activity interferes with the large DG c-Fos activation following acquisition relative to the effect on reacquisition. The behavioral results support this theory because there was a general impairment of freezing in acquisition and reacquisition by ethanol administration, but acquisition showed the most significant impairment in freezing due to ethanol administration.

The ethanol-administration-induced increase in c-Fos expression in the IL, however, is likely not involved in acquisition impairment, as the IL is more critical involved in extinction expression (Laurent & Westbrook, 2009a; Peters et al., 2009) and c-Fos was not found to be elevated following acquisition in the IL here. It is important to note again here that the upregulation of c-Fos found in these brain region (Kozell et al., 2005; Ryabinin et al., 1995) does not necessitate that these regions are critically involved in AEW or administration and these interpretations represent a simplistic view of the role of c-Fos in neural and functional activity. Thus, the neurobiological implication of the alcohol results on fear learning detailed are all correlative, but they do add more to the small body of knowledge on the neurobiology of rapid reacquisition and how it differs with initial acquisition.

IV. Rapid Reacquisition as a Model of Relapse to Mild CS-US Re-pairings in PTSD

In addition to further characterizing the behavior and neurobiology of rapid reacquisition relative to acquisition and other post-extinction phenomena, these studies also showed how rapid reacquisition could be used to model PTSD. In Chapter 1, I discussed how rapid reacquisition mimics certain symptoms of PTSD. The findings here added to this PTSD model by showing that the rapid reacquisition of extinguished contextual fear happened follow a single re-pairing of context and shock. Mild reconditioning resulted in a massive freezing response relative to initial conditioning with identical parameters that persisted into long-term enhanced freezing. This result is comparable to the exaggerated responses that those with PTSD show to mild stressors, as well as their hypervigiliance for anything trauma-related or perceived as a threat (Pitman, 1989; Pitman et al., 1996; Rothbaum & Davis, 2003). Additionally, the rapid re-emergence of fear is similar to post-treatment relapse of fear behavior in PTSD patients.

The persistence of the rapid reacquisition effect relative to other post-extinction CR outcomes is particularly relevant for PTSD. Two of the defining attributes of PTSD are persistent re-experiencing of the trauma and symptoms that last for at least one month (DSM-V). I demonstrated that rapid reacquisition of contextual fear persisted up to two weeks later in both mice and rats. The persistence of rapid reacquisition makes it an ideal model of some aspects of PTSD relative to other post-extinction CR phenomena, like spontaneous recovery.

Rapid reacquisition is particularly important for individuals with PTSD that experience repeated traumas of a similar nature, like combat veterans or victims of sexual abuse. Although not recognized by the DSM-V, the PTSD that results from repeated traumas has even been classified as an alternative form of PTSD, called complex PTSD (Herman, 1992). Complex PTSD is said to include symptoms of pervasive personality disturbances, alterations in mood and cognition that can result in dissociative states, chronic pain, and inability to regulate impulses. Thus, a model that includes re-exposures to a fearful experience with a persistent alteration in responses to CS-related stressors can start to capture some of the aspects of PTSD following multiple traumas.

Additionally, rapid reacquisition shares similarities to other popular rodent models of PTSD. Stress-enhanced fear learning (SEFL) is one such model that shows persistently enhanced fear learning to a single context-shock association after massive footshock in another context (Rau & Fanselow, 2009). The model shows that once an animal has undergone repeated footshock, they show an exaggerated response and hypervigilance during new fear learning, similar to rapid reacquisition. The model suggests that the animals are now in an altered state of stress that favors enhanced fear learning. However, this model shows a general increase in fear learning to any stressor that does not need to be specific to the initial massive footshock learning, which is equated with "trauma." Conversely, I showed that the rapid reacquisition effect was specific to the context in which animals originally learned the context-shock association. Thus, SEFL is an ideal model to study a general persistent enhancement in exaggerated stress responses,

whereas rapid reacquisition is more suited to the study of exaggerated response to trauma-related cues that persists even after treatment.

Additionally, the findings of AEW's and ethanol administration's differential effects on acquisition and reacquisition can start to inform the impact of ethanol use on fear memory in humans and provide some preclinical insight into aspects that lead PTSD-AUD comorbidity. Initial ethanol administration impaired acquisition more than reacquisition, which could suggest that ethanol administration is protective during the formation of fear memories, but it also suggests that normal adaptive fear memory is impaired by administration of ethanol. Likewise, AEW's selective impairment of rapid reacquisition could suggest that withdrawal from alcohol can prevent the rapid relapse into fear memory. Or like ethanol administration, AEW may impair the natural reacquisition of fear, which would enhance over repeated CS-US pairings. Further characterization of AEW and ethanol administration interactions with different phases of memory in rodents and humans would help clarify when alcohol is most detrimental to those with PTSD. However, more chronic forms of ethanol use and withdrawal are necessary to model AUD-like phenotypes and their interactions with fear learning, as a single administration or withdrawal of ethanol does not model AUD.

The neurobiology of rapid reacquisition found here also corresponds with some of the neurological characteristics of PTSD. The hippocampus has shown involvement in PTSD symptoms. The hippocampus size has been noted to be significantly smaller in those with PTSD, especially in individuals who have experienced repeated trauma (Bremner et al., 1997; Gilbertson et al., 2002; Gurvits

et al., 1996; Stein, Koverola, Hanna, Torchia, & McClarty, 1997). Accordingly, I found that the hippocampus showed increased activity during rapid reacquisition. Also, the BNST is also starting to be recognized as a structure involved in PTSD (Avery, Clauss, & Blackford, 2015; Somerville, Whalen, & Kelley, 2010), particularly in those with the dissociative characteristics described by complex PTSD (Rabellino et al., 2018). Thus, the sensitivity of rapid reacquisition to BNST inactivation suggests that rapid reacquisition captures some of the aspects of PTSD following multiple traumas. Animal models of PTSD have also indicated the importance of the BNST (Bangasser, Santollo, & Shors, 2005; Rodríguez-Sierra, Goswami, Turesson, & Paré, 2016). Overall, the rapid reacquisition effect is a useful tool for studying PTSD symptoms.

V. Overall Limitations

There are several important caveats to the studies conducted here. First, while rapid reacquisition generally occurred in each experiment, there were several experiments in which the effect did not occur or was not as robust across rodent strain. Rapid reacquisition of contextual fear did not appear after massive extinction of conditioned behavior in mice. This outcome was expected based on findings from several studies that found reacquisition was not rapid following massive extinction (Bouton, 1986; Bouton, Woods, & Pineño, 2004a; Leung et al., 2007). Yet, rapid reacquisition was also not seen in alcohol naïve mice following moderate extinction (Chapter 3, Experiment 6), which suggests that there are particular circumstances that allow rapid reacquisition of contextual fear. However, similar uneven results are

seen in spontaneous recovery. Following a delay in extinction, spontaneous recovery does not always occur and could be limited to specific circumstances (Rescorla, 2004b).

Additionally, while rapid reacquisition did occur in rats regardless of extinction strength, the effect was sometimes less robust than what was seen in mice (Chapter 2, Experiment 2A & 2B). However, the finding of rapid reacquisition was replicated in five separate cohorts of rats (Chapter 2, Experiments 2A & 2B, Chapter 4, Experiments 7, 8, & 9), displaying that it was not a one-time occurrence.

Second, the persistence of rapid reacquisition was only examined in Chapter 2, but not when rapid reacquisition behavior interacted with ethanol or neurobiological manipulation. As noted in Chapter 2, retesting the same animals in both the short-term and long-term retention tests could have biased their behavior on the long-term test to be similar to the first test (Stafford & Lattal, 2009). Additionally, rapid reacquisition can be impaired by a variety of manipulations (AEW and BNST inactivation), so the conditions under which the persistency of enhanced freezing following reconditioning occurs should be examined in more depth.

Third, it is difficult to determine in my studies if post-extinction reconditioning is reactivating or both reactivating and strengthening the original CS-US association due to the design of my rapid reacquisition studies. I used a between-subjects comparison of animals undergoing conditioning and reconditioning at the same time and did not investigate the within-subjects comparison of freezing following the first acquisition shock to the reconditioning shock at two different time points. While the comparison used here has the obvious advantage of a common test, alterations in

the rate of CR to context-shock pairing and re-pairing in each animal was not easily discerned. However, previous reports showing that reconditioning leads to a strengthening of the CS-US association (Rescorla, 2001), suggest that reconditioning is likely enhancing the original context-shock association.

Additionally, the use of reconditioning parameters (3 min context exposure with one shock) that were not identical to initial conditioning (12 min context exposure with four shocks) could confound reacquisition and new learning. It is possible that during reconditioning, rodents formed a new associative memory between the context and a single shock rather than reactivating and strengthening the original conditioning association. This theory is unlikely to be correct as reconditioning led to rapid reacquisition of contextual fear that did not appear like a new mild context conditioning association. Also, the reconditioning parameters were chosen to avoid all animals freezing at a behavioral ceiling that would prevent observation of group differences in contextual fear. Yet, it will be important to examine the difference in reacquisition and acquisition that follows strong reconditioning parameters in future work.

Fourth, state-dependent learning was not addressed as a potential confound in my findings. As discussed in Chapter 3, ethanol administration can operate as an internal stimuli that can be paired with a learning contingency (Cunningham, 1979; Lattal, 2007). Thus, in my experiments, AEW could have also created an internal stimulus that signaled the operation of conditioning, extinction, or reconditioning contingencies. Additionally, BNST inactivation could have also acted as an interoceptive context in which reconditioning occurred. Perhaps, if retention of

reacquisition memory was tested under AEW or BNST inactivation, instead of drug-free, the facilitation of rapid reacquisition might show state-dependent expression. Yet, this outcome could also suggest that AEW could be inactivating the BNST to cause rapid reacquisition impairments.

Fifth, several of my conclusions about the neurobiology of rapid reacquisition are indirect or correlational. Both the proposed regions affected by ethanol administration and AEW could have differential involvement in phases of contextual fear learning, but I did not directly study which regions were affected by ethanol and the corresponding changes in conditioned behavior. Thus, I cannot conclude from the ethanol studies what regions specifically led to impaired contextual fear memory. Similarly, enhanced markers of activity following the different phases of contextual fear learning in Chapter 4 pinpointed regions that could be important for reacquisition of contextual fear (like the hippocampus). Yet, the IHC results suggested only a correlation of a regions activity with learning and did not show a direct role of a region in acquisition or reacquisition. I did functionally probe the role of the amygdala and BNST in rapid reacquisition by inactivating both regions prior to reconditioning. However, inactivating a region prior to a conditioning session can impair not just learning, but also other confounding factors like attention, sensation, and motivational state. Thus, it will be important to examine the inactivation of these regions immediately following learning to examine consolidation of learning specifically. Additionally, some of my neurobiology findings (like the potential lack of amygdala involvement in rapid reacquisition) require more power, replication, and

subregion differentiation to fully understand each region's role in rapid reacquisition relative to acquisition.

Finally, contextual fear conditioning and rapid reacquisition of fear do not encapsulate the complexities of PTSD, but rather model the formation of a fear memory that can be preferentially enhanced by a mild re-pairing of context and fear. Additionally some findings of PTSD contradict the rapid reacquisition results detailed here. One study showed that PTSD patients tend to generalize fear across stimuli and are sensitized by stress in a fear potentiated startle paradigm (Grillon & Morgan, 1999), which contradicts my findings of context-specific rapid reacquisition. Also, in another study, those with PTSD following multiple traumas had blunted defensive and autonomic responses relative to those with PTSD following one trauma (McTeague et al., 2010), which contrasts with the enhanced freezing response following mild reconditioning in my research.

Similarly, withdrawal following a single exposure to ethanol does not model AUD, which is a complex disorder that involves uncontrollable use of ethanol despite consequences (De Witte, Pinto, Ansseau, & Verbanck, 2003; Heilig, Egli, Crabbe, & Becker, 2010; Koob & Le Moal, 2008; DSM-V). Therefore, a single experience of ethanol withdrawal and a formation of a relatively mild fear memory (when compared to PTSD) do not mimic the human pattern of comorbidity. However, these findings do provide insight into how initial withdrawal from ethanol use and fear memory might interact to alter disorder progression and likelihood of comorbidity.

VI. Future Directions

There are several avenues for future research given the results and caveats of these studies. First, in order to better understand how rapid reacquisition of contextual fear is occurring, it is important to examine if reconditioning causes a reactivation and strengthening of the original context-shock association. An examination of the within-subjects difference in rate of CR during acquisition and reacqusition could inform this question. Additionally, a study to see if reconditioning could result in stronger CR return in animals that received initial acquisition well beyond a behavioral asymptote relative to the initial acquisition parameters used here (12 min context exposure with 4 shocks for one or two days) could be telling as well.

Second, examining the role of contextual renewal and sequential learning in rapid reacquisition of fear conditioning is necessary to better characterize the phenomena. This experiment would necessitate using discrete cued, rather than contextual, fear reconditioning. Switching the CS type could alter many of the findings here, as contextual conditioning results in more sustained fear responses compared to cued fear conditioning. But using a discrete cue in multiple contexts would also better elucidate the role of context in rapid reacquisition.

Third, research on the exact conditions of contextual fear reconditioning that can prevent rapid reacquisition or cause slow reacquisition in rats is critical. I was unable to find a behavioral manipulation that prevented rapid reacquisition of contextual fear, but others have shown that it can occur (Leung et al., 2007). Conducting a study with even more massive extinction or using the design from

Leung et al. (2007) to replicate their findings is a logical next step. If the parameters that cause rapid versus slow reacquisition of CR are fully characterized, the field will have a better understanding of the outcomes of post-extinction reconditioning and insight into outcomes that suggest some decrement of the original CS-US association (slow reacquisition).

Fourth, examining the effects of more chronic ethanol administration and withdrawal on rapid reacquisition of contextual fear could make a better model of PTSD-AUD comorbidity. Repeated administration and withdrawal of ethanol more closely resembles the human condition of AUD and the effects of AEW on contextual fear memory were quite mild (only rapid reacquisition, but not acquisition or extinction, of contextual fear was impaired by AEW). Further, chronic withdrawal has been found to have the opposite effect on rapid reacquisition (Bertotto et al., 2006) relative to the effect of AEW found here (chronic withdrawal enhances, while AEW impairs, rapid reacquisition of fear). However, studies examining the chronic effects of alcohol on rapid reacquisition will have to be carefully designed. If chronic ethanol exposure occurs prior to any learning, the effect on reacquisition could be due to an effect of the ethanol on acquisition and extinction, not reacqusition, as was seen in Bertotto et al., 2006.

Fifth, the investigation of the functional necessity of the fear circuit in rapid reacquisition needs to be expanded to other regions. Using pharmacological inactivation of the BNST and amygdala, I began to demonstrate a differential role for each in rapid reacquisition. However, the hippocampus and mPFC were not similarly probed. The hippocampus in particular should be studied for its role in rapid

reacquisition of contextual fear, as a couple of my correlative results (both the c-Fos and AEW studies) suggest that the hippocampus is involved in rapid reacquisition. Additionally, the hippocampus has been implicated in other forms of rapid spatial reacquisition (Winocur et al., 2005), making it an likely candidate for a role in rapid reacquisition of contextual fear. The mPFC on the other hand showed little to no indication of being important for rapid reacquisition in my studies. However, the region is critical for regulating the expression of conditioned behavior (IVidal-Gonzalez et al., 2006) and has been implicated in other forms of reacquisition (Oswald et al., 2008; Willcocks & McNally, 2013; Zheng et al., 2013), so the mPFC also deserves further exploration for its involvement in rapid reacquisition of contextual fear. Further, the subregions of each region as well as their connections with one another for their role in rapid reacquisition will be an important goal of future studies. Additionally, better characterizing the circumstances of reconditioning that involve and do not involve amygdala activity would be beneficial to clarify its role in rapid reacquisition.

VII. Final Conclusions

In summation, the dissertation work herein adds much needed characterization of rapid reacquisition of contextual fear conditioning. The novel findings included the first demonstrations of rapid reacquisition in mice, selective AEW-induced impairment of rapid reacquisition in mice, and BNST inactivation impairment of rapid reacquisition in rats. Importantly, this work displays that rapid reacquisition is a distinctive form of post-extinction CR return that shows behavioral

and neurobiological characteristics important for studying aspects of PTSD and the processes of memory updating.

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