# EXTINCTION OF RECENT FEAR: BEHAVIORAL AND NEURAL MECHANISMS

**by**

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To the ones who helped me to where I am today

獻給所有幫助過我的人

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# CHAPTER I

## INTRODUCTION

Fear is an evolutionarily preserved emotion to help organisms avoid threats in the future and to improve chances of survival (LeDoux, 1996; Mineka and Ohman, 2002). It activates defensive behaviors such as escape, immobility, and aggression within a split-second, which is critical in environments where disasters are life threatening and occur without warning (Fanselow and Lester, 1988). However, failure to suppress fear may lead to certain severe anxiety disorders, such as panic, obsessive-compulsive, phobia, and post-traumatic stress disorders (PTSD) (Bouton et al., 2001; Rothbaum and Davis, 2003). In a given year, about 40 million (18%) American adults are affected by these common psychiatric disorders (Kessler et al., 2005). An example quoted in a booklet published by National Institute of Mental Health (NIMH) about anxiety disorders, illustrated a person with a specific phobia of flying.

"I'm scared to death of flying, and I never do it anymore. I used to start dreading a plane trip a month before I was due to leave. It was an awful feeling when that airplane door closed and I felt trapped. My heart would pound, and I would sweat bullets. When the airplane would start to ascend, it just reinforced the feeling that I couldn't get out. When I think about flying, I picture myself losing control, freaking out, and climbing the walls, but of course I never did that. I'm afraid of crashing or hitting turbulence. It's just that feeling of being trapped. Whenever I've thought about changing jobs, I've had to think, 'Would I be under pressure to fly?' These days I only go places where I can drive or take a train. My friends always point out that I couldn't get off a train traveling at high

speeds either, so why don't trains bother me? I just tell them it isn't a rational fear." (NIMH, 2007)

As illustrated above, most adults with phobias realize that their fears are irrational. However, they still find that facing, or even just the thought of facing, the situation will bring severe anxiety. Specific phobias are defined as "excessive or unreasonable fears of circumscribed objects or situations, which are avoided or endured with dread." (Craske, 1999) Not like commonly occurring fears, phobias can be profound enough to interfere with normal life functions; thus, psychotherapy is usually pursued. Among the treatments of anxiety disorders, exposure-based behavioral therapy has been extensively used for specific phobias (Craske, 1999). Individuals with specific phobias are guided to the objects or situations that are feared, through pictures or tapes, computer-generated virtual realities, or face-to-face conversation (Rothbaum et al., 2000; Gros and Antony, 2006; Rothbaum et al., 2006). Supported and accompanied by therapists in face of the feared situations and sometimes in combination with pharmacological treatments, exposure-based behavioral therapy has been proven effective to suppress irrational fears on phobias (Davis et al., 2006; Gros and Antony, 2006; Hofmann et al., 2006).

It is not surprising, then, that understanding the psychological and neurobiological mechanisms of normal and pathological fear learning, expression, and more importantly, fear suppression, has long attracted interest in basic research. While some stimuli, such as height and snakes, provoke innate fear (Mineka and Ohman, 2002), it is believed that learning through Pavlovian conditioning helps shape the development of fear to certain initially neutral objects and situations that may signal threat or danger (Mineka, 1979).

Thus, by using Pavlovian fear conditioning in rats as the animal model, the current dissertation examines the behavioral and neural mechanisms of extinction to recent fear.

## Pavlovian Fear Conditioning and Extinction

Pavlovian fear conditioning is one of the most frequently used behavioral paradigms to study the neurobiology of fear learning and memory (LeDoux, 2000). It has been used in a variety of species, such as marine slugs (Carew et al., 1981; Walters et al., 1981), fish (Xu and Davis, 1992; Eisenberg and Dudai, 2004), rabbits (Pascoe and Kapp, 1985; Sebastiani et al., 1994; Poremba and Gabriel, 1997; McEchron et al., 2000), rats (LeDoux, 2000; Maren, 2001a; Pare et al., 2004), and humans (LaBar et al., 1998; Milad et al., 2005; Phelps and LeDoux, 2005; Delgado et al., 2008). In this form of learning, organisms associate a neutral conditioned stimulus (CS), such as a tone or a light, with an aversive unconditioned stimulus (US), such as a mild electric shock. Unconditioned stimuli are biologically relevant stimuli capable of evoking innate unconditioned responses (UR), such as vocalization. After a few pairings, CS presentations alone elicit a variety of conditioned responses (CR), including freezing (Blanchard and Blanchard, 1972; Fanselow, 1980; Fendt and Fanselow, 1999), potentiated startle (Davis, 1989; Davis et al., 2003), ultrasonic vocalization (Choi and Brown, 2003; Lindquist et al., 2004), elevated heart rate or arterial blood pressure (Romanski and LeDoux, 1992a, b; Antoniadis and McDonald, 1999), and heightened stress hormone release (Sullivan et al., 2004). Fear conditioning is a robust and enduring form of learning: fear is readily

acquired after as few as one single CS-US pairing (Blanchard and Blanchard, 1972; Davis, 1989; Maren, 2001b), and lasts more than a year after training (Gale et al., 2004).

The stimuli used in fear conditioning paradigms vary considerably. Conditioned stimuli could be discrete as tones (Maren, 2001a), lights (Davis et al., 1993), colored shapes (Phelps et al., 2004), or the context in which conditioning occurs (Maren and Holt, 2000). Unconditioned stimuli also vary according to the species under study, including footshock in rats (Maren, 2001a), eyelid shock in rabbits (Kapp et al., 1979), and wrist shock in humans (Phelps et al., 2004). In the current experiments, discrete tones served as CSs and mild footshocks as USs in all experiments in rats. The CR of interest is freezing, defined as the absence of all motor activity except that required for breathing. Before conditioning, when rats were first placed into the chambers, they showed little to no freezing but rather tended to engage in exploratory behavior. During conditioning, delivery of tone-shock pairings typically resulted in activity bursts and vocalization (UR) followed by freezing (CR), which gradually reached an asymptote with repeated conditioning trials. After conditioning, fear acquired was assessed by freezing to tones in the absence of the footshocks.

From a clinical perspective, what is of more importance is not how fear is generated, by how it is inhibited. After rats acquire conditioned fear, repeated presentations of the CS alone result in a decrement of fear, a process called extinction (Pavlov, 1927). Extinction learning is not as rapid or as stable as the original fear learning. It usually requires a greater number of training trials to reduce fear. It is generally agreed that extinction is not an "unlearning" of the CS-US association, but a "new learning" of an inhibitory CS-'No US' memory. This notion is supported by

numerous studies showing that extinguished fear can be recovered, suggesting the original CS-US memory is not lost during extinction (Pavlov, 1927; Bouton, 2004; Bouton et al., 2006). Extinguished CRs "spontaneously recover their full strength after a longer or shorter period of time …", as first described by Pavlov (1927). Extinguished CRs can be reinstated by the delivery of a US in the context in which extinction was taken place (Rescorla and Heth, 1975; Bouton et al., 2006). Finally, extinguished CRs renewed when the CSs are simply presented outside the extinction context (Corcoran and Maren, 2001; Bouton, 2004; Corcoran and Maren, 2004).

As stated above, extinction is not the erasure of the CS-US acquired during conditioning, but a result in the formation of new inhibitory memory of CS-'No US' acquired during extinction. As a result, extinction training renders the meaning of the CS ambiguous: it predicts the US during conditioning, but not during extinction (Bouton and Ricker, 1994). Argued by Bouton and his colleagues, such ambiguity is gated by extinction context to decide which association is retrieved (Bouton, 2002; Bouton et al., 2006). Context can be the physical context in which conditioning and extinction occurred, interoceptive context of the organism such as its emotional state, presence of drugs, hunger, etc., or temporal context (retention interval between extinction and retrieval) that is gradually changing and affecting the perception of the organism on the overall environment (Bouton et al., 2006). Context here refers to any cues present in the environment, including multimodal sensory stimuli, the relationship among these stimuli, etc., around the subjects under study. Extinction memory retrieval of CS-'No US' is context dependent because it is the second thing learned about the CS (Swartzentruber and Bouton, 1992). As in Bouton's (2002) words, "the learning and memory system

encodes the second thing learned about a stimulus as a conditional, context-specific exception to the rule." Thus, extinction context acts as a negative occasion-setter, in which the presence of the overall context itself predicts that its target (CS, in this situation) leads to 'No US' retrieval.

## Neurobiology of Fear Conditioning and Extinction

The amygdala, a brain structure located in the temporal lobe, is the critical locus for the acquisition, storage, and expression of fear memories (LeDoux, 2000; Davis and Whalen, 2001; Maren and Quirk, 2004; Pare et al., 2004; Sigurdsson et al., 2007). With regard to fear conditioning, there are three main nuclei of interests: the basolateral complex (BLA), the central nucleus (CeA), and the intercalated cells (ITC) that lie in between the BLA and CeA (LeDoux, 2000; Maren, 2001a; Pare et al., 2004).

The BLA, composed of the lateral (LA), basolateral (BL), and basomedial (BM) nuclei, is the sensory interface of the amygdala (LeDoux et al., 1990). Auditory CS and footshock US information converge here. It receives auditory information from the medial geniculate nucleus of the thalamus (Clugnet and LeDoux, 1990; Clugnet et al., 1990; Romanski and LeDoux, 1992a) and auditory cortex (Romanski and LeDoux, 1992a; Li et al., 1996; Doyere et al., 2003). Information about the footshock is conveyed through the posterior intralaminar nucleus of the thalamus and the insular cortex (Shi and Davis, 1999; Brunzell and Kim, 2001; Lanuza et al., 2004). The BLA not only receives both CS and US information, it is also a site for associative plasticity. Single neurons in the LA respond to both auditory CS and footshock US stimulations (Romanski et al., 1993), and fear conditioning induces long-term potentiation (LTP) in the LA (McKernan

and Shinnick-Gallagher, 1997; Rogan et al., 1997). The BLA lesions or pharmacological inactivation blocks fear acquisition (LeDoux et al., 1990; Sananes and Davis, 1992; Fanselow and Kim, 1994; Campeau and Davis, 1995; Maren et al., 1996a; Maren et al., 1996b; Goosens et al., 2000; Schafe et al., 2000; Goosens and Maren, 2001, 2003) and fear expression (Sananes and Davis, 1992; Maren et al., 1996a; Maren et al., 1996b; Fendt, 2001; Lee et al., 2001), suggesting its critical role in fear conditioning. Single-unit studies also reveal that neurons in the LA encode fear memories (Quirk et al., 1995; Quirk et al., 1997; Maren, 2000; Pare and Collins, 2000; Repa et al., 2001; Goosens et al., 2003).

The CeA, composed of the medial (CeAm) and lateral (CeAl) divisions, is the motor interface of the amygdala. Neurons in CeAm project to hypothalamic and brainstem areas that are in control of many fear CRs, such as freezing (periacqueductal gray), fear potentiated startle (nucleus reticularis pontis caudalis), glucocorticoid release (paraventricular nucleus of the hypothalamus and bed nucleus of the stria terminalis), increased respiration (parabrachial nucleus), and increase heart rate and blood pressure (lateral hypothalamus) (LeDoux, 2000; Pitkanen, 2000; Maren, 2001a). Stimulation of the CeA evokes fear (Kapp et al., 1982; Iwata et al., 1987) and Lesions of the CeA abolish numerous CRs, including freezing (Iwata et al., 1986; Goosens and Maren, 2001). The CeA is suggested as the final common output structure of CRs (LeDoux et al., 1988), as lesions downstream to the CeA only result in specific, but not the overall, impairment in CR expression.

The ITC cells are GABAergic interneurons lie in between the BLA and the CeA. They gate the transmission between these two nuclei. In some cases, stimulating the BLA activates the ITC cells and results in feed-forward inhibition of the CeA (Royer et

al., 1999). In other cases, however, activated ITC cells project onto a second group of ITC cells, which results in disinhibition of the CeA (Pare et al., 2003; Pare et al., 2004). Within the amygdala, there are several pathways transferring sensory information from the BLA to the motor output of the CeA. Fear expression could be driven through the CeAm by direct excitation via LA-BL-CeAm (Smith and Pare, 1994; Pare et al., 1995) and LA-CeAl-CeAm (Smith and Pare, 1994; Jolkkonen and Pitkanen, 1998), or indirect disinhibition via LA-ITC-CeAm as stated above (Figure 1.1).

It has been suggested that CS-US association acquired during fear conditioning resides within the amygdala, and that the extinction memory may require the recruitment of another inhibitory brain structure (Davis and Myers, 2002). One candidate is the medial prefrontal cortex (mPFC), which is involved in the inhibition of inappropriate behaviors. The mPFC has been implicated in the reduction of fear responses: rats with the mPFC lesions, specifically the infralimbic (IL) region, require more training to extinguish fear responses (Morgan et al., 1993; Morrow et al., 1999). The IL receives direct excitatory projections from the BL (Conde et al., 1995; Herry et al., 2008), which has been proposed to mediate the plasticity required for extinction memory formation. It sends projections back to the amygdala targeting mainly the BLA (ventromedial LA and rostral BL) and a subset of the ITC neurons (McDonald et al., 1996), supporting its potential role of suppressing the CeAm output through the inhibitory ITC cells (Figure 1.2A) (Royer et al., 1999; Pare et al., 2004; Likhtik et al., 2008). Indeed, lesions of the IL (Quirk et al., 2000; but see Gewirtz et al., 1997; Garcia et al., 2006), and pharmacologically inactivating the IL immediately before (Santini et al., 2004; Sierra-Mercado et al., 2006; Burgos-Robles et al., 2007) or after (Hugues et al., 2004; Hugues et al., 2006; Burgos-Robles et al., 2007; Quirk and Mueller, 2008) extinction leads to impaired extinction retrieval subsequently. Moreover, physiological correlates, such as

increased evoked responses to CSs, increased short latency bursts, and learning related LTP, have been observed in the IL neurons after extinction training (Herry and Garcia, 2002; Milad and Quirk, 2002; Burgos-Robles et al., 2007; Hugues and Garcia, 2007).

## Erasure of Fear?

Behaviorally, fear memory spontaneously recovers with the passage of time (Pavlov, 1927), reinstates following unsignaled US in the relevant context (Bouton et al., 2006), and renews when tested outside the extinction context (Corcoran and Maren, 2004). Neurobiologically, extinction recruits an inhibitory circuit including the mPFC (Maren and Quirk, 2004; Pare et al., 2004). All the arguments suggest that extinction is a new learning, not unlearning, process. Then, is erasure of the fear memory possible?

Consolidation of fear memory requires time. It is considered a process in which protein synthesis independent and labile short-term memory is transformed into stable long-term memory resistant to further manipulation (Schafe et al., 2001). The LTP induction within the BLA is considered the cellular mechanisms of fear acquisition and consolidation (Fanselow and LeDoux, 1999; Maren, 1999; Blair et al., 2001; Sigurdsson et al., 2007). Calcium influx through glutamate N-methyl-D-aspartate (NMDA) receptors and L-type voltage-gated calcium channels (L-VGCCs) (Bauer et al., 2002) are important for some forms of the LTP induction, which initiate the downstream cascades of intracellular events, such as gene expression and protein synthesis (Maren, 1999; Blair et al., 2001; Schafe et al., 2001; Sigurdsson et al., 2007). This process has a time-limited role that happens at late or immediately after fear acquisition.

In most experiments examining extinction, CS alone trials are usually given 24 hours after fear conditioning. At this time, fear memory is fully consolidated, the CS-US association is well established, and moreover, suppression of fear engages the inhibitory neural circuitry. As stated above that consolidation of fear memory requires time within a limited temporal frame after fear acquisition, during which the memory trace is labile and subject to be disrupted, then it raises the question of whether giving CS alone trials shortly after conditioning "reverses" the learning process and breaks the CS-US association, which leads to the erasure of the fear memory? Myers et al. (2006) tested this idea using fear potentiated startle as the measurement, and found supportive evidence that fear memory can indeed be erased when CS alone trials were given shortly after conditioning. On the other hand, McNally and Westbrook (2006) demonstrated that using freezing as the index, short conditioning to retention test intervals, compared to long ones, generated more fear during test. Thus, the timing of extinction on long-term fear suppression remains controversial.

## Specific Aims and Hypotheses

To test the hypothesis that CS alone trials given at different time intervals after conditioning initiates different fear suppression mechanisms, "new learning" at longer intervals (Figure 1.2A) and "unlearning" at shorter intervals (Figure 1.2B), in Chapter II we gave immediate (10 min) and delayed (24 hr) extinction trials after fear was acquired. In contrast to our hypothesis, immediate extinction did not erase the fear memory. Immediate extinction administered minutes after aversive experience generated acute fear

suppression, but unlike delayed extinction, such suppression was not maintained when tested the next day. Moreover, our results suggested that the level of fear before extinction intervention interfered with the efficacy of extinction: high onset fear due to recent aversive experience led to poor long-term fear suppression.

The results in Chapter II suggested that immediate extinction after conditioning did not erase the fear memory, but in fact failed to suppress the long-term fear. We thus hypothesize that immediate extinction failed to result in a long-term extinction memory. To test this hypothesis, in Chapter III we explored the possible mechanisms underlying this deficit by assessing the suppression of fear to a CS immediately after extinction training and the context-specificity of fear after both immediate and delayed extinction training. Our results indicated that immediate extinction produced a short-lived and context-independent suppression of conditional freezing. Taken together the results of Chapter II and Chapter III, these results suggested that first, fear was not unlearned under immediate extinction due to high onset fear, and second, fear suppression under immediate extinction may be due to a short-term, context-independent habituation process, rather than extinction *per se*.

Based on the behavioral results stated above, we hypothesize that immediate extinction failed to generate the long-term fear suppression because the inhibitory neural circuitry, specifically the IL, was not engaged. However, the effects of IL lesions on the retention of extinction memory are inconsistent. In Chapter IV, we first examined experimental parameters that might influence the effects of IL lesions on the retention of extinction. Our results revealed that that contextual fear present before extinction influences the effects of IL lesions. Moreover, different strains of rats exhibit different

levels of contextual fear, resulting in strain differences in the influence of IL lesions on extinction.

To further explore the possibility that immediate extinction failure is related to prefrontal cortical dysfunction, in Chapter V, single-units were simultaneously recorded from neurons in the IL and the prelimbic (PrL) divisions of the mPFC during immediate and delayed extinction sessions after auditory fear conditioning. Our results revealed that conditioning and extinction did not alter the spontaneous firing rate of either the IL or the PrL neurons. However, delayed, but not immediate, extinction produced an increase in both spontaneous and trial-related bursts in the IL. These results suggested that the IL function is dampened under immediate extinction. We then test the hypothesis that deficits in immediate extinction could be overcome by driving IL activity during extinction. Our results showed that such deficits were rescued with microinfusions of either a GABA receptor antagonist or an NMDA receptor partial agonist into the IL. These data reveal that engaging the infralimbic cortex during extinction is necessary for long-term fear suppression.

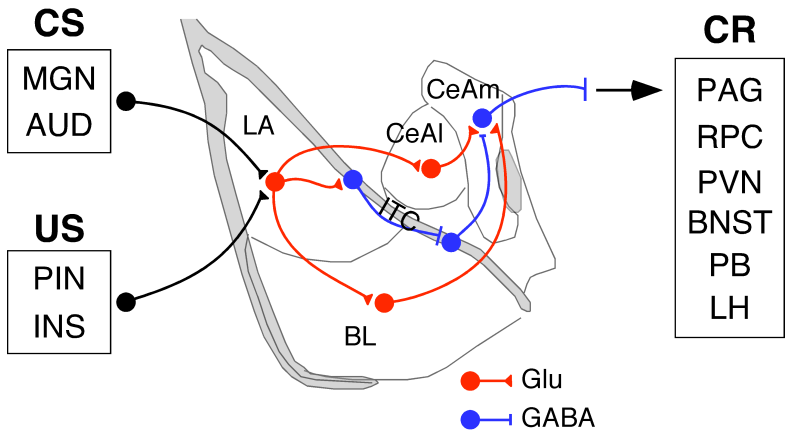


**Figure 1.1. Neuroanatomy of Pavlovian fear conditioning circuitry.**
The sensory input interface of the basolateral amygdala complex (BLA; consisting of the lateral, LA; and basolateral, BL) is where the CS and US information converge and become associated. The LA receives excitatory glutamatergic auditory CS information from the medial geniculate nucleus of the thalamus (MGN) and the auditory cortex (AUD). The pathway(s) for conveying information about the aversive US to the LA is still under investigation, however some suggest that the posterior intralaminar nucleus of the thalamus (PIN) and the insular cortex (INS) are involved. The LA neurons have projections to the lateral division of the central nucleus of the amygdala (CeAl), which then has connections with the medial division of the CeA (CeAm). The LA neurons also project to the BL, which then send input to the CeAm. The motor output interface of CeAm sends afferent projections to many brainstem areas that control the expression of fear CRs, such as the periacqueductal gray (PAG; freezing behavior), nucleus reticularis pontis caudalis (RPC; fear-potentiated startle), paraventricular nucleus of the hypothalamus (PVN) and bed nucleus of the stria terminals (BNST; glucocorticoid release), parabrachial nucleus (PB; increased respiration), and the lateral hypothalamus (LH; increases in heart rate and blood pressure). In addition, during fear conditioning, neurons from the LA excite inhibitory intercalated cells (ITC), which lie in between of the BLA and the CeA. These ITC cells then project onto a second population of ITC cells and this second population of ITC cells makes direct connections with the CeAm, which disinhibits the CeAm. This figure was adapted from Swanson (2004).

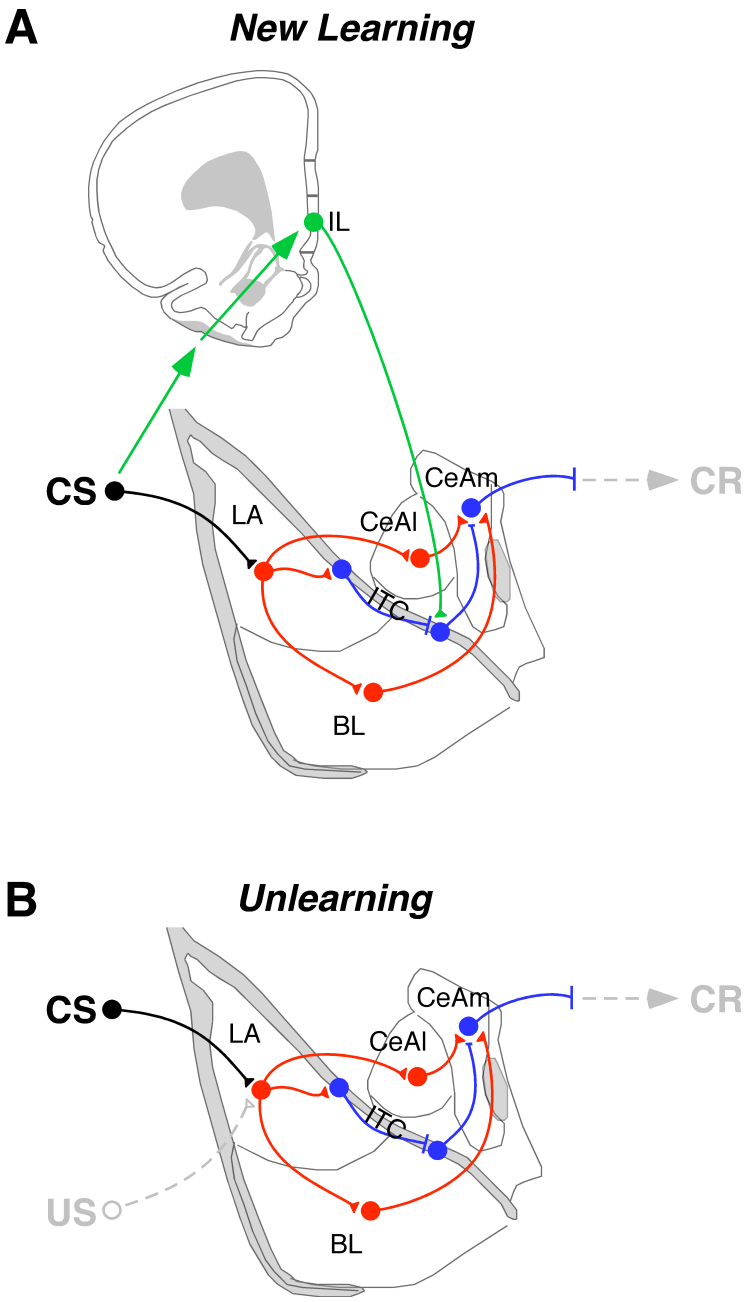


**Figure 1.2. Models of fear suppression after extinction training.**
A. "New learning" model of giving CS alone trials at longer intervals (24 hr) after conditioning: CR is suppressed due to the inhibitory learning of IL projections onto ITC neurons, which regulates the information flow from the BLA to the CeA without erasing the original fear memory. B. "Unlearning" model of giving CS alone trials at short intervals (10 min) after conditioning: CR is suppressed due to the break of the CS-US association, which erases the original fear memory. This figure was adapted from Swanson (2004).

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# CHAPTER II

## RECENT FEAR IS RESISTANT TO EXTINCTION

Traumatic events such as military combat, motor vehicle accidents, or sexual assault can lead to debilitating psychological disturbances, including post-traumatic stress disorder (PTSD) (McNally, 2003). Although PTSD is estimated to develop in less than ten percent of individuals experiencing trauma in the general population (Breslau et al., 1998), it presents at significantly higher rates in individuals exposed to extremely traumatic events, such as combat. For example, rates of PTSD as high as 17 % have been reported in military personnel three to four months after returning from combat (Hoge et al., 2004). It is not surprising then that traumatic events exact an incredible toll on mental health, affecting millions of people worldwide.

Because of the staggering costs and consequences of PTSD and other anxiety disorders, clinical interventions to reduce the long-term consequences of psychological trauma are essential. As a first line of defense against the development of mental illness in the aftermath of a traumatic event, it has been argued that early interventions, such as psychological debriefing, are critical to manage the stress response to trauma (Everly and Mitchell, 1999; Campfield and Hills, 2001). In a typical debriefing session, victims of a traumatic event are encouraged to talk about their experience in a supportive group

setting and this is presumed to facilitate psychological recovery from the trauma. Although early intervention is intuitively reasonable, there has been considerable work challenging the efficacy of debriefing in curbing the development of PTSD after trauma (McNally et al., 2003). Moreover, there is little work systematically examining whether early interventions, whatever form they take, are more effective than delayed interventions in reducing the incidence of psychopathology after trauma (Gray and Litz, 2005). Indeed, intervening too early, particularly when the intense and acute stress of the experience has not waned, might even exacerbate relapse of fear (Bisson et al., 1997; McNally et al., 2003; Rothbaum and Davis, 2003; Gray and Litz, 2005). Nonetheless, recent work in rats suggests that an early intervention may produce more effective fear suppression than a delayed intervention (Myers et al., 2006).

In the present study, we sought to compare the efficacy of early or delayed interventions in reducing fear associated with a traumatic event. To address this question, we used an animal model of traumatic fear, Pavlovian fear conditioning in rats (Davis, 1998; LeDoux, 2000; Maren, 2001; Fanselow and Poulos, 2005). In this form of associative learning, innocuous stimuli (i.e., conditional stimuli, CSs) that predict aversive events (i.e., unconditional stimuli, USs) come to yield fear responses themselves. This type of learning may be involved in the development of pathological fear in patients with a variety of anxiety disorders, including post-traumatic stress disorder and panic disorder (Grillon et al., 1996; Bouton et al., 2001; Rau et al., 2005). Extinction training, in which CSs are presented without the US, suppresses conditioned responses (CRs) learned during fear conditioning. Considerable evidence indicates that fear conditioning and extinction yield excitatory and inhibitory memories, respectively,

and these memories compete with each other for expression in behavior. In most cases, extinction does not erase fear memory. Nonetheless, extinction is an important component of exposure therapy in humans, and is emerging as a powerful model for understanding the mechanisms of fear suppression relevant to the treatment of anxiety disorders (Bouton, 1988; Myers and Davis, 2002; Rothbaum and Schwartz, 2002; Barad, 2005; Milad et al., 2006). Hence, this behavioral paradigm affords many advantages, because it allows us to precisely control both the nature of the traumatic event (i.e., conditioning), as well as the timing of the intervention (i.e., extinction) in a clinically relevant model of traumatic fear.

## Materials and Methods

*Subjects*

The subjects were 240 male Long-Evans rats (Harlan Sprague Dawley, USA) weighing between 250-330 g. They were housed in individual cages with 14-h light/10-h dark cycle (lights on at 7:00 am), and allowed food and water ad libitum. During the first five days, they were handled for 10 sec to habituate them to the experimenter.

*Apparatus*

Eight identical observation chambers (30 x 24 x 21 cm; MED-Associates, St. Albans, VT) were used in all experiments. The chambers were constructed of aluminum and Plexiglas and were situated in sound-attenuating cabinets located in a brightly lit and isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center-to-center). Rods were wired to a shock source and

solid-state grid scrambler (MED-Associates) for the delivery of footshock US (0.5 sec; 1 mA). A speaker mounted outside a grating in one wall of the chamber was used for the delivery of acoustic CS (2 sec; 80 dB; 2 kHz). Illumination, odor, and ambient noise were manipulated to create two distinct contexts for some of the experiments.

Each conditioning chamber rested on a load-cell platform that was used to record chamber displacement in response to each rat's motor activity, allowing therefore detection of freezing behavior. Freezing was determined during each 1-min inter-trial interval after the CS offset during conditioning, extinction, and the retention test, and during the minutes preceding the first CS presentation during extinction training.

*Behavioral Procedures*

Rats were submitted to three phases of training: fear conditioning, extinction, and an extinction retention test. All of these phases were conducted in the same context in Experiments 1-3 and Experiment 5; in Experiment 4 fear conditioning was conducted in a different context than extinction and retention testing. For fear conditioning, rats received one (Experiment 4) or five (Experiments 1-3, and 5) tone-footshock trials (60 s inter-trial interval) beginning 3 min after being placed in the chambers. For extinction (EXT), rats received 45 tone-alone presentations (60 s ISI) either 15 min (IMMED) or 24 hours (DELAY) after conditioning (again with a 3-min baseline preceding the extinction trials). Rats that received immediate extinction trials were transported home 2 min after the last footshock and returned to the conditioning context (Experiment 1-3, 5) or a novel context (Experiment 4) 15 min later for extinction. Rats in the delay condition received extinction training 24 hours after conditioning in the conditioning context (Experiments 1-3, and 5) or a novel context (Experiment 4). In Experiment 3, rats received either 45

(60 s or 12 s ISI) or 225 (12 s ISI) extinction trials 15 min after conditioning; time in the extinction context was equated among the groups. In Experiment 5, the rats were placed in a novel context and were either shocked (SHOCK) or not (NO-SHOCK) 15 min before returning to the conditioning chamber for delayed extinction trials. Under the no-extinction condition (NO-EXT), rats were placed in the chamber for the same amount of time as the EXT rats but were not exposed to the tone CS. Two days after conditioning, all rats were returned to the extinction context and exposed to five CS-alone presentations 3-min after placement in the chambers. Retention test freezing was averaged across the five CS trials and subtracted from the 3-min baseline. All behavioral data are expressed as means ± standard error of the means (SEM).

## Results

Experiment 1: Immediate or delayed extinction after fear conditioning.

The first experiment was aimed at comparing the efficacy of extinction training at two different times after fear conditioning. We were particularly interested in whether an early intervention delivered minutes after fear conditioning would produce superior extinction relative to a standard delayed intervention (24 hours). Rats were submitted to a standard fear conditioning procedure in which an auditory CS was paired with a noxious footshock US in a novel chamber. After either a short (15 min) or long (24 h) delay, half of the animals received 45 extinction trials in which the CS was presented alone; the other half of the animals remained in the chambers without the presentation of either the CS or US (these animals served as a no-extinction control group). Forty-eight

hours after conditioning, rats were tested for their fear to the CS by assessing freezing behavior, which is manifest as somatomotor immobility (except for breathing). For this retention test, rats were once again returned to the conditioning chambers and presented with five auditory CSs.

Freezing behavior during the conditioning session is shown in Figure 2.1A. There were very low levels of freezing behavior before the first conditioning trial; freezing behavior only emerged after the first conditioning trial and steadily increased in frequency thereafter. During the extinction session (Figure 2.1B), which was conducted in the conditioning context either 15 min (immediate) or 24 hours (delayed) after fear conditioning, group differences emerged. All animals exhibited high levels of fear prior to the onset of extinction trials, a consequence of fear conditioned to the testing context. However, recently conditioned rats exhibited significantly higher levels of freezing behavior *before* the first extinction trial compared to rats in the delayed extinction groups [$F(1, 60) = 20.7$, $p < 0.0001$]. Once extinction training commenced, CS presentations yielded robust freezing behavior in both the immediate and delayed extinction groups, and there was an equivalent decline in freezing in both groups across the session [extinction x interval x block, $F(8, 480) = 1.9$]. Shock-induced sensitization of fear contributed to the elevation of fear in the immediate groups, which potentiated fear above and that generated by context fear alone in the delay groups. Rats that were placed in the boxes 15 minutes after conditioning, but not exposed to the CS, exhibited a similar pattern of freezing behavior to animals in both of the extinction groups [interval x extinction interaction: $F(1, 60) = 16.0$, $p < 0.0005$].

Despite similar levels of fear reduction during the extinction session, rats receiving immediate or delayed extinction training differed with respect to their retention of the extinction memory (Figure 2.1C). Forty-eight hours after conditioning, only rats that had received the delayed extinction procedure exhibited a significant reduction in freezing relative to non-extinguished controls when presented with the CS [interval x extinction: $F(1, 60) = 10.6$, $p < 0.002$; Figure 2.1D]. Hence, fear memories exhibited substantial spontaneous recovery (i.e., a return in conditional responding with the passage of time after extinction) after an early intervention, but remained inhibited in rats with a 24 h delay between conditioning and the extinction intervention.

Experiment 2: Retention testing with a common extinction-test interval.

The different levels of extinction in the immediate and delayed groups cannot be explained by the time elapsed between fear conditioning and retention testing; this interval was held constant in both groups. However, the design of Experiment 1 confounded the interval between extinction training and the retention test. That is, animals in the immediate extinction group were tested 48 hours after extinction, whereas those in the delayed group were tested only 24 hours after extinction. It is possible that the longer extinction-test interval in the immediate group allowed for more spontaneous recovery of fear than the shorter extinction-test interval in the delay group. In Experiment 2, we examined this possibility by equating the extinction-test interval in the immediate and delayed extinction groups. The experiment was identical to Experiment 1, except that rats in both the immediate and delayed groups were tested 48 hours after extinction training.

Behavior during the conditioning and extinction sessions was similar to that reported in Experiment 1 (not shown). As shown in Figure 2.2, rats in the immediate extinction condition exhibited significantly weaker extinction than those animals in the delayed extinction condition. Planned comparisons indicated that only rats in the delayed extinction condition exhibited significant extinction relative to their no-extinction controls [$p < 0.05$]. Thus, early extinction trials failed to yield long-term extinction even when the extinction-test interval was equated among the immediate and delayed groups.

Experiment 3: Massed or distributed extinction trials immediately after fear conditioning.

Recent work suggests that massed extinction training [delivering CS alone trials with a short inter-stimulus interval (ISI)] produces more robust long-term extinction than extinction training with distributed trials (Cain et al., 2003). In Experiment 3, we examined the possibility that delivering many more massed extinction trials might enable extinction in the immediate groups. To this end, we replicated the immediate condition in Experiment 1 (45 trials with a 1-min ISI), and also examined groups receiving either 45 or 225 massed extinction trials (12-sec ISI); the time all animals spent in the conditioning context was equated across the groups (i.e., animals in the short ITI groups were left in the boxes after their extinction trials). Animals in all groups exhibited similar decrements in freezing behavior during the extinction training session (not shown). However, as shown in Figure 2.3, neither massing the extinction trials (45 trials; 12 sec ISI) nor increasing the number of extinction trials (225 trials; 12 sec ISI) yielded long-term retention of extinction relative to the no-extinction controls. These data reveal that

neither massed nor distributed (Experiment 1 and 2) extinction trials yield long-term fear suppression when delivered shortly after training.

Experiment 4: Reducing fear before immediate extinction.

In Experiment 1, we observed much higher levels of fear before the onset of extinction training among rats in the immediate condition compared to those in the delayed condition (see Figure 2.1B, baseline). This is likely due to the sensitization of fear produced by recent shock summating with fear conditioned to the context. It has recently been reported in both rats and humans that the arousal of fear prior to extinction training can interfere with the development of long-term extinction (Lovibond et al., 2000; Morris et al., 2005). We therefore investigated whether the different levels of fear at the outset of extinction training in the immediate and delayed groups contributed to their different levels of long-term extinction in these groups. In Experiment 4, rats were submitted to the identical behavioral procedures as in Experiment 1, except that they received only a single conditioning trial (Figure 2.4A) and extinction training and testing were conducted outside of the conditioning context. The goal of these manipulations was to reduce the level of fear before the onset of extinction training.

As shown in Figure 2.4B (see baseline), reducing the number of conditioning trials and shifting the context between conditioning and extinction greatly reduced freezing behavior at the outset of extinction training in both immediate and delay groups. Importantly, reducing fear before the onset of extinction training yielded robust extinction, even in the immediate extinction group [Figure 2.4C; extinction, $F(1,60) = 8.9$, $p < 0.005$; extinction x interval, $F(1,60) = 0.6$]. Therefore, early extinction is

effective in producing long-term fear suppression when fear is relatively low at the onset of extinction training. In fact, extinction obtained under these conditions did not exhibit spontaneous recovery in a retention test conducted one week after the first retention test (data not shown). This is consistent with a recent study showing that early extinction training produces a lasting fear suppression that does not show either spontaneous recovery or renewal upon a change in context (Myers et al., 2006).

Experiment 5: Arousing fear before delayed extinction.

If the level of fear at the outset of extinction training influences the long-term retention of extinction, then arousing fear prior to a delayed extinction intervention should impair extinction memory. To test this hypothesis, we examined whether arousing fear prior to a delayed extinction intervention compromises long-term extinction. Rats were submitted to the same procedures as the delayed groups in Experiment 1, except that they were exposed to additional unsignaled footshocks in a novel context 15 min before the extinction session.

Conditioning proceeded normally in all of the rats (Figure 2.5A). As shown in Figure 2.5B, exposing rats to footshock 15 min prior to extinction training elevated their levels of fear before the onset of the extinction trials [$F(1,28) = 32.6$, $p < 0.0001$]. Rats that received extinction trials 15 min after unsignaled shock decreased their fear over the course of the extinction session and reached similar levels of freezing to rats that received unsignaled shock but did not receive extinction trials. Nonetheless, rats in the EXT /SHOCK condition showed substantial recovery when tested 24 hr later. As shown in Figure 2.5C, only rats that were not shocked prior to extinction training exhibited a

normal reduction in fear during the retention test [planned comparisons, $p < 0.05$]. Collectively, these results indicate that the level of acute fear at the time of the extinction intervention determines both the nature and extent of extinction memory. Moreover, these experiments indicate that the conditioning-extinction interval *per se* is not the critical factor regulating the efficacy of extinction, but that recent fear appears to mitigate long-term extinction memory.

## Discussion

The major finding of the present work is that long-term extinction is minimal when extinction training is conducted shortly after fear conditioning in rats. This deficit in long-term extinction appears to be more related to the level of fear present at the outset of extinction training, rather than the interval between conditioning and extinction *per se*. These results indicate that attempts to extinguish fear shortly after a traumatic experience may not be effective, particularly if the trauma is particularly extreme.

Interestingly, recent work by Davis and colleagues in another fear-conditioning paradigm in rats (fear-potentiated acoustic startle) has revealed that the properties of extinction also depend on the interval in between conditioning and extinction (Myers et al., 2006). In this study, short intervals between conditioning and extinction yielded a form of extinction that was both enduring (i.e., it did not spontaneously recover with the passage of time) and insensitive to context shifts that normally attenuate extinction. Although there was a trend for weaker extinction with their early intervention, our observations would appear to be at odds with the relatively robust extinction observed by

these investigators with early extinction. But this disparity can be explained when one considers that Davis and colleagues used relatively weak footshocks during conditioning, which is typical in the fear-potentiated startle paradigm. Although these investigators did not measure fear during the extinction session, it is reasonable to assume that their conditioning procedure limited fear prior to the extinction session. And, as we observed in Experiment 4, an early intervention does yield extinction if the conditioning procedure does not arouse fear before the extinction session; early interventions only appear to fail when there are high levels of fear at the outset of extinction training. It is also possible that the greater number of conditioning trials used by Davis and colleagues influenced subsequent extinction. Together these reports reveal that both the nature and magnitude of long-term extinction depend on an interaction between the timing of extinction relative to conditioning and the level of fear present when extinction trials are delivered (Rothbaum and Davis, 2003). This interaction has clinical relevance because it suggests that an early intervention may be optimal after mild trauma, but that a delayed intervention may be more suitable after a severe trauma.

A key question from a theoretical point of view is whether the arousal of fear before extinction training interferes with extinction learning (i.e., learning an inhibitory CS-US association), the consolidation of the extinction memory, or the later retrieval of the extinction memory. Because rats do reduce their level of fear to the CS during extinction training (independently of when extinction trials are administered relative to training), it is unlikely that they simply fail to encode inhibitory associations. Therefore, the decrement in long-term extinction is either a failure to consolidate the extinction memory or a generalization decrement from extinction to testing that interferes with the

retrieval of the extinction memory. The latter possibility is particularly compelling insofar as there is substantial evidence that the inhibitory associations acquired during extinction are modulated by both time and context (Bouton, 1993; Bouton et al., 2001; Maren and Quirk, 2004). Indeed, some theoretical accounts of spontaneous recovery after extinction predict that there will be greater spontaneous recovery of conditional responding when the interval between conditioning and extinction is short (Spear, 1971; Devenport, 1998). Consistent with this view, Rescorla has recently reported that there is greater spontaneous recovery to a CS that is extinguished 1-day versus 8-days after conditioning in an appetitive conditioning paradigm (Rescorla, 2004). Although these results are consistent with what we have observed in the present experiments, it is unlikely that the interval between conditioning and extinction alone accounts for our results. As we have shown, a critical variable, at least in our hands, is the level of fear present before the delivery of extinction trials. Nonetheless, it is reasonable to suggest that the deficit in long-term extinction in both cases is related to a failure to retrieve the extinction memory during the retention test. This possibility awaits further examination.

It is important to note that other models of associative learning actually predict that either short intervals between conditioning and extinction or high levels of background fear (aroused by another excitatory CS or a fearful context, for example) will enhance extinction (Rescorla and Wagner, 1972; Wagner, 1981). In Wagner's SOP (sometimes opponent process) model, for example, inhibitory associations between the CS and US are more likely to occur if CS-alone trials occur shortly after exposure to the US. And in the Rescorla-Wagner model, high levels of fear before the onset of extinction should strongly predict footshock when the CS is presented, resulting in especially large

decrements in associative strength to the CS when it is presented in the absence of the US. There is some evidence for the latter effect in appetitive conditioning procedures (Rescorla, 2000), and it has recently been shown that compound presentation of excitatory CSs during extinction yields greater extinction than extinction of either element alone (Rescorla, 2006). Therefore, it will be important to use other measures (e.g., summation, retardation) to determine whether CSs that undergo extinction shortly after conditioning under high levels of fear gain any inhibitory value, and if so, the retrieval processes that work against the expression of that inhibition during retention testing.

From a neurobiological perspective, it is surprising that our early extinction manipulation did not produce more effective extinction. Indeed, it is well known that memories (including fear memories) are most susceptible to disruption within an hour of encoding (McGaugh, 2000; Schafe et al., 2001). Neurobiological studies (Lin et al., 2003a; Lin et al., 2003b) have recently shown that extinction training reverses some of the biochemical changes that develop during conditioning in brain structures such as the amygdala that are essential for fear conditioning (Davis, 1998; LeDoux, 2000; Maren, 2001; Fanselow and Poulos, 2005). And, as already noted, delivering CS-alone trials shortly after fear conditioning can produce a form of extinction that appears to be more an erasure of fear memory than an acquisition of inhibition (Myers et al., 2006). Thus, the influence of CS-alone trials on fear memory may be determined by the degree to which those trials either disrupt cellular consolidation of the conditioning memory or engage new inhibitory learning that permits extinction. By this view, the present experiments suggest that high levels of fear prevent CS-alone trials delivered shortly after

conditioning from disrupting cellular consolidation, and it remains to be seen how these conditions influence the inhibitory associations learned during extinction training.

Psychological interventions are not always effective when administered shortly after a traumatic event (Bisson et al., 1997; McNally et al., 2003; Rothbaum and Davis, 2003; Gray and Litz, 2005). The present work indicates that this may be the result of recent fear interfering with either the consolidation or retrieval of long-term extinction memories. Of course, we have not examined the longevity of fear suppression obtained with delayed extinction training (we assessed behavior up to 48 hours after extinction), and understanding the factors that contribute to a suppression of fear lasting weeks to months is important when developing clinical interventions. Likewise, in our experiments, interposing a twenty-four hour delay in the delivery of the intervention was sufficient to enable fear suppression (lasting at least 2 days). Whether a 24 hr delay is always optimal is not clear, and our data suggest that this interval will critically depend on the duration and extent of acute stress associated with trauma. Indeed, for people that experience severe trauma, this interval may extend beyond days or even weeks (Pennebaker, 1999). Clearly, the modulation of extinction by concurrent levels of fear and stress has important implications for optimizing clinical interventions for psychological trauma in humans.

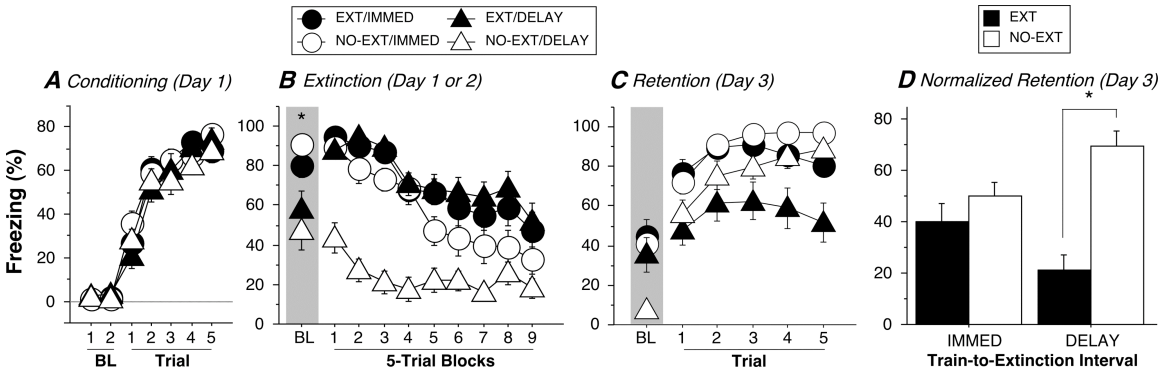


**Figure 2.1. Immediate or delayed extinction after fear conditioning.**
A. Freezing behavior on the conditioning day. Data are 1-min averages for the period before (baseline, BL) and after each of five tone-shock conditioning trials. B. Freezing behavior during the extinction session, which occurred either 15 min (IMMED) or 24 h (DELAY) after conditioning. Control rats did not receive CS presentations during extinction (NO-EXT). C. Freezing behavior during the retention test 48 hours after conditioning. D. Baseline freezing data were averaged and subtracted from the average freezing across test trials to yield normalized freezing for the retention test data shown in C. All data are means (± SEM).

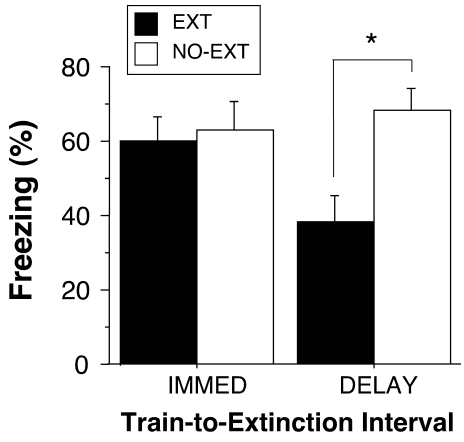


**Figure 2.2. Retention testing with a common extinction-test interval.**
Freezing behavior during the retention test 48 hours after extinction; the extinction-test interval was equated in rats that were extinguished either 15 min (IMMED) or 24 h (DELAY) after conditioning; control rats did not receive CS presentations during extinction (NO-EXT). Data were normalized as in Figure 1D. All data are means (± SEM).

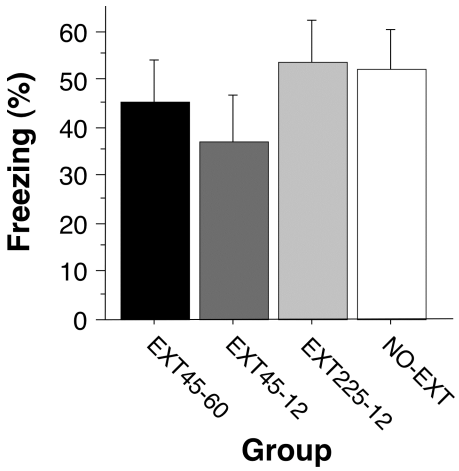


**Figure 2.3. Massed or distributed extinction trials immediately after fear conditioning.**
Rats received 45 or 225 extinction trials 15 min after fear conditioning. For two groups of rats (EXT45-12 and EXT225-12), the extinction trials were massed (12 sec ISI); the EXT45-60 group was treated identically to that in Experiment 1 (45 trials; 60 sec ISI). Total time in the extinction context was equated in all of the groups. The graph displays freezing behavior during the retention test 24 hours after extinction. Data were normalized as in Figure 1D. All data are means (± SEM).

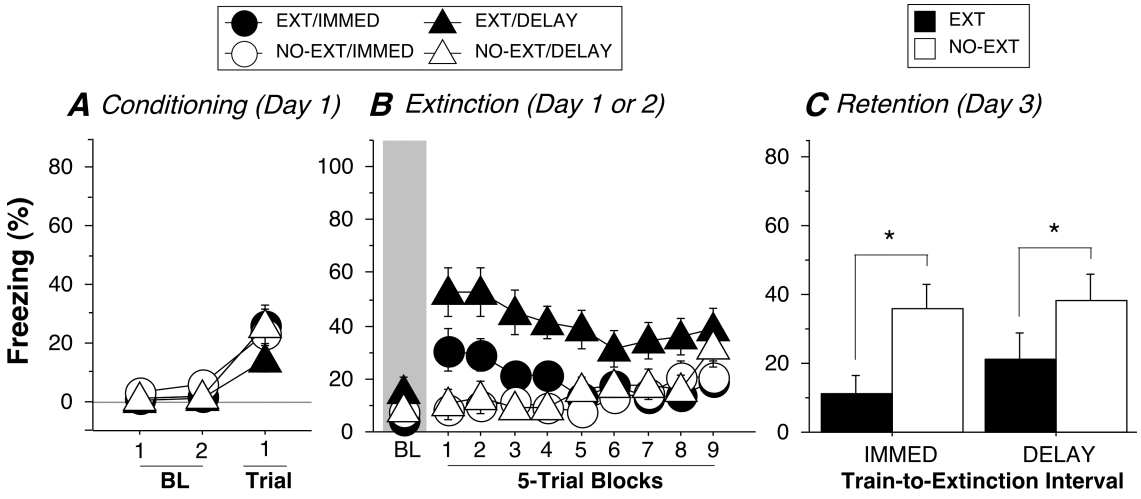


**Figure 2.4. Reducing fear before immediate extinction.**
A. Freezing behavior on the conditioning day. Data are 1-min averages for the period before (baseline, BL) and after a single tone-shock conditioning trials. B. Freezing behavior during the extinction session in a novel context, which occurred either 15 min (IMMED) or 24 h (DELAY) after conditioning. Control rats were exposed to the context but did not receive CS presentations during extinction (NO-EXT). C. Freezing behavior during the retention test 48 hours after conditioning; data from the retention test were normalized as in Figure 1D. All data are means (± SEM).

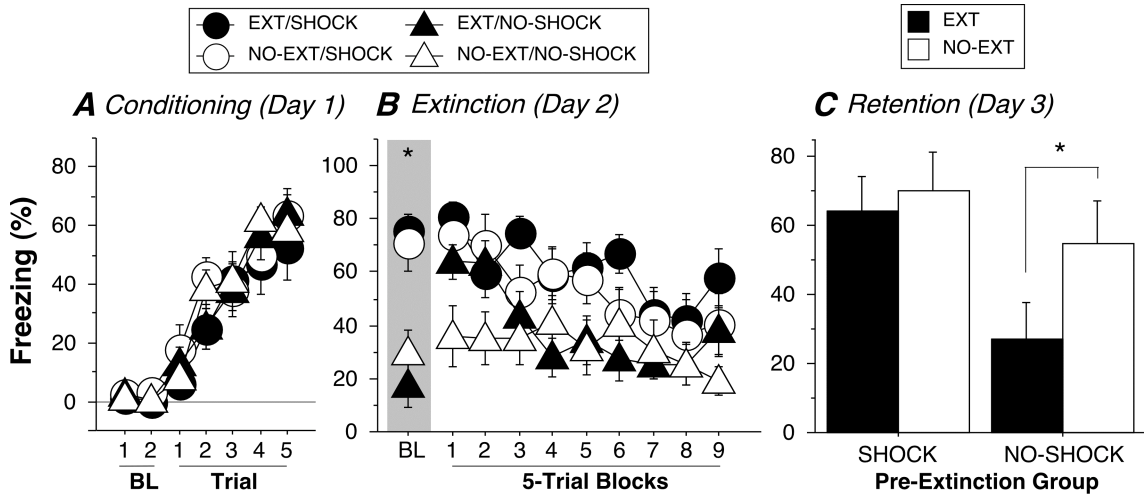


**Figure 2.5. Arousing fear before delayed extinction.**
A. Freezing behavior on the conditioning day. Data are 1-min averages for the period before (baseline, BL) and after each of five tone-shock conditioning trials. B. Freezing behavior during the extinction session. Extinction training was conducted in the context in which the rats had been conditioned a day earlier. Fifteen minutes before the extinction session, rats received either five unsignaled footshocks (SHOCK) in a novel context or exposure without shock (NO-SHOCK) in that context. Control rats did not receive CS presentations during extinction (NO-EXT). C. Freezing behavior during the retention test 48 hours after conditioning. All data are means (± SEM).

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# CHAPTER III

## EARLY EXTINCTION AFTER FEAR CONDITIONING YIELDS A CONTEXT-INDEPENDENT AND SHORT-TERM SUPPRESSION OF CONDITIONAL FREEZING IN RATS

Pavlovian fear conditioning and extinction are important behavioral models for studying the brain mechanisms underlying the acquisition, storage, retrieval, and suppression of traumatic fear (LeDoux, 2000; Maren, 2001; Kim and Jung, 2005; Maren, 2005). In this procedure, an emotionally neutral stimulus, such as a tone, is paired with an aversive stimulus (US), such as an electric footshock. After a few tone-footshock pairings, the previous neutral tone becomes a potent conditioned stimulus (CS) and acquires the ability to elicit fear responses, such as freezing (CR). However, with repeated presentations of the CS-alone the previously acquired CR gradually subsides, a process called extinction (Davis et al., 2003; Maren and Quirk, 2004; Kim and Jung, 2005; Myers and Davis, 2007). The behavioral processes and the underlying neural mechanisms of extinction have attracted extensive attention in contemporary research of learning and memory (Bouton et al., 2006). Indeed, it has been suggested that failure to extinguish fear may contribute to posttraumatic stress disorder (PTSD) (Bouton et al., 2001; Rothbaum and Davis, 2003). To avoid the possible long-term consequences and

costs of PTSD or other anxiety disorders, clinical interventions are essential. While early interventions may manage the stress response to trauma, their efficacy has been challenged because the acute intense stress of the traumatic experience might actually exacerbate relapse of fear (McNally et al., 2003; Rothbaum and Davis, 2003; Gray and Litz, 2005). Thus, it is essential to learn when these interventions generate the best long-term extinction of fear responses.

In a recent study, we demonstrated that delivering extinction trials shortly after fear conditioning yields poor long-term fear reduction (Maren and Chang, 2006; but see Myers et al., 2006). We observed that conditional freezing decreased during extinction training, but recovered completely 24 hours later. This was true even when we gave 225 massed extinction trials 15 min after fear conditioning. However, in these experiments the within-session decrease in fear in rats that underwent extinction was similar to that in rats that were not exposed to extinction trials. Thus, it is unclear to what extent the short-term fear suppression we observed was due to a loss of fear to the context, the auditory CS, or both. It is also not clear whether fear suppression was due to extinction or, alternatively, another learning process such as habituation.

To examine these issues further, in the present study we first assessed fear suppression to the auditory CS after immediate extinction by probing CS fear 15 minutes after extinction training. In a second experiment, we examined whether short-term fear suppression to the CS is renewed outside the extinction context, as context-specificity is one of the hallmarks of extinction (Bouton, 2002; Ji and Maren, 2007). In the third and fourth experiments, we examined the temporal delay necessary between conditioning and extinction to yield long-term suppression of fear. In our previous work (Maren and

Chang, 2006), all phases of training were conducted in the same context. Therefore, fear to the context decreased conditional freezing to the tone, particularly when extinction occurred shortly after conditioning, a time at which sensitized context fear was high. In an effort to isolate fear to the tone CS during extinction, we conducted extinction and test sessions in a context that was different from the conditioning context (i.e., an ABB procedure, where each letter denotes the context used for conditioning, extinction, and test, respectively). Our results reveal that delivering CS-alone trials shortly after fear conditioning produces a short-lived and context-independent suppression of freezing. This fear suppression may be due to a short-term, context-independent habituation process, rather than extinction. Furthermore, poor long-term extinction occurs even when the extinction trials were administered up to six hours after conditioning.

## Materials and Methods

Experiment 1: Does immediate extinction training produce short-term decrements in fear to an auditory CS?

*Subjects and behavioral apparatus*

The subjects were 16 Male Long-Evans rats (250-330 g; Blue Spruce) obtained from a commercial supplier (Harlan Sprague Dawley, USA). They were housed in individual cages with 14-h light/10-h dark cycle (lights on at 7:00 am), and allowed food and water ad libitum. During the first 5 days, they were handled for 10 sec to habituate them to the experimenter.

Eight identical observation chambers (30 x 24 x 21 cm; MED-Associates) were used in all experiments. The chambers were constructed of aluminum (side walls) and Plexiglas (rear wall, ceiling, and hinged front door) and were situated in sound-attenuating cabinets located in a brightly lit and isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center-to-center). Rods were wired to a shock source and solid-state grid scrambler (MED-Associates) for the delivery of footshock US (0.5 sec; 1 mA). A speaker mounted outside a grating in one wall of the chamber was used for the delivery of acoustic CS (2 sec; 80 dB; 2 kHz).

Each conditioning chamber rested on a load-cell platform that was used to record chamber displacement in response to each rat's motor activity and acquired on-line using Threshold-Activity software (MED-Associates). The output of each chamber's load cell was set to a gain that was optimized for detecting freezing behavior (somatomotor immobility, except that necessitated by breathing). Load-cell amplifier output (-10 to +10 V) from each chamber was digitized. Absolute values of the load-cell voltages were then computed and multiplied by 10 to yield a scale that ranged from 0 to 100. For each chamber, load-cell voltages were digitized to 5 Hz, yielding one observation every 200 msec. Freezing was quantified (Maren, 1998) by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity = 10). We score an observation as freezing if it fell within a continuous group of at least five observations that were all less than the freezing threshold. Thus, freezing was only scored if the rat was immobile for at least 1 sec. Freezing was determined during each 1 min inter-trial interval after the CS offset during conditioning, extinction, probe, and the

retention test, and during the minutes preceding the first CS presentation during extinction training.

Two distinct contexts were used in this experiment. For the first context (context A), a 15 W houselight mounted opposite the speaker was turned on, and room lights remained on. The chambers were cleaned with a 1% acetic acid solution. To provide a distinct odor, stainless steel pans containing a thin layer of this solution were placed underneath the grid floors before the rats were placed inside. Ventilation fans in each chest supplied background noise (65 dB). Rats were transported to this context in white plastic boxes. For the second context (context B), all room and chamber houselights were turned off. A pair of 40 W red lights provided illumination. Additionally, the doors on the sound-attenuating cabinets were closed, the ventilation fans were turned off, and the chambers were cleaned with 1% ammonium hydroxide solution. Also, stainless steel pans containing a thin layer of the same solution were placed underneath the grid floors before the rats were placed inside to provide a distinct odor. Rats were transported to this context in black plastic boxes.

*Procedure*

Rats were submitted to four phases of training: fear conditioning, extinction, probe, and extinction retention test. For fear conditioning, rats received five tone-footshock trials (60 s inter-trial interval) beginning 3 min after being placed in the chambers (context A). Fifteen minutes later, rats received 45 tone-alone presentations for fear extinction (EXT, n = 8) in the other context (context B). For no-extinction controls (NO-EXT, n = 8), rats were placed in the chamber for the same amount of time but were not exposed to the tone CS. Another 15 minutes after immediate extinction, all animals were

returned to extinction context and presented with one single probe CS. Two days following conditioning, all rats were returned to the extinction context again and exposed to five CS-alone presentations.

*Data analysis*

All behavioral data are expressed as means and standard error of the means (SE) and analyzed by analysis of variance (ANOVA). *Post hoc* comparisons in the form of Fisher's PLSD tests were performed after a significant F ration.

Experiment 2: Does immediate extinction training produce a context-specific suppression of conditional fear?

*Subjects and behavior apparatus*

The subjects were 64 adult male Long-Evans rats (250-330 g) obtained and housed as described in Experiment 1. The conditioning chambers described in Experiment 1 comprised the behavior apparatus.

Three distinct contexts were used in this experiment. The first two were the same as described in Experiment 1. For the third context (Context C), the room lights were on and the houselights and fans were off. Rubber and black plastic sheets were placed above the rods. The chambers were cleaned with a 70% ethanol solution. To provide a distinct odor, stainless steel pans containing a thin layer of this solution were placed underneath the grid floors before the rats were placed inside. Rats were transported to this context in white plastic boxes with beddings on the floor.

*Procedure*

Rats were submitted to three phases of training: fear conditioning, extinction, and retrieval testing (ABC renewal testing). For fear conditioning, rats received five tone-footshock trials in context A. Fifteen minutes (IMMED) or 24 hours (DELAY) after conditioning, rats were presented with 45 tones either in context B or context C for fear extinction. Another fifteen minutes after extinction, all rats were presented with 5 tones in context B for renewal testing. The day before conditioning, the animals were exposed to the non-extinction context in order to familiarize them with each test context. The actual contexts for extinction and testing were counterbalanced across groups, yielding a total of four groups in a 2 x 2 (extinction time x test context) design. The four groups were IMMED/SAME, IMMED/DIFF, DELAY/SAME, and DELAY/DIFF, with 16 rats per group. The labels SAME and DIFF referred to whether the CS was tested in a same context as the extinction context (SAME) or in a different context from the extinction context (DIFF).

*Data analysis*

The average percentage of freezing during two-trial during early extinction (first twotrials), late extinction (last two trials), and early renewal (first two trials) were used for the data analyses, as described in Experiment 1. In order to test the renewal effect, rats failed to show extinction at late extinction trials, that is, the freezing level remained the same or even higher than early trials, were excluded for further analyses. It leads to the number of animals in each group as following: IMMED/SAME = 11, IMMED/DIFF = 13, DELAY/SAME =12, and DELAY/DIFF = 12.

Experiment 3: How much time must elapse after conditioning for extinction training to yield long-term fear suppression?

*Subjects and behavior apparatus*

The subjects were 64 adult male Long-Evans rats (250-330 g) obtained and housed as described in Experiment 1. The conditioning chambers described in Experiment 1 comprised the behavior apparatus.

*Procedure*

Rats were submitted to three phases of training: fear conditioning, extinction, and extinction retention test. For fear conditioning, rats received five tone-footshock trials in context A. Fifteen minutes (15min), one hour (1hr), six hours (6hr), or 24 hours (24hr) later, rats received 45 tone-alone presentations for fear extinction (EXT, n = 8 per condition) in the other context (context B). For no-extinction controls (NO-EXT, n = 8 per condition), rats were placed in the chamber for the same amount of time but were not exposed to the tone CS. Two days following conditioning, all rats were returned to the extinction context again and exposed to 45 CS-alone presentations.

*Data analysis*

Data analyses were performed as described in Experiment 1.

Experiment 4: Is the immediate extinction deficit due to longer extinction-retention test interval?

*Subjects and behavior apparatus*

The subjects were 32 adult male Long-Evans rats (250-330 g) obtained and housed as described in Experiment 1. The conditioning chambers described in Experiment 1 comprised the behavior apparatus.

*Procedure*

Rats were submitted to three phases of training: fear conditioning, extinction, and extinction retention test. For fear conditioning, rats received five tone-footshock trials in context A. Fifteen minutes (IMMED) or 24 hours (DELAY) later, rats received 45 tone-alone presentations for fear extinction (EXT, n = 8 per condition) in the other context (context B). For no-extinction controls (NO-EXT, n = 8 per condition), rats were placed in the chamber for the same amount of time but were not exposed to the tone CS. Two days following extinction, all rats were returned to the extinction context again and exposed to 45 CS-alone presentations.

*Data analysis*

Data analyses were performed as described in Experiment 1. Four animals were excluded from the final analyses because they failed to show evidence of successful conditioning (n = 3), or failed to show evidence of extinction (n = 1); these animals exhibited levels of freezing that were more than two standard deviations above or below their group means. Thus, the number of animals in each group as following: IMMED/EXT = 7, IMMED/No-EXT = 8, DELAY/EXT =7, and DELAY/No-EXT = 6.

## Results

Experiment 1: Does immediate extinction training produce short-term decrements in fear to an auditory CS?

Previous work from our laboratory has revealed similar decrements in freezing during extinction training in animals exposed to either the CS or the context alone (Maren and Chang, 2006). The present experiment aimed to assess whether decrements in fear in rats receiving CS extinction trials involved a loss of fear to the CS itself, as opposed to a loss of context fear.

Rats were submitted to a standard fear conditioning procedure in which an auditory CS was paired with a noxious footshock US in a novel chamber. After a 15-minute delay, rats were placed in another novel context where half of the animals received 45 extinction trials in which the CS was presented alone (EXT) while the other half of the animals remained in the chambers without the presentation of either the CS or US (NO-EXT); these rats served as the non-extinguished control group. Fifteen minutes after extinction, both groups were returned to the extinction context and a single CS was presented to assess freezing to the CS shortly after immediate extinction and to avoid possible extinction in the EXT group due to CS presentations. Forty-eight hours after extinction, rats were tested for their fear to the CS again by returning them to the extinction context and presenting five auditory CSs.

Freezing behavior during the conditioning session is shown in Figure 3.1A. Freezing levels were very low before the first conditioning trial, and then increased in frequency thereafter. There was an equivalent increase in freezing across trials in both groups [extinction × trials, $F(6,84) < 1$]. Freezing behavior during the extinction session is shown in Figure 3.1B. As reported in our previous study (Maren and Chang, 2006),

recently conditioned rats exhibited high levels of freezing before the first extinction trial [EXT = 64.4 ± 11.1, NO-EXT = 73.3 ± 8.9; $F(1,14) < 1$], probably due to shock-induced sensitization of fear. This was evident even though rats experienced a context shift in between conditioning and extinction. Freezing behavior in both groups showed an equivalent decline in freezing across the sessions [extinction × block, $F(9,126) = 1.05$].

Despite the similar reduction in freezing in the EXT and NO-EXT groups, rats that had received CS-alone extinction trials showed significantly greater short-term suppression of fear to the CS in the probe trial (Figure 3.1C). Before the CS onset, EXT and NO-EXT groups showed equivalent and low freezing levels [EXT = 19.1 ± 9.8, NO-EXT = 8.5 ± 3.4; $F(1,14) = 1.005$, $p = 0.33$]. Relative to NO-EXT rats, freezing levels were significantly lower in the EXT group compared to non-extinguished controls when presented with a single CS fifteen minutes after extinction. [$F(1,14) = 7.81$, $p < 0.02$]. However, fear in the EXT animals exhibited substantial spontaneous recovery when tested 24 hours after extinction; there was no significant difference between the baseline freezing before tone onset [EXT = 15.5 ± 8.2, NO-EXT = 13.9 ± 8.0; $F(1,14) <1$], or between the groups in the long-term retention test [$F(1,14) = 3.17$, $p = 0.09$; Figure 3.1D]. Thus, immediate extinction generated a significant, but short-lived, suppression of conditional fear.

Experiment 2: Does immediate extinction training produce a context-specific suppression of conditional fear?

A hallmark of extinction is its context specificity; extinguished fear returns or "renews" when the CS is presented outside the extinction context (Bouton, 2002; Ji and

Maren, 2007). In the previous experiment, we examined the efficacy of short-term extinction by giving a probe right after immediate extinction trials. Our results showed that there was a short-lived suppression of freezing to the CS shortly after extinction. In the present experiment, we further examine whether this short-lived suppression in freezing exhibits context specificity. Rats were conditioned to five tone-shock trials in a novel context. Fifteen minutes (IMMED) or 24 hours (DELAY) after conditioning, they received 45 extinction trials in another context. To access the context dependence of the short-term fear suppression, five tones were presented either inside (SAME) or outside (DIFF) the extinction context fifteen minutes after extinction training.

Behavior during the conditioning session was similar to that reported in Experiment 1 (data not shown). Freezing behavior in the IMMED and DELAY groups during early extinction, late extinction, and retrieval testing is shown in Figure 3.2. Freezing levels were high at the beginning of extinction (Early Ext) and decreased significantly at later trials (Late Ext) in both groups. However, when tested 15 minutes after extinction, freezing levels in IMMED group remained low regardless of test context (Figure 3.2A, Test), while freezing in the DELAY group was significantly higher outside than inside the extinction context (Figure 3.2B, Test). There was no significant interaction between test period and renewal context in the IMMED group [$F(2,44) < 1$], while the interaction reached significance in the DELAY group [$F(2,44) = 4.01$, $p < 0.03$]. Thus, early extinction yields a context-independent suppression of freezing when tested shortly after extinction.

Experiment 3: How much time must elapse after conditioning for extinction training to yield long-term fear suppression?

In the previous two experiments, we demonstrated that immediate extinction soon after conditioning generates a short-lived and context-independent suppression of fear. In the present experiment, we further examined the relationship of the timing of extinction relative to conditioning by giving extinction trials at different delays after conditioning. Rats were trained with five tone-footshock pairings in one context. They next received 45 extinction trials 15 min, 1 hr, 6 hr, or 24 hr later in a different context, and control groups at each time point received no extinction training. All rats were tested to 45 tone-alone presentation 48 hours after conditioning.

Behavior during the conditioning and extinction sessions was similar to that reported in Experiments 1 and 2 (data not shown). Freezing behavior during the entire retention session is shown in Figure 3.3. There was no significant difference in freezing levels for non-extinguished controls at different delays [$p = 0.06$], so they were collapsed into a single NO-EXT group ($n = 32$). During baseline, there was no significant difference in freezing for 15min, 1hr, 6hr, and 24hr [$p > 0.05$], while NO-EXT was significantly lower than 15min and 1hr groups [$ps < 0.05$]. During the first five tones, only animals receiving extinction trials 24 hours after conditioning demonstrated significant long-term extinction of fear memory: there was no difference in freezing levels of NO-EXT, 15min, 1hr, and 6hr groups, and the freezing level of the 24hr group was significantly lower than any of the other groups [$ps < 0.04$]. Interestingly, animals in the 15min, 1hr, and 6hr groups demonstrated savings of extinction training, that is, their freezing levels in these groups declined faster compared to the NO-EXT controls, while

the 24hr group remained low across whole session. There was a significant main effect of delay [$F(4,59) = 5.77$, $p = 0.0005$] and a significant interaction in delay and block [$F(36,531) = 4.57$, $p < 0.0001$]. Thus, administering extinction trial up to six hours after conditioning failed to extinguish long-term fear response, but all groups exhibited savings during subsequent extinction.

Experiment 4: Is the immediate extinction deficit due to a longer extinction-retention test interval?

In the previous experiment, we demonstrated that giving extinction trials up to six hours after conditioning failed to extinguish long-term fear response. However, animals receiving extinction trials up to six hours after conditioning were tested 48 hrs after extinction, while animals in the 24hr group were tested 24 hrs after extinction. It is possible that the 24hr group showed less freezing during the retention test because of the more limited opportunity for spontaneous recovery after extinction compared to the other groups. Thus, in this experiment, we equated the extinction-retention test interval for both immediate (15min) and delayed (24hr) groups: all animals were tested 48 hrs after extinction training.

Behavior during the conditioning and extinction sessions was similar to that reported in Experiments 1, 2, and 3 (data not shown). Figure 3.4 shows the average freezing during the first five trials of the retention test. There was a significant interaction between delay and extinction [$F(1,24) = 4.34$, $p < 0.05$]. Planned comparisons indicated that only rats in the delayed extinction condition exhibited significant extinction relative to their no-extinction controls [$p < 0.05$]. Thus, the greater

recovery of fear after an immediate extinction procedure in Experiment 3 was not due to the longer extinction-test interval used in that experiment. This confirms our previous report that immediate extinction produces only a short-term fear suppression that rapidly recovers within 24 hours after extinction (Maren and Chang, 2006).

## Discussion

In the present study, we examined the properties of short-term fear suppression after immediate extinction and the time course between conditioning and the delivery of CS-alone trials required for long-term fear suppression. Our major finding is that delivering CS-alone trials soon after fear conditioning generates a short-lived and context-independent suppression of fear. Long-term extinction was minimal when CS-alone trials were administered shortly after conditioning (15 min), and this effect persisted up to six hours after fear conditioning. These results further support the view that early intervention shortly after a traumatic experience may not be effective in producing long-term fear suppression.

A critical question that emerges from these experiments is what psychological process underlies the temporary and context-independent suppression of conditional freezing to an auditory CS when extinction trials are administered soon after conditioning. One possibility is that short-term habituation, rather than extinction, accounts for the response loss we have observed during the presentation of CS-alone trials shortly after fear conditioning. Consistent with this possibility, short-term habituation often exhibits spontaneous recovery over 24-hour retention intervals

(Thompson and Spencer, 1966; McSweeney and Swindell, 2002). Unlike long-term extinction, short-term habituation is typically not context-dependent (Bouton, 2004). That is, extinction is associated with a loss of responding that is specific to the extinction context, while habituation is associated with a loss of responding in any test context. In the present experiments, we observed that exposure to CS-alone trials shortly after fear conditioning resulted in a context-independent loss of fear, while delayed extinction induced a long-lived and context-dependent suppression in fear response. This suggests that different processes mediate fear under these conditions: the former mediated by habituation and the latter by extinction.

According to this view, extinction learning itself may have been impaired when extinction training was conducted soon after conditioning. According to Wagner's SOP model (Wagner, 1981), for example, a CS presented during extinction training will excite conditional responding when it activates a representation of the US in the A2 state. Subsequent presentations of the CS will promote inhibitory (extinction) learning when CS activity in A1 coincides with US activity in A2. As we have seen, however, conditional fear is in the IMMED condition is substantial prior to the delivery of the first CS-alone extinction trial. Hence, it is possible that elements of both the CS and US are primed in A2 (perhaps by contextual stimuli common to the conditioning context or the recent delivery of CSs and USs during conditioning). This arrangement would retard inhibitory (or excitatory) learning between the CS and US. Short-term reductions in conditional responding as the CS might then result from additional self-generated priming of the CS to the A2 state. After a delay, we would posit that fewer CS and US

elements are represented in the A2 state, and conditions therefore favor coincident activation of the CS to A1 and US to A2 that promotes extinction.

Interestingly, we found that the fear suppression generated by delayed CS-alone trials was context-specific within 15 minutes of the end of the extinction session. To our knowledge, this is the first report of fear renewal within minutes of the end of extinction training. Indeed, most experiments have examined renewal effects at least 24 hours after the end of extinction (Bouton et al., 2006). Insofar as time (both intertrial interval effects, and the passage of time between extinction and retention) has been posited to regulate memory retrieval after extinction (Morris et al., 2005; Bouton and Garcia-Gutierrez, 2006), these results indicate that a shift in environmental context that occurs within the temporal context of extinction session is sufficient to yield robust renewal of fear. Indeed, both short- and long-term extinction memories exhibit context-dependence as long as extinction commences at least 24 hours after conditioning.

The neurobiological basis for the long-term extinction of fear has received considerable attention in recent years (Bouton et al., 2006; Quirk et al., 2006; Ji and Maren, 2007; Quirk and Mueller, 2008). Interactions among the hippocampus, the medial prefrontal cortex (mPFC), and the amygdala are thought to play a role in limiting the expression of fear responses. For example, there is growing evidence that the mPFC is positioned to reduce amygdala output by exciting inhibitory interneurons in the intercalated nuclei (Quirk et al., 2000; Milad and Quirk, 2002; Pare et al., 2004; Santini et al., 2004). Moreover, the hippocampus may modulate this circuitry to gate when and where extinction is expressed (Thierry et al., 2000; Hobin et al., 2003; Sotres-Bayon et al., 2004), a feature that is critical for the contextual regulation of fear (Hobin et al.,

2003; Maren, 2005). With regard to the present experiments, it is not clear whether this circuit is also engaged by the delivery of CS-alone trials shortly after fear conditioning. One possibility is that neuronal activity in the LA is suppressed during the extinction session without actually engaging the inhibitory circuits stated above. Indeed, amygdala activity in response to novel stimuli, including acoustic tones, is known to rapidly habituate (LaBar et al., 1998; Herry et al., 2007), and this habituated neuronal response might reduce the behavioral expression of fear under some conditions (Kamprath and Wotjak, 2004). Interestingly, the cellular mechanisms within the amygdala for early and late extinction may be different (Herry et al., 2006).

Another issue we explored in the present experiments is the amount of time that must elapse between conditioning and the delivery of CS-alone trials to promote long-term extinction. Our results revealed that even up to 6 hours after conditioning the delivery of extinction trials produced a minimal long-term suppression of conditional freezing the following day. Although we were not able to obtain reliable extinction when CS-alone trials were delivered within 6 hours of conditioning, it should be noted that in both rats (Quirk et al., 2000; Milad and Quirk, 2002; Myers et al., 2006) and humans (Phelps et al., 2004; Kalisch et al., 2006; Milad et al., 2007) long-term extinction has been reported within an hour of fear conditioning. However, in these studies, relatively weak unconditioned stimuli were used, and the level of fear aroused during conditioning may be critical in determining the sensitivity of the fear memory to early extinction. Indeed, we found that reducing the number of conditioning trials enabled extinction shortly after conditioning (Maren and Chang, 2006).

Despite showing deficits in long-term extinction, all of the groups that underwent extinction in the third experiment exhibited faster response loss upon subsequent extinction training relative to rats extinguished for the first time. This indicates that there was savings for the original extinction training, even though it was not evident in the degree of fear suppression at the outset of the retention test. This pattern of savings is also observed during the expression of long-term habituation, which is typically manifest as a more rapid rate of habituation relative to naïve controls (Carew et al., 1972). Hence, more rapid fear loss during the second exposure to CS-alone trials is consistent with the possibility that a habituation, rather than an extinction, process mediates response loss after early extinction (Thompson and Spencer, 1966). An alternative explanation of the savings we observed is that the extinction learning under immediate extinction was impaired but not totally abolished, which led to a faster fear loss during retention test.

In our previous work, we argued that arousal as a result of returning to the context of recent trauma (shock in this case) or associative fear of the context may have led to the failure of long-term fear suppression in the immediate extinction rats (Maren and Chang, 2006). In these experiments, all of the behavioral sessions were conducted in the same context (i.e., AAA, where each letter denotes conditioning, extinction, and retention test, respectively), and contextual fear may have contributed to the failure to extinguish recent fear. However, in the present experiments, we extinguished the animals outside the conditioning context (ABB design) in an attempt to minimize the influence of context fear on extinction. Even under these conditions recent shock sensitized fear in the novel context, resulting in high baseline freezing levels before the onset of the extinction trials. Although we cannot entirely rule out the contribution of context fear to early extinction

impairments, it seems more likely that they are due to the recent sensitization of fear, rather than associative fear to the context per se.

Early psychological interventions after a traumatic event are not always effective (Bisson et al., 1997; Rothbaum and Davis, 2003; Gray and Litz, 2005). The present work indicates that CS-alone exposure soon after conditioning yields a short-term habituation rather than a long-term extinction of the fear response. These results suggest that delayed interventions may be more effective in reducing pathological fear. Understanding the factors that contribute to the long-term suppression in fear memory is the next important step in developing optimal clinical interventions for psychological trauma in humans.

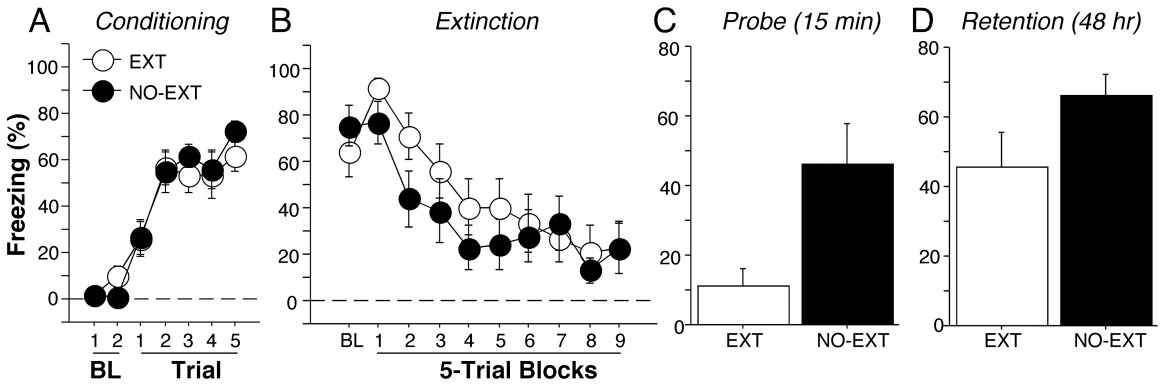


**Figure 3.1. Probe CS after immediate extinction.**
A. Percentage of freezing behavior during conditioning. Data are 1-min averages for the periods before (baseline, BL) and after each of five tone-shock conditioning trials. B. Percentage of freezing behavior during the extinction session, which occurred 15 min after conditioning. Control rats did not receive CS presentations during extinction (NO-EXT). C. Percentage of freezing behavior during the probe CS 15 min after extinction. Baseline freezing data were averaged and subtracted from the freezing level during probe CS to yield normalized freezing. D. Normalized average percentage of freezing across test trials during retention test 48 hours after conditioning. All data are means ± SEM.

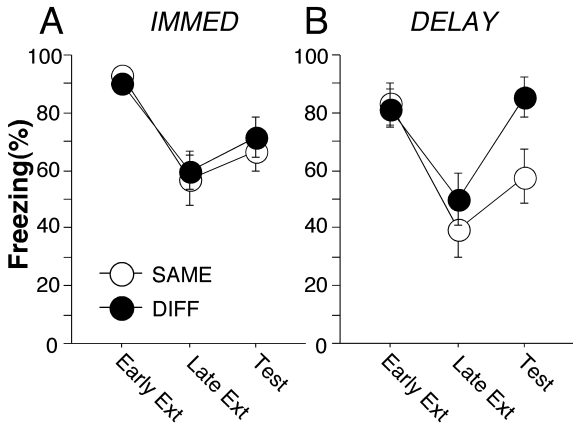


**Figure 3.2. Renewal of fear response immediately after immediate or delayed extinction.**
Shown are averaged two-trial block percentage of freezing levels during early extinction, late extinction, and early renewal in immediate (A) and delayed (B) extinction conditions. All data are means ± SEM.

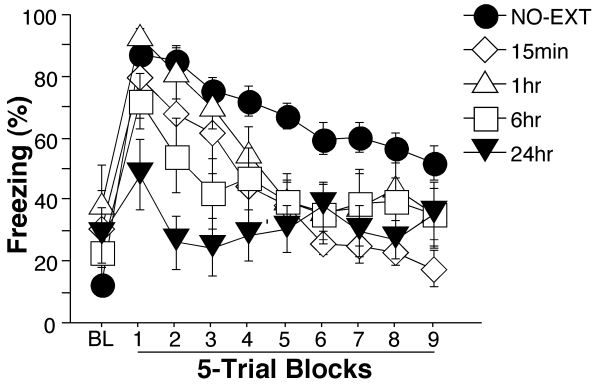


**Figure 3.3. Percentage of freezing levels during retention test after different delay between conditioning and extinction sessions.**
All data are means ± SEM.

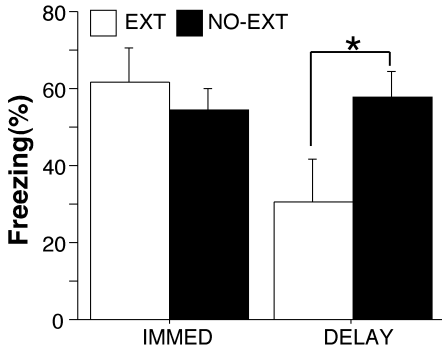


**Figure 3.4. Percentage of freezing levels during retention test with equated extinction-retention test intervals.**
All data are mean ± SEM.

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# CHAPTER IV

## CONTEXTUAL FEAR REGULATES THE EFFECT OF INFRALIMBIC CORTEX LESIONS ON FEAR EXTINCTION IN RATS

Extinction is a form of new learning in which presentation of a conditioned stimulus (CS) in the absence of the US reduces conditional responding to that CS (Pavlov, 1927; Bouton, 2002, 2004; Bouton et al., 2006). Considerable interest has emerged in the neurobiological mechanisms of extinction, insofar as impairments in extinction may contribute to a variety of anxiety disorders, including post-traumatic stress disorder (PTSD) (Bouton et al., 2001; Rothbaum and Davis, 2003). One brain structure that has been implicated in the extinction of learned fear is the medial prefrontal cortex (mPFC), specifically the infralimbic cortex (IL) (Quirk et al., 2006; Quirk and Mueller, 2008). Robust projections from IL to inhibitory interneurons located in the intercalated nuclei (ITC) of the amygdala (McDonald et al., 1996) make it perfectly positioned for regulating amygdala output after extinction learning (Likhtik et al., 2008).

In support of this possibility, several studies indicate that IL manipulations influence the extinction of fear. For example, intra-IL infusions of protein synthesis inhibitors (Santini et al., 2004), NMDA receptor antagonists (Burgos-Robles et al., 2007; Laurent and Westbrook, 2008), or sodium channel blockers (Sierra-Mercado et al., 2006),

prior to extinction leads to poor retrieval of extinction memory the following day without affecting acquisition of extinction *per se*. Intra-IL infusions of NMDA receptor antagonists (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2009) or MAPK inhibitors (Hugues et al., 2004; Hugues et al., 2006) immediately after extinction also leads to impaired extinction retrieval, suggesting that consolidation of extinction memory involves post-training molecular cascades in IL. Physiological correlates of extinction have been observed in IL (Herry and Garcia, 2002; Milad and Quirk, 2002; Burgos-Robles et al., 2007; Hugues and Garcia, 2007) and electrical stimulation of IL enhances extinction (Milad and Quirk, 2002; Milad et al., 2004). Moreover, inhibitory interneurons in the amygdala that receive input from the IL are involved in the expression of extinction (Likhtik et al., 2008).

Despite mounting evidence for a role for IL in fear extinction, studies examining the effect of IL lesions on extinction have yielded inconsistent results. Although several studies have found impaired retention of extinction with IL lesions (Morgan et al., 1993; Quirk et al., 2000; Lebron et al., 2004), other studies have not (Gewirtz et al., 1997; Farinelli et al., 2006; Garcia et al., 2006). One factor that could account for this discrepancy is the size of the mPFC lesions. Studies employing focal IL lesions have typically found impairments in extinction retrieval (Quirk et al., 2000; Lebron et al., 2004), while larger lesions in mPFC including IL and prelimbic cortex (PrL) tend not to affect extinction (Gewirtz et al., 1997; Morgan et al., 2003; Farinelli et al., 2006; Garcia et al., 2006). Because IL and PrL have opposite influences on the expression of learned fear (Vidal-Gonzalez et al., 2006; Corcoran and Quirk, 2007), larger lesions including IL and PrL may produce different results than IL lesions alone. In addition, the majority of

the studies that found effects of IL lesions on extinction used albino rats as subjects (Morgan et al., 1993; Quirk et al., 2000; Morgan et al., 2003; Lebron et al., 2004; Farinelli et al., 2006).

A recent study from our laboratory failed to find effects of IL lesions on the extinction of conditioned freezing to an auditory CS (Garcia et al., 2006). This failure to observe an influence of IL lesions on extinction may have been due to the large size of the lesions (which included PrL) or the use of hooded (i.e., Long-Evans) rats. To address this possibility, the present experiments compared the effects of focal IL lesions on the extinction of conditioned freezing to an auditory CS in Sprague-Dawley (SD) and Long-Evans (LE) rats.

## Materials and Methods

Experiment 1: Do strain differences influence the effects of IL lesions on fear extinction?

*Subjects*

Two strains of rats were used in this experiment: 48 male Long-Evans (LE) rats (250-330 g; Blue Spruce) from a commercial supplier (Harlan Sprague Dawley, USA) and 48 male Sprague-Dawley (SD) rats (250-330 g) from another commercial supplier (Hilltop, USA). They were housed in individual cages with 14-h light/10-h dark cycle (lights on at 7:00 am), and allowed food and water ad libitum. During the first five days, they were handled for 10 sec to habituate them to the experimenter. All experiments were carried out in accordance with guidelines approved by the University of Michigan University Committee on Use and Care of Animals.

*Surgery*

Rats received pre-conditioning bilateral infralimbic cortex lesions (IL; AP: +2.8 mm; ML: ±0.5 mm; DV: -5.2 mm relative to bregma) or sham surgeries for control groups (SH-E and SH-NE; extinction and no-extinction, respectively). In both cases, rats were anesthetized with sodium pentobarbital (Nembutal, 65 mg/kg, ip), treated with atropine (0.04 mg/kg, i.p.) and placed in a stereotaxic frame for electrolytic lesions with stainless-steel electrodes insulated with epoxylite except for 0.3 mm at the tip. Lesions were made with anodal, constant direct current (0.3 mA, 5 sec), and the incision was closed with stainless-steel wound clips. The rats were allowed to recover for 7 days.

*Behavioral apparatus*

Eight identical observation chambers (30 x 24 x 21 cm; MED-Associates) were used in all experiments. The chambers were constructed of aluminum (side walls) and Plexiglas (rear wall, ceiling, and hinged front door) and were situated in sound-attenuating cabinets located in a brightly lit and isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center-to-center). Rods were wired to a shock source and solid-state grid scrambler (MED-Associates) for the delivery of footshock US. A speaker mounted outside a grating in one wall of the chamber was used for the delivery of acoustic CS.

Each conditioning chamber rested on a load-cell platform that was used to record chamber displacement in response to each rat's motor activity and acquired on-line using Threshold-Activity software (MED-Associates). The output of each chamber's load cell was set to a gain that was optimized for detecting freezing behavior (somatomotor immobility, except that necessitated by breathing). Load-cell amplifier output (-10 to

+10 V) from each chamber was digitized. Absolute values of the load-cell voltages were then computed and multiplied by 10 to yield a scale that ranged from 0 to 100. For each chamber, load-cell voltages were digitized to 5 Hz, yielding one observation every 200 msec. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity = 10). We score an observation as freezing if it fell within a continuous group of at least five observations that were all less than the freezing threshold. Thus, freezing was only scored if the rat was immobile for at least 1 sec (Maren, 1998).

Two distinct contexts were used in Experiment 1 and 2. For the first context (context A), a 15 W houselight mounted opposite the speaker was turned on, and room lights remained on. The chambers were cleaned with a 1% acetic acid solution. To provide a distinct odor, stainless steel pans containing a thin layer of this solution were placed underneath the grid floors before the rats were placed inside. Ventilation fans in each chest supplied background noise (65 dB). Rats were transported to this context in white plastic boxes. For the second context (context B), all room and chamber houselights were turned off. A pair of 40 W red lights provided illumination. Additionally, the doors on the sound-attenuating cabinets were closed, the ventilation fans were turned off, and the chambers were cleaned with 1% ammonium hydroxide solution. Also, stainless steel pans containing a thin layer of the same solution were placed underneath the grid floors before the rats were placed inside to provide a distinct odor. Rats were transported to this context in black plastic boxes.

*Procedure*

Rats were submitted to three phases of training: fear conditioning, extinction, and extinction retention test. In each phase, trials began 3 min after being placed in the chambers. All phases were conducted in context A. There were 16 animals in each group for each strain (IL, SH-E, and SH-NE; LE and SD).

On Day 1, rats received five conditioning trials consisted of tones (30 sec, 80 dB, 4k Hz) that coterminated with footshocks (1 mA, 0.5 sec) (variable ITI ranging from 90-150 sec, with an average = 120 sec). On Day 2, rats received 20 tone-alone presentations for fear extinction (IL and SH-E). For no-extinction controls (SH-NE), rats were placed in the chamber for the same amount of time but were not exposed to the tone CS. On Day 3, all rats were exposed to another 20 CS-alone presentations for retention test.

Freezing was determined during each 30 sec tone period during conditioning, extinction, and retention test. Baseline freezing to context was assessed during the minutes preceding the first CS presentation.

*Histology*

Histological verification of lesion location was performed after behavioral testing. Rats were perfused across the heart with 0.9% saline followed by a 10% formalin solution. After extraction from the skull, brains were post-fixed in 10% formalin solution for two days, at which time the solution was replaced with a 10% formalin and 30% sucrose solution until sectioning. Sections (45 μm thick) were cut on a cryostat (-20°C), and wet mounted on glass microscope slides with 70% ethanol. After drying, sections stained with 0.25% thionin for visualization of lesions.

*Data analysis*

All behavioral data are expressed as means and standard error of the means (SE) and analyzed by analysis of variance (ANOVA). *Post hoc* comparisons in the form of Fisher's PLSD tests were performed after a significant F ration.

Experiment 2: Do LE rats with IL lesions show impaired retrieval of extinction memory with a context shift after conditioning?

*Subjects*

The subjects were 58 adult male Long-Evans rats (250-330 g) obtained and housed as described in Experiment 1.

*Surgery and behavioral apparatus*

Surgery and behavioral apparatus were identical to those described in Experiment 1.

*Procedure*

All procedures were identical to those described in Experiment 1, except that after conditioning (context A), rats were extinguished and tested in another context (context B). There were 20 rats in IL, and 19 rats each in SH-E and SH-NE controls.

*Histology and data analysis*

Histology and data analyses were performed as described in Experiment 1.

## Results

Experiment 1: Do strain differences influence the effects of IL lesions on fear extinction?

In this experiment, we examined the influence of focal electrolytic IL lesions on the extinction of conditioned freezing to an auditory CS in SD and LE rats. We used a conditioning and extinction procedure that has previously been shown to be sensitive to IL lesions in SD rats (Quirk et al., 2000; Lebron et al., 2004). As in previous studies, lesions of the IL were made prior to fear behavioral training.

*Histology*

A representative IL lesion is depicted in Figure 4.1. Only rats with focal bilateral IL lesions were included in the final data analyses. Animals were included if their lesion produced substantial IL damage in at least two of three coronal sections (+3.2 mm, +2.8 mm, and +2.15 mm relative to bregma). For the LE strain, three rats in the IL group were excluded with one combined into SH-E group for no lesion at all. This yielded the following group sizes: IL (n = 13), SH-E (n = 17), and SH-NE (n = 16). For the SD strain, two rats in the IL group were excluded and three rats died during surgery. This yielded the following group sizes: IL (n = 14), SH-E (n = 14), and SH-NE (n = 15).

*Behavior*

Freezing behavior during the tone CS across all behavioral phases is shown in Figure 4.2, with SD and LE strains in the upper and lower panels, respectively. Freezing behavior was low before the first conditioning trial (Figure 4.2A1 and 4.2B1), and then increased in magnitude thereafter. There was a significant main effect of strain [$F(1,83) = 15.1$, $p = 0.0002$], a significant main effect of trial block [$F(2,166) = 623.9$, $p < 0.0001$], and a significant interaction between strain and trial block [$F(2,166) = 17.5$, $p < 0.0001$]. Planned comparison revealed that between strains, there was a significant

difference in freezing behavior only on the last trial block [$F(1,87) = 36.3$, $p < 0.0001$], suggesting that at the end of conditioning, SD rats spent more time freezing than LE rats.

Despite the fact that SD rats acquired higher levels of freezing at the end of conditioning, LE rats showed significantly higher freezing to the conditioning context before the first CS trial during extinction (Figure 4.2A2 and 4.2B2; BL periods). There was a significant main effect of strain [$F(1,83) = 18.8$, $p < 0.0001$]. Moreover, the effects of IL lesions across different trial blocks differed in the two strains (Figure 2A2 and 2B2; tone CS periods). There was a significant main effect of strain [$F(1, 83) = 8.7$, $p = 0.0042$], a significant main effect of group [$F(2,83) = 23.6$, $p < 0.0001$], a significant two-way interaction between group and trial blocks [$F(18, 747) = 5.0$, $p < 0.0001$], and a significant three-way interaction among strain, group, and trial blocks [$F(18, 747) = 3.3$, $p < 0.0001$]. *Post hoc* analyses revealed that LE rats showed higher freezing than SD rats [$p < 0.05$]. Moreover, rats with IL lesions showed the highest level of freezing and SH-NE the lowest; SH-E rats exhibits intermediate level of freezing [all $ps < 0.05$]. Planned comparison revealed that at the end of extinction, all groups in both strains showed equivalent and low freezing levels [$F(5,83) = 1.5$, $p = 0.2$], demonstrating good within-session extinction in IL and SH-E animals in both strains.

Freezing behavior during the first 12 CSs of the test session is shown in Figure 4.2A3 and 4.2B3. Similar to the extinction session, LE rats showed significantly higher freezing to the context than SD rats before the first test trial. There was a significant main effect of strain [$F(1,83) = 10.2$, $p = 0.002$] (Figure 4.2A3 and 4.2B3; BL periods). Also similar to the extinction session, the effects of IL lesions across different trial blocks differed in the two strains (Figure 4.2A3 and 4.2B3; tone CS periods). There was a

significant main effect of strain [$F(1, 83) = 7.0$, $p = 0.01$], a significant main effect of group [$F(2,83) = 46.0$, $p < 0.0001$], a significant two-way interaction between group and trial blocks [$F(22, 913) = 5.5$, $p < 0.0001$], and a significant three-way interaction among strain, group, and trial blocks [$F(22, 913) = 2.8$, $p < 0.0001$]. Planned comparison revealed that during the first tone CS trial, there was a significant difference in freezing behavior across all groups [$F(5,83) = 5.5$, $p = 0.0002$]. There was a strain difference in spontaneous recovery with control LE rats showing more spontaneous recovery than SD rats [$p < 0.05$], suggesting that LE rats are more resistant to extinction than SD rats. Moreover, the effects of IL lesions also differed between the two strains during the first tone CS trial. For SD rats, IL and SH-NE rats showed equivalent freezing levels [$p = 0.15$] that were significantly higher than SH-E animals [both ps $< 0.05$], suggesting failed retrieval of extinction memory in IL rats during early test trials. For LE rats, there was no significant difference in freezing levels among all groups [all ps $> 0.05$].

The effect of IL lesions on the recall of extinction in SD rats was transient. Planned comparison revealed that during the second tone CS trial, there was a significant difference in freezing behavior across all groups [$F(5,83) = 11.5$, $p < 0.0001$]. However, for both the SD and LE rats, IL and SH-E animals showed equivalent and significantly lower freezing than their SH-NE controls, respectively [all ps $< 0.05$]. For SD rats, there were no longer differences in freezing levels among the groups by the $11^{th}$ trial [all ps $> 0.05$], while for LE rats, IL and SH-E animals showed equivalent and significantly lower freezing than SH-NE [both ps $< 0.05$] until the last trial in test session. Thus, IL lesions only impaired the retrieval of extinction memory in SD rats, and this impairment was most pronounced in the earliest trials of the extinction session.

Experiment 2: Do LE rats with IL lesions show impaired retrieval of extinction memory with a context shift after conditioning?

In Experiment 1, we show that focal IL lesions impair extinction retrieval in SD, but not LE rats. Interestingly, LE rats exhibited much higher levels of contextual fear prior to the onset of extinction training, and this may have impaired both extinction learning and IL function (Correll et al., 2005; Izquierdo et al., 2006; Maren and Chang, 2006; Akirav and Maroun, 2007). We therefore hypothesized that the effect of IL lesions on extinction recall may be influenced by the degree of contextual fear at the outset of extinction training. If so, reducing contextual fear in LE rats prior to extinction might facilitate extinction in control rats and unmask an effect of IL lesions. To test this hypothesis, we repeated the same procedures in Experiment 1 in LE rats, but conducted extinction in a different context from conditioning in an effort to reduce contextual fear before extinction.

*Histology*

The criteria are the same as described in Experiment 1. On the basis of the histological results, 5 of 20 IL rats were excluded. This yielded the following group sizes: IL (n = 15), SH-E (n = 19), and SH-NE (n = 19).

*Behavior*

Freezing behavior during the conditioning session is shown in Figure 4.3. Freezing behavior was low before the first conditioning trial (Figure 4.3A), and then increased in magnitude thereafter. There was an equivalent increase in freezing across trials in all groups [group × trial, $F(4,100) < 1$].

Freezing behavior during the extinction session is shown in Figure 4.3B. Baseline freezing levels before tone onset were equivalent among groups [$F(2,50) < 1$]. The context shift between conditioning and extinction significantly lowered freezing levels before CS onset in LE rats relative to Experiment 1. We compared the pre-extinction freezing levels in Experiment 1 and 2 and found a significant main effect of group [$F(2,133) = 10.4$, $p < 0.0001$] in the ANOVA. An additional analysis revealed that contextual freezing in LE rats in Experiment 2 (with a context shift) was similar to that in SD rats in Experiment 1 [$p = 0.63$] and was significantly lower than that in LE rats [both $ps < 0.05$].

During the extinction session in Experiment 2, there was a significant main effect of group [$F(2,50) = 15.4$, $p < 0.0001$], a significant main effect of trial block [$F(10,500) = 18.3$, $p < 0.0001$], and a significant interaction between group and trial block [$F(20,500) = 3.2$, $p < 0.0001$]. Freezing behavior was equivalent between rats in the IL and SH-E groups [$p = 0.15$], which were both significantly higher than SH-NE rats [both $ps < 0.05$]. At the end of extinction, IL and SH-E rats showed equivalent [$p = 0.33$] but significantly higher freezing levels than SH-NE animals [both $ps < 0.05$].

Freezing behavior during the first 12 tones during the retention test is shown in Figure 4.3C. During the pre-CS period, there was a significant main effect of group [$F(2,50) = 6.8$, $p = 0.0023$]. Rats with IL lesions showed significantly higher freezing levels than rats in the SH-E and SH-NE groups [both $ps < 0.05$], which did not differ from one another [$p = 0.19$]. This suggests that IL lesions may have caused spontaneous recovery of context extinction, or alternatively elevated second-order conditioning to the extinction context. After CS onset, there was a significant main effect of group [$F(2,50)$

= 5.5, p = 0.007], a significant main effect of trial [F(12,600) = 20.9, p < 0.0001], and a significant interaction between group and trial [F(24,600) = 6.0, p < 0.0001]. Freezing levels were significantly higher in SH-NE rats compared to SH-E rats [p < 0.05]; rats with IL lesions exhibited intermediate levels of freezing that did not differ from either group [both ps > 0.05]. However, a *post hoc* analysis revealed that on the second test trial SH-NE and IL rats had equivalent freezing levels that were significantly higher than SH-E rats [both ps < 0.05]. Thus, by shifting context between conditioning and extinction, contextual fear before extinction was diminished and extinction in the controls was facilitated. Under these conditions, LE rats with IL lesions exhibited a transient, but reliable, impairment in the retrieval of extinction early in the retention test.

## Discussion

In the present study, we examined the effects of focal IL lesions on the retrieval of extinction memory in SD and LE rats. Our results reveal that IL lesions impair the retrieval of extinction memory in SD, but not LE rats, when conditioning and extinction occur in the same context. When LE rats were conditioned and extinguished in different contexts, IL lesions yielded a modest impairment in the retrieval of extinction memory. Higher levels of pre-extinction fear and weaker long-term extinction in LE rats, both of which were mitigated by a shift in context, appeared to be responsible for the failure to detect an IL lesion effect in the first case. We suggest that high levels of fear at the outset of extinction training may interfere with IL function and yield a transient and IL-

independent fear suppression, similar to that observed when extinction training is conducted soon after fear conditioning (Maren and Chang, 2006).

An interesting outcome of the present study was that identical conditioning procedures produced different levels of contextual fear in LE and SD rats. Compared to SD rats, LE rats exhibited significantly greater levels of contextual fear at the outset of the extinction session, and this appeared to produce a less durable extinction. An interaction between contextual fear and extinction has also been observed when extinction training occurs soon after fear conditioning or when reminder shocks elevate fear prior to a delayed extinction session (Maren and Chang, 2006). When contextual fear at the outset of extinction training was equated in LE and SD rats, IL lesions produced similar deficits in extinction in both strains. This suggests that strain *per se*, is not likely a factor in observing deficits in extinction after IL lesions. Hence, failures to observe extinction deficits in previous studies with albino rats and weak conditioning protocols are probably due to the size of large mPFC lesions including both IL and PrL (Gewirtz et al., 1997; Morgan et al., 2003; Farinelli et al., 2006; Garcia et al., 2006).

As we have suggested, the failure to detect an effect of IL lesions in LE rats in Experiment 1 may have been related to the high degree of spontaneous recovery of fear in the control rats. The impairment in long-term extinction by elevated fear at the outset of extinction training may be related to impaired prefrontal cortical function. A considerable body of work indicates that the IL is involved in extinction learning (Pare et al., 2004; Maren, 2005; Bouton et al., 2006; Quirk and Mueller, 2008). There is also considerable evidence that acute or chronic stressors disrupt prefrontal cortical function. For example, an acute stressor causes dendritic retraction in IL and impairs fear

extinction (Izquierdo et al., 2006). Stress-related impairments in PFC function may influence extinction by influencing PFC-amygdala interactions (Correll et al., 2005). In the present study, extinction in LE rats and its sensitivity to IL lesions were related to the level of fear present at the beginning of the extinction session. We propose that high levels of fear compromise IL function, and additionally, that any extinction obtained under these conditions is independent of the IL. One structure that might compensate for the PFC is the hippocampus, which has direct projections to the amygdala (Maren and Fanselow, 1995) and is involved in the acquisition of extinction under some conditions (Corcoran et al., 2005). Of course, stress impairs hippocampal function as well (McEwen and Sapolsky, 1995; Kim and Yoon, 1998; Akirav and Richter-Levin, 1999), so further work is required to understand the how stress influences extinction circuits beyond the hippocampus and PFC.

In conclusion, we have demonstrated that contextual fear before extinction training regulates the effects of IL lesions on long-term extinction retrieval. As contextual fear is influenced by strain, the salience of contextual stimuli, and the CS-US contingency, it is likely to be an important factor that contributes to the efficacy of extinction and the engagement of the IL in this process. This may be especially critical for clinical studies as the results suggest the stress level aroused during exposure therapy may interfere the efficacy of the long-term extinction in patients.

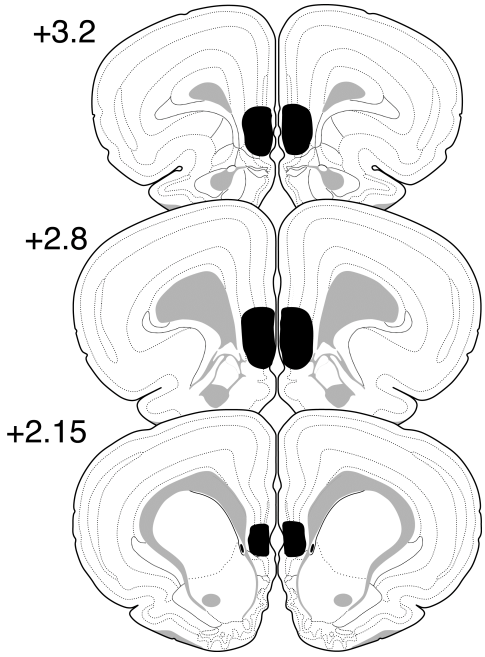


**Figure 4.1. Schematic illustration of a representative bilateral IL lesion.** This figure was adapted from Swanson (2004).

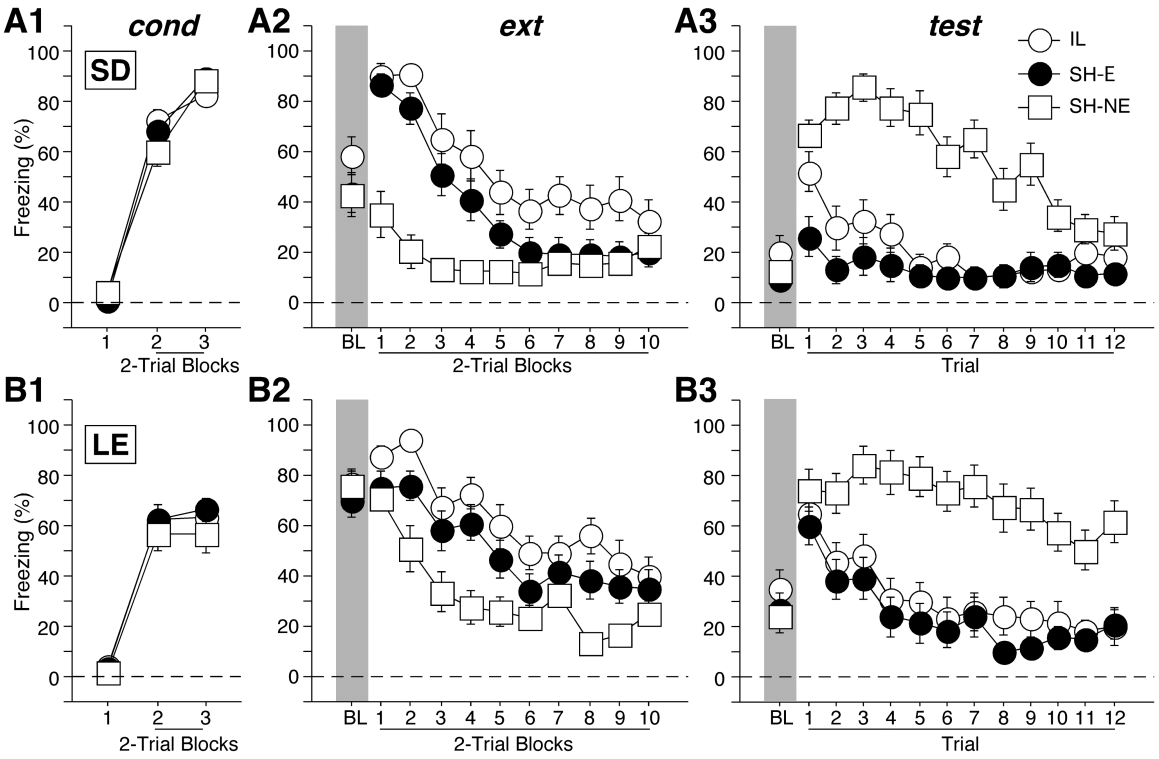


**Figure 4.2. Conditional freezing during conditioning, extinction, and retention testing for Sprague-Dawley (A) and Long-Evans (B) rats.**
SH-NE rats did not receive CS presentations during extinction. All data are percentage of freezing behavior during tone periods and baseline. All data are means ± SEM.

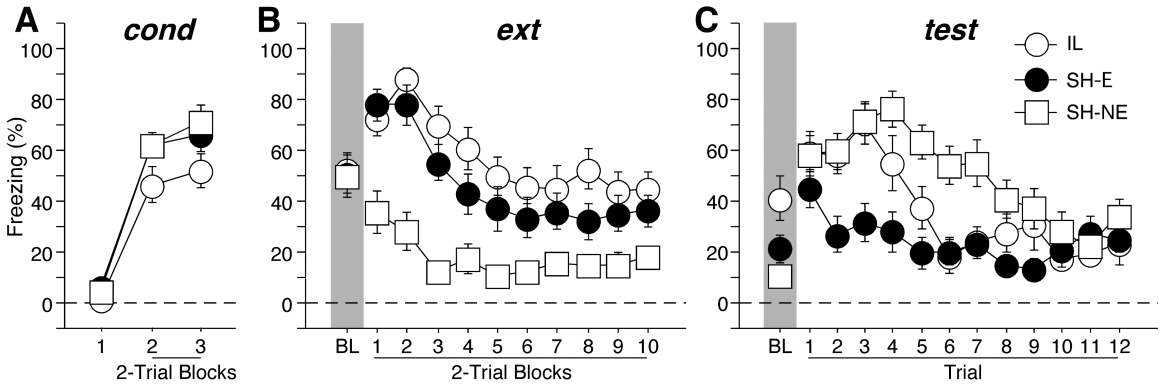


**Figure 4.3. Conditional freezing during conditioning, extinction, and retention testing.**
All labels the same as in Figure 4.2. All data are means ± SEM.

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# CHAPTER V

## PREFRONTAL CORTICAL RESCUE OF FEAR EXTINCTION

Failure to extinguish fear memory is a core feature of several anxiety disorders, including panic disorder, specific phobia, and post-traumatic stress disorder (Rasmusson and Charney, 1997; Rosen and Schulkin, 1998; Myers and Davis, 2002; Wessa and Flor, 2007; Muigg et al., 2008). In rats, extinction has been studied extensively using Pavlovian fear conditioning procedures (LeDoux, 2000; Pare et al., 2004; Maren, 2005). That is, after a conditioned stimulus (CS) has been paired with an aversive unconditioned stimulus (US), repeated presentations of the CS alone leads to a loss of conditioned fear. This loss of fear is labile, recovering with the passage of time and with changes in context (Pavlov, 1927; Bouton et al., 2006; Myers and Davis, 2007). Hence, extinction procedures do not erase fear memory, but yield a new safety memory that inhibits fear under certain conditions.

In recent years, considerable progress has been made in understanding the neural circuitry underlying fear extinction (LeDoux, 2000; Maren and Quirk, 2004; Pare et al., 2004). Indeed, the acquisition and expression of extinction memories involves a distributed neural circuit that includes the amygdala, medial prefrontal cortex (mPFC), and hippocampus. Specifically, the basolateral complex of the amygdala is required for

the acquisition of extinction memory (Falls et al., 1992; Lu et al., 2001; Lin et al., 2003; Quirk and Mueller, 2008), the mPFC is involved in the consolidation and expression of long-term extinction memory (Quirk et al., 2000; Milad and Quirk, 2002; Santini et al., 2004; Burgos-Robles et al., 2007; Quirk and Mueller, 2008) and the hippocampus regulates the context-dependent retrieval of extinction memories (Corcoran and Maren, 2001, 2004; Bouton et al., 2006; Hobin et al., 2006).

Although considerable progress has been made in understanding the behavioral and neurobiological mechanisms underlying extinction in experimental models (Bouton et al., 2006; Garakani et al., 2006; Myers and Davis, 2007), less progress has been made in understanding the nature and causes of extinction impairments that are believed to contribute to psychopathology in humans. Interestingly, we have found that a recently acquired fear is especially difficult to extinguish (Maren and Chang, 2006; Chang and Maren, 2009). In addition, delayed extinction (which is normally effective) is disrupted by delivering footshock shortly before the extinction session (Maren and Chang, 2006). Other investigators have also reported that extinction is impaired by stress (Akirav and Maroun, 2007) and is difficult to obtain in anxious inbred mouse strains (Muigg et al., 2008). Interestingly, acute stress causes dendritic retraction in the mPFC (Izquierdo et al., 2006) and chronic stress has been shown to influence mPFC control over amygdala excitability (Correll et al., 2005).

Collectively, these data suggest that stress-related impairments in mPFC function may contribute to the immediate extinction deficit. To test this hypothesis, we characterized neural activity in the infralimbic (IL) and prelimbic (PrL) divisions of the mPFC during immediate and delayed extinction using electrophysiological recordings *in*

*vivo*. Specifically, we assessed local field potentials, spontaneous spike firing, and CS-evoked activity in each region during the acquisition and expression of extinction. We find that successful extinction is associated with development of neuronal bursting in the infralimbic cortex, and that pharmacologically driving infralimbic cortex rescues the impairments in extinction observed soon after fear conditioning.

## Materials and Methods

Experiment 1: Neural activity in the medial prefrontal cortex during immediate or delayed extinction

*Subjects*

The subjects were 8 Male Long-Evans rats (>400 g; Blue Spruce) obtained from a commercial supplier (Harlan Sprague Dawley, USA). They were housed in individual cages with 14-h light/10-h dark cycle (lights on at 7:00 am), and allowed food and water ad libitum. During the first 5 days, they were handled for 10 sec to habituate them to the experimenter. All experiments were carried out in accordance with guidelines approved by the University of Michigan University Committee on Use and Care of Animals.

*Electrophysiology*

Each rat was implanted a chronic headstage with 18 individually drivable tetrodes aimed at different target areas: the medial prefrontal cortex (infralimbic and prelimbic cortex) and the amygdala (lateral, basolateral, and central nucleus). Skull screws were placed in contact with several cortical regions to record EEG signal (above left

hippocampus), as a reference to LFP/EEG signal (1 mm posterior to lambda), or as ground (posterior lateral skull ridge). Additional screws were implanted as anchors.

Data acquisition was performed using a 96 channel amplifier system (Boston University Electronics Design Facility) and acquired on-line using DataWave software (DataWave Technologies, Longmont, CO). EEG and LFP signals were amplified with gain of 5000, filtered at 1–600 Hz, and digitized at 12.5 KHz. Single unit spikes were amplified with gain of 10000, filtered at 300 Hz to 6 kHz, and digitized at 31.25 kHz. Tetrodes were progressively lowered into target area across several days based on estimated distances from atlas, and stopped moving at least three days prior to the starting of behavioral procedures, which was ten days after surgery. Data were continuously acquired across the behavior procedures and stored for further analyses.

Single units with signal-to-noise ratio above 2 were detected off-line using Datawave software, and then manually discriminated and clustered in Offline Sorter software (Plexon Inc., Dallas, TX). Data were then imported to NeuroExplorer software (NEX Technologies, Littleton, MA) for analyses of firing rate, inter-spike intervals, peri-event time histogram, bursting, and power spectrum.

*Behavioral apparatus*

One standard rodent conditioning chamber (30 x 24 x 21 cm; MED-Associates; as described in experiment 2) was modified to accommodate electrophysiological recording. It rested on a load-cell platform that was used to record chamber displacement in response to each rat's motor activity. The load cell amplifier output was digitized at 5Hz and acquired on-line using DataWave software (DataWave Technologies, Longmont, CO).

Two distinct contexts were used in this experiment. For the first context (context A), a 15 W houselight mounted opposite the speaker was turned on, and room lights remained on. The chamber was cleaned with a 1% acetic acid solution. To provide a distinct odor, a stainless steel pan containing a thin layer of this solution was placed underneath the grid floors before the rat was placed inside. The ventilation fan in chest supplied background noise (65 dB). For the second context (context B), the room lights were turned dim and the chamber houselight was turned off. Additionally, the door on the sound-attenuating cabinet was closed, the ventilation fan was turned off, and the chamber was cleaned with 1% ammonium hydroxide solution. Also, a stainless steel pan containing a thin layer of the same solution was placed underneath the grid floors before the rat was placed inside to provide a distinct odor.

*Behavioral procedures*

Rats were submitted to four phases of training: baseline tone exposure, fear conditioning, extinction, and extinction retention test. Conditioning trials began 3 min, while all others began 10 min, after being placed in the chamber. EEG, LFPs, and unit activities were acquired during baseline exposure, extinction, and test.

On Day 1, rats received 10 tone-alone (2 sec, 80 dB, 10kHz) presentations for baseline tone exposure (BL, context A). Depending on group, rats received five conditioning trials consisted of tones that coterminated with footshocks (1 mA, 0.5 sec) (60 sec inter-trial interval (ITI)) either 10 min after baseline exposure on Day 1 (DELAY, n = 4) or 10 min before extinction (IMMED, n = 4) on Day 2 in another context (context B). On Day 2, all rats received 50 tone-alone presentations for fear extinction (EXT,

context A). On Day 3, all rats were returned to the extinction context (context A) again and exposed to another 50 CS-alone presentations for retention test (TEST).

Freezing was determined during each 60 sec ITI after the CS offset during baseline exposure, conditioning, extinction, and retention test, and during the minutes preceding the first CS presentation for pre-tone freezing to context.

*Histology*

At the end of experiments, current (20 μA, 20 s) was passed through electrode tips to create small marker lesions. Rats were then perfused through the heart with 0.9% saline followed by a 10% formalin solution. After extraction from the skull, brains were post-fixed in 10% formalin solution for two days, at which time the solution was replaced with a 10% formalin and 30% sucrose solution until sectioning. Sections (45 μm thick) were cut on a cryostat (-20°C), and wet mounted on glass microscope slides with 70% ethanol. After drying, sections stained with 0.25% thionin for visualization of lesions.

*Data analysis*

All behavioral data are expressed as means and standard error of the means (SE) and analyzed by analysis of variance (ANOVA) in 10-trial blocks during BL, early/late EXT (first and last 10), and TEST (first 10) unless specified otherwise. *Post hoc* comparisons in the form of Fisher's PLSD tests were performed after a significant F ration.

Tone-evoked response for each unit was summed across 10 CS trials in different behavioral phases and post-CS activity was normalized to the 2 sec pre-CS baseline (200ms bins) to generate standard scores (Z-scores) during 2 sec tone period. A burst was defined as three or more consecutive spikes with an interval of less than 30 ms between the first two spikes and less than 50 ms in subsequent spikes.

Experiment 2: Disinhibiting IL with picrotoxin rescues immediate extinction deficits

*Subjects*

The subjects were 36 adult male Long-Evans rats (250-330 g) obtained and housed as described in Experiment 1.

*Surgery*

Rats received pre-conditioning implants with a single 26 gauge stainless-steel guide cannulae (Plastic One, Roanoke, VA). All rats were anesthetized with sodium pentobarbital (Nembutal, 65 mg/kg, ip), treated with atropine (0.04 mg/kg, i.p.) and placed in a stereotaxic frame. Stereotaxic coordinates were: AP +2.8 mm, ML +1.0 mm, DV -4.1 mm relative to bregma, with an 11° angle toward the midline in the coronal plane. Rats were allowed to recover for 7 days.

*Behavioral apparatus*

Eight identical observation chambers (30 x 24 x 21 cm; MED-Associates) were used in all experiments. The chambers were constructed of aluminum (side walls) and Plexiglas (rear wall, ceiling, and hinged front door) and were situated in sound-attenuating cabinets located in a brightly lit and isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center-to-center). Rods were wired to a shock source and solid-state grid scrambler (MED-Associates) for the delivery of footshock US. A speaker mounted outside a grating in one wall of the chamber was used for the delivery of acoustic CS.

Each conditioning chamber rested on a load-cell platform that was used to record chamber displacement in response to each rat's motor activity and acquired on-line using

Threshold-Activity software (MED-Associates). The output of each chamber's load cell was set to a gain that was optimized for detecting freezing behavior (somatomotor immobility, except that necessitated by breathing). Load-cell amplifier output (-10 to +10 V) from each chamber was digitized. Absolute values of the load-cell voltages were then computed and multiplied by 10 to yield a scale that ranged from 0 to 100. For each chamber, load-cell voltages were digitized to 5 Hz, yielding one observation every 200 msec. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity = 10). We score an observation as freezing if it fell within a continuous group of at least five observations that were all less than the freezing threshold. Thus, freezing was only scored if the rat was immobile for at least 1 sec.

Two contexts as described in Experiment 1 were used in this study.

*Behavioral procedure*

Rats were submitted to three phases of training: fear conditioning, extinction, and extinction retention test. In each phase, trials began 3 min after being placed in the chambers.

On Day 1, rats received five conditioning trials consisted of tones (2 sec, 80 dB, 2k Hz) that coterminated with footshocks (1 mA, 0.5 sec) (60 sec inter-trial interval (ITI); context A). One hour afterward, rats received 45 tone-alone presentations for fear extinction (PIC, n = 12; SAL-E, n = 12) in the other context (context B). For no-extinction controls (SAL-NE, n = 12), rats were placed in the chamber for the same amount of time but were not exposed to the tone CS. On Day 2, all rats were returned to

the extinction context (context B) again and exposed to another 45 CS-alone presentations for retention test.

Freezing was determined during each 60 sec ITI after the CS offset during conditioning, extinction, and retention test, and during the minutes preceding the first CS presentation for baseline freezing to context.

*Picrotoxin infusion*

One day before conditioning, rats were acclimated to the infusion procedure by transporting them to the infusion room in identical white 5-gallon buckets. Their dummy cannulas were replaced and the infusion pumps (Harvard Apparatus) were activated. After 5 min, the pumps were stopped and the animals were returned to their home cages.

One hour after conditioning, the rats were transported to the infusion room as described above and infused with either picrotoxin (100 ng in 0.5 μL of sterile saline at 0.1 μL/min; Sigma, St. Louis, MO) or sterile saline (0.9%; 0.5 μl at 0.1 μL/min; SAL-E and SAL-NE). After the infusion, 1 min was allowed for diffusion before the internal cannulas were removed. Clean dummy cannulas were then inserted into the guide cannulas, and rats were immediately transported to the conditioning chambers, where they received extinction trials.

*Histology and data analysis*

Histology and behavioral data analyses were performed as described in Experiment 1.

Experiment 3: Facilitating IL with D-cycloserine rescues immediate extinction deficits

*Subjects*

The subjects were 36 adult male Long-Evans rats (250-330 g) obtained and housed as described in Experiment 1.

*Surgery and behavioral apparatus*

Surgery and behavioral apparatus were identical to those described in Experiment 2.

*Behavioral procedure*

All procedures were identical to those described in Experiment 2, except that D-cycloserine (DCS) was infused before extinction.

*DCS infusion*

Rats were acclimated to the infusion procedures as described in Experiment 2.

One hour after conditioning, the rats were transported to the infusion room and infused with either DCS (10 μg in 0.5 μL of sterile saline at 0.1 μL/min; Sigma, St. Louis, MO) or sterile saline (0.9%; 0.5 μl at 0.1 μL/min; SAL-E and SAL-NE). After the infusion, 1 min was allowed for diffusion before the internal cannulas were removed. Clean dummy cannulas were then inserted into the guide cannulas, and rats were immediately transported to the conditioning chambers, where they received extinction trials.

*Histology and data analysis*

Histology and behavioral data analyses were performed as described in Experiment 1.

## Results

Experiment 1: Neural activity in the medial prefrontal cortex during immediate or delayed extinction

Fear extinction has been reported to increase the bursting of neurons in the infralimbic division of the medial prefrontal cortex (Burgos-Robles et al., 2007), as well as yielding increases in spike firing to extinguished CSs (Milad and Quirk, 2002). In the present experiment, we explored the possibility that immediate extinction fails to yield long-term fear suppression because elevated bursting and/or CS-evoked firing do not emerge in the IL when extinction is conducted soon after conditioning. To address this issue, we implanted multiple, drivable tetrodes in the IL and PrL division of the mPFC and characterized the neural correlates of immediate and delayed extinction in awake, behaving rats.

*Histology*

Unit recording sites in IL and PrL are illustrated in Figure 5.1. The total number of neurons recorded in each area and behavioral session is summarized in Table 5.1. All units were treated as independent neurons across each of the behavioral sessions.

*Behavior*

All behavioral sessions are shown in Figure 5.2. Freezing behavior was low during the baseline (BL) recording session prior to fear conditioning. Once extinction (EXT) trials commenced, all animals exhibited equivalent and high levels of conditioned freezing early in the EXT session. Extinction training reduced the levels of conditional freezing during the EXT session in both the IMMED and DELAY rats. However, this

loss of fear was only maintained 24 hours later among rats in the DELAY condition; fear in rats in the IMMED condition recovered to pre-extinction levels during the retention test (TEST) as previously reported (Maren and Chang, 2006; Chang and Maren, 2009). There was a significant main effect of group [$F(1,6) = 15.1$, $p < 0.01$], a significant main effect of behavioral phase [$F(3,18) = 25.9$, $p < 0.0001$], and a significant interaction between group and behavioral phase [$F(3,18) = 3.8$, $p < 0.05$]. *Post hoc* comparisons revealed that freezing behavior between the two groups were equivalent during BL and early/late EXT sessions [all $ps > 0.05$]. During the TEST session, freezing levels of rats in the IMMED condition were significantly higher than those in the DELAY condition [$p < 0.05$].

We previously reported that freezing is elevated prior to the onset of EXT in IMMED relative to DELAY rats (Maren and Chang, 2006). We also observed this pattern of behavior in the present experiment (Figure 5.3A). Compared to DELAY rats, IMMED rats exhibited high levels of freezing prior to the onset of the first EXT trial [$F(1,6) = 10.5$, $p = 0.02$]. As we have previously suggested, elevated pre-EXT freezing in the IMMED rats may result from sensitization of fear by recent footshock.

*Electrophysiology*

*Local field potentials*. Prior to the onset of EXT training, rats in the IMMED condition exhibited a sensitized fear response that may have interfered with the development of long-term extinction. It is possible that the different behavioral states of rats in the IMMED and DELAY conditions are related to different levels of prefrontal cortical arousal. Neocortical arousal is cholinergically mediated and is characterized by suppression of delta waves (1-4 Hz) (Jasper and Tessier, 1971; Buzsaki et al., 1988; Kapp

et al., 1994). Indeed, amygdala stimulation can suppress neocortical delta activity through its projections to cholinergic neurons of the nucleus basalis (Price and Amaral, 1981; Grove, 1988; Kapp et al., 1994). Therefore, we hypothesized that sensitized fear in the IMMED rats, presumably a result of amygdala hyperactivity, would be accompanied by a suppression of delta activity in the medial prefrontal cortex.

Averaged power spectra recorded from the IL during the EXT session for animals in each experimental group are shown in Figure 5.3B; average power in the delta range (1-4 Hz) is shown in the inset. Supporting our hypothesis, delta activity was suppressed in IMMED rats compared to DELAY controls before the first EXT trial was delivered (pre EXT). Delta activity was equally suppressed in both groups during early EXT, but recovered only in DELAY animals during late EXT. There was a significant main effect of behavioral phase on delta power [$F(2,10) = 19.0$, $p = 0.0004$] and a significant interaction between group and behavioral phase [$F(2,10) = 4.0$, $p = 0.05$]. *Post hoc* comparisons revealed that delta power was significantly lower in IMMED rats during pre and late EXT [both $ps < 0.05$], with no significant difference during the early EXT [$p > 0.05$]. Thus, IMMED and DELAY rats were behaviorally and physiologically in different states before extinction training: IMMED rats were more aroused compared to their DELAY controls.

*Spontaneous and trial-related spike bursting*. Previous studies have shown that spike bursting in the IL is a neural correlate of effective extinction in rats (Burgos-Robles et al., 2007; Mueller et al., 2008). We therefore hypothesized that bursting in IL neurons may be reduced in rats receiving immediate extinction trials. To address this issue, we

examine single-unit discharges in the medial prefrontal cortex (IL and PrL) during immediate and delayed extinction.

As shown in Table 5.1, we recorded ~ 40 units in the IL (mean = 41, range from 28 to 47) and ~ 50 units in the PrL (mean = 55, range from 43 to 70) across three behavioral sessions. Waveform analysis (Bartho et al., 2004) suggested that all of the neurons we recorded were primarily projections neurons with wide half peak and peak-valley durations (mean = 164.5 ± 1.0 μs and 453.9 ± 3.2 μs, respectively; under our filter settings). Two neurons recorded in the PrL with narrow durations (half peak < 120 μs, peak-valley < 200 μs), presumably interneurons, were excluded from further analyses. All neurons displayed low spontaneous firing rates (< 3 Hz), which is characteristic of projection neurons (Pare and Gaudreau, 1996; Collins and Pare, 1999; Bartho et al., 2004; Berke et al., 2004).

As a first step in characterizing the pattern of spike firing in the prefrontal cortex, we constructed inter-spike interval (ISI) histograms for each behavioral phase. This ISI analysis allowed us to examine whether extinction changes the frequency of short-latency spike events that would be expected if there were an increase in burst-mode firing. Indeed, we found significant differences in the frequency of events with inter-spike intervals less than 30 ms and therefore focused our analysis on these events. The percentage of both short inter-spike interval (ISI) events (< 30 ms) and spontaneous firing rates during different behavioral phases in IL and PrL are shown in Figure 5.4. For IL, there were no significant differences in the percentage of short ISI events between the groups during the BL session [$F(1,62) = 2.0$, $p = 0.16$]. However, differences between the IMMED and DELAY groups were evident during the EXT session and were reflected

in fewer short ISI events in IMMED compared to DELAY rats. Interestingly, both IMMED and DELAY rats exhibited an increase in short ISI events over the course of the EXT session, and this pattern was maintained during the TEST session 24 hours later. These observations were confirmed by a significant main effect of group [$F(1,84) = 7.3$, $p = 0.008$] and a main effect of early/late EXT [$F(1,84) = 9.0$, $p = 0.0036$], but no significant interaction between group and early/late EXT [$F(1,84) < 1$]. During the TEST session, there was a main effect of group [$F(1,82) = 6.6$, $p = 0.01$]. Unlike the short ISI events in IL, the spontaneous firing rates of IL neurons in each group were equivalent across all behavioral phases [all $ps > 0.05$]. Thus, the difference in the percentage of short ISI events during the EXT and TEST session was not due to an overall increase in spike firing, but the change in firing patterns, in the IMMED group. In contrast to the IL, there were no differences in either short ISI events or spontaneous firing between the IMMED and DELAY groups in any behavioral phase in the PrL [all $ps > 0.05$].

The previous analyses indicate that effective extinction in DELAY rats is associated with an increase in short ISI events in the IL, and this effect was impaired in IMMED rats. This suggests that IMMED extinction does not engage burst-mode firing in the IL. To examine this issue further, we characterized IL bursting using a slightly modified criterion as previously described, that is, the occurrence of three or more consecutive spikes with an ISI of less than 30 ms (instead of 25 ms, in order to keep it consistent with the short ISI event analysis stated above) between the first two spikes and less than 50 ms for subsequent spikes (Shi and Zhang, 2003; Burgos-Robles et al., 2007). For this analysis, we examined the frequency of bursts during both the CS period and the 1-min ITI following the CS in each behavioral phase. As shown in Figure 5.5, IL

bursting varied as a function of the behavioral session primarily among rats in the DELAY group. During the BL session, bursting was similar in IMMED and DELAY rats. Differences in bursts started to emerge during early EXT session: whereas the IL bursts frequency in DELAY rats increased from the BL to the EXT session, it marginally decreased in rats in the IMMED condition. Moreover, although both IMMED and DELAY rats showed elevated bursts during the 2-s period, such increase was sustained only in DELAY rats over the 1-min ITI. During the TEST session, differences in the IMMED and DELAY rats were maintained. Interestingly, IMMED rats began to show an increase in bursting relative to the extinction session; this mirrored the increase in bursting experienced by the DELAY rats on their first effective extinction session. As the divergent IL bursts mode emerged early during EXT (Figure 5.4A1 and 5.5B1) and sustained during TEST (Figure 5.4A1 and 5.5C), it suggested that elevated IL bursts may be essential for the acquisition of extinction and the long-term fear suppression.

*Tone-evoked spike firing*. Studies have shown that IL, but not PrL, neurons exhibit increases in transient evoked firing rate to extinguished CSs when fear is suppressed after extinction (Milad and Quirk, 2002; Maren and Quirk, 2004). Moreover, electrical microstimulation of IL that mimics CS-evoked spike firing suppresses freezing in rats have not been extinguished (Milad and Quirk, 2002; Milad et al., 2004). We thus hypothesized that impaired extinction in the IMMED rats might be reflected by a failure of IL neurons to increment their firing to an extinguished CS.

To examine this issue, we characterized CS-evoked single-unit in both IL and PrL neurons during each behavioral phase. Figure 5.6 illustrates the normalized CS-evoked response during the 200 ms period after CS onset averaged across all the neurons

recorded in the IL and PrL. On average, the magnitude of the CS-evoked response in both the IL and PrL was low. In fact, of all the units recorded, only 13 out of 245 neurons in the IL and 13 out of 327 neurons in the PrL would have met our standard criterion ($z > 3$ within any 50-ms bin within 200 ms of CS onset) for tone responsivity that we have used in earlier reports (Maren, 2000; Goosens et al., 2003; Hobin et al., 2003). As shown in Figure 5.6, IL neurons from both IMMED and DELAY rats exhibited an increases in CS-evoked spike firing in the EXT session relative to the BL, and this pattern was sustained during the TEST session [$F(2, 229) = 3.1$, $p < 0.05$]. There was no interaction between group and behavioral phase [$F(2, 229) = 1.3$, $p = 0.26$ ]. *Post hoc* comparisons revealed that CS-evoked responses were significantly higher in the EXT and TEST sessions compared to the BL in both the IMMED and DELAY rats [both $ps < 0.05$]. In contrast, there was no influence of behavioral training on CS-evoked firing in the PrL [$F(2,321) < 1$].

These results are not consistent with earlier reports indicating an increase in CS-evoked spike firing in IL after extinction (Milad and Quirk, 2002; Maren and Quirk, 2004). To examine this issue further, we conducted another analysis focusing on only those IL neurons that exhibited an increase in CS-evoked spike firing ($z > 0$ within 200 ms of CS onset) during any behavioral session. Average peri-event time histograms of spike firing in these neurons are shown in Figure 5.7. During the BL session, there was no significant difference in CS-evoked spike firing among the groups [$F(1,9) < 1$]. During the EXT session, both IMMED and DELAY rats showed increased CS-evoked firing, which subsided only in DELAY group during the course of the EXT session. There was no significant difference in CS-evoked firing among the groups [$F(1,28) < 1$],

but there was a main effect of early/late during the session [$F(1,28) = 6.9$, $p = 0.01$]. Planned comparisons revealed that CS-evoked firing reliably decreased only in the DELAY group [$F(1,11) = 6.1$, $p = 0.03$]. During the TEST session, this difference was maintained and CS-evoked firing was significantly higher in the IMMED group [$F(1,29) = 4.5$, $p = 0.04$]. Thus, animals in the DELAY group that successfully extinguished fear exhibited lower levels of CS-evoked firing than animals in the IMMED group that did not exhibit extinction. Indeed, CS-evoked spike firing in the IL appeared to correlate more strongly with the expression of fear than extinction. In contrast to the IL, PrL neurons exhibited CS-evoked firing in all behavioral sessions, but no significant difference between groups in any phases [all $ps > 0.05$; Figure 5.8].

Experiment 2: Picrotoxin infusion into the IL rescues the immediate extinction deficit

The results from Experiment 1 suggest that the immediate extinction deficit may be caused by abnormal physiological activity of IL neurons, at least with regard to the pattern of spike bursts. Reducing $GABA_A$ receptor-mediated inhibition greatly enhances neocortical activity and produces synchronized bursting (Connors, 1984; Chagnac-Amitai and Connors, 1989; Metherate and Cruikshank, 1999). We therefore examined whether increasing IL activity by antagonizing IL $GABA_A$ receptors would rescue the immediate extinction deficit.

*Histology*

On the basis of the histological results, four of 36 rats were excluded. This yielded the following group sizes: PIC (n = 10), SAL-E (n = 10), and SAL-NE (n = 12). IL cannula placements for rats included in the data analyses are depicted in Figure 5.9A.

*Behavior*

Freezing behavior during the conditioning session is shown in Figure 5.9B. Freezing behavior was very low before the first conditioning trial, and then increased in frequency thereafter. There was an equivalent increase in freezing across trials in all groups [group × trial, $F(10,145) < 1$].

Freezing behavior during the extinction session is shown in Figure 5.9C. There was a significant main effect of group [$F(2,29) = 9.8$, $p = 0.0006$], a significant main effect of trial block [$F(9,261) = 6.7$, $p < 0.0001$], and a significant interaction between group and trial block [$F(18,261) = 11.9$, $p < 0.0001$]. Interestingly, picrotoxin infusions into the IL completely eliminated the expression of freezing during the extinction session. *Post hoc* comparisons revealed that freezing levels in SAL-E rats were significantly higher than those in both SAL-NE and PIC rats [both $ps < 0.05$], with no significant difference between the latter groups [$p > 0.05$]. At the end of extinction, however, there was no difference in freezing among the groups [$F(2,29) < 1$], demonstrating within-session extinction in SAL-E animals.

Freezing behavior during the retention test is shown in Figure 5.9D. There was a significant main effect of group [$F(2,29) = 4.8$, $p = 0.02$], a significant main effect of trial block [$F(9,261) = 23.4$, $p < 0.0001$], and a significant interaction between group and trial block [$F(18,261) = 2.3$, $p = 0.0024$]. *Post hoc* comparisons revealed that freezing levels in PIC rats were significantly lower than SAL-NE [$p < 0.05$], while there was no significant difference in freezing levels between SAL-E and SAL-NE [$p > 0.05$]. Hence,

immediate extinction failed to produce long-term fear suppression, but this deficit was overcome by intra-IL picrotoxin infusions prior to the extinction session.

Experiment 3: Facilitating IL with D-cycloserine rescues the immediate extinction deficit

Extinction learning depends on NMDA receptor-mediated plasticity (Falls et al., 1992; Santini et al., 2001; Walker et al., 2002). Blocking NMDA receptors in IL during delayed extinction impaired long-term recall (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2009). We therefore assessed whether facilitating IL NMDA receptors with D-cycloserine (DCS), a partial agonist of the NMDA receptor, would rescue the immediate extinction deficit.

*Histology*

Two of 36 rats were excluded due to failed shock delivery during conditioning. This yielded the following group sizes: DCS (n = 12), SAL-E (n = 10), and SAL-NE (n = 12). IL cannula placements for rats included in the data analyses are depicted in Figure 5.10A.

*Behavior*

Freezing behavior during the conditioning session is shown in Figure 5.10B. Freezing behavior was very low before the first conditioning trial, and then increased in frequency thereafter. There was an equivalent increase in freezing across trials in all groups [group × trial, $F(10,155) < 1$].

Freezing behavior during the extinction session is shown in Figure 5.10C. There was a significant main effect of group [$F(2,31) = 6.5$, $p = 0.0044$], a significant main effect of trial block [$F(9,279) = 19.6$, $p < 0.0001$], and a significant interaction between

group and trial block [$F(18,279) = 4.0$, $p < 0.0001$]. *Post hoc* comparisons revealed that freezing levels of SAL-NE were significantly lower than both SAL-E and DCS [both ps $< 0.05$], with no significant difference between the later two [$p > 0.05$]. At the end of extinction, however, there was no difference in freezing levels among all groups [$F(2,31) < 1$], demonstrating within-session extinction in DCS and SAL-E animals.

Freezing behavior during the retention test is shown in Figure 5.10D. There was a significant main effect of group [$F(2,31) = 4.8$, $p = 0.02$], a significant main effect of trial block [$F(9,279) = 23.0$, $p < 0.0001$], and a significant interaction between group and trial block [$F(18,279) = 2.4$, $p = 0.0017$]. *Post hoc* comparisons revealed that freezing levels in DCS-treated rats were significantly lower than SAL-NE and SAL-E [both ps $< 0.05$], while there was no significant difference in freezing levels between the latter two groups [$p > 0.05$]. As in Experiment 2, immediate extinction did not produce long-term fear suppression, but IL infusions of DCS overcame this deficit and produced a significant facilitation of extinction.

## Discussion

In the present study, we used electrophysiological recordings and drug microinfusions in the medial prefrontal cortex to examine neural correlates of the immediate extinction deficit in rats. Our results reveal that immediate extinction trials fail to engage the medial prefrontal cortex in the same manner as delayed extinction trials. That is, immediate extinction was not associated with neuronal bursting in the IL and CS-evoked activity in the IL remained elevated throughout both extinction and

retention testing. This dysregulation of neuronal firing in rats undergoing immediate extinction may have been related to levels of neocortical arousal insofar as delta wave activity was reduced in the medial prefrontal cortex of rats undergoing immediate extinction. Pharmacological manipulations of IL with either picrotoxin or D-cycloserine prior to the extinction session rescued the immediate extinction deficit. These data suggest that the immediate extinction deficit is caused by compromised IL function shortly after fear conditioning.

In previous studies, extinction was reported to increase the bursting of neurons in the IL after extinction training was complete (Burgos-Robles et al., 2007; Mueller et al., 2008). Together with the sensitivity of extinction to post-training pharmacological manipulations of IL (Sotres-Bayon et al., 2009), these data have been argued to support a role for IL bursting in the consolidation of extinction memories (Santini et al., 2004; Burgos-Robles et al., 2007; Mueller et al., 2008). Consistent with the view, we observed that IL bursting emerged during the extinction session in rats in both the immediate and delayed extinction conditions. This suggests that the acquisition of extinction engages IL bursting, and that this bursting persists during the post-extinction period to presumably foster the consolidation of extinction. Although it is not entirely clear how IL bursting fosters the acquisition and consolidation of extinction memory, it may foster local synaptic plasticity in the IL (Buzsaki et al., 2002; Burgos-Robles et al., 2007), which might play a role in integrating of hippocampal and amygdala inputs (Garcia et al., 1999; Herry and Garcia, 2002; Barrett et al., 2003; Corcoran et al., 2005; Bouton et al., 2006; Herry et al., 2008). IL bursting might also drive activity among inhibitory intercalated

cells in the amygdala that are involved in the inhibition of fear (Lisman, 1997; Pare et al., 2004).

In addition to reduced IL bursting in rats undergoing immediate extinction, we found that CS-evoked activity in the IL was elevated early during extinction training and did not dissipate during the course of the extinction session in rats undergoing immediate extinction. Interestingly, CS-evoked activity in rats undergoing delayed extinction dissipated during the extinction session and remained low during the retention test. This result was unexpected insofar as an earlier report found that CS-evoked responses in the IL were minimal before extinction training, and increased in magnitude after extinction. (Milad and Quirk, 2002; Maren and Quirk, 2004). Indeed, the pattern of CS-evoked firing that we have observed in the present study is more consistent with the firing properties of a subpopulation of tone-responsive neurons recently described in the PrL (Burgos-Robles et al., 2009). We also observed sustained CS-evoked responses in the PrL (Figure 5.8), but their response was not related to extinction. The reasons for these disparities are not clear, but it suggests that CS-evoked activity in IL neurons may reflect both the acquisition of conditional fear, as well as its extinction. In either case, however, the present data suggest that neuronal activity in the IL is dysregulated in rats that fail to extinguish fear relative to those that extinguish normally.

What might account for dysregulated mPFC activity in rats undergoing immediate extinction? We have previously suggested that the stress engendered by a recent traumatic event might yield the immediate extinction deficit. Indeed, the present data indicate that rats in the immediate extinction condition are behaviorally and physiologically more aroused than those in the delay condition. This stress-induced

arousal appears to compromise the function of mPFC circuits involved in extinction learning. Indeed, several studies have found that stress impairs IL function and impairs extinction (Izquierdo et al., 2006; Maroun, 2006; Akirav and Maroun, 2007; Muigg et al., 2008). Stress-induced impairments in IL function may be related to hyperactivity of amygdala circuits that project to the mPFC (Maroun and Richter-Levin, 2003). Hence, the neural circuits involved in the generation and suppression of fear may antagonize one another, with the subcortical expression of fear responses dominating the acute response to trauma and the emergence of cortical fear suppression appearing only after the acute stressor has subsided.

Interestingly, the cortical dysregulation associated with recent fear could be overcome pharmacologically. We found that the immediate extinction deficit could be attenuated by infusing either a GABA antagonist (picrotoxin) or an NMDA receptor agonist (D-cycloserine) into the IL. Picrotoxin releases IL from local inhibition and increases bursting (Connors, 1984; Chagnac-Amitai and Connors, 1989; Metherate and Cruikshank, 1999), while D-cycloserine may augment synaptic potentiation in IL (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2009). The facilitation of extinction learning by intra-IL DCS infusions is particularly exciting insofar as DCS has been used successfully as an adjunct to exposure-based therapies (Ressler et al., 2004; Hofmann et al., 2006).

There is considerable debate in the clinical literature about the appropriate timing of therapeutic interventions for psychological trauma (Bisson et al., 1997; Everly and Mitchell, 1999; Campfield and Hills, 2001; McNally et al., 2003; Rothbaum and Davis, 2003; Gray and Litz, 2005). Recent work in animal models suggests that early

interventions may not yield long-term fear suppression (Maren and Chang, 2006; Norrholm et al., 2008; Schiller et al., 2008; Woods and Bouton, 2008; Chang and Maren, 2009). We now show that the function of neural circuits involved in encoding extinction memory is impaired shortly after an acute trauma, and that this impairment can be reversed by pharmacologically activating the medial prefrontal cortex during extinction training. This suggests strategies for increasing the efficacy of early interventions for trauma and increasing the likelihood of long-term fear suppression.

**Table 5.1. Number of neurons recorded in each behavior session.**

| | | Behavior session | | |
|---|---|---|---|---|
| Group | Brain structure | BL | EXT | TEST |
| DELAY | IL | 28 | 43 | 47 |
| | PrL | 52 | 57 | 70 |
| IMMED | IL | 36 | 44 | 47 |
| | PrL | 43 | 50 | 55 |

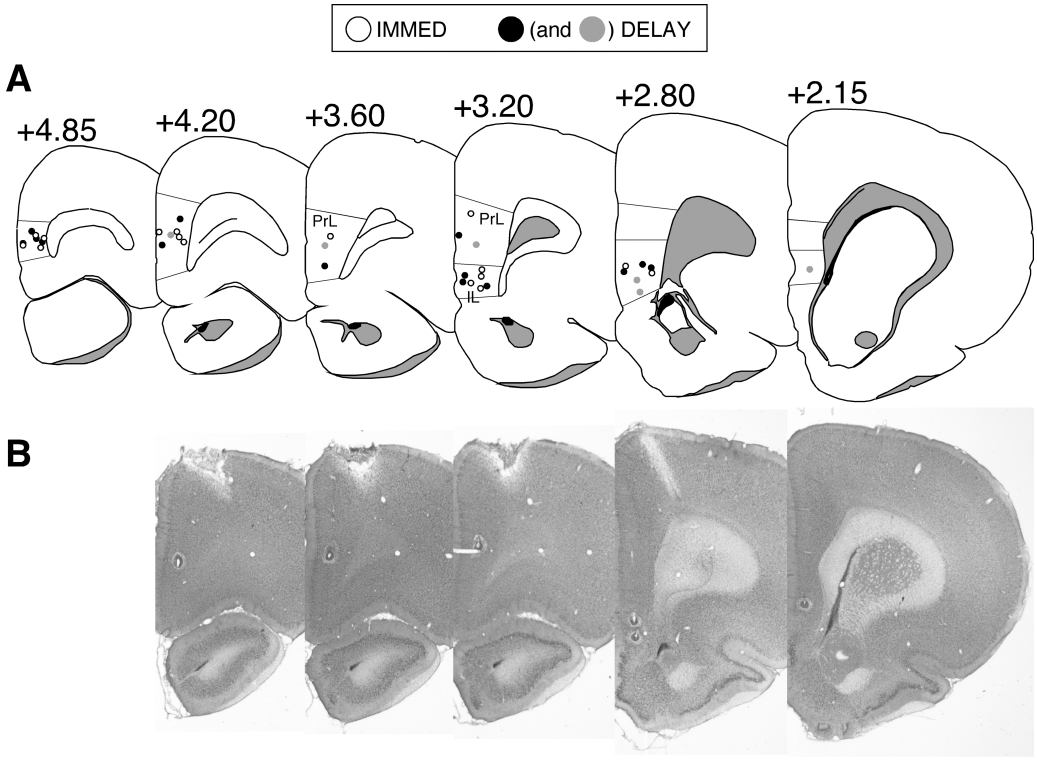


**Figure 5.1. Anatomical placement of tetrodes.**
(A) Coronal sections representing all of the tetrode placements included in the data analysis. (B) Serial sections from one DELAY animal showing tetrodes in both the IL and PrL; these placements are shown as filled gray circles in (A). This figure was adapted from Swanson (2004)

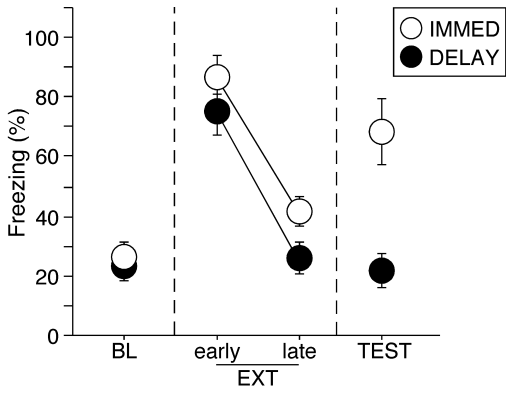


**Figure 5.2. Immediate extinction fails to generate long-term fear suppression.** Freezing levels were significantly higher in IMMED rats compared to their DELAY controls during the retention test ($p < 0.05$).

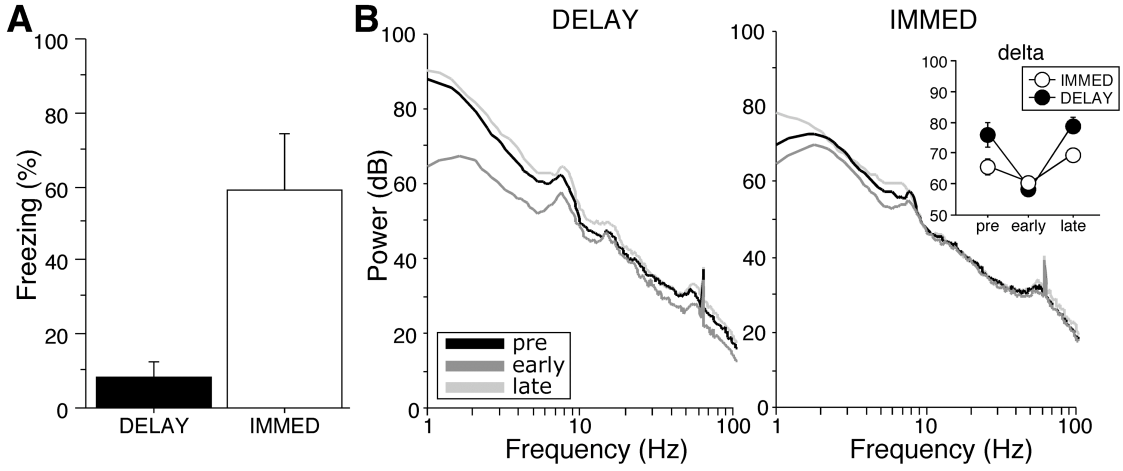


**Figure 5.3. Rats in the immediate extinction condition were behaviorally and physiologically aroused before extinction training.**
(A) Pre-extinction freezing levels were significantly higher in IMMED compared to DELAY rats. (B) Averaged power spectrum of IL during the extinction session (pre-extinction, early extinction, or late extinction), with delta frequencies (1-4 Hz) in the inset. Delta power was significantly suppressed during the pre-extinction period ($p < 0.05$).

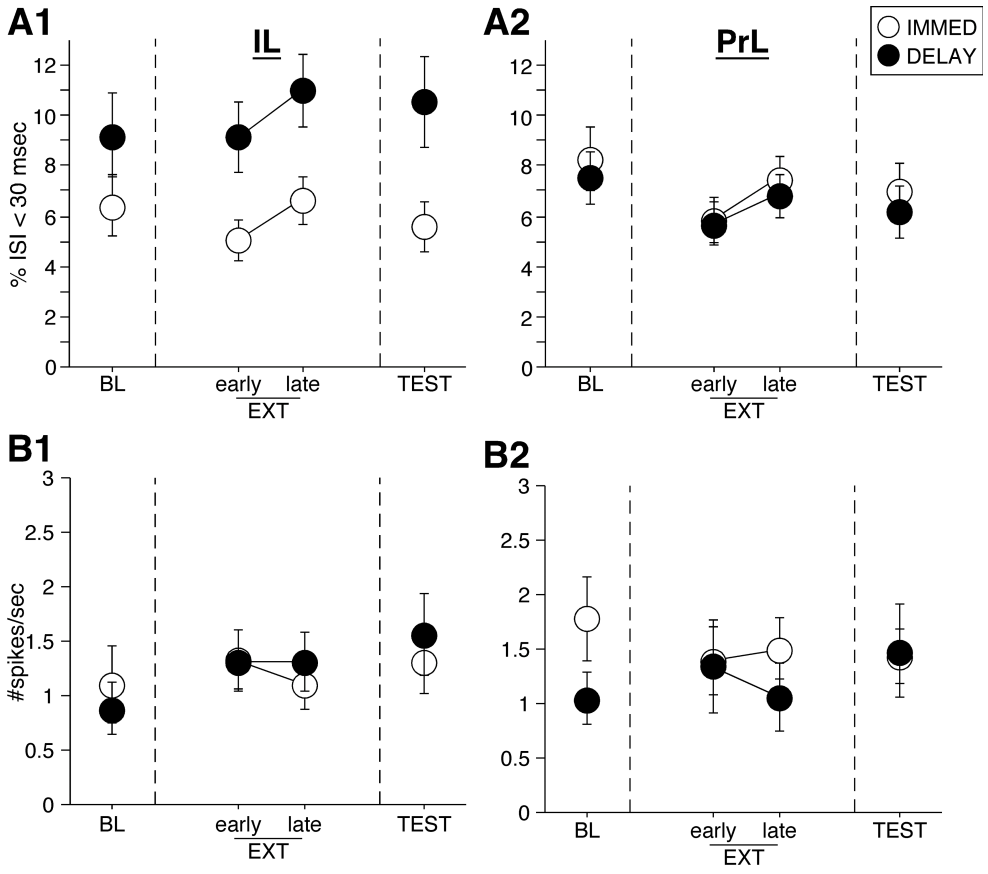


**Figure 5.4. Immediate extinction reduces IL bursting.**
(A1) Short ISI events were significantly less frequent in IMMED compared to their DELAY control during the extinction (EXT) and test (TEST) sessions (both ps < 0.05). Extinction training also increased the number of short ISI events in both the IMMED and DELAY conditions. (B1) There was no significant difference between groups in spontaneous firing rates across different behavioral phases (all ps > 0.05). (A2, B2) There was no significant difference between groups in PrL for short ISI events or spontaneous firing rates across different behavioral phases (all ps > 0.05).

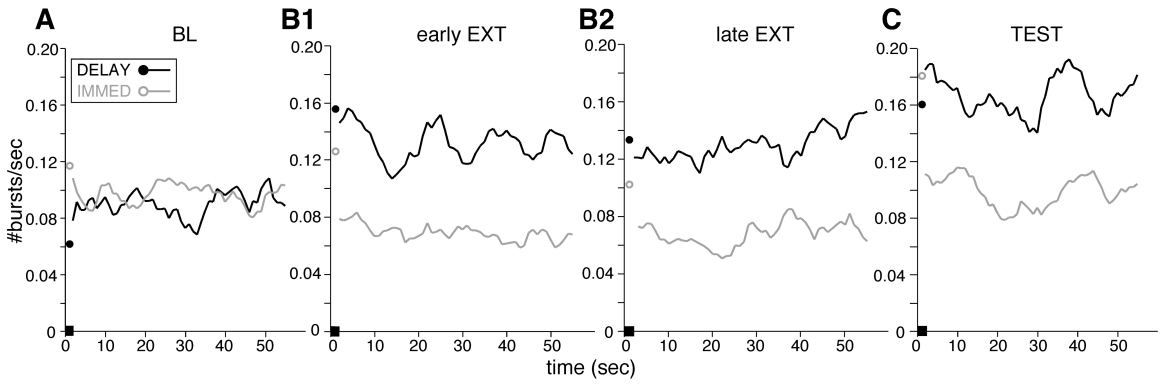


**Figure 5.5. Trial-related IL bursting.**
IL bursting was greater in rats in the DELAY compared to the IMMED extinction conditions, and this difference was sustained across the entire ITI period. Events were smoothed with a moving average of 3 sec.

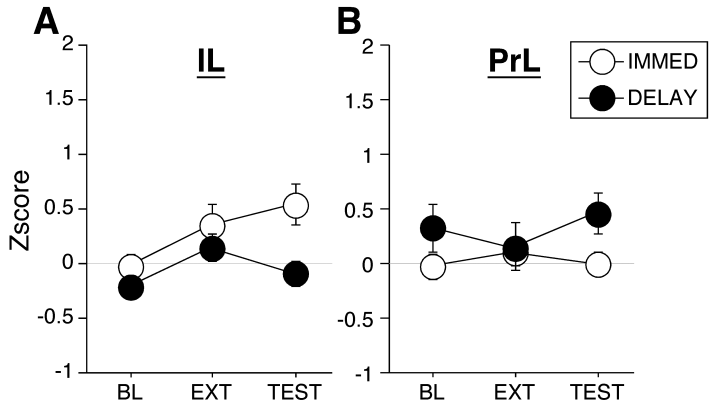


**Figure 5.6. Averaged short-latency CS-evoked neuronal responses in the IL during behavioral training.**
(A) Normalized CS-evoked response in IL neurons were averaged during the 200ms period after CS onset. CS-evoked responses were significantly higher during early EXT and TEST compared to BL (both ps $< 0.05$) in IMMED and DELAY rats. (B) Normalized tone-evoked onset response in PrL neurons. No significant changes across different behavioral phases ($p > 0.05$).

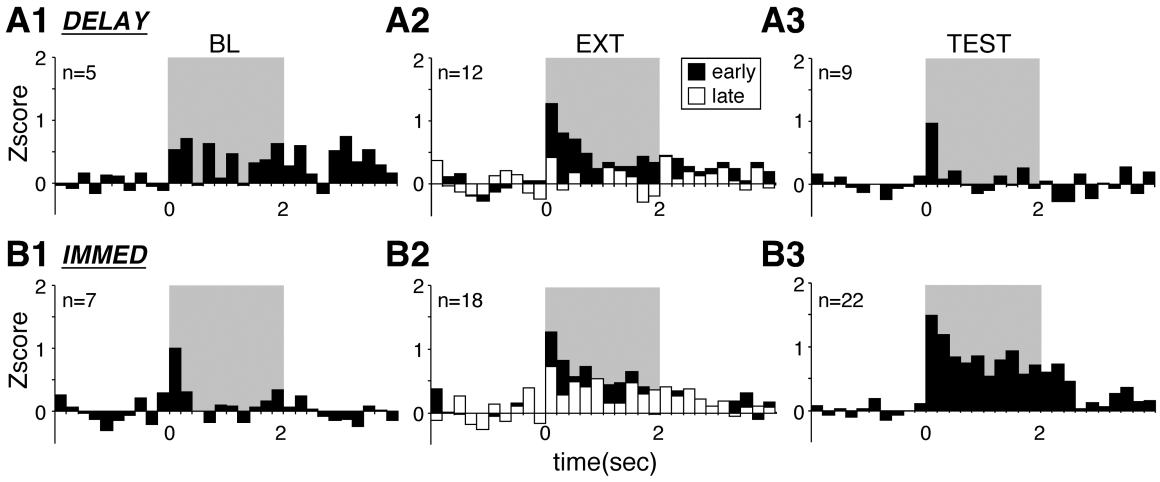


**Figure 5.7. Peri-event time histograms illustrating CS-evoked activity in the IL during behavioral training.**
The number of neurons contributing to each average (Z > 0 within 200 ms) are indicated in the panels. (A1, B1) There was no difference between the IMMED and DELAY groups in firing to tones during BL ($p > 0.05$) and early EXT (A2, B2). During the extinction session (A2, B2), only DELAY rats decreased their firing to the CS ($p < 0.05$). (A3, B3) Firing to tones was significantly higher in IMMED than their DELAY controls during TEST ($p < 0.05$).

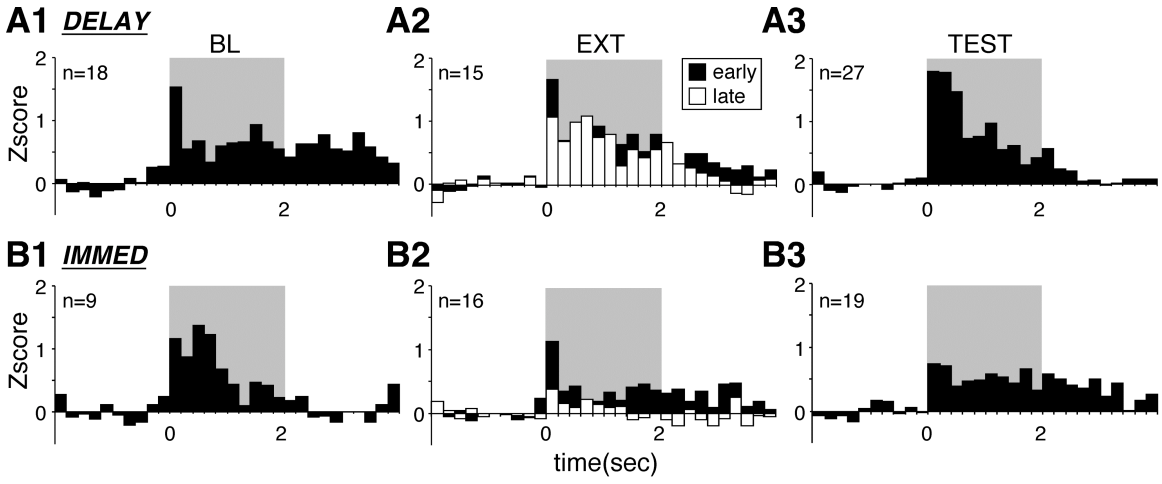


**Figure 5.8. Peri-event time histograms illustrating CS-evoked activity in the PrL during behavioral training.**
The number of neurons contributing to each average (Z > 0 within 200 ms) are indicated in the panels. There were no differences between groups in firing to tones in any behavioral sessions (all ps > 0.05).

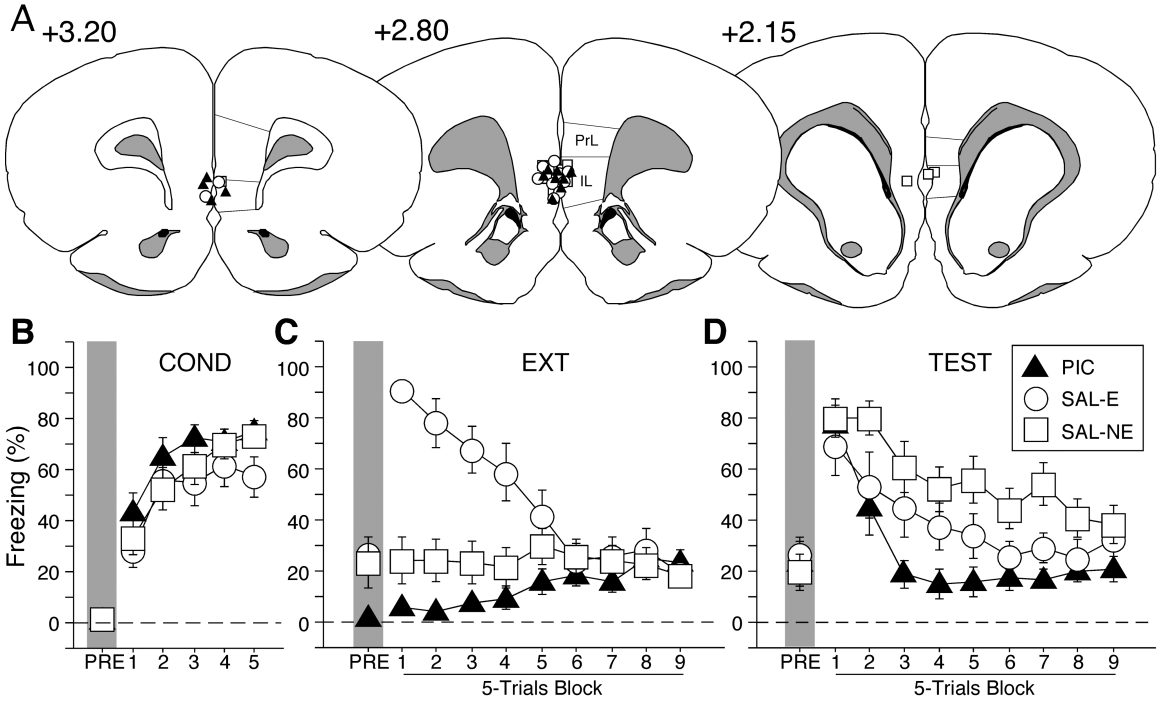


**Figure 5.9. Intra-IL picrotoxin infusions rescue the immediate extinction deficit.** (A) Cannula placements of all animals included in data analysis. (B) All animals equivalently acquired fear at the end of conditioning ($p > 0.05$). (C) Picrotoxin infusion blocked fear expression during EXT. (D) Picrotoxin infused animals showed faster drop in freezing levels compared to both SAL-E and SAL-NE controls. This figure was adapted from Swanson (2004)

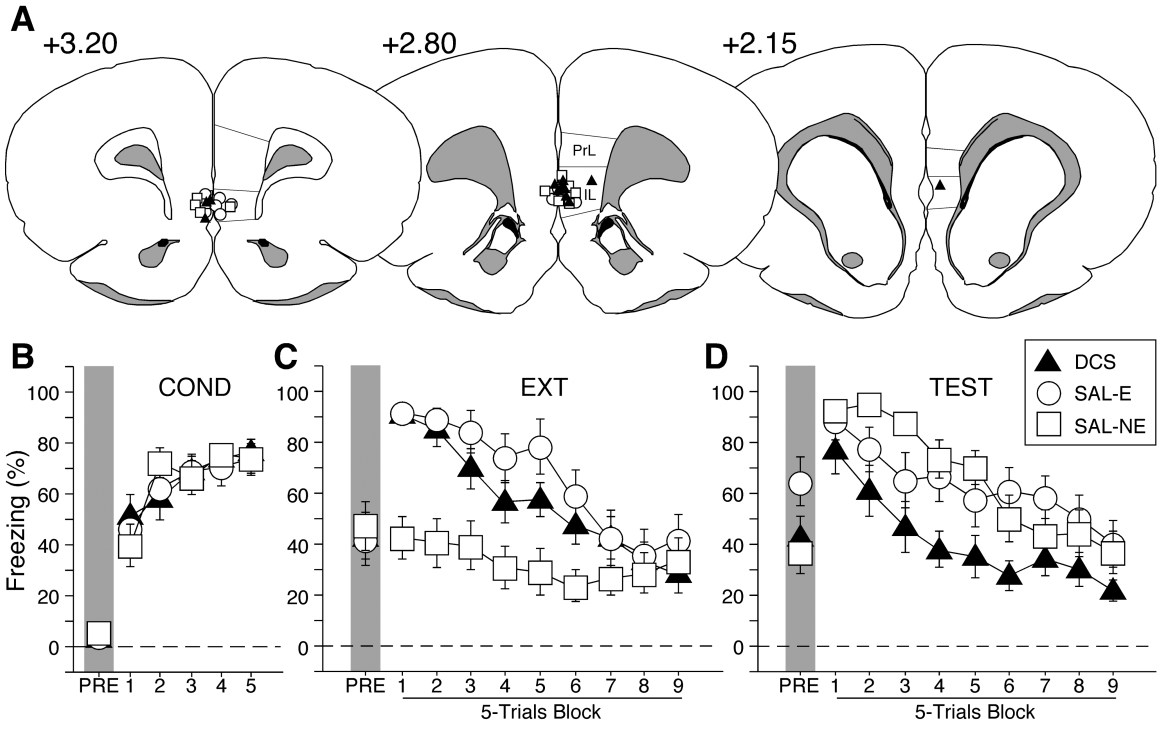


**Figure 5.10. Intra-IL D-cycloserine infusions rescue the immediate extinction deficit.** (A) Cannulae placements of all animals included in data analysis. (B) All animals equivalently acquired fear at the end of conditioning ($p > 0.05$). (C) DCS infusion animals retained normal freezing behavior during EXT. (D) DCS infused animals showed faster drop in freezing levels compared to both SAL-E and SAL-NE controls. This figure was adapted from Swanson (2004)

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# CHAPTER VI

# CONCLUSION

## Summary of Findings

In the current thesis, we tested the hypothesis that CS-alone trials given at different time intervals after conditioning initiates different fear suppression mechanisms: "new learning" at longer intervals and "unlearning" at shorter intervals. Contrary to our hypothesis, fear was not unlearned under immediate extinction. This deficit was related to high levels of sensitized fear at the onset of extinction in recently shocked rats (Chapter II). Although immediate extinction failed to produce long-term fear suppression, it did produce a short-term suppression that exhibited properties of context-independent habituation, rather than extinction (Chapter III). We next demonstrated that the immediate extinction deficit was due to dysfunction of the infralimbic division of the medial prefrontal cortex (IL) (Chapter IV and V), and was rescued by pharmacological manipulations that facilitate neuronal activity and synaptic plasticity in the IL (Chapter V). Taken together, these results suggest that the IL is important for the acquisition of long-term extinction, and that recent fear suppresses IL function leading to an immediate extinction deficit.

## Erasure of Fear… Impossible?

In a recent study, Myers and colleagues (2006) demonstrated that fear memory could be erased when CS-alone trials were presented immediately after conditioning in rats. This phenomenon has also been reported in humans (Norrholm et al., 2008). These results suggest that early extinction erases fear memory ("unlearning"), whereas delayed extinction results in a new inhibitory memory that transiently suppressed fear ("new learning"). However, we have found that not only does early extinction fail to erase fear memory, but rather it fails to yield extinction at all. Several other laboratories have confirmed this outcome and have also found that immediate extinction produces a less durable fear suppression than delayed extinction (Alvarez et al., 2007; Schiller et al., 2008; Woods and Bouton, 2008; Chang and Maren, 2009). This has been demonstrated in rats using freezing (Maren and Chang, 2006; Schiller et al., 2008; Chang and Maren, 2009) or conditioned emotional response (CER) (Woods and Bouton, 2008) as indices of fear, and in humans using the skin-conductance response (Schiller et al., 2008) or acoustic startle (Alvarez et al., 2007) as the measurement of fear. There are still other reports that demonstrate that immediate extinction produces long-term extinction, but shows normal renewal, reinstatement, or spontaneous recovery (Quirk, 2002; Phelps et al., 2004; LaBar and Phelps, 2005; Kalisch et al., 2006; Dirikx et al., 2007); these studies were not designed to parametrically compare the timing of extinction on its efficacy. These studies indicate that immediate extinction procedures do not erase fear memory, and in some cases, fail to produce extinction at all.

Along with other reports showing impaired long-term fear suppression under immediate extinction, the Myers et al. (2006) and the Norrholm et al. (2008) studies are relatively unique, and the reason for their uniqueness remains unclear at this point. One potential explanation is that the measure of fear in these studies, fear-potentiated startle, is sensitive to erasure behaviorally and neurobiologically (but see Alvarez et al., 2007). The other possibility, as suggested in Chapter II, is that "new learning" or "unlearning" under immediate extinction is related to the level of fear acquired during conditioning, as well as the state of the animal during extinction (Maren and Chang, 2006). The latter hypothesis requires further investigation.

The resistance of fear to immediate extinction is interesting in the light of a vast body of evidence that memory is labile for hours after it is acquired (Maren, 1999; Schafe et al., 2001; Sigurdsson et al., 2007). The sensitivity of newly acquired memories to disruption may involve reversal of learning-related changes in the physiology of neural circuits. For example, low-frequency stimulation is capable of depotentiating synapses that have undergone long-term potentiation (LTP), which is considered a cellular mechanism of fear acquisition and consolidation (Zhou and Poo, 2004). Moreover, depotentiation is more readily induced at short intervals following LTP induction (Staubli and Chun, 1996), suggesting that "unlearning" is plausible, at least physiologically. Indeed, depotentiation can be induced in the amygdala in vitro (Lin et al., 2003a; Lin et al., 2005), and its induction shares some key features with fear extinction in behaving rats measured by fear potentiated-startle: both can be blocked by NMDAR and L-VGCC channel antagonists (Lin et al., 2003c; Lin et al., 2003b) and both are sensitive to manipulations targeting downstream intracellular events (Lin et al., 2003a; Lin et al.,

2003c; Lin et al., 2003b; Cannich et al., 2004; Lin et al., 2005). Taken together, the literature favors the conclusion that fear memory is not erased under immediate extinction. However, whether fear memory might be erased under some conditions remains an open question.

## Stress, is it the Key?

The behavioral results in Chapter II and III suggest that immediate extinction not only fails to erase fear memories, but also fails to suppress fear memory at all. This has led to the question of why these animals fail to extinguish fear memory. We hypothesize that immediate extinction fails to induce long-term fear suppression because it does not engage the neural circuitry required to learn a new inhibitory memory.

We focused our analysis on the medial prefrontal cortex, which has been implicated in extinction learning. After extinction, appropriate fear expression requires the interaction between the IL and the amygdala, which is also regulated by context presumably via a hippocampal-dependent mechanism (Bouton et al., 2006; Quirk and Mueller, 2008). Under stress, the function of the IL is dampened and results in impaired extinction (Akirav et al., 2006; Izquierdo et al., 2006; Maroun, 2006; Muigg et al., 2008), while the function of the amygdala is facilitated and results in enhanced fear (Southwick et al., 1999; Maroun and Richter-Levin, 2003; Rodriguez Manzanares et al., 2005). Moreover, post-conditioning consolidation of fear memory is enhanced by stress hormones (Corodimas et al., 1994; Zorawski and Killcross, 2002; Hui et al., 2004; McGaugh, 2004; Rodrigues et al., 2009). Because stressful experiences are important

events to learn, stress could act to facilitate defensive behavior that under threat, higher-order behaviors mediated by medial prefrontal cortex (mPFC) are shut down to allow automatic subcortical control of fear mediated by the amygdala to be preserved (Maroun and Richter-Levin, 2003; Maren, 2007; Arnsten, 2009).

As demonstrated earlier, rats were behaviorally (Chapter II and V) and physiologically (Chapter V) aroused when extinction trials were delivered shortly after fear conditioning. This behavioral and neural state may facilitate subcortical maintenance of the fear memory at the expense of the higher-order control of IL inhibitory circuits (Maroun and Richter-Levin, 2003; Arnsten, 2009). Although we did not directly measure the levels of the stress hormones, sensitized fear shortly after fear conditioning and consequent increases in freezing behavior suggest that the output from the medial division of the central nucleus of the amygdala (CeAm) is increased. This would presumably be accompanied with increased glucocorticoid release through the paraventricular nucleus (PVN) and bed nucleus of the stria terminalis (BNST), and may therefore enhance the consolidation of the fear memory. Thus, the levels of circulating glucocorticoids may play an important role in determining the efficacy of extinction procedures.

However, in a recent study, Woods and colleagues (2008) argued that the level of fear during extinction is not responsible for the immediate extinction deficit. They used CER to index fear and reported higher levels of conditional suppression during delayed rather than immediate extinction. This suggests that fear was greater in the delay, compared to immediate rats. However, it is worth noticing that in the CER paradigm, the animals were food-deprived and were motivated to press bars for food. The fact that

under immediate extinction, the bar-pressing behavior was less suppressed, suggests that mPFC mediated suppression of inappropriate behavior is compromised (Dalley et al., 2004). Thus, these results support the notion that under immediate extinction, the animals fail to engage the inhibitory circuit of the mPFC.

## Fear in the Circuit

Pharmacological enhancement of medial prefrontal activity during immediate extinction results in better long-term recall of extinction when tested 24 hr afterward (Chapter V). However, neither manipulation totally restored the immediate extinction deficit: intra-IL infusion of picrotoxin and D-cycloserine showed equivalent and high levels of freezing during early test trials. It is worth noting that under our manipulation, the overall state of the animal was not changed. Thus, even if the inhibitory circuit of the IL was manually engaged, the subcortical maintenance of the fear memory within the amygdala may still be preferred under the influence of stress hormone. It is also possible that simply engaging the IL was not enough. The firing timing and pattern of the IL neurons could be critical (Milad and Quirk, 2002; Burgos-Robles et al., 2007). Of course, the IL is not the only brain structure involved in establishing extinction memory, so the incomplete rescue we obtained may reflect compensation by other brain structures involved in extinction (Bouton et al., 2006; Quirk and Mueller, 2008). One of the targets where extinction is taken place is the amygdala itself (Davis et al., 2003). Fear extinction is impaired by blocking NMDA receptors function (Falls et al., 1992) and inhibiting $GABA_A$ receptor insertion (Lin et al., 2009) in the amygdala, suggesting the local

plasticity is critical. The new learning model summarized in Figure 1.2A suggests that the IL regulates information flow from the basolateral amygdala (BLA) to the CeA without erasing the original fear memory trace. However, there is evidence that modulation could happen upstream of the CeA within the BLA. Indeed, the lateral amygdala (LA) firing to the auditory CSs decreased over the course of extinction (Quirk et al., 1995; Repa et al., 2001), and is modulated by context in a hippocampus-dependent manner (Hobin et al., 2003; Maren and Hobin, 2007). Electrical stimulation of the hippocampus induces synaptic plasticity in the amygdala (Maren and Fanselow, 1995), suggesting a plausible direct interaction between the hippocampus and the amygdala. The IL could also potentially contribute to the modulation within the BLA by its projections here (McDonald et al., 1996).

Taken together, here we propose the idea that appropriate fear expression and suppression at the right time and right place after extinction requires coordinated function among the IL, the hippocampus, and the amygdala at the circuit level (Figure 6.1). Moreover, the proper function of each area is modulated by endocrine and hormone systems by the overall state of the animal. Dysfunction of any structures involved could potentially lead to unwanted pathological fear.

## Future Directions

In the current study, we presented the results that delayed extinction is more efficacious in suppressing long-term fear than immediate extinction. The immediate extinction deficit is due, at least partially, to the failure to engage the inhibitory extinction

circuit of the IL. However, there are questions not clear at this point and await further investigation.

One argument we raised in this study is that stress is one key factor in deciding the direction of new learning or unlearning under immediate extinction. This argument requires parametric analyses on the relationship of the amount of fear acquired during conditioning and its impact on long-term fear extinction. Moreover, it also raises the question: if stress is well-controlled under immediate extinction, are we able to initiate the unlearning process of the fear memory, and/or engaging the inhibitory extinction circuit of the IL? In a recent study (Rodriguez-Romaguera et al., 2009), the authors showed that suppressing the effect of stress hormones by blocking the noradrenergic β-receptor with systemic administration of its antagonist, propranolol, lowered within-session fear without long-term effects on extinction recall. However, they used the delayed extinction design, and the null effect in long-term fear could be a result of a floor effect of the already low fear during test in the control group. Two other recent studies suggested that fear memory could be erased during reconsolidation either behaviorally (Monfils et al., 2009), or with propranolol (Kindt et al., 2009), after a consolidated long-term memory was reactivated and became labile again. It would be interesting to see the effect of propranolol on immediate extinction when the acute fear is well controlled and the memory trace is labile.

In this study, we characterized only the medial prefrontal cortex, leaving all other potential structures within the extinction circuit, including the amygdala and the hippocampus, unexplored. Local synaptic plasticity within the sensory interface of the amygdala, specifically the BLA, is especially of interest. The hippocampus (Canteras

and Swanson, 1992; Maren and Fanselow, 1995) and the IL (McDonald et al., 1996) both directly project onto the BLA. There is evidence showing extinction-related changes in LA neuronal activity (Quirk et al., 1995; Repa et al., 2001; Hobin et al., 2003; Maren and Hobin, 2007), which may require the feedback (Quirk et al., 1995) and feed-forward (Li et al., 1996; Woodson et al., 2000) interaction between projection neurons and local interneurons and modulated by the hippocampus (Maren and Hobin, 2007). Thus, under immediate and delayed extinction, how BLA local plasticity is modulated by local interneurons and/or direct hippocampal and IL inputs awaits further investigation. There is also mounting evidence showing coordinated interactions among different brain structures during different learning paradigms (Bauer et al., 2007; Paz et al., 2008; Popescu et al., 2009). However, how different structures within the extinction circuit communicate to one another is unclear.

In conclusion, studying the function of each brain area within the extinction circuits, their interactions, and how they are modulated by different states of the animal, will extend our knowledge from pre-clinical animal research to future clinical treatment in pathological fear (Miller and McEwen, 2006).

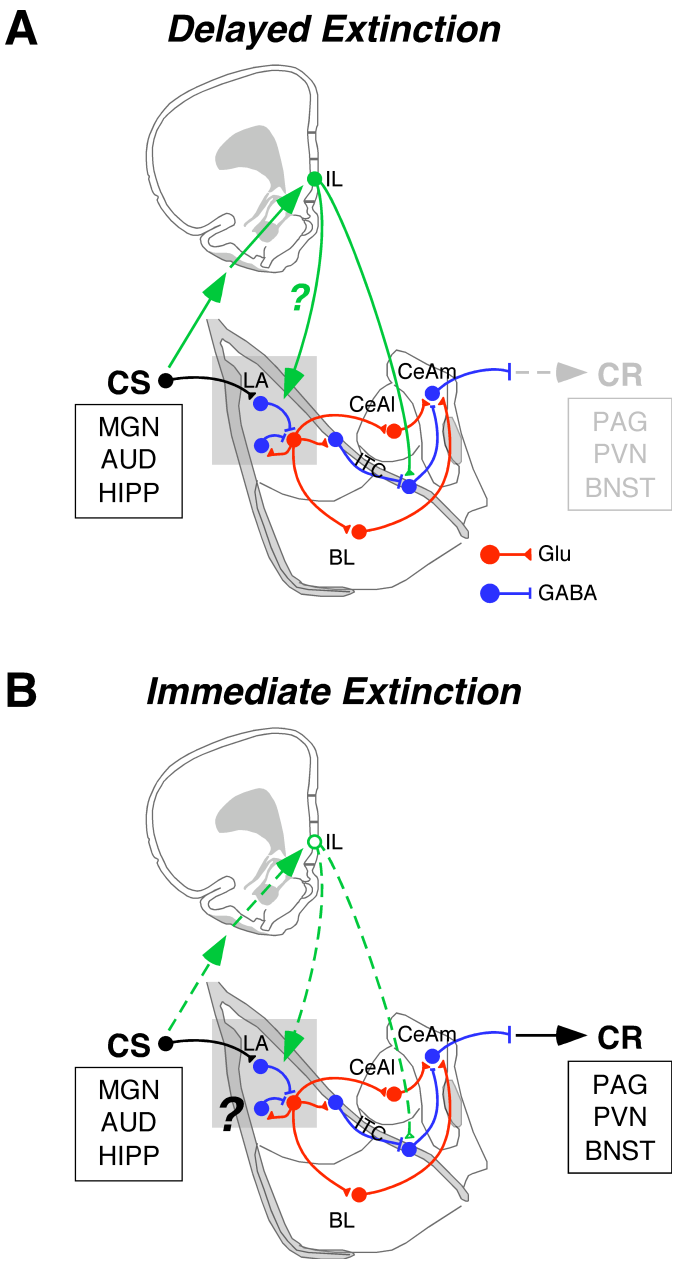


**Figure 6.1. Revised models of fear suppression after delayed and immediate extinction training.**
A. Fear is suppressed after delayed extinction. Despite the inhibitory extinction circuit of the IL, there are evidences showing that extinction also happens locally within the BLA, modulated by contextual information from the hippocampus (HIPP). The role of the projections from the IL to the BLA in extinction modulation remains unclear. B. Immediate extinction failed to suppress long-term fear. The IL was not engaged. What happened within the local circuit of the amygdala requires further investigation. This figure was adapted from Swanson (2004).

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